



Determination of β -Galactosidase Activity using the FL600™ Microplate Fluorescence Reader

Quantitation of *b*-galactosidase (*b*-gal) enzymatic activity is a commonly used determination in cellular and molecular biology. Fluorometric assays using fluorescein di-*b*-D-galactopyranoside (FDG) or 4-methylumbelliferone *b*-D-galactopyranoside (MUG) as substrates for *b*-galactosidase are described.

Introduction

Transient expression of cloned DNA sequences into mammalian cells is a commonly used tool in molecular biology for the investigation of transcriptional activity of eukaryotic promoters. However, in most cases the natural product of the promoter cannot be assayed in a quantitative fashion. In these instances, the promoter is joined to a reporter gene, which encodes for a protein with a unique enzymatic activity that can be assayed easily. The level of gene expression is then monitored by determination of that unique enzymatic activity.

The LacZ gene from *Escherichia coli* is frequently used as a reporter gene for detecting the expression of recombinant fusion gene studies and for monitoring the efficiency with transient or stable transfections of mammalian, yeast and bacterial cells. A translational in-frame fusion of the gene of interest and the LacZ gene puts lacZ expression under control of the promoter of interest. The activity of the promoter can then be assayed by measuring *b*-galactosidase activity. Because *b*-galactosidase has a high turnover rate and is absent in mammalian cells it serves as a very useful and sensitive reporting tool for gene expression.

The enzymatic product of the LacZ gene, *b*-galactosidase, catalyses the hydrolysis of *b*-D-galactosides, such as lactose, into their component sugars by hydrolysis of the terminal non-reducing *b*-D-galactose residues (Figure 1A). The substrate specificity of the enzyme is such that a variety of different substrates, each with a *b*-D-galactopyranoside moiety, can be acted upon. Investigators have taken advantage of the enzyme's substrate specificity by synthesizing compounds which when hydrolyzed by *b*-galactosidase result in a measurable product. Although the most commonly used chromogenic substrate, *o*-nitrophenyl galactopyranoside (ONPG) results in a colored product upon hydrolysis, it is relatively insensitive and requires long incubations. Fluorescence based assays, which utilize substrates that fluoresce upon hydrolysis, have been employed to provide increased sensitivity. Two such compounds are fluorescein di-*b*-D-galacto-pyranoside (FDG) and 4-methylumbelliferone *b*-D-galacto-pyranoside (MUG).

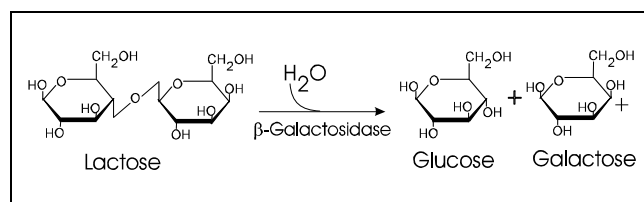


Figure 1. Enzymatic hydrolysis of b-D-galactosides by b-galactosidase. b-Galactosidase catalyses the hydrolysis of terminal non-reducing b-D-galacto-pyranoside residues from disaccharides such as lactose.

