

DetectaGene™ Blue CMCG *lacZ* Gene Expression Kit (D-2921) For Detecting β -Galactosidase Activity in Living Cells

Quick Facts

Storage upon receipt:

- -20°C
- Protect from light

Abs/Em: 372/470 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 with genomic regulatory elements, the resulting DNA constructs can be introduced into cells of interest and the reporter gene product can then be 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 used for detection of genes sequentially 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} Under physi-

ological 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.

To overcome the limitations of these substrates, scientists at Molecular Probes have developed the DetectaGene Blue *lacZ* Gene Expression Kit with a unique β -galactosidase substrate that yields a bright blue fluorescent product with greatly improved cellular retention. The fluorogenic substrate in our DetectaGene Blue *lacZ* Gene Expression Kit is 4-chloromethylcoumarin β -D-galactopyranoside (CMCG, 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 CMCG–glutathione adduct is subsequently converted to a bright blue fluorescent product. Because peptides do not readily cross cellular membranes, the resulting 7-hydroxycoumarin–glutathione adduct is well retained, even in cells that are kept at 37°C . Molecular Probes' researchers have found that *lacZ*-positive CRE BAG 2 cells loaded with $200\ \mu\text{M}$ CMCG are highly fluorescent. Moreover, the CMCG-stained cells were found to be fluorescent even after incubation at 37°C for 18 hours in fresh medium.

The DetectaGene Blue Kit also includes stock solutions of phenylethyl β -D-thiogalactopyranoside (PETG), chloroquine diphosphate and propidium iodide. PETG is a competitive inhibitor of β -galactosidase that can be added to terminate reactions prior to analysis. Chloroquine may be used to lower 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.

Materials

Contents

- **DetectaGene Blue substrate reagent** (Component A), 100 μL of 10 mM 4-chloromethylcoumarin β -D-galactopyranoside (CMCG) 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 $150\ \mu\text{M}$ propidium iodide in water.

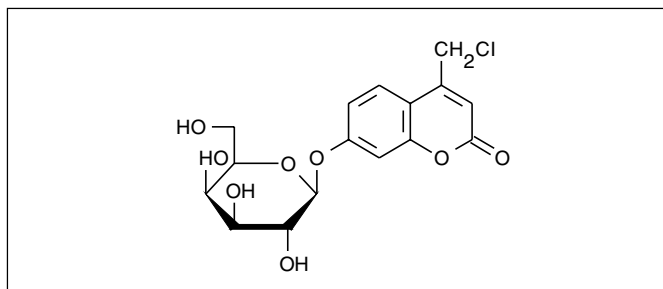


Figure 1. Structure of CMCG.

Using the protocol provided, this kit provides sufficient reagents for at least 50 tests when loading cells in suspension and 25 tests when loading adherent cells.

Storage and Handling

The stock CMCG reagent is stable for several months if stored frozen. 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. The CMCG reagent can be diluted fivefold with distilled water to facilitate this distribution. *Do not keep the CMCG working solution at elevated temperatures for extended periods, as spontaneous hydrolysis may occur.* **Note:** The presence of a pronounced blue-green color in the CMCG 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 frozen at -20°C. Minimize exposure to light.

Experimental Protocol

The DetectaGene Blue CMCG *lacZ* Gene Expression Kit can be used for either fluorescence imaging or flow cytometric analysis of β -galactosidase-containing cells. Detection by flow cytometry using the DetectaGene Blue CMCG substrate requires a flow cytometer that can provide UV excitation. The following protocol includes the basic methodology for preparing adherent cells or cells in suspension, staining them with CMCG and detecting fluorescence in a fluorescence microscope or flow cytometer. Also described are methods for using the competitive inhibitor, PETG, to slow or completely block β -galactosidase activity and methods for using chloroquine to lower the background from endogenous lysosomal β -galactosidase activity, which is present in some cells. This protocol should serve as a guideline and may require modification based on specific experimental requirements.

Preparing 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

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

1.2 Resuspend the cells in Staining Medium to approximately 10^7 cells/mL (note **B**) and pipet 100 μ L 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, (Component C)); concentrations greater than 1 mM may be deleterious to cells³ (note **C**). Proceed to step 1.3 immediately, or put cells on ice.

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

1.4 Immediately before use, prepare 100 μ L of 200 μ M CMCG substrate working solution in deionized water from the 10 mM stock solution (Component A) (notes **D** and **E**). Warm the solution at 37°C for about 10 minutes.

1.5 Combine 100 μ L of pre-warmed CMCG substrate working solution with 100 μ L of pre-warmed cells suspended from step 1.3. Mix rapidly and *thoroughly*. Return the sample to the 37°C water bath for 2 minutes. **Note:** The optimal working concentration of CMCG substrate must be determined experimentally. The recommended 200 μ M working concentration suggested may have to be varied based on the method of loading (note **E**) and the level of β -galactosidase activity in cells.

1.6 Stop the CMCG loading at the end of 2 minutes by adding 1.8 mL of Staining Medium to the 200 μ L volume of CMCG and cell suspension.

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

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

Loading Adherent Cells

2.1 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, (Component C)); concentrations greater than 1 mM may be deleterious to cells³ (note **C**).

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

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

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

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

2.6 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 further intracellular hydrolysis of the substrate by treatment with PETG (see note **G**).

2.7 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 off the supernatant and resuspend the cells in Staining Medium.

Analysis

Flow Cytometry

Set up and calibrate the flow cytometer to detect coumarin, propidium iodide and forward scatter according to standard procedures.⁴ Use unstained cells of the same type you are analyzing to set the background autofluorescence compensation⁵ (note **H**).

Fluorescence Microscopy

Fluorescence is detected using standard coumarin or DAPI filter sets.

Notes

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

[B] 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] 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).

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

[E] The loading procedure described in steps 1.5 and 2.4 subjects cells to hypotonic shock in order to facilitate substrate entry. This treatment may not be necessary for some cell types. For loading under isotonic conditions, prepare the CMCG working solution in staining medium instead of distilled water and increase the incubation time from 2 to about 30 minutes.

[F] Propidium iodide is impermeant to the plasma membrane and selectively labels the nuclei of dead cells with red fluorescence.

[G] Competitive inhibition of β -galactosidase by PETG (Component B) can be used to terminate CMCG turnover prior to analysis. After terminating CMCG loading 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.

[H] Some endogenous constituents of cells give rise to broad bandwidth autofluorescence when excited by the argon 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.⁵

References

1. Proc Natl Acad Sci USA 85, 2603 (1988); 2. Cytometry 12, 291 (1991); 3. Exp Cell Res 136, 327 (1981); 4. Parks, D.R. *et al.* in *The Handbook of Experimental Immunology 4th edition*, D.M. Wier, Ed., Blackwell, Edinburgh (1986) pp 29.1–29.21; 5. Cytometry 8, 114 (1988).

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

Cat #	Product Name	Unit Size
D-2921	DetectaGene™ Blue CMCG <i>lacZ</i> Gene Expression Kit	1 kit

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