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FluoReporter® lacZ/Galactosidase Quantitation Kit (F-2905)

Quick Facts

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

Ex/Em of reaction product: 386/448 nm

Introduction

The *E. coli lacZ* gene, encoding β-D-galactosidase, is extensively used as a reporter gene for detecting the expression of recombinant fusion genes and for monitoring transfection efficiency in mammalian, yeast and bacterial cells. Although mammalian cells do contain β-galactosidases, they are generally lysosomal enzymes with low pH optima and therefore exhibit low activity at neutral pH. Combining this with the fact that *E. coli* β-galactosidase has a high turnover rate, the enzyme can be detected at very low levels, making it a sensitive reporter of gene expression.

The FluoReporter® *lacZ*/Galactosidase Quantitation Kit (F-2905) contains the improved fluorogenic substrate CUG for the sensitive detection of β-galactosidase activity. The advantages of this substrate over the commonly used 4-methylumbelliferyl β-D-galactopyranoside (MUG) substrate include its higher aqueous solubility and increased fluorescence efficiency, resulting in a lower threshold of detection. Molecular Probes' scientists have demonstrated a practical detection limit of 0.5 picograms of β-galactosidase using this FluoReporter kit and a fluorescence microplate reader. Although this kit was originally developed for assaying mammalian cell extracts, it is very useful for enzyme-linked immunosorbent assays (ELISAs)[1] that employ β-galactosidase conjugates, as well as for assaying bacterial[2] and yeast cell extracts, prepared using conventional protocols.[3-6] Each kit supplies sufficient reagents for at least 1000 tests using 96-well microplates.

Materials

Reagents Supplied

- **Substrate reagent** (Component A), 3 mL of 40 mM 3-carboxy-umbelliferyl β-D-galactopyranoside (CUG) in 100 mM sodium phosphate buffer (pH 7.0), 1 mM $MgCl_2$ and 110 mM β-mercaptoethanol
- **Reference standard** (Component B), 1 mL of 10 mM 7-hydroxycoumarin-3-carboxylic acid in dimethylformamide

Storage and Handling

The CUG substrate reagent should be stored frozen at -20°C; the storage temperature for the reference standard is not critical. Both components should be protected from light. When stored properly, these solutions are stable for at least six months. CUG solutions should not be exposed to room temperature for extended periods of time as spontaneous hydrolysis will occur. If the CUG substrate exhibits a high fluorescent background signal prior to enzymatic hydrolysis, then this reagent may have deteriorated during storage and is likely no longer suitable for use.

Materials Required but Not Provided

- Reaction buffer
- Stop buffer
- β-galactosidase (for calibration curve, if desired)

Protocol

Overview

The FluoReporter *lacZ*/Galactosidase Quantitation Kit provides a sensitive method for measuring the level of β-galactosidase activity in solution or in cell extracts. Enzymatic cleavage of the fluorogenic β-galactosidase substrate CUG yields 7-hydroxycoumarin-3-carboxylic acid, a highly fluorescent product that can be quantitated in a fluorescence microplate reader.

The CUG substrate and an aliquot of cell extract or other β-galactosidase–containing solution are added to each microplate well. After an incubation period, the reaction is terminated by adding stop buffer, which raises the pH of the sample. The fluorescence of each sample is then measured using a fluorescence microplate reader. The fluorescence signal can be converted into picograms of β-galactosidase by using a standard curve, if desired. This assay has a linear detection range of about 0.5 to over 1000 pg β-galactosidase (Figure 1). The lower detection limit corresponds to about ten *lacZ*-positive NIH 3T3 cells per well. The following protocol describes the assay of mammalian cell extracts using a fluorescence microplate reader and may require some modification when assaying bacterial or yeast cell extracts[1-4] or β-galactosidase conjugates in an ELISA.[5]

Solution Preparation

1.1 Prepare a reaction buffer containing 0.1 M sodium phosphate, pH 7.3, 1 mM $MgCl_2$ and 45 mM β-mercaptoethanol. Approximately 10 mL of reaction buffer are needed per 96-well microplate. Additional buffer is needed for preparing enzyme dilution buffer, which is used when generating a standard curve.

1.2 If generating a standard curve, prepare enzyme dilution buffer by adding bovine serum albumin (BSA) at a final concentration of 1 mg/mL to approximately 1 mL of reaction buffer.

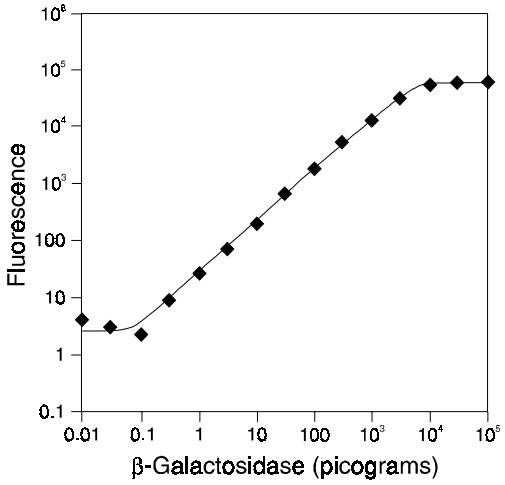

Figure 1. *Example of a standard curve using the FluoReporter® lacZ/ Galactosidase Quantitation Kit.*

1.3 To generate a standard curve, prepare a fresh 1 µg/mL solution of β-galactosidase solution in enzyme dilution buffer. In addition, make 10-fold, 100-fold, 1000-fold and 10,000-fold dilutions of this enzyme solution in enzyme dilution buffer (note **A**). These solutions are stable for several hours at room temperature. In the enzyme assay, 10 µL of each of these solutions will be pipetted into an individual microplate well, yielding 10 ng, 1 ng, 100 pg, 10 pg and 1 pg β-galactosidase standards, respectively. For increased accuracy, we recommend performing assays in triplicate.

