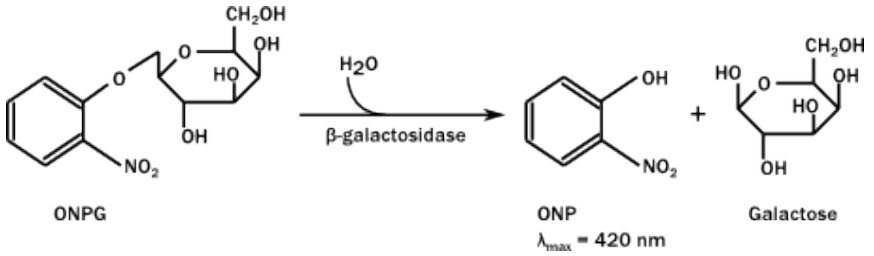
Background

β-Galactosidase is encoded by the *lacZ* gene of the *lac* operon in *E. coli.* It is a large (120 kDa, 1024 amino acids (http://www.ecocyc.org/ECOLI/NEW-IMAGE?type=ENZYME&object=BETAGALACTOSID-CPLX)) protein that forms a tetramer. The enzyme's function in the cell is to cleave lactose to glucose and galactose so that they can be used as carbon/energy sources. The synthetic compound o-nitrophenyl-β-D-galactoside (ONPG) is also recognized as a substrate and cleaved to yield galactose and o-nitrophenol, which has a yellow color. When ONPG is in excess over the enzyme in a reaction, the production of o-nitrophenol per unit time is proportional to the concentration of β-Galactosidase; thus, the production of yellow color can be used to determine enzyme concentration.



So, why do we care? Usually, experiments are designed so that the β-Galactosidase concentration in the cell is a readout for some aspect of a system being studied. For example, an investigator may fuse a promoter to the *lacZ* gene and use β-Gal levels as a readout for promoter activity under various conditions. In 1972, Jeffrey Miller published "Experiments in Molecular Genetics" which contained a protocol for determining the amount of β-Gal with ONPG. Because of this, ONPG/β-Gal assays are referred to as "Miller" assays, and a standardized amount of β-Gal activity is a "Miller Unit".

$$1\ Miller\ Unit = 1000\ x\ \frac{(Abs_{420} - (1.75\ x\ Abs_{550}))}{(t\ x\ v\ x\ Abs_{600})}$$

where:

- Abs_{420} is the absorbance of the yellow o-nitrophenol,
- Abs_{550} is the scatter from cell debris, which, when multiplied by 1.75 approximates the scatter observed at 420nm,
- t = reaction time in minutes,
- v = volume of culture assayed in milliliters,
- Abs_{600}[†] reflects cell density.

[†]Note that this value is different for each spectrophotometer used and should be calibrated by plating dilutions of known Abs_{600} cultures to determine the colony-forming units per Abs_{600}. Also note that because of this, and numerous other possible reasons, although one person's Miller Unit should be equivalent to someone else's, this is most

likely not always the case. *It is therefore critical that you include positive and negative controls, in parallel to your experimentals, whenever doing β-Gal assays.*

Protocol

The protocol allows more samples to be measured with less manipulation. In short, the protocol consists of measuring the cell density of a culture of bacteria (Abs_{600}), then removing an aliquot of the cells from the cuvette and mixing them with a "permeabilization" solution that contains detergent that disrupts the cell membranes (but leaves the β-Gal intact). This kills the cells and stops translation. After incubation, an ONPG "substrate" solution is added and the yellow color is allowed to develop. A "stop" solution is then added and the absorbance of o-nitrophenol is measured.

1. Grow cultures under whatever conditions you wish to test.
2. During growth, pre-measure 80 µL aliquots of **permeabilization solution** into 1.5 mL microfuge tubes and close them.
3. Measure Abs600 and RECORD IT!
4. Remove a 20 µL aliquot of the culture and add it to the 80 µL of permeabilization solution.

The sample is now stable for several hours. This allows you to perform time-course experiments.

