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ImaGene Red™ C_{12}RG lacZ Gene Expression Kit (I-2906)

Quick Facts

Storage upon receipt:
- –20°C
- Desiccate
- Protect from light

Abs/Em of the reaction product: 571/585 nm

Introduction

Introduction of cloned DNA constructs into cultured cells and transgenic organisms has become a standard experimental methodology for defining the mechanisms that regulate gene transcription. Continued progress in the study of gene expression depends on development of improved analytical methods that permit sensitive detection and analysis of genetic products. In present analytical techniques, transcription from the transfected promoter is monitored by RNA analysis or by the detection of an encoded protein product. In these methods, certain genes, called reporter genes, are incorporated into the recombinant expression construct. These 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 *Escherichia coli* β-D-galactosidase gene (*lacZ*) has been extensively studied and utilized in this manner.

5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal, B-1690, B-22015) is widely used for detection of genes sequentially fused in the frame of the *lacZ* gene. The sequentially fused gene is really the gene of interest and is the gene whose expression is proportional to the β-galactosidase activity. When X-gal is cleaved, an intensely blue halogenated indoxyl precipitate is formed that, while effective in identifying transformed cells by imaging techniques, is toxic to viable cells and, being non-fluorescent, is not useful for flow cytometric analysis and sorting of viable cells. The fluorescent β-galactosidase substrate, fluorescein di-β-D-galactopyranoside (FDG, F-1179), has been used for a sensitive flow cytometric β-galactosidase assay.[1,2]

Scientists at Molecular Probes have developed several unique fluorogenic β-galactosidase substrates, some of which are now available in our ImaGene Green™ C_{12}FDG and ImaGene Red™ C_{12}RG *lacZ* Gene Expression Kits (I-2904 and I-2906, respectively). The ImaGene Green and ImaGene Red β-galactosidase substrates differ from FDG in that they are lipophilic fluorescein or resorufin derivatives that can easily cross most cell membranes under physiological culture conditions at ambient temperature or 37°C.[3] After incubation for minutes to hours, depending on the level of β-galactosidase expression, green or red fluorescence of cells expressing the *lacZ* gene product can be visualized by standard fluorescence imaging and flow cytometry instrumentation.[4]

The ImaGene Red C_{12}RG *lacZ* Gene Expression Kit (I-2906) permits detection of *lacZ* expression in single cells by detecting the enzymatic hydrolysis of dodecylresorufin β-D-galactopyranoside (C_{12}RG, Figure 1) to the highly fluorescent product, 2-dodecylresorufin.

Cells are cultured in a suitable medium and loading is accomplished by adding a solution of the ImaGene Red C_{12}RG β-galactosidase substrate to the cells and incubating for a suitable period (20 minutes to several hours). The substrate enters the cells and, following hydrolysis by β-galactosidase, forms a red fluorescent product that remains inside the cell for hours to days, including through cell divisions. The reaction can be stopped by addition of the competitive inhibitor, phenylethyl β-D-thiogalactopyranoside (PETG). Fluorescence is typically detected by fluorescence microscopy, although flow cytometry can also be used. At least a semi-quantitative indication of the enzyme activity in single cells can be obtained by flow cytometry, by digital imaging or in a fluorescence microplate reader.

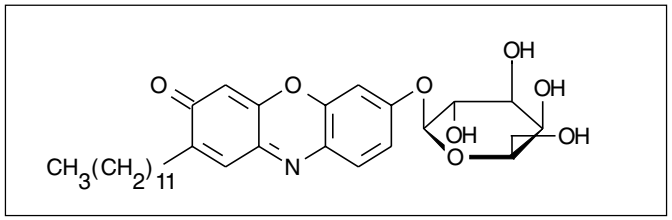

Figure 1. *Structure of C_{12}RG.*

Materials

Materials Provided

- **ImaGene Red C_{12}RG substrate reagent** (Component A), 1.4 mL of 5 mM C_{12}RG substrate in 70% DMSO/30% ethanol (v/v). Before withdrawing samples, inspect the stock solution for possible precipitation; occasionally a precipitate will form upon storage. The precipitate can be dissolved easily by warming the vial to 37–50°C in a water bath for a few minutes and mixing. The substrate provided will prepare about 210 mL of 33 µM C_{12}RG staining medium, which should be sufficient for about 100–200 tests, depending on the volume used for each experiment.
- **PETG reagent** (Component B), 1.0 mL of 50 mM phenylethyl β-D-thiogalactopyranoside in water. The reagent is stable for several months when stored at 4°C.
- **Chloroquine reagent** (Component C), 1.0 mL of 30 mM chloroquine diphosphate in water. The reagent is stable for several months when stored at 4°C.