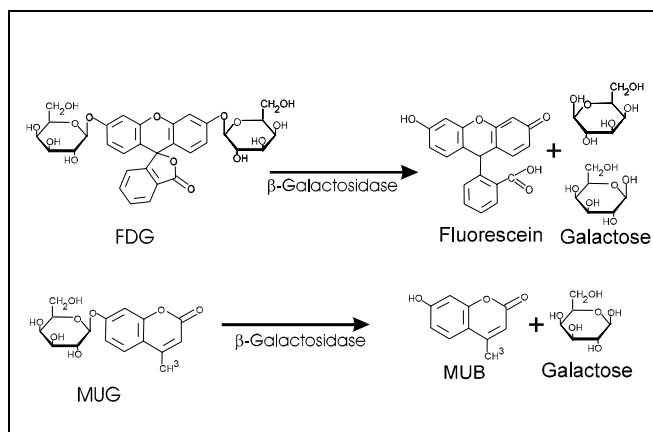


Figure 2. Hydrolysis of FDG or MUG to fluorescent moieties by b-galactosidase. Fluorescein di- β -D-galactopyranoside (FDG) is hydrolyzed to the fluorescent compound, fluorescein, and two galactose residues by b-galactosidase. Methylumbelliferone β -D-galacto-pyranoside (MUG) is hydrolyzed to the fluorescent molecule, 4-methylumbelliferone (MUB) and galactose. FDG and MUG, when hydrolyzed form fluorescein and 4-methylumbelliferone respectively along with galactose (Figure 2). Fluorescein emits light maximally at 514 nm when excited with light at 491 nm (3) whereas the precursor molecule FDG does not (4). Therefore, the increase in fluorescence at these wavelengths can be used to monitor b-galactosidase when FDG is used as a substrate. 4-methylumbelliferone, the hydrolysis product of MUG, has an excitation peak at 372 nm with a fluorescence emission peak at 445 nm.

Materials and Methods

Fluorescein di- β -D-galactopyranoside (FDG), catalogue number F-1179, and 4-methylumbelliferone β -D-galactopyranoside (MUG), catalogue number M-1489, were purchased from Molecular Probes (Eugene, Oregon). The 96 well microplates, catalogue number CFCPN9610, were purchased from Biosearch (Framingham, Massachusetts). b-Galactosidase enzyme (cat. # G-6008), sodium phosphate, magnesium chloride and 2-mercaptoethanol were obtained from Sigma Chemical Company (St. Louis, MO).

The b-galactosidase assay was performed according to Sanbrook et al. (1) except that the substrate utilized was FDG or MUG rather than ONPG. Briefly, 100 μ l of samples or standards in distilled water was placed in each well of a 96-well microplate. The assay was initiated by the addition of 100 μ l of 2X assay buffer. Assay buffer was prepared previously as a 2X stock solution and stored frozen at -20°C . Assay buffer (1X) consists of 100 mM sodium phosphate, pH 7.0; 1 mM MgCl_2 ; 50 mM b-mercaptoethanol; and 0.5 mg/ml of either FDG or MUG in distilled water. Kinetic experiments were performed as described above, with fluorescence determinations beginning immediately upon the addition of reaction buffer. Lyophilized b-galactosidase enzyme was reconstituted with distilled water to stock concentration of 500 U/ml. Enzyme dilutions were made fresh daily and stored on ice until assayed. A series of enzyme dilutions ranging from 0 to 5 U/ml of b-galactosidase (b-gal) were then made using distilled water as the diluent, where one unit of enzyme will hydrolyze 1.0 mmole of ONPG to ONP and galactose per min. Fluorescence determinations were made using a FL600 fluorescence microplate reader (Bio-Tek Instruments, Winooski, VT) with the reader controlled by an external PC running KC4 data reduction software (Bio-Tek Instruments, Winooski, VT). Fluorescein production by the hydrolysis of FDG was monitored using a 485 nm, 20 nm bandwidth, excitation filter and a 530 nm, 25 nm bandwidth emission filter. The sensitivity setting varied as required and the data collected from the bottom. Experiments that utilized MUG as the substrate were read using a 360 nm, 40 nm bandwidth, excitation filter and a 460 nm, 40 nm bandwidth

emission filter. Likewise, the sensitivity setting varied as required and the data collected from the bottom.

Results

b-Galactosidase activity was measured using two different substrates. FDG, when hydrolyzed by b-galactosidase is converted into fluorescein and two galactose molecules, while MUG is hydrolyzed into 4-methyl-umbelliferone and galactose. In either case, the result is the formation of a fluorescent compound by the action of b-galactosidase.

Endpoint fluorescence determinations were made for b-galactosidase enzyme concentrations ranging from 0.0 to 10 U/ml with MUG as the substrate. As demonstrated in Figure 3, concentrations of b-gal enzyme from 0 to 3 U/ml result in a linear increase in fluorescence when the MUG substrate concentration is 0.5 mg/ml. Enzyme concentrations above 3 U/ml result in greater fluorescence values, but the increase is no longer linear. Increasing the reaction time of the assay from 5 minutes to 10 minutes resulted in greater fluorescence values for all enzyme concentrations. For example, the fluorescence at 10 minutes for the 1 U/ml sample increased from 21,437 RFUs at 5 minutes to 34,666 RFUs at 10 minutes. However, the endpoint response loses linearity at a lower enzyme concentration. With either a 5 or 10 minute incubation a 4-parameter logistic best fit of the data was necessary to describe the data. The plateau in fluorescence is most likely the result of substrate depletion by enzymatic activity. When the V_{max} of each reaction is plotted against enzyme concentration a linear response is observed (Figure 4). Again, the leveling off of the reaction rate with higher enzyme concentrations is the result of substrate depletion.

