ASSAY OF β-GALACTOSIDASE IN YEAST

(Obtained from John Stebbins, Triezenberg lab)

There are two basic methods for the *in vitro* assay of B-galactosidase activity from yeast. They differ mainly in the method of preparing the material for assay. In the first method (Rose and Botstein, 1983), a crude extract is prepared, and the activity is normalized to the amount of protein assayed (determined by Bradford protein assays). In the second method (Guarente 1983), the cells are permeabilized to allow the substrate to enter the cells, and the activity is normalized to the number of cells assayed (determined by measuring the OD_{600} of the cells). The former method is preferable when comparing cells that are grown under very different conditions or that have different genetic backgrounds. The latter method is adapted from the assay for E. coli and is particularly suited for changing levels of activity within a single strain.

Method I: Assay of Crude Extracts

The objective:

To grow a culture of cells to a concentration of 1 X 10^7 – 2 X 10^7 cells per ml (OD_{600} = 0.8 - 1.0) in an appropriate liquid medium at an appropriate temperature (usually 30^{o}C). If the hybrid gene is expressed from an autonomous plasmid, the appropriate medium is usually a synthetic dropout (e.g., SD ΔLEU), which selects for the presence of the plasmid. One of the reasons for trying to harvest the yeast cultures at similar cell concentrations is that it has been found that certain yeast promoters are repressed when the cells enter into stationary phase (e.g., the *ADC1* promoter; John Stebbins, personal communication). When analyzing multiple constructs the easiest way to do this is to try and synchronize the growth of all yeast cultures so that cells can be harvested within a relatively narrow window of time. To do this, a 5 ml culture is first allowed to grow overnight. The OD_{600} of this culture is determined after the first night's overnight growth. Then a known quantity of the 5 ml culture is added to a larger (10ml) volume of liquid media and the 10ml culture is then allowed to grow until an OD_{600} = 0.8 to 1.0 is reached. During the exponential growth phase, normal laboratory yeast strains double every ~90 minutes or so in rich media (YPAD) and every ~140 minutes in synthetic media. Lets say you wanted to harvest your yeast cells at OD_{600} = 1.0 at noon (Day 3) and the OD_{600} of the five ml culture at 4:00 PM the day before (Day 2) was 1.0; how much volume of the 5 ml culture would you need to add? The ΔT is 20 h and yeast double ~ every 2.5h (140 min), so that is approximately 8 doublings the yeast will go through. That means at the beginning you want the OD_{600} of the 10ml culture volume to be ~ 0.004; to get this you need to add 40μl: (10ml X OD_{600} 0.004 = 0.040ml X OD_{600} 1.0). However after inoculation into the 10ml culture volume the yeast will first go through a lag phase where not much growth is apparent until they reach log phase (exponential growth). Thus for all practical purposes I have found that adding ~4 X of the volume I actually calculate usually gets me in the ballpark. Thus I would add 200μl. Another way to do this is to just blindly add 200μl from your 5ml overnight (without even taking an OD_{600} reading).

Yeast β-gal assays

The following protocol assumes that you have yeast colonies on plates from which you can pick single colonies into selectable media.

Yeast Cell Growth

Day 1:

1. Pick three single/individual colonies of each construct to be analyzed into 5ml of an appropriate medium (e.g., SD ΔLEU). Grow overnight at 30°C.

   (Each colony will be analyzed separately for β-gal activity from which the mean and standard deviation/error will be determined.)

Day 2:

2. Add the appropriate volume of the 5 ml overnight culture to the 10ml of fresh medium. Grow overnight.

Day 3:

3. First thing in the morning take 1.0 ml out of the 10ml culture and determine the OD_{600} (discard the portion of sample that is removed from the 10ml culture). Allow the yeast to continue growth until the OD_{600} is 0.8-1.0.

Yeast Cell Harvest

4. Transfer 5ml of the 10ml culture volume to a 16 X 125 disposable culture tube. Place tubes on ice to stop yeast cell growth.

   Note: Keep the cells on ice from this point on.

5. Spin the 5ml culture volume in the clinical centrifuge on high (2000 rpm) for ~5 min. Aspirate or decant off the supernatant.

6. Add 1.0 ml sterile dH_2O to the cells and resuspend. Transfer cells to a 1.5ml microfuge tube.

7. Spin in a microfuge to pellet the cells.

8. Discard the supernatant. Resuspend the cells in 250 µl of breaking buffer. The cells can now be frozen at –20°C and assayed at a later date.

   Note: All of the following steps can be performed in a 1.5 ml microfuge tube.

9. If the cells were frozen, thaw them on ice. Add glass beads until the beads reach a level just below the meniscus of the liquid. Add 12.5 µl of PMSF stock solution.

10. Vortex six times at top speed in 15-second bursts. Chill on ice between bursts.

11. Add 250µl of breaking buffer and mix well. Withdraw the liquid extract after plunging the tip of a 1000 µl pipettor to the bottom of the tube.

12. Clarify the extract by centrifuging for 15 minutes in a microfuge. Transfer the clarified supernatant to a new microfuge tube. Extract may be stored at –20°C.

