3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone (800) 325-5832 (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

ProductInformation

b-GALACTOSIDASE (GAL) FLUORESCENT ACTIVITY DETECTION KIT

Product No. **GAL-F**

Technical Bulletin MB-550
November 1998

TECHNICAL BULLETIN

Product Description

Reporter genes are "markers" widely used for analysis of mutationally altered genes as well as gene regulation and localization. The expressed reporter genes are detected by biochemical activity assay, immunological assay or by histochemical staining of tissue sections or cells[1].

The *E. coli* β-Gal (β-galactosidase) gene is extensively used as a gene fusion marker for analysis of gene expression in eukaryotes. The *E. coli* β-galactosidase enzyme hydrolyzes D-galactose from various β-galactosides. The compound, 4-methylumbelliferyl-galactoside (4-MU-Gal) is a β-Gal substrate which upon hydrolysis produces the fluorescent 4-methylumbelliferone (4-MU). It is used in this fluorescent activity detection test designed for transfected cells or tissues expressing the *E. coli* β-Gal enzyme[2].

The β-galactosidase hydrolyzes the 4-MU-Gal to galactose and the fluorescent compound 4-MU. The reaction is stopped with sodium carbonate solution; raising the pH also serves to enhance the fluorescence of the 4-MU. The 4-MU can be excited at 365 nm; it's emission maximum is at 455 nm. *E. coli* β-galactosidase serves as a positive control enzyme.

This kit includes all buffers, substrate and reagents required for highly sensitive, easy to perform quantitative activity assays.

Unit Definition

One unit of β-galactosidase will release one pmole of 4-MU from 4-MU-Gal per minute at pH 7.0 and 37 °C.
Note: This unit definition is different from Sigma's usual units for β-galactosidase.

Reagents Provided

This kit is sufficient for 200 tests.

- 5X Lysis Buffer, Product No. L5784 — 25 ml
  250 mM HEPES, pH 7.4, containing 25 mM CHAPS
- 2X Assay Buffer, Product No. F4672 — 10 ml
  200 mM sodium phosphate buffer, pH 7.3, containing 2 mM $MgCl_2$ and 100 mM β-mercaptoethanol
- 4-Methylumbelliferyl β-D-Galacto-pyranoside, (4-MU-Gal substrate), Product No. M1633 — 10 mg
- 4-Methylumbelliferone Standard, (4-MU), Product No. M1508 — 25 mg
- β-Galactosidase Positive Control From *E. coli,* Product No. G4538 — 100 µl
- 5X Stop Solution, Product No. S5930 — 100 ml
  1 M sodium carbonate

<u>Reagents and Equipment Required but Not Provided</u>
(Sigma product numbers are given where appropriate)

- Temperature controlled 37 °C water bath
- 10 mm x 75 mm glass test tubes
- Microcentrifuge tubes
- Fluorimeter and cuvets
- Dimethyl sulfoxide (DMSO), Product No. D8418
- Reagents for determination of protein concentration

Precautions and Disclaimer
Sigma's β-Galactosidase Fluorescent Activity Detection Kit is for laboratory use only. Not for drug, household or other uses.

Storage
Store kit at –20 °C.

Preparation Instructions
Preparation of working solutions

1. Lysis buffer:
   Mix thawed 5X lysis buffer thoroughly before diluting 1:5 with deionized water.

2. Substrate solution:
   Dissolve 4-MU-Gal at a concentration of 11.4 mg/ml (34 mM) in DMSO. Store solution at –20 °C up to one year.

3. 2X Assay buffer with substrate:
   Mix thawed 2X assay buffer thoroughly. To 0.5 ml 2X assay buffer add 25 µl of substrate solution (34 mM 4-MU-Gal solution). Use freshly prepared solutions.

4. Calibration standard:
   Dissolve 2.0 mg 4-MU in 10 ml deionized water to make a 1 mM stock solution. Dilute 100 µl of the stock solution to a volume of 10 ml with deionized water (1:100) to make a 10 µM intermediate stock solution. Dilute 50 µl of the intermediate stock solution to a volume of 5 ml with 1X stop solution to make the 100 nM working solution. Use freshly prepared solutions.

5. Positive control enzyme solution:
   Dilute 10 µl β-galactosidase positive control to a volume of 1 ml with 1X lysis buffer. Dilute a 10 µl aliquot of the first dilution to a volume of 1 ml with 1X lysis buffer. Dilute a 10 µl aliquot of the second dilution to a volume of 1 ml with 1X lysis buffer to give a working control solution ($1:10^6$ dilution). Use freshly prepared solutions.

6. Stop solution:
   Mix thawed 5X stop solution thoroughly before diluting 1:5 with deionized water.

Fluorimeter settings:
Set fluorimeter at 365 nm excitation, 455 nm emission, 5 nm slit width. Zero the instrument using 2 ml stop solution.