Storage

To avoid frequent freezing and thawing, aliquot the substrate reagent into several small storage containers and store frozen

until use. This will minimize potential decomposition of the substrate. Presence of a pronounced red color in the ImaGene Red C_{12}RG β-galactosidase substrate reagent or a high fluorescent background in the cells, as compared to previously obtained results, may indicate that the reagents no longer meet appropriate standards for use. The reagents are stable for at least six months, provided the recommended storage conditions are observed.

Protocol

Overview

The following protocol describes the methodology for culture of cells transformed with the β-galactosidase reporter gene, introduction of the ImaGene Red C_{12}RG β-galactosidase substrate into these cultured cells and preparation of the cell culture for imaging. Our suggested initial conditions may require modifications based on the expression level of the cells, permeability of the substrate into the cells or tissue and background from intrinsic cellular β-galactosidase. Procedures are given for preparing the cell cultures and staining them with the ImaGene Red C_{12}RG β-galactosidase substrate, as well as the general setup of the fluorescent microscope for imaging the stained cells. Methods are described for using a selective inhibitor to lower the background endogenous lysosomal β-galactosidase activity that is present in some cell types and a competitive inhibitor to slow or completely block β-galactosidase activity.

Cell Preparation and Staining

1.1 Prepare a sterilized working reagent: Dilute the required amount of the ImaGene Red C_{12}RG substrate reagent 1:150 (v/v) with appropriate sterile culture medium (notes **A**, **B**) to a concentration of 33 µM. Warm to 37°C for about 10 minutes to obtain a working solution. *Do not keep the substrate working solution at 37°C for extended periods, as spontaneous hydrolysis may occur.* Resorufins are sensitive to thiols so omit these from the medium during incubation with this substrate. Alternatively, a 10X concentrated solution (330 µM) can be prepared and diluted 10-fold (v/v) to obtain the required 33 µM working solution. The 10X concentrated solution can be stored for 48 hours at 4°C or longer in the freezer. Prepare the substrate-containing media by filtering through a sterile 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.

1.2 Prepare the cells for staining:

Adherent cells: Grow the cells on coverslips inside a petri dish in an appropriate culture medium and condition to desired confluency (note **C**). Remove the medium from the dish and cover the cells with prewarmed (37°C) 33 µM substrate-containing culture medium from the working solution prepared above (note **D**). Incubate the cell cultures under desired conditions for 20 minutes to one hour. The coverslip with cells can be removed at any desired time after 20 minutes to one hour and examined for fluorescence intensity (note **E**).

Suspension cells: Centrifuge to obtain a cell pellet and aspirate the supernatant. Then resuspend the cells gently in prewarmed (37°C) 33 µM substrate-containing culture medium (note **F**). Incubate the cell suspension for 15–30 minutes under desired conditions. Centrifuge and resuspend the pellet in fresh culture medium or calf serum. Plate the cells on a slide, cover with a coverslip, seal and examine the fluorescence.

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

2.1 Add 1 part chloroquine reagent to 100 parts culture media to obtain a 300 µM solution and prepare cell cultures as described above. Incubate the cells for 30 minutes at 37°C.

2.2 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.

β-D-Galactosidase Inhibition by PETG Reagent (note I)

Prepare and stain cell cultures as described in steps 1.1 and 1.2. 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 is now strongly inhibited, and the samples can be examined at a convenient time.

Fluorescence Microscope Setup and Calibration

The ImaGene Red C_{12}RG 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 visualized with a rhodamine filter set that permits excitation at about 570 nm and detection of emission near 585 nm.

Interpretation of Results

Uniform staining of β-galactosidase–positive cells (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 minutes to one hour after incubation with the substrate.[3] 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 red patches 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 endogenous 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 of the commonly used culture medium preparations should be acceptable. D-MEM containing 10% calf serum has been successfully used. In any case, it is preferable, when possible, to prepare the ImaGene Red C_{12}RG 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 33 µM loading concentration is recommended as an initial condition that may require optimization depending on *lacZ* expression levels and other cell type–dependent factors. Increased concentrations (60 µM) and longer incubation times should be tested in cases where fluorescent staining of *lacZ*-positive cells is weak.

[**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 the section on inhibition of endogenous β-galactosidase activity and notes **G** and **H** below).

[**D**] Alternatively, the 10X concentrated solution can be added directly to the original medium containing cells in a ratio of 1 part concentrate to 9 parts medium. This may serve to minimize potential damage to the cells.

[**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 this ratio by varying the amount of staining solution.

[**G**] Some mammalian cell types have endogenous β-galactosidase activities in their lysosomes that can interfere with measurement of β-galactosidase 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 minutes to 2 hours), then the washing step is optional, since 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-galactosidase in mammalian cells. It 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.** FASEB J 5, 3108 (1991); **4.** Biotech Bioeng 42, 1113 (1993); **5.** Genes and Development 6, 591 (1992).

Product List

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

Cat #	Product Name	Unit Size
I-2906	ImaGene Red™ C_{12}RG *lacZ* Gene Expression Kit	1 kit

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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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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)
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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

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