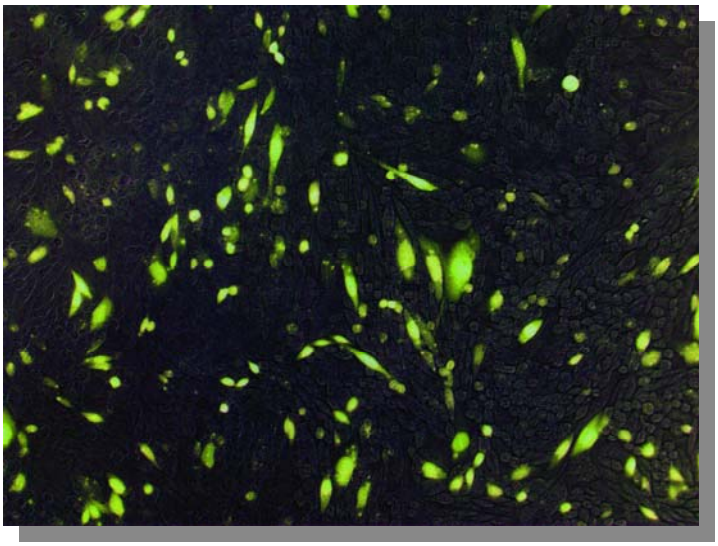
PRODUCT INFORMATION SHEET



MARKERGENE™
IN VIVO LACZ β-GALACTOSIDASE DETECTION KIT
(PRODUCT M0259)

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In vivo lacZ β-Galactosidase Detection Kit

(Product M0259)

NOTE: The following information is given as a viable methodology for use of the MarkerGene™ in vivo lacZ β-Galactosidase Detection Kit. The user may determine their own best conditions for use dependent on the specific conditions present in their experiment. This information is subject to change without notice. Copyright. 2007.

I. OVERVIEW

One of the most common reporter genes used in molecular biology applications is the E. coli lacZ gene that codes for an active subunit of β-galactosidase in vivo. Since this enzyme is generally absent in normal mammalian, yeast, some bacterial and even plant cells, it can be detected at very low levels, and since the enzyme has a wide substrate specificity, monitoring lacZ expression (and therefore co-expressed genes or promoter efficiency) has become routine to the point of detection of as few as 5 copies of β-galactosidase per cell. Here, we use the β- galactoside analog fluorescein di-β-D-galactopyranoside (FDG) in a protocol that sensitively distinguishes lacZ+ cells from lacZ- cells[1].

Although chromogenic assays of ß-galactosidase activity (i.e. X-Gal) are useful, the recent application of the fluorogenic substrate fluorescein di-β-D-galactopyranoside (FDG) has been shown to be several orders of magnitude more sensitive[2]. Because of this high sensitivity, use of FDG allows quantitation of lacZ expression in single, viable eukaryotic cells, whereas other assays often result in dead cells. In addition, because of its high water solubility and detection limits, the FDG substrate has found extensive use in automated ELISA type assay systems[3]. This β-galactosidase detection kit provides reagents and protocol to perform up to 250 assays.

II. MATERIALS

A.) **Fluorescent Substrate Reagent**. The fluorescent substrate fluorescein di-β-D-galactopyranoside is provided as a 10mM solution in (8:1:1) H2O:EtOH:DMSO. This reagent should be kept at –20ºC (frozen) until use. Heating of this reagent by any means (hot air gun, ultrasonic bath, etc.) should be avoided as this may cause decomposition of the reagent. When diluting this reagent to working solutions, the dilution solvent (water, buffer, medium) should be cooled to OºC (ice-bath) prior to use.

B.) **Reference Standard**. 1.0mL of 2mM fluorescein in distilled water is provided as a reference standard.

C.) **Inhibitors**.

i.) Chloroquine. 1mL of 30mM Chloroquine in distilled water.

ii.) Phenylethyl β-D-1-thiogalactoside. 1mL of 50mM PETG in distilled water.

Storage and Handling. Fluorescent reagents and fluorescent labeling solutions or samples should be handled with care, kept cold (ice-bath) when not in use, and stored frozen (-20ºC). In case of contact with skin or eyes, wash thoroughly with soap and cold-water. Reagents should be stable for at least 6 months following purchase. Unstable background fluorescence readings for blank samples will indicate decomposition. These materials are intended for research purposes only. Use in drug or manufacturing processes is strictly prohibited. Please contact us for information on use or licensing.