    (If the activity is in the particulate fraction, the unclarified supernatant can be used and the assay mixture may be clarified later in step 12.)

β-gal assays:

Label the tubes in which the reaction will occur. (The most convenient tubes for this assay are the 13 X 100 disposable culture tubes.)

13. Add 10-100 µl of extract to 0.9 ml of Z buffer. Adjust the volume to 1 ml with breaking buffer.

14. Incubate the mixture in a water bath at 28°C for 5 minutes.

15. Initiate the reaction by adding 0.2 ml of ONPG stock solution. Note precisely the time that the addition is made. Incubate at 28°C until the mixture has acquired a pale yellow color (10 – 30 min).

16. Terminate the reaction by adding 0.5 ml of Na_2CO_3 stock solution. Note precisely the time that the reaction is terminated. Measure the optical density at 420 nm.

When working with many samples the easiest thing to do is to initiate and terminate reactions at a convenient interval. For example, I add 0.2 ml of ONPG to my first sample tube at T = 0 sec, 0.2 ml of ONPG to my second sample tube at T = 10 sec and so on until ONPG has been added to all of my samples. Then I terminate the reactions in the same way; e.g. sample #1 at 30 min 0-sec, sample #2 at 10 min 10-sec, etc.

17. Using the Beckman DU 640 spec housed in Al Barta's lab measure the optical density at 420 nm (this can be done using the *vis* light source; i.e., the *uv* is not required). Do this similar to the way you do the Bradford assays:

    (1) Turn on the Spec as described under spec operations and let go through its initialization phase (about 2 minutes).

    (2) Turn on the *vis* lamp

    (3) Set all ODs (in this instance to 420)

    (4) Choose the sipper

    (5) Place the sipper cuvette into the slot at the **Back** of the cuvette holder (furthest from you)

(6) Condition the flow cell as described under spec operations.

(7) Run (**FLUSH**) several mls of ddH_2O through.

(8) Fill the blank by inserting the sipper tube into one of your blank solutions and press the FILL BLANK button. It takes about 30 seconds for the machine to complete this process.

(9) Run (**FLUSH**) several mls of ddH_2O through.

(10) Insert the sipper tube into the solution to be read and press the FILL READ button.

(11) After this is complete, **FLUSH** several mls of ddH_2O through.

(12) Insert the sipper tube into the next solution to be read and press the FILL READ button.

(13) Repeat this cycle, **FLUSH**ing several mls of ddH_2O through between each sample reading

Read several of your blank samples as well. Usually I do this after reading all of my samples. Most of the protein samples come out in the range 0.09 to 0.6. The numbers that we get from the blank will be averaged and then that average will be subtracted from each samples' OD_{420} reading.

Be sure to print this and to annotate the readings with the sample. This then will get entered into an excel spreadsheet.

Bradford Assays:

18. Measure the protein concentration in the extract using the dye-binding assay of Bradford (1976):

    a. Dilute the Bradford reagent fivefold in dH_2O (1 part Bradford: 4 parts dH_2O). Filter the diluted reagent through Whatman 540 paper (or equivalent, I use the Millipore filtration unit).

    b. Add 10-20 µl of the protein extract sample to 1 ml of the diluted reagent and mix. Measure the blue color formed at 595 nm. (Use disposable plastic cuvettes to prevent the formation of a blue film.)

       Note: The Beckman DU 640 spec housed in Williams 2___A is equipped with a sipper; thus the sample can be sucked right out of a microfuge tube directly into the spec eliminating the need to use cuvettes.

    c. Prepare a standard curve using a serial dilution series (0.1-1.0 mg/ml) of a known protein sample concentration; e.g., BSA dissolved in dissolved in breaking buffer.

       Typical extracts prepared in this fashion contain 0.5-1 mg/ml of protein.

Yeast β-gal assays

Using the sipper with Bradford reagent is always a little bit tricky. This is because the Bradford reagent will stick to the tubing. To minimize this problem I do the following:

(1) Turn on the Spec as described under spec operations and let go through its initialization phase (about 2 minutes).

(2) Turn on the vis lamp

(3) Set all ODs (in this instance to 595)

(4) Choose the sipper

(5) Place the sipper cuvette into the slot at the **Back** of the cuvette holder (furthest from you)

(6) Condition the flow cell as described under spec operations.

(7) Run (**FLUSH**) several mls of ddH_2O through.

(8) Run (**FLUSH**) several mls of the Bradford reagent through. You'll notice that the tubing picks up a blue color. This is good because it will help to minimize variability later.

(9) Run (**FLUSH**) several mls of ddH_2O through.

(10) Now fill the blank by inserting the sipper tube into one of your blank solutions (be sure there is a minimum of 1.0ml of blank, usually I add some of a blank to another to be sure when that sipper sucks, it gets what it needs) and press the FILL BLANK button. It takes about 30 seconds for the machine to complete this process.

(11) Run (**FLUSH**) several mls of ddH_2O through.

(12) Insert the sipper tube into the solution to be read and press the FILL READ button.

(13) After this is complete, **FLUSH** several mls of ddH_2O through.

(14) Insert the sipper tube into the next solution to be read and press the FILL READ button.

