Bacterial Overexpression

DAY 1

1. Start 5 ml overnight cultures from single colony picks. Grow in shaking incubator at 37 degrees in LB + amp.

2. Autoclave 200 ml LB and a 250 mL erhlenmeyer flask for each clone. Also autoclave a glass graduated cylinder for measuring the LB into the flasks in the morning. Add amp to LB. Leave the LB in the 37 degree plate incubator overnight to get the broth warm.

DAY 2

3. The next morning, draw 2 ul of over-night culture and run a PCR to check for the presence of the insert. Alternatively, do this at the end of the 3-hour grow-out.

4. Dilute overnight into 200ml LB + amp and grow at 37 degrees in a shaking incubator at 200 rpm until OD ~ .5 (2-3 hours from room temperature LB). Save some LB for blank!

5. Add 500uL 200mM IPTG solution to the 200ml culture. Put back in shaker at 37 degrees.

   *** To make 5 ml of 200 mM IPTG stock: Add .23831 g IPTG powder (-20 deg) in 5 ml H_2O.

6. After three hours, aliquot samples into labeled 50ml tubes and spin down in swing-rotor at 4 degrees for 20 minutes. Get out most of the LB as possible.

7. Store at -80 degrees.

Preparing bulk protein for enzyme assays

Lysis Buffer – **Make this the day of.** Make a little extra for the amount of reactions you need to do.

Per 10 ml: Per 2.1 ml:

- 7.85 ml H_2O
- 500 ul 1M HEPES (pH – 7.3)
- 87 mg NaCl
- 1000 ul 200mM PMSF (in isoprop)
- 100 ul 100 mM TPCK (in EtOH)
- 50 ul 200 mM TLCK (in H_2O)

Stocks –

- 100 mM TPCK -> dissolve .035185 g TPCK in 1 ml EtOH
- 200 mM TLCK -> dissolve .036931 g TLCK in 1 ml H_2O

Lysozyme – Make this the day of.

Need 5 ul of 100mg/ml lysozyme per sample. E.g. dissolve .0021 g in 21 ul water.

1. Defrost pellet on ice for 20-30 minutes. As pellet is defrosting, remove the layer of thawed water that accumulates above the pellet.

2. The DFR assay works well working off protein extracted from pellets of one 50-ml tube.

3. First, add 500uL lysis buffer per sample. Then add 5 ul lysozyme solution per sample. Flick to re-suspend pellet.

4. Set swing-rotor temperature to 4 degrees.

5. Incubate on ice for 30 minutes with occasional mixing.

6. Spin down 20 minutes at 4 degrees and transfer the super-natant to pre-chilled eppendorf. Yield is generally ~1.5 ml crude bacterial protein from 2 tubes of cells.

Enzyme function assays

Reaction conditions: *We do half reactions. The only issue is that NADPH comes in glass vials – enough for one full reaction. Therefore, you need to do an even number of half reactions. (Resuspend NADPH in enough Tris for one full reaction, then split into two reactions.*

For a full reaction – 1 ml total

1 umol substrate (=.304 mg DHQ), dissolved in 20 ul EtOH
1 umol NADPH (=1 vial), dissolved in 670 ul .1 M Tris.
6 umol Glucose-6-Phosphate = 1.7 mg, dissolved in 40 ul .1 M Tris.
1 U Glucose-6-Phosphate-dehydrogenase = .25 mg, dissolved in 20 ul .1 M Tris
250 ul bacterial extract

Today I'm doing 4 half reactions. (Each is 500 ul).
I'll need…

2 umol DHQ (=.000608 g) dissolved in 40 ul EtOH. *Add 10 ul to each reaction*.
2 vials NADPH, each dissolved in 670 ul .1 M Tris. *Add 335 ul to each reaction.*
12 umol G-6-P (=.0034 g) dissolved in 80 ul .1 M Tris. *Add 20 ul to each reaction.*
2 U G-6-P-dh (=.0005 g) dissolved in 40 ul .1 M Tris. *Add 10 ul to each reaction*.
125 ul bacterial extract.

Performing enzyme function assays

1. Set up reaction in the following order:
   Resuspend NADPH and pipette 335 ul into each screw-