III. ASSAY CONDITIONS

It is recommended that measurements be made in duplicate, if possible, and that the approximate concentration range of the fluorescent probe be adjusted for optimum signal and sensitivity. Previous studies have indicated that the labeling of cells is virtually independent of the initial fluorescent probe concentration in the range of about 100 pM - 2 mM. Since staining may be somewhat time dependent, a time course for the experiments should also be generated for initial trials. The emission of the highly fluorescent product fluorescein is monitored at 512 nm using excitation at 488 nm (argon ion laser). The user is asked to consult with the manufacturer (or instrument manual) for the particular instrument in use for appropriate filter set(s) needed for monitoring at these wavelengths. Typical epifluorescence microscopic analysis is performed using an excitation filter, a dichroic filter and emission filter for fluorescein fluorescence. For flow cytometric analysis, the FACS instrument is typically equipped with bandpass filter for monitoring fluorescein fluorescence.

To normalize data, each cell suspension or plate is monitored at exactly the same time (20 min.) after equilibration with the probe. The product-forming rate is dependent on the concentration of incorporated probe in the cell, but with concentrations of the probe above approximately 2 µM, enzyme kinetics are typically independent of initial concentration. The working range of the assay will need to be determined for each individual experiment. Adjust working concentrations accordingly. A blank prepared with all reagents, substituting corresponding lacZ-cells should be run in parallel if possible. Alternately, a blank prepared with all reagents except cells (substituting water or media) can be run to determine background fluorescence readings for each experiment.

IV. PROTOCOL

(For mammalian cells – See Notes (1) and (2) for bacteria and yeast cells)

1.) Healthy cells expressing the lacZ gene are harvested from exponential growing cultures. (Note: unhealthy cells or those grown to confluence often exhibit high background staining due to endogenous galactosidase activity or low pH conditions).

2.) Adherent cells are trypsinized by standard protocols, spun down to a weak pellet.

3.) Warm the cell pellet in a 37ºC water bath for 10 minutes prior to adding staining media. [see also step 10 below] For other cell types, adjust physical staining methodology accordingly.

4.) Add an equal volume of pre-warmed staining media to the cell pellet (typically about 100 to 200 µL of the fluorescein di-β-D-galactopyranoside substrate reagent (100 µM to 2 mM concentration)/100-200 µL of cell pellet). The staining media is prepared by diluting the FDG reagent to 100 µM to 2 mM concentration (i.e. 1:100 to 1:5 respectively for 100 µM to 2 mM) with ice-cold media (non-serum), then warming to 37ºC.

5.) Mix the cell suspension thoroughly and rapidly.

6.) Incubate this reaction mixture for 20-25 minutes at 37ºC. (Note: Longer incubation periods may be used with appropriate controls to guard against high background fluorescence from decomposition at these temperatures.) (see also step 11 below]

7.) FDG loading is terminated by addition of 1.8 mL ice-cold media (or Phosphate Buffered Saline containing 10 mM Hepes, 4% Fetal Calf Serum (FCS), pH 7.3, and 1 pg/mL propidium iodide (optional). (Note: The propidium iodide helps to identify dead (propidium iodide bright red) cells in such assays). [see also step II below]

8.) It may be best to keep cells on ice until viewed, either by FACS analysis or by conventional microscopic analysis.

9.) Read fluorescence at 512 nm using an appropriate excitation filter for excitation at 488 nm. Use reference standard for optimizing spectrometer conditions.

10.) In case high levels of endogenous β-galactosidase are noticed, cells are resuspended in media (from step 7) at approximately 10^7 cells per mL containing 300 µM chloroquine. After incubating at 37ºC for 20 min., the cells are centrifuged and staining is continued as described earlier (step 4).

11.) For cells with high lacZ β-galactosidase activity, FDG loading is terminated by adding ice- cold media (from step 7 above) containing the reversible β-galactosidase inhibitor phenylethyl thiogalactoside (PETG) at 1 mM concentration (40 µL of 50 mM PETG/1.8 mL media).

Note (1): When performing this assay using bacterial cells, make the following procedural modifications:

1.) Pellet the cells from liquid culture by centrifugation at *10,000 x g.*
2.) Resuspend cells in sterile distilled water (1 mL)
3.) Rather than preparing a staining media, add substrate reagent so that final concentration is 1mM (110 µL per 1 mL cell suspension)
4.) Incubate cells at 37ºC for 30 min. with moderate shaking.
5.) Perform FACS as per manufacturer's instructions. Also see Step 9 for proper wavelength.

Note (2): When performing this assay using yeast cells, cells must first be permeabilized. See reference (1) below for further details.

V. WHOLE CELL MICROPLATE ASSAY *(For mammalian cells)*

1.) Healthy cells (adherent or non-adherent) are cultured in multi-well tissue culture plates (such as BD Falcon clear, flat bottom plates; with 6, 12, or 96-wells). Plate wells should also be prepared that contain no cells as a control. Ideally, a sufficient number of wells should be prepared so that assays can be performed in triplicate.