In terms of detecting small quantities of enzyme, the MUG fluorometric assay can detect as little as 1.95×10^{-5} U/ml b-gal enzyme activity, with statistical significance, after a 60 minute incubation (data not shown). As expected, allowing the reaction to continue for a total of 120 minutes increases the detection limit. With a two hour incubation, at ambient temperature, 9.78×10^{-6} U/ml can be distinguished from the 0.0 U/ml blank. When corrected for the volume of sample in each well, as little as 1×10^{-6} Units of enzyme per well can be distinguished from a reaction buffer-only blank.

The generation of fluorescence when using FDG as the substrate was investigated. When samples containing 0.5 U/ml of enzyme are incubated with 0.5 mg/ml FDG in reaction buffer and the fluorescence plotted as a function of time, an increase in fluorescence is observed (Figure 5). This increase in fluorescence is sigmoidal in shape, with relatively less fluorescence being generated in the first 10 minutes as compared to that generated from 10 min to 60 min. At longer time points the curve begins to flatten somewhat, indicating that the FDG substrate is no longer in excess.

When V_{mean} values are calculated from kinetic data generated over 120 minutes and plotted against enzyme concentration (0-0.5 U/ml) using KC4, a linear relationship is observed (Figure 6). Using a 4-parameter logistic fit of these data an equation describing this relationship can be used with a high degree of confidence, as the coefficient of determination (R^2) is 0.9997. When data from enzyme concentrations up to 0.25 U/ml are considered, the V_{mean} is linear. Linear regression analysis of this subset of the data results in a coefficient of determination (R^2) of 0.998 (data not shown).

The relationship between fluorescence and enzyme concentrations with various incubation times was investigated for FDG. Increasing the time allowed for the reaction to take place results in a generalized increase in signal for each enzyme concentration tested (Figure 7). For example, fluorescence signal of 0.5 U/ml samples increased over 300% when the reaction time was increased from 30 minutes to

120 minutes. However, the relationship between enzyme concentration and fluorescence is no longer linear with a reaction time of 120 minutes. With longer incubation times (90-120 min.) the curve describing the increase in fluorescence begins to flatten at the higher enzyme concentrations. Shorter incubation times (30-60 min.), while resulting in lower levels of fluorescence, maintain linearity over a greater concentration range (Figure 7). The detection limits were also determined for the FDG assay. Using FDG at 0.5 mg/ml as the substrate, one can detect as little as 1.95×10^{-5} U/ml b-gal enzyme activity with statistical significance, after a 60 minute incubation (data not shown). When corrected for the volume of sample in each well, as little as 2×10^{-6} Units of enzyme per well can be distinguished from a reaction buffer-only blank.