1. After the last sample is taken, move the samples and the **Substrate solution** to the 30 °C warm room for 20-30 minutes.
2. Add 600 µL of **Substrate solution** to each tube and NOTE THE TIME OF ADDITION.
3. After sufficient color has developed, add 700 µL of **Stop solution,** mix well, and NOTE THE STOP TIME.
4. After stopping the last sample (some may take longer than others, but generally they are done in 30-90 minutes), transfer the tubes to a microfuge and spin for 5-10 minutes at full speed.
5. Carefully remove the tubes from the centrifuge and transfer solution from the TOP of the tubes to your cuvette(s). You are trying to avoid having particulate material in the cuvette so that scattering will not influence the reading.
6. Record Abs420. This should be less than 1 and greater than 0.05. If it's a bit outside of this range, don't sweat it. [Note, these are the typical limits of detection of a standard spectrophotometer.]

Calculate Miller Units as:

$$1000\ x\ \frac{(Abs420)}{(Abs600\ x\ (volume\ [0.02\ mL])\ x\ reaction\ time)}$$

Note if you are careful with your pipetting, you can skip the Abs550 step and assume that you have no particulates that could lead to scattering.

Comments:
When you are measuring β-Gal activity of a strain in a particular medium for the first time, you will likely need to optimize this protocol. Some general tips:

- See if you can get reproducible data when the yellow color is somewhere between *just detectable* and *just about the color of LB* before adding the stop solution.
- Make three separate measurements for each culture and average them.
- Miller recommends that the OD420nm reading should ideally be 0.6-0.9.

Plastic cuvettes have been known to give bad optics. You may consider doing the reads in a flat-bottom, clear 96-well plate. This will also allow for high-throughput readings! You should pipet between 150 – 200 µL of your solution into each well. [Note, you can also measure Abs600 this way too!]

One of the greatest contributions to error will be your estimate of reaction time. By having the reactions conditions set so that it takes about an hour, the time errors become insignificant. If you need to slow the reaction, you can use fewer cells and increase the amount of permeabilization buffer so the volume is still 100 µL.

RECIPES

Permeabilization Solution

You need 80 µL per sample.

100 mM dibasic sodium phosphate (Na_2HPO_4)
20 mM KCl
2 mM $MgSO_4$
0.8 mg/mL CTAB (hexadecyltrimethylammonium bromide)
0.4 mg/mL sodium deoxycholate
5.4 µL/mL beta-mercaptoethanol

To make 250 mL of Permeabilization Solution:

Mix the following in a media bottle
125 mL 0.2 M dibasic sodium phosphate (Na_2HPO_4)*
5 mL 1 M KCl*
0.5 mL 1 M $MgSO_4$*
200 mg CTAB (solid, found in chemicals cabinet under "C")
25 mL 4 mg/mL sodium deoxycholate*

Make sure all of the CTAB is dissolved. Pour the contents into a graduated cylinder and bring the solution to 250 mL with MQ-H2O. Filter sterilize the solution and store at room temperature. [Note that the beta-mercaptoethanol is added to an aliquot just before use – DO NOT ADD beta-mercaptoethanol to the stock bottle of Permeabilization Solution!]

Substrate solution

You need 600 µL per sample.

60 mM Na_2HPO_4
40 mM NaH_2PO_4
1 mg/mL o-nitrophenyl-β-D-Galactoside (ONPG)
2.7 µL/mL β-mercaptoethanol

To make 200 mL substrate solution

Mix the following in a clean media bottle:
60 mL 0.2 M Na_2HPO_4*
40 mL 0.2 M NaH_2PO_4*
200 mg ONPG (found in the fridge)

Make sure all of the ONPG is dissolved. Pour the contents into a graduated cylinder and bring the solution to 200 mL with MQ-H2O. Make 10 mL aliquots (in 15 mL tubes) and store them at – 20 °C. [Note that the beta-mercaptoethanol is added to an aliquot just before use – DO NOT ADD beta-mercaptoethanol to the stock bottle of Permeabilization Solution!]

Stop solution
You need 700 µL per sample.

1 M Sodium Carbonate (Na_2CO_3; MW 105.99)

To make 50 mL, dissolve 5.3 g of sodium carbonate in 50 mL MQ-H2O.

The high pH of the stop solution denatures the β-Gal and approximately doubles the yellow color of the reaction.

*All of these stock solutions can be made by dissolving the appropriate amount of solid (use the MW noted on the container, when appropriate), which can be found in the alphabetized chemicals cabinet, in MQ-H_2O. Typically, the solution is then filter-sterilized. Use the empty / existing bottle of stock solution to guide you in terms of how much to make up and how to label the stock solution after you've made it.