Procedure
The reaction is performed in lysis/assay buffer containing 0.85 mM 4-MU-Gal at 37 °C. The reaction is initiated by the addition of the 2X assay buffer with substrate, and is stopped by the addition of cold 1X stop solution.

A. Calibration Procedure
Since the activity of β-galactosidase in the extract is unknown, perform a preliminary assay to find the proper dilution factor so that the measured values fall within the fluorimeter's detection range.

1. Add 20 µl of cell extract (sample volume) to a microcentrifuge tube.
2. Add 80 µl of lysis buffer.
3. Pre-incubate 1-2 minutes at 37 °C.
4. Initiate reaction with the addition of 100 µl of 2X assay buffer with substrate.
5. Cap tube and incubate at 37 °C for 1 hour.
6. Transfer a 20 µl aliquot to 2 ml of cold 1X stop solution and place in an ice bath.
7. Measure the fluorescence intensity (FI) of the sample.

If the values obtained are higher than the upper limit of the instrument, dilute the sample in stop solution until measurements are on scale. Calculate the dilution factor. If values obtained are below the detection range of the instrument, repeat the reaction with a larger volume of cell extract.

B. Multiple Reaction Kinetic Assay Procedure

Complete the Calibration Procedure in order to optimize the sample dilution or the sample volume. The procedure described below is an example for cell extract that has to be diluted by a factor determined from the calibration procedure.

REAGENT	SAMPLE ASSAY	POSITIVE ENZYME CONTROL ASSAY	NEGATIVE CONTROL ASSAY
Sample extract	20 µl	---	---
Diluted β-galactosidase positive control	---	20 µl	---
Lysis buffer	80 µl	80 µl	100 µl
2X assay buffer with substrate	100 µl	100 µl	100 µl

1. Prepare one vial containing 2.0 ml stop solution for each time point and place in an ice bath.

2. Dilute the sample cell extract by the dilution factor determined in the calibration procedure.

3. Pipet 20 µl of the diluted sample extract to sample assay tube and 20 µl of diluted β-galactosidase positive control to positive control assay tube.

4. Add the amount of lysis buffer indicated in the above table to each assay tube. Note: In the event that a volume of cell lysate other than 20 µl is used, adjust the amount of lysis buffer added to give a total reaction volume of 100 µl.

5. Mix and equilibrate for 1-2 minutes at 37 °C.

6. Add 100 µl 2X assay buffer with substrate to each assay tube to initiate the reaction.

7. Immediately remove 20 µl aliquots (volume per test) from all three reaction mixes into 2 ml stop solution as the zero minute test points. Keep the tubes on ice.

8. Cap tubes and incubate at 37 °C.

9. Transfer additional 20 µl aliquots at 10 minute intervals to vials containing 2 ml cold stop solution.

10. Prepare 4-MU standard dilutions of 10, 20, 40, 60, 80 and 100 nM in stop solution. Include a minimum of 5 data points in order to generate a standard curve. 10 nM equals 20 pmol in a 2 ml cuvet.

11. Measure fluorescence intensity (FI) of all samples, positive enzyme and negative controls and 4-MU standard dilutions.

C. Calculations

1. Draw a calibration curve of 4-MU standards FI versus pmol 4-MU.
2. Calculate FI per pmol 4-MU.
3. Plot sample, negative control and positive enzyme control FI versus time.
4. Calculate FI per minute for sample, negative control and positive enzyme control.
5. Subtract the negative control FI per minute value from the sample and positive enzyme control values.
6. Determine the protein concentration of the sample extract.
7. Calculate β-galactosidase activity of extract in pmol 4-MU per minute per μg protein (units per μg protein), according to the following equations.
8. Positive control enzyme activity is used for confirmation that the enzymatic system is working properly.

Sample calculations

$$\textbf{Activity of extract} \text{ (pmol MU/min/mg protein)} = \frac{\text{FI/min}}{\text{FI/pmole MU}} \times \frac{\text{Dilution factor}}{\text{Extract conc., (mg protein/ml)}} \times 500/\text{ml}^*$$

$$\textbf{500/ml}^* = \frac{\text{Reaction volume (0.2 ml)}}{\text{Sample volume (0.02 ml) x Vol. per test (0.02 ml)}}$$

500/ml factor is calculated according to the following parameters: Reaction volume = 0.2 ml, Sample volume = 0.02 ml (20 μl), and Volume per test = 0.02 ml (20 μl). In the event that the sample volume is different than 20 μl (0.02 ml), adjust the factor accordingly.

References

1. Kain, S.R. & Ganguly, S., "Uses of Fusion Genes in Mammalian Transfection", in Current Protocols in Molecular Biology, Vol. 1, Suppl. 36, Ausubel, F.M. *et al.*, eds. (John Wiley & Sons, New York, 1996) p. 9.6.1
2. Young, D.C., *et al.*, Anal. Biochem., **215**, 24-30 (1993)