1.4 Prepare a 1.1 mM working solution of the CUG substrate reagent by diluting 275 µL of the 40 mM stock solution (Component A) with 9.73 mL of reaction buffer. CUG solutions should not be exposed to room temperature for extended periods of time as spontaneous hydrolysis will occur. Approximately 10 mL of CUG working solution are needed for a 96-well microplate. The working solution can be stored frozen at -20°C for at least six months.

1.5 If desired, prepare a 0.1 mM working solution of the 7-hydroxycoumarin-3-carboxylic acid reference standard by diluting the stock solution (Component B) 100-fold with reaction buffer. The reference standard can be used to normalize fluorescence, allowing assays performed at different times, or on different instruments, to be compared to a single standard curve.

1.6 Prepare a stop buffer containing 0.2 M Na_2CO_3. Approximately 5 mL of stop buffer are needed for a 96-well microplate.

Enzyme Assay

2.1 Carefully pipet 10 µL cell extract into individual microplate wells (note **B**). It may be necessary to dilute the cell extracts prior to performing this assay, depending on the level of β-galactosidase present. For more accurate results, we recommend performing each assay in triplicate.

2.2 If generating a standard curve, pipet 10 µL of each of the purified β-galactosidase solutions prepared in step 1.3 into individual microplate wells.

2.3 Pipet 10 µL of reaction buffer into a microplate well to serve as a blank.

2.4 Add 100 µL of the CUG working solution (prepared in step 1.4) to each well.

2.5 If desired, pipet 100 µL of the 0.1 mM reference standard (prepared in step 1.5) into an empty well. The reference standard can serve as an instrument-independent control. Normalization of the fluorescence signals with the reference standard allows a single standard curve to be used for assays performed at different times, even if performed on different instruments or with different instrument settings. The reference standard can also be used to convert the fluorescence signal into moles of product.

2.6 Incubate the microplate for 30 minutes at room temperature. For comparison to a previously generated standard curve, incubation time is critical — the same incubation time and temperature should be used to ensure accurate quantitation.

2.7 Add 50 µL of stop buffer (prepared in step 1.6) to each well. In addition to terminating the reaction, the stop buffer causes an increase in the fluorescence of the product.

2.8 Measure the fluorescence of the solution in each well using a suitable fluorescence microplate reader fitted with an excitation filter centered at about 390 nm and an emission filter centered at about 460 nm (notes **C**, **D**).

Analysis of Results

3.1 To generate a standard curve, first subtract the fluorescence of the blank from that of each of the samples containing the purified β-galactosidase solutions. If the standard curve will be used for comparison with assays performed at a later date, divide the background-subtracted fluorescence of the β-galactosidase standards by the background-subtracted fluorescence of the reference standard. Plot the resulting corrected fluorescence intensities versus enzyme amount on a log–log scale. Note that the values for enzyme amount must be adjusted to compensate for the purity of the enzyme preparation. Alternatively, fluorescence can be plotted versus units of β-galactosidase activity. A standard curve (without reference standard normalization) should resemble the one shown in Figure 1.

3.2 Analyze the fluorescence of the samples by subtracting the fluorescence of the blank from that of each sample. If a reference standard is being used, divide the corrected fluorescence by the background-subtracted fluorescence of the reference standard. Use the standard curve to determine the amount of β-galactosidase in each well.

Notes

[A] Additional intermediate dilutions (e.g. 3-, 30-, 300- and 3000-fold dilutions) can be made to create a more complete standard curve, if desired.

[B] Cell extracts from mammalian cells can be prepared using a freeze–thaw cell extraction protocol or by treatment of the cells with a detergent, such as Triton® X-100. Alternatively,

Triton X-100 can be added to the reaction buffer and the assay procedure altered to allow for cell lysis within the microplate. For instance, 10 µL of a cell suspension can be added to 90 µL of reaction buffer prepared with 0.1% Triton X-100 (v/v) and the microplate incubated at room temperature for 10 minutes to allow for cell lysis. The enzyme reaction can then be initiated by the addition of 10 µL an 11 mM solution of CUG (which is 10-fold higher than the concentration of the working solution prepared in step 1.4). Bacterial and yeast cell extracts prepared using conventional methods[1-4] can also be assayed with the kit.

[C] These excitation and emission wavelengths may not correspond to standard filter sets on all microplate readers. Other filters can be used (e.g. excitation at 360 nm), but sensitivity will be diminished.

[D] The fluorescence should be measured within 15 minutes of adding the stop buffer. If comparing samples to a previously generated standard curve, the same time interval between stopping the reaction and reading the fluorescence should be used.

References

1. Anal Biochem 118, 102 (1981); **2.** Infect Immun 61, 5231 (1993); **3.** *Short Protocols in Molecular Biology, Second Edition*, F.M. Ausubel *et al.*, Eds., Greene Publishing Associates and John Wiley & Sons, New York (1992) pp. 13–27; **4.** Sambrook, J., Fritsch, E.F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual, Second Edition*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989) p. 16.66; **5.** Methods Enzymol 101, 181 (1983); **6.** *Experiments in Molecular Genetics*, J.H. Miller, Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1972) p. 352.

Product List

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

Cat #	Product Name	Unit Size
F-2905	FluoReporter® *lacZ*/Galactosidase Quantitation Kit *1000 assays*	1 kit

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