

High-throughput β -Galactosidase and β -Glucuronidase Assays Using Fluorogenic Substrates

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[Abstract] β -galactosidase and β -glucuronidase enzymes are commonly used as reporters for gene expression from gene promoter-*lacZ* or *uidA* fusions (respectively). The protocol described here is a high-throughput alternative to the commonly used Miller assay (Miller, 1972) that utilises a fluorogenic substrate (Fiksdal *et al.*, 1994) and 96-well plate format. The fluorogenic substrates 4-Methylumbelliferyl β -D-galactoside (for β -galactosidase assays) (Ramsay *et al.*, 2013) or 4-Methylumbelliferyl β -D-glucuronide (for β -glucuronidase assays) (Ramsay *et al.*, 2011) are cleaved to produce the fluorescent product 4-methylumbelliferon. Cells are permeabilized by freeze-thawing and lysozyme, and the production of 4-methylumbelliferon is monitored continuously by a fluorescence microplate reader as a kinetic assay. The rate of increase in fluorescence is then calculated, from which relative gene-expression levels are extrapolated. Due to the high sensitivity fluorescence-based detection of 4-methylumbelliferon and the high density of time points collected, this assay may offer increased accuracy in the quantification of low-level gene expression. The assay requires small sample volumes and minimal preparation time. The permeabilisation conditions outlined in this protocol have been optimised for Gram-negative bacteria (specifically *Escherichia coli* and *Serratia*), but is likely suitable for other organisms with minimal optimisation.

Materials and Reagents

1. Bacteria cell culture
2. 4-Methylumbelliferyl β -D-galactoside (Life Technologies, catalog number: M-1489MP) (for β -galactosidase assays)
3. 4-Methylumbelliferyl β -D-glucuronide (Life Technologies, catalog number: M-1490) (for β -glucuronidase assays)
4. Phosphate-Buffered Saline Tablets (Life Technologies, InvitrogenTM, catalog number: 00-3002)
5. Lysozyme from chicken egg white (Sigma-Aldrich, catalog number: L7651)
6. 200x stock solution for 4-Methylumbelliferyl β -D-galactoside or 4-Methylumbelliferyl β -D-glucuronide (see Recipes)

7. Final working reagent of 4-Methylumbelliferyl β -D-galactoside or 4-Methylumbelliferyl β -D-glucuronide (see Recipes)

Equipment

1. Fluorescence microplate reader, for example: Gemini XPS Fluorescence Microplate Reader (Molecular Devices) or Infinite 200 PRO (Tecan Group Ltd.)
2. 96-Well microplates (Thermo Fisher Scientific, catalog number: 269787)
3. Flat-bottomed clear 96-well microplates (low autofluorescence and/or absorbance at 365 and 445 nm is preferable)
4. Multi-channel pipette(s) (12 or 8 channel) capable of dispensing 10 μ l and 100 μ l
5. Ultra-low temperature freezer (-70 °C)

Procedure

1. Record the OD₆₀₀ of the samples to be assayed. It is recommended that the OD₆₀₀ of the samples to be analysed are in the 0.1-1 OD₆₀₀ range. Dilute the samples in growth media to give an OD₆₀₀ in the 0.1-1 range. Depending on the range of gene expression levels observed, the dilution factor may need to be optimised by analysing multiple dilutions.
2. Collect 100 μ l of the diluted samples (0.1-1 OD₆₀₀ range) to be assayed and freeze at -70 °C in a 96-well microplate (the "master plate"). Once all samples are collected, return plate to the -70 °C and leave to freeze overnight.

*For example: For a time-course experiment in *E. coli*, 100 μ l samples could be collected every hour for 12 h in a single microplate, returning the microplate to the -70 °C after collecting each sample. This microplate now acts as the "master plate", from which replicate assays can be carried out at a later date if desired. Negative media-only controls can also be added to the master plate in free wells.*

3. After all samples are collected (make sure all samples have been fully frozen at -70 °C), defrost the master plate in the 37 °C incubator with the lid off (to avoid cross-contamination via condensation).
4. When the master plate has fully defrosted, aliquot 10 μ l from each well into a new microplate (the "assay plate") using a multichannel pipette and place the assay plate in the -70 °C for at least 15 min.
5. Pre-warm the microplate reader to 37 °C prior to carrying out the assay and initialise the microplate reader software so it is ready to go (see Notes about microplate reader settings for example settings).
6. Prepare the final working reagent.