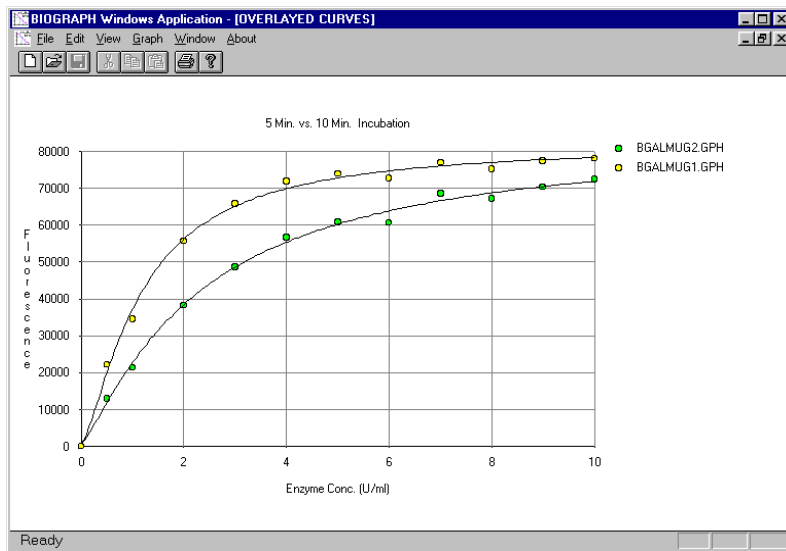


Figure 3. Fluorescence vs. b-galactosidase enzyme concentration using either 5 or 10 minute incubations. b-galactosidase concentrations from 0 to 10 U/ml were incubated with reaction buffer containing MUG (0.5 mg/ml) as the substrate. Production of 4-methylumbelliferone by hydrolysis of MUG was monitored using a FL600 fluorescence microplate reader with a 365 nm, 40 nm bandwidth, excitation filter and a 460 nm, 40 nm bandwidth emission filter at a sensitivity setting of 80. Note the values represent the average of eight determinations at each time point. End point determinations were made at 5 minutes and 10 minutes.

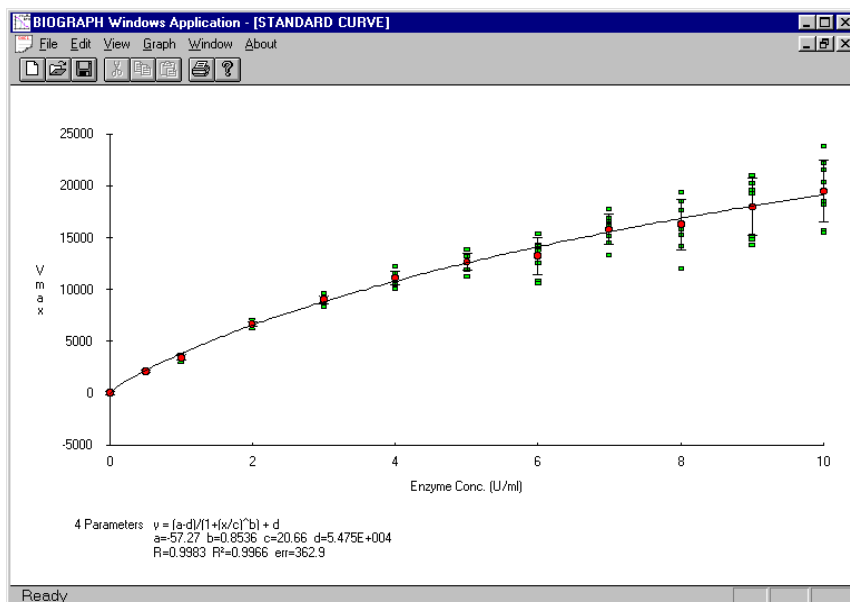


Figure 4. Comparison of Vmax and enzyme concentration using MUG as the substrate. The fluorescence of b-galactosidase assays was determined kinetically and the Vmax of the reactions were determined and plotted against enzyme concentration using KC4 data reduction software. Note that a 4-parameter logistic best fit was used to describe the data.