(15) Repeat this cycle, **FLUSH**ing several mls of ddH_2O through between each sample reading.

Read several of your blank samples as well. Usually I do this after reading all of my samples. Most protein samples come out at 0.2 to 0.6. A lot of times when you run a blank sample invariably as high as 0.02 (or higher). I cannot seem to get around this. Thus these numbers that we get from the blank will be averaged and then that average will be subtracted from each samples' OD_{595} reading.

Be sure to print this and to annotate the readings with the sample. This then will get entered into an excel spreadsheet.

(15) When you are completely done it usually takes a bit of effort to rid the tubing of the Bradford reagent sticking to it. Usually I can accomplish this by two cycles of first rinsing with several mls of ddH_2O, then flushing several mls of Methanol through, then rinsing with ddH_2O, then the Trace Clean solution, then rinsing with ddH_2O…

19. Express the specific activity of the extract according to the following formula:

$$\frac{OD_{420} \text{ X } 1.7}{0.0045 \text{ x protein x extract volume x time}}$$

OD420 is the optical density of the product, o-nitrophenol, at 420 nm. The factor 1.7 corrects for the reaction volume. The factor 0.0045 is the optical density of a 1 nmole/ml solution of o-nitrophenol. Protein concentration is expressed as mg/ml. Extract volume is the volume assayed in ml. Time is in minutes. Specific activity is expressed as nmoles/minute/mg protein.

Yeast β-gal assays

Method II:Permeabilized cell assay

1. Grow the cells as above. Measure the OD_{600} of the culture and harvest $1 \times 10^6 - 1 \times 10^7$ cells by centrifugation as above.

2. Discard the supernatant. Resuspend the cells in 1 ml of Z buffer.

3. Add 3 drops of chloroform and 2 drops of 0.1% SDS. Vortex at top speed for 10 seconds.

4. Preincubate the samples at 28°C for 5 minutes. Start the reaction by adding 0.2 ml of ONPG as above.

5. Stop the reaction by adding 0.5 ml of Na_2CO_3 stock solution when the sample in the tube has developed a pale yellow color. Note the amount of time elapsed during the assay. Remove the cell debris by centrifuging for 10 minutes in a microfuge and then discarding the pellet.

6. Measure the OD_{420} of the reactions.

7. Express the activity as β-galactosidase units:

$$\frac{OD_{420}}{OD_{600} \text{ of assayed culture} \times \text{volume assayed} \times \text{time}}$$

OD_{420} is the optical density of the product, o-nitrophenol. OD_{600} is the optical density of the culture at the time of assay. Volume is the amount of the culture used in the assay in ml. Time is in minutes.

Yeast β-gal assays

Materials

Appropriate liquid medium

Breaking buffer:
100mM Tris-HCl (pH 8)
1mM Dithiothreitol (DTT)
20% Glycerol

Glass beads:
Sigma cat # G8772 = acid-washed, 425-600 nm glass beads. A/C and dry.

PMSF (Phenylmethylsulfonyl fluoride)

Stock	For 1 ml
PMSF (100mM)	17.4mg
2-propanol (iso)	1.0 ml
Store at –20°C	

or

AEBSF (4-(2-amino-ethyl)- benzenesulfonyl fluoride hydrochloride), which is a non-toxic alternative to PMSF)

AEBSF (100mM)	23.9mg
dH_2O	1.0 ml
Store at –20°C	

Z buffer (Miller 1972):

	Stock	Final []
$Na_2HPO_4 \cdot 7H_2O$	16.1g	60 mM
$NaH_2PO_4 \cdot H_2O$	5.5g	40 mM
KCl	0.75g	10 mM
$MgSO4 \cdot 7H_2O$	0.246g	1 mM
dH_2O to a final volume of:	1 liter	
pH to 7. Store at 4°C.		

Right before using add 2-Mercaptoethanol to 50 mM:

2ME	2.7 ml (per liter Z buffer)

ONPG (o-nitrophenyl-β-D-galactoside) stock solution:
4 mg/ml in Z buffer. Store at –20°C.

Na_2CO_3 stock solution:

Yeast β-gal assays

1 M in dH_2O (53g/500ml)

Bradford reagent (BioRad)

dH_2O

Whatman 540 paper or equivalent (Millipore ____)

Disposable plastic cuvettes (not needed; the Beckman DU 640 spec has a sipper).

0.1-1 mg/ml bovine serum albumin (BSA) in breaking buffer
Chloroform
0.1% SDS

Yeast β-gal assays

Safety notes

Nitric acid is volatile and should be used in a hood. Concentrated acids should be handled with great care; gloves and a face protector should be worn.

Phenylmethylsulfonyl fluoride (PMSF) is extremely destructive to the mucus membranes of the respiratory tract, the eyes, and the skin. It may be fatal if inhaled, swallowed, or absorbed through the skin. In case of contact, immediately flush eyes or skin with copious amounts of water and discard contaminated clothing.

Chloroform is irritating to the skin, eyes, mucous membranes, and upper respiratory tract. It should only be used in a chemical hood. Gloves and safety goggles should also be worn. Chloroform is a carcinogen and may damage the liver and kidneys.

References

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