Note: Prepare immediately prior to use and avoid prolonged exposure to light (i.e. use the same day). Make enough for 100 μ l per reaction.

7. Defrost the assay plate in the 37 °C incubator with the lid off (to avoid cross-contamination via condensation) for at least 10 min and/or until any visible ice crystals have dissolved in all wells.
8. Place yourself within close distance of the microplate reader. Dispense 100 μ l of working reagent into each well of the 96-well microplate (or just wells containing samples) using a multichannel pipette. Try to minimize the time between dispensing reagent to each well. Immediately place the microplate in the plate reader with the lid off and start the program. Samples containing large amounts of β -galactosidase and β -glucuronidase can saturate the assay within the first 10 min, therefore it is important to capture reads quickly after the addition of substrate.

Note: For highly expressing samples that saturate the detector early, data is still recoverable. However as there are likely to be fewer timepoints with a linear increase in fluorescence the rate estimation may be less accurate. Sample dilution will improve accurate measurement of these samples.

9. Extract the rate of increase in fluorescence from the reads using the microplate software or export to excel (see Figure 1):
 - a. Plot the relative fluorescence intensity (the machine carries out internal fluorescence normalisation, hence it is "relative" fluorescence intensity) over time (per second is usually convenient). Choose a linear portion of the graph with the steepest slope (V_{max}) from which to extrapolate the rate (in excel, the equation = SLOPE() can be used). This will provide relative fluorescence units per second (RFU/sec). Some platereader software will calculate V_{max} automatically and in real time during the assay. If the graph is curved or very noisy, you may have problems with cell permeabilization or the fluorescence detector gain settings, respectively (see Troubleshooting below).
 - b. Optional: Subtract the average RFU/sec of negative control wells (media-only with final working reagent) from all samples (in practice this value is usually zero or very small and so this step is usually not necessary).
 - c. Normalise to optical cell density (OD_{600}) recorded in step 1. This will give you units of RFU/sec/ OD_{600} .
 - d. Values can be normalized to account for sample volume used (i.e. Divided by 0.01 ml to give values as RFU/sec/ml/ OD_{600}), however this normalization is somewhat arbitrary if the same volumes (10 μ l) are always used. Alternatively, standard curves with known concentrations of purified β -galactosidase or β -glucuronidase can be used to estimate actual units of enzyme concentration.

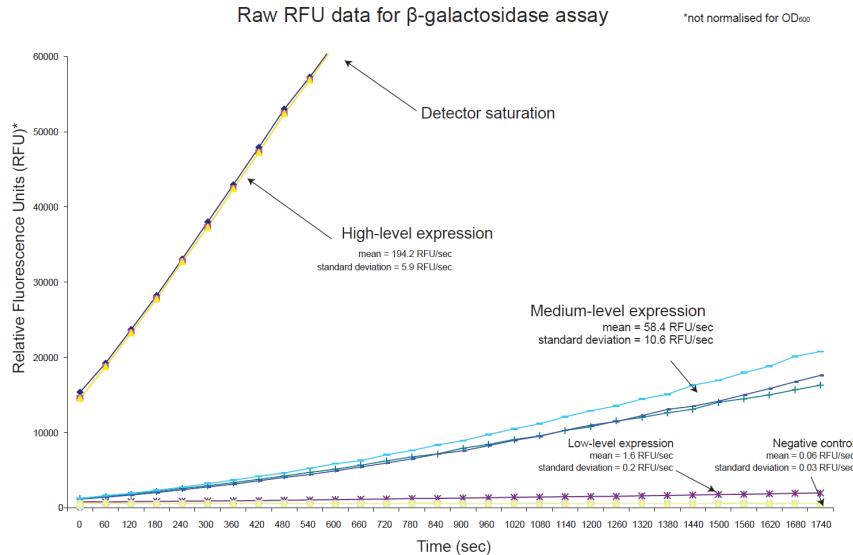


Figure 1. Plot of raw relative fluorescence units over time. Biological triplicate samples were analysed by the β -galactosidase assay over a 30-min period, as described above. Mean slope and standard deviation for the three replicates are indicated next to each group. The highly-expressed samples saturate the detector after 600 sec. Statistically significant low-level expression is also detected from the lowly-expressed sample compared to the negative media-only (with final reagent mix) control. Final values for publication/presentation are normalised to OD₆₀₀.