2.) Prior to performing assay, all media should be replaced with serum-free growth medium (such as DMEM or RPMI 1640) without antibiotics. Presence of serum or antibiotics in the medium may affect results. Cells may be incubated in the serum-free medium for several hours (up to 24 hours) prior to analysis for ß-galactosidase activity.

3.) Add the Fluorescent Substrate Reagent (Part 0259-001) to wells so that final concentration in the medium is 500µM (1/20 volume of medium in well). Reference Standard (Part 0259-002) may also be added to additional wells at varying concentrations, for use in optimizing reading conditions or creating a calibration curve.

4.) Record fluorescence (EX/EM: 490/514) using a microtiter plate reader with appropriate excitation and emission filter sets. Fluorescence may either be recorded continuously, or at defined intervals, ranging from several minutes to several hours. When measurements are not being taken, cells should be placed in an incubator (37^{o}C) with adequate CO_2 and humidification.

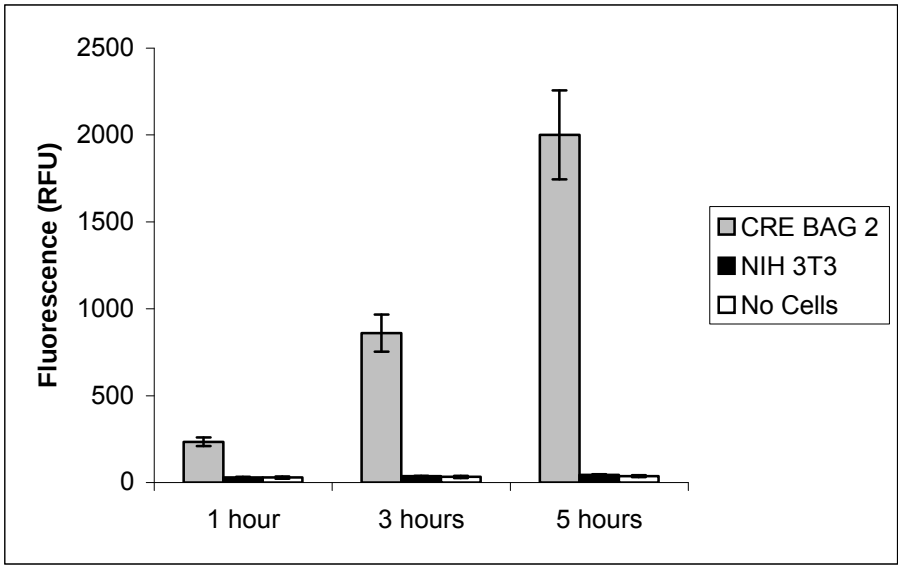

Figure 1: Two adherent mouse fibroblast tumor cell lines, CRE BAG 2 (*lacZ* stable transformants), and NIH 3T3 (*lacZ* negative) were cultured to 50% confluence in 12-well tissue culture plates (BD Falcon clear, flat bottom). Media was replaced with Dulbecco's Modified Eagles Medium (DMEM) containing no serum or antibiotics (1mL). Plate wells were also prepared containing medium only (no cells). Cells were incubated (37^{o}C, 5% CO_2) for 24 hours. Fluorescent Substrate Reagent (Part 0259-001) was added to all wells to a final concentration of 500µM (50µL). Fluorescence was recorded using a Perkin Elmer HTS7000 Plus Bio Assay Reader, using 485nm excitation and 535nm emission filters. Readings were taken at 1, 3, and 5 hour intervals. Assays were performed in triplicate and averaged, error bars represent standard error.

REFERENCES:

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3.) Roederer M., Fiering S., Herzenberg L.A. (1991) "FACS-Gal: flow cytometric analysis and sorting of cells expressing reporter gene constructs." Methods: Comp. Meth. Enzymol. **2**: 248.

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M0259 KIT CONTENTS			
DESCRIPTION	**QUANTITY**	**PART NO.**	**STORAGE**
REAGENTS			
FLUORESCENT SUBSTRATE REAGENT 10 mM SOLUTION (8:1:1) H2O:ETOH:DMSO	1 X 1.0mL VIAL	0259-001	F,L
REFERENCE STANDARD 2 mM FLUORESCEIN	1 X 1.0mL VIAL	0259-002	F,L
INHIBITORS			
CHLOROQUINE 30 mM	1 X 1.0mL VIAL	0259-003	F
PETG 50 mM	1 X 1.0mL VIAL	0259-004	F
DOCUMENTATION			
MSDS SHEETS	2	0259-005/6	N/A
PRODUCT INFORMATION SHEET	1	0259-007	N/A

Notes: F=store at or below -20^{o}C; C=store cold (4^{o}C); L=light sensitive; T=avoid repeat freeze/thaw; R=read protocol;instructions carefully prior to use.

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