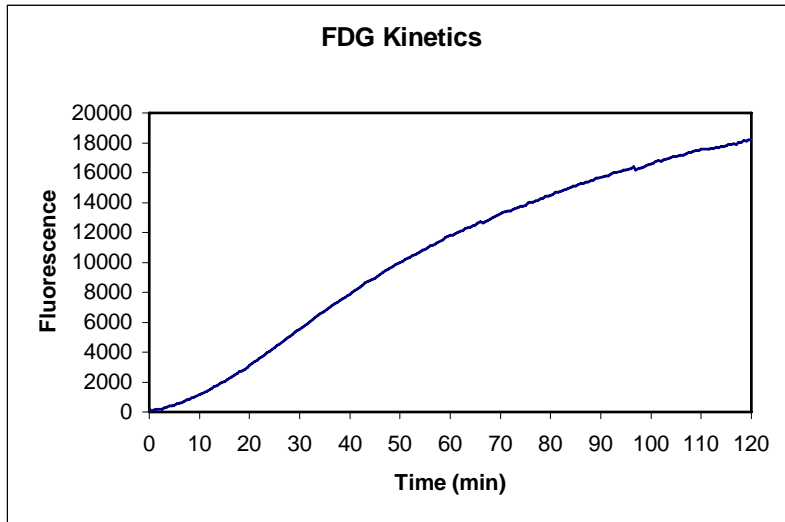


Figure 5. Increase in fluorescence over time when using FDG as the substrate. Reactions containing 0.5 U/ml b-galactosidase were incubated with reaction buffer containing 0.5 mg/ml of FDG substrate and the fluorescence was determined kinetically with readings every 30 seconds. Fluorescein production by the hydrolysis of FDG was monitored using a FL600 fluorescence microplate reader with a 485 nm, 20 nm bandwidth, excitation filter and a 530 nm, 25 nm bandwidth emission filter at a sensitivity setting of 50. Note the values represent the average of eight determinations at each time point.

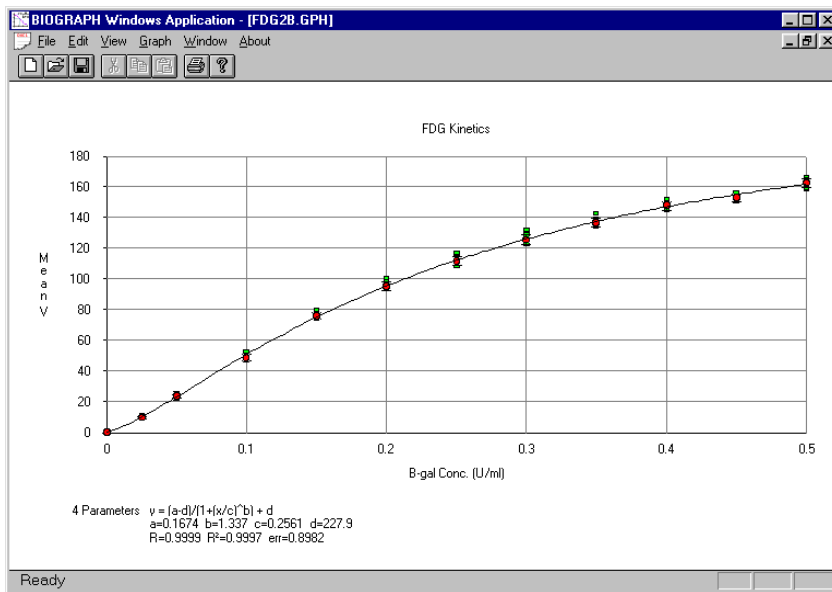


Figure 6. Average reaction rate (Mean V) vs. b-galactosidase concentration. The average change of fluorescence over 120 minutes was determined using KC4 and plotted against b-galactosidase concentration. Reactions contained 0.25 mg/ml FDG substrate and fluorescence was determined using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Note that each data point represents the mean of eight determinations.

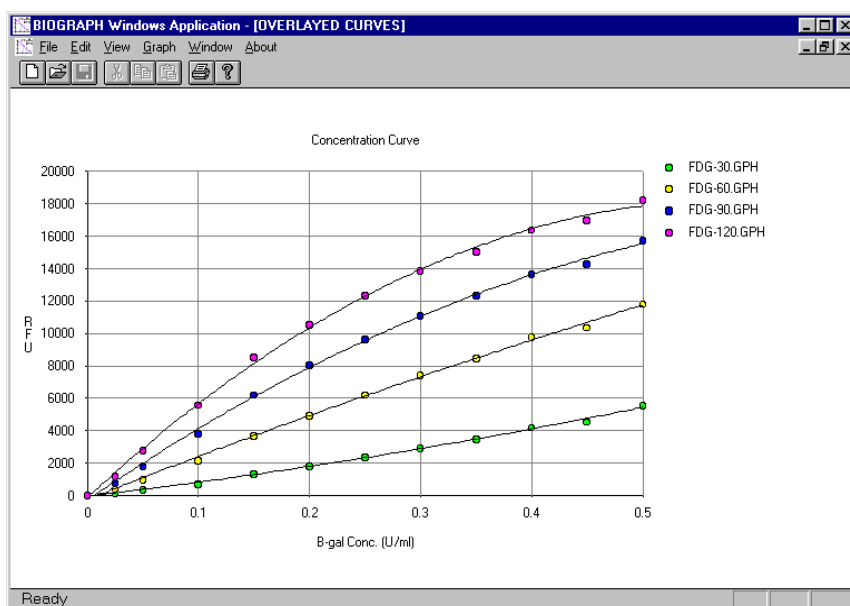


Figure 7. Fluorescence vs. b-galactosidase concentration curve. The fluorescence of reactions containing various concentrations of b-galactosidase and FDG substrate was determined at 30, 60, 90, and 120 min. after the initiation of the reaction. Note that each data point represents the mean of eight determinations.

Discussion

The ability to quantitate b-galactosidase activity is of particular importance in molecular biology. Because the LacZ gene is not normally present in mammalian cells, it can be used as a reporter for gene expression. It is also used in many instances as an internal control for transfection assays. The chromogenic substrate ONPG has historically been used to quantitate levels of b-galactosidase activity. However, this substrate is relatively insensitive, requiring high concentrations of enzyme and long reaction times to produce sufficient signal for detection. The use of fluorogenic substrates, such as FDG or MUG, provides several orders of magnitude greater sensitivity than the colorimetric substrates (5).

The non-linear relationship between fluorescence and time when using FDG as the substrate is most likely the result of stepwise sequential nature of hydrolysis of FDG to fluorescein. The conversion of FDG to fluorescein and galactose takes place in two steps. FDG is initially hydrolyzed by b-galactosidase to fluorescein monogalactoside (FMG), which is not fluorescent, and one molecule of galactose. FMG is further hydrolyzed to the fluorescent moiety, fluorescein, with the release of a second galactose residue. Because the turnover rate for hydrolysis of FDG to FMG is relatively slow ($1.9 \text{ mmole min}^{-1} \text{ mg}^{-1}$) when compared to the rate of conversion of FMG to fluorescein ($22.7 \text{ mmole min}^{-1} \text{ mg}^{-1}$) one would expect a delay in the production of fluorescence. Fluorescence would only be generated after FMG is subsequently hydrolyzed to fluorescein (6). When using FDG as the substrate for endpoint b-gal determinations, it is advisable to avoid using very short incubation times (e.g. less than 10 minutes), particularly if high sensitivity is required. Longer reaction times result in greater sensitivity due to the greater fluorescence signal response. MUG does not have a multistep hydrolysis in order to generate a fluorescent signal and may be more appropriate if a very rapid and high sensitivity assay is desired.

Maintaining an appropriate relationship between the catalytic enzyme concentration, substrates, and incubation time is paramount in obtaining appropriate linearity in the assay. For example, the plateau of fluorescence seen with endpoint reactions at higher enzyme concentrations indicates that either shorter incubation times or lower enzyme concentrations would provide superior linearity. Alternatively, higher substrate concentrations can be used to maintain

linearity, but is eventually limited by the solubility characteristics of the substrate. Fortunately the reaction with either FDG or MUG does not require the addition of a stop solution to enhance the signal. Therefore if lower than expected b-galactosidase activity is present upon an initial determination, the reaction can be allowed to continue for a longer period of time and read a second or even a third time when sufficient signal is present.

The prevention of background hydrolysis of the substrate can increase the sensitivity of the assays. Because FDG and MUG are subject to hydrolysis in aqueous solutions, care should be taken to prevent it from occurring. Hydrolysis can be minimized by first dissolving the substrate in a 1:1 mixture of DMSO and ethanol, the powder should easily dissolve and the subsequent solution can then be added slowly to cold water. Likewise, stock solutions of reaction buffer containing substrate should be aliquoted and stored frozen at -20°C until needed to prevent hydrolysis.

In the past such b-gal determinations have been performed using a conventional tube fluorometer. This method usually entailed using a tube or cuvette to perform the analysis resulting in a very low throughput. The ability to use the FL600 microplate fluorescence reader to perform this analysis allows this routine procedure to be performed on 96 samples in a matter of seconds leading to a tremendous increase in productivity and throughput.

References

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