ab189816

LacZ beta Galactosidase
Intracellular Detection Kit

Instructions for Use

For the detection of beta-galactosidase using Microplate or FACS Assay

This product is for research use only and is not intended for diagnostic use.
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1. Introduction

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.

Abcam’s LacZ beta Galactosidase Intracellular Detection Kit (ab189816) uses the β-galactoside analog fluorescein di-β-D-galactopyranoside (FDG) in a protocol that sensitively distinguishes lacZ+ cells from lacZ- cells. Although chromogenic assays of β-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. 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.
Abcam’s LacZ beta Galactosidase Intracellular detection kit (ab189816) provides reagents and protocol to perform up to 250 assays.

Emission: 512 nm.

Excitation: 488 nm.
2. Protocol Summary

FACS Protocol

- Harvest cells
- Spin cells down to form pellet
- Add warm staining media
- Incubate
- Add ice-cold media
Read fluorescence

Microtitre plate format

Pipette cells into 96 well plate

Replace media with serum-free media

Add Substrate Reagent

Incubate

Read fluorescence
3. Kit Contents

<table>
<thead>
<tr>
<th>Components</th>
<th>Amount</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescent Substrate Reagent</td>
<td>1 mL</td>
<td>Store at or below -20°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Light sensitive</td>
</tr>
<tr>
<td>Reference Standard</td>
<td>1 mL</td>
<td>Store at or below -20°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Light sensitive</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>1 mL</td>
<td>Store at or below -20°C</td>
</tr>
<tr>
<td>PETG</td>
<td>1 mL</td>
<td>Store at or below -20°C</td>
</tr>
</tbody>
</table>

4. 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. High background fluorescence readings for blank samples will indicate decomposition. Avoid exposure of Substrate Reagent and Reference Standard to light.
5. Additional Materials Required

- 96 or 384-well black wall/clear bottomed microplates
- Fluorescence microplate reader
- PBS or HHBS
- CO₂ Incubator
- Flow Cytometer
6. Assay Protocol

A. 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 labelling 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 minutes) 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.

B. Protocol

1. Mammalian Cells

- 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).

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

- Warm the cell pellet in a 37°C water bath for 10 minutes prior to adding staining media. For other cell types, adjust physical staining methodology accordingly.

- 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 Fluorescent Substrate 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 37°C.

- Mix the cell suspension thoroughly and rapidly.

- Incubate this reaction mixture for 20-25 minutes at 37°C. 
  *Note: Longer incubation periods may be used with appropriate controls to guard against high background fluorescence from decomposition at these temperatures.*

- 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.*

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

- Read fluorescence at 512 nm using an appropriate excitation filter for excitation at 488 nm. Use Reference Standard for optimizing spectrometer conditions.
In case high levels of endogenous β-galactosidase are noticed, cells are resuspended in media at approximately $10^7$ cells per mL containing 300 μM Chloroquine. After incubating at 37°C for 20 minutes, the cells are centrifuged and staining is continued as described earlier.

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

2. Bacterial Cells

When performing this assay using bacterial cells, make the following procedural modifications:

- Pellet the cells from liquid culture by centrifugation at 10,000 x g.
- Resuspend cells in sterile distilled water (1 mL)
- Rather than preparing a staining media, add Fluorescent Substrate Reagent so that final concentration is 1mM (110 μL per 1 mL cell suspension)
- Incubate cells at 37°C for 30 min. with moderate shaking.
- Perform FACS analysis.
3. **Yeast Cells**

When performing this assay using yeast cells, cells must first be permeabilized.

4. **Suspension Cells**

When using suspension cells, such as bone marrow cells freshly isolated, it has been shown that incubation with the Fluorescent Substrate Reagent for 1 min at 37ºC followed by cooling on ice 20 minutes prior to FACS analysis gave optimal staining results. Your results may vary.
C. **Quick Test Assay**

A quick to set up assay that can be used to determine if cells show LacZ activity perhaps to check for transfection efficiency or in other situations whenever the question is if LacZ is present in the cells. The assay can be performed after routine sub culturing of cell lines with as little as 5000 cells, e.g. those remaining after seeding of new vessels.

- The sample containing cell suspension is pipetted into individual microtiter plate wells in duplicate for each cell/tissue sample (150 μL/well). Include two wells for blanks (150 μL/well of fresh media, no cells).

- Add 50 μL 10 mM Fluorescent Substrate Reagent to each well. Mix thoroughly.

- Read fluorescence at 460 nm using an appropriate excitation filter for excitation at 390 nm at regular intervals (e.g. 10 minutes) for approximately 2 hours. Use Reference Standard for optimizing spectrometer conditions.

- Subtract fluorescence from the blank well(s) from each sample well. Average the readings of duplicate samples. Graphs can then be plotted of increase in fluorescence over time.
D. **Whole Cell Microplate Assay (for mammalian cells)**

- Healthy cells (adherent or non-adherent) are cultured in multi-well tissue culture plates (such as 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.

- 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.

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

- 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°C) with adequate CO₂ and humidification.

7. Data Analysis

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 (clear, flat bottom). Media was replaced with Dulbecco’s Modified Eagles Medium (DMEM) containing no serum or antibiotics (1 mL). Plate wells were also prepared containing medium only (no cells). Cells were incubated (37°C, 5% CO₂) for 24 hours. Fluorescent Substrate Reagent was added to all wells to a final concentration of 500 μM (50 μL). Fluorescence was recorded 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.

8. Troubleshooting

1. Can this kit be used with live tissue samples?

*Live cell tissue staining of tissues with our product M0250 (FDG) or Kit M0259 has been accomplished in many tissue types. Cells or tissues are bathed in a solution of FDG (0.5 to 2 mM concentration) for a period of time, from 30 minutes to 2 hours. Cells or tissues are then washed with buffer or media prior to observation. Keeping the cells or tissues cold (4 degrees C) will help prevent translocation of the fluorescent signal.*

2. Will this kit work with fixed tissues?

*FDG will work best in live cell or tissue staining. Once fixed, the tissues become much more permanent. Although this does not affect the enzyme reaction very much, it does cause the fluorescent product (fluorescein) to leak from the stained tissues easily. However, some people have had success in staining fixed tissues with FDG, and of course, there are many methods of live tissue staining. Please see our website for more information about tissue staining with FDG.*
For further technical questions please do not hesitate to contact us by email (technical@abcam.com) or phone (select “contact us” on www.abcam.com for the phone number for your region).
UK, EU and ROW
Email: technical@abcam.com | Tel: +44-(0)1223-696000

Austria
Email: wissenschaftlicherdienst@abcam.com | Tel: 019-288-259

France
Email: supportscientifique@abcam.com | Tel: 01-46-94-62-96

Germany
Email: wissenschaftlicherdienst@abcam.com | Tel: 030-896-779-154

Spain
Email: soportecientifico@abcam.com | Tel: 911-146-554

Switzerland
Email: technical@abcam.com
Tel (Deutsch): 0435-016-424 | Tel (Français): 0615-000-530

US and Latin America
Email: us.technical@abcam.com | Tel: 888-77-ABCAM (22226)

Canada
Email: ca.technical@abcam.com | Tel: 877-749-8807

China and Asia Pacific
Email: hk.technical@abcam.com | Tel: 108008523689 (中國聯通)

Japan
Email: technical@abcam.co.jp | Tel: +81-(0)3-6231-0940

www.abcam.com | www.abcam.cn | www.abcam.co.jp