Notes about microplate reader settings

1. Universal settings:

Temperature	37 °C
Excitation wavelength	360-365 nm
Emmission wavelength	445-460 nm
Total assay time	10-30 min depending on expression level

Additional settings may be optional on some machines, however shaking between each read is recommended if available.

2. Specific machine settings:

Gemini XPS Fluorescence Microplate Reader

Temperature:	37 °C
Autoshake:	on
Read type:	kinetic read of 30 min, read intervals 1 min
Excitation:	360 nm
Emission:	450 nm
Cut-off:	435 nm

Reads:	8 reads/well
PMT:	High
Calibrate:	On

Infinite 200 PRO

Select full plate read

Nuclon flat-bottom transparent

lid mode	off
Temperature	37 °C
Number of cycles	30
Kinetic time interval	1 min
Shaking duration	5 sec
Shaking amplitude	1 mm
Shaking mode	Linear
Excitation	360 nm
Emission	460 nm
Plate read mode	Top
Gain	Manual Gain mode
Gain	85
Lagtime	0
Integration time	20
Multiple reads per well	No

Troubleshooting

1. Excitation/emission

4-methylumbellifерone has an excitation peak at 365 nm and emission peak at 448 nm. However the peak absorption and emission spectra observed on your particular machine and the settings available to you may vary slightly. Additionally, autofluorescence and/or absorbance from the media and/or microplate may require you to select wavelengths outside the peaks. It is best to optimize for your setup by carrying out an emission/excitation wavelength scan with a positive control, such as a sample with known β -galactosidase or β -glucuronidase activity. In our experience, LB, TY and minimal medium and Nunc MicroWell Flat-bottomed 96-Well Microplates do not generate any problems with autofluorescence. It is possible that a particular organism may generate products that fluoresce in this assay. Excitation/emission wavelength scans of samples

containing appropriate negative control strains (lacking *lacZ* or *uidA*) may reveal if this is the case.

2. Cell permeabilization
In our hands this assay is very sensitive to the degree of cell permeabilization. In this protocol, optimized for Gram-negative bacteria, efficient cell permeabilization is achieved by freeze-thawing the samples twice between -70 and 37 °C and through the addition of high concentrations of lysozyme in the assay buffer. If cells are not completely permeabilized or become increasingly permeabilized throughout the assay, the RFU/sec plot will appear curved rather than linear. You may need to optimise the protocol to allow efficient permeabilization of your samples.
3. Microplate reader gain
Some microplate readers automatically adjust the detector gain over the entire plate or for each individual well. This can increase the dynamic range of the reader, as ideally both very lowly and very highly fluorescent samples can be analysed in the same plate. However for some microplate readers, the gain may need to be set manually. If fluorescence is not detected at all then the gain is likely too low. If the gain is too high, samples will saturate the detector almost immediately after the assay begins. To determine the optimal gain for your assay and machine, take samples from your most highly expressed samples and your most lowly expressed samples and carry out trial assays, adjusting the gain with each iteration. Choose a level at which lowly expressed samples have a detectable increase and the highest expressed sample doesn't saturate the detector before 10 min.

Recipes

1. 200x stock solution for 4-Methylumbelliferyl β-D-galactoside or 4-Methylumbelliferyl β-D-glucuronide Dissolve 4-Methylumbelliferyl β-D-galactoside or 4-Methylumbelliferyl β-D-glucuronide in DMSO at 50 mg/ml.
Store in small aliquots away from light at -70 °C. Avoid repeated freeze-thawing.
2. Final working reagent of 4-Methylumbelliferyl β-D-galactoside or 4-Methylumbelliferyl β-D-glucuronide
Dilute 200x stock to 1x in PBS buffer containing 2 mg/ml lysozyme

References

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