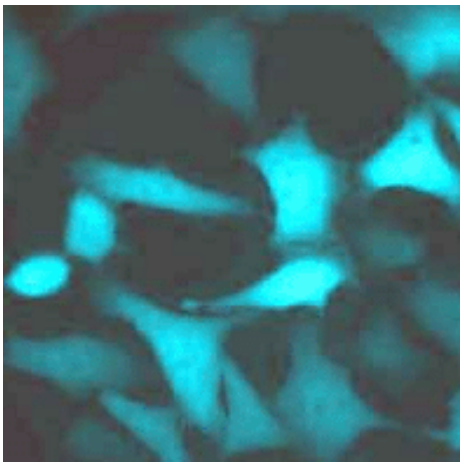
Product Information Sheet



MarkerGene™ FACS Fluorescent Blue *lacZ* β-Galactosidase Detection Kit

(Product M0255)

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NOTE: The following information is given as a viable methodology for use of the Marker Gene™ FACS Blue *lacZ* β-Galactosidase Detection Kit. The user may determine their own best conditions for use dependent on the specific conditions present in their experiment.

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 by FACS analysis[1].

Although chromogenic assays of β-galactosidase activity (i.e. X-Gal) have use, application of the fluorogenic substrate 3-carboxyumbelliferyl β-D-galactopyranoside (CUG) (blue fluorescence) combined with Fluorescence Activated Cell Sorting (FACS) analysis has been shown to be several orders of magnitude more sensitive[2]. In addition, because of its high water solubility and detection limits, the CUG substrate has found extensive use in automated ELISA type assay systems.[3] This assay system is especially useful for dual-labeling experiments where the cells to be analyzed area also labeled with a fluorescein-based probe (FITC-labeled antibody, fluorescein-based substrate, etc.).

This β-galactosidase detection kit provides reagents and protocol to perform up to 500 automated (10 x 96 well microtiterplate) assays.

II. MATERIALS

A.) Substrate Reagent. 0.5mL of 50mM CUG in distilled water. Dilute with 2.0 ml of the REACTION BUFFER to prepare the reagent for use in step 3 below.

B.) Reference Standard. 0.5mL of 20µM CU in absolute methanol. Dilute with STOP BUFFER for spectrometer standardization as outlined in step 6 below.

C.) Inhibitor. 1.0mL of 30mM Chloroquine in distilled water.

D.) Buffer Solutions. (not provided) Prepare buffer solutions as described below. Prepare REACTION BUFFER containing 100mM sodium phosphate buffer, pH 7.0 with 1mM MgCl, 10mM β-mercaptoethanol and 0.1 % Triton X-100. Prepare STOP BUFFER containing 500mM glycine buffer pH 12.0) and 10mM EDTA. Adjust pH with conc. NaOH (10 M).

E.) Storage and Handling. Fluorescent reagents and fluorogenic substrates should be handled with care, kept cold (ice-bath) when not in use, and stored frozen (-20°C). 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. High 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. ENZYME ASSAY (Microtiterplate Format).

It is recommended that a calibration curve be prepared using known concentrations of purified β-galactosidase enzyme in the approximate concentration range of the unknown analyte. Since the conversion of the fluorogenic substrate (CUG) releases the fluorophore 7-hydroxycoumarin-3 carboxylic acid (CU), the emission of this highly fluorescent product is monitored at 460 nm using excitation at 390 nm. To normalize data, each enzyme reaction is terminated at exactly the same time (20 min.) using a stop buffer of high pH (12.0). The enzyme assay has a typical working range from about 1-1000 picograms. Adjust enzyme concentrations accordingly.

1.) The sample containing cell lysate, purified enzyme, or cell suspension is pipetted into individual microtiterplate wells with serial dilutions in duplicate for each concentration and for each cell/tissue sample (20 - 50 µL/well). Include two wells for blanks (20 - 50 µL/well reaction buffer).

2.) Add 100 µL reaction buffer to each well. Incubate/mix for a few minutes to make sure the reaction system is homogeneous.

3.) Add 50 µL 10 mM CUG reagent to each well. Mix thoroughly by manual agitation. NOTE: Lower concentration of the CUG reagent (1-5 mM) are routinely used for lower enzyme concentrations.

4.) Incubate for 20 min, at a fixed temperature (normally 25°C). NOTE: If lower substrate concentrations are used, incubation times may need to be adjusted proportionally.

5.) Add 100 µL stop buffer to each well. Wait for 10 min. Emission readings can now be made at any time up to 3 hours after stopping the reaction. Store unread plates (4°C) covered by parafilm or plastic wrap if they are not to be read immediately.

6.) Read fluorescence at 460 nm using an appropriate excitation filter for excitation at 390 nm. Use reference standard for optimizing spectrometer conditions. NOTE: A 1:50 dilution with stop buffer will usually be sufficient.

7.) Subtract fluorescence from the blank well(s) from each sample well. Average the readings of duplicate samples.

8.) Generate a calibration curve using purified enzyme samples by plotting normalized' fluorescence vs. β-galactosidase concentration (log-log).

9.) Using the calibration curve from above, determine the concentration of β-galactosidase in samples by comparison, and extrapolate this data to determine concentration of the enzyme in the original cell/tissue suspension.

10.) Additional information on these and alternate protocol conditions is given in the references [4-8].

IV. FACS 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 carboxyumbelliferone is monitored at 445 nm using excitation at 396 nm. 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 coumarin (AMC) fluorescence. For flow cytometric analysis, the FACS instrument is typically equipped with bandpass filter for monitoring coumarin 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.

V. FACS PROTOCOL

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 3-carboxyumbelliferyl-β-D-galactopyranoside (CUG) substrate reagent (100 µM to 2 mM concentration)/100-200 µL of cell pellet). The staining media is prepared by diluting the CUG reagent to between 200 µM to 2 mM concentration (i.e. 1:12500 to 1:25 respectively for 200 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.) CUG 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, if not read immediately.

9.) Read fluorescence at 460 nm using an appropriate excitation filter for excitation at 390 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, CUG loading can be 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).

REFERENCES:

1.) Nolan, et al., Proc. Natl. Acad. Sci. USA **85** (1988) 2603.
2.) Methods:Companion to Meth. Enzymol. **2** (1991) 248.
3.) Burd, J.F., Meth. Enzymol. 74C (1981) 79.
4.) Fierer, J., et al., Infection and Immunity **61** (1993) 5231.
5.) Armenta, R., et al., Anal. Biochem. **146** (1985) 211.
6.) Place, J.D., Antimicro. Agents Chemother. **24** (1983) 240.
7.) Li, T.M., et al., Epilepsia **23** (1982) 391.
8.) Johnson, P.K., et al., Clin. Chem. **27** (1981) 1087.

M0255 KIT CONTENTS			
DESCRIPTION	**QUANTITY**	**PART NO.**	**STORAGE**
REAGENTS			
FLUORESCENT SUBSTRATE REAGENT 50 mM CUG IN DISTILLED WATER	1 X 0.5 mL VIAL	0255-001	F,L
REFERENCE STANDARD 20µM CU IN ABSOLUTE METHANOL	1 X 0.5 mL VIAL	0255-002	F,L
INHIBITORS			
30 mM CHLOROQUINE IN DISTILLED WATER	1 X 1.0mL VIAL	0255-003	F
DOCUMENTATION			
MSDS SHEETS	3	0255-004/5/6	N/A
PRODUCT INFORMATION SHEET	1	0255-007	N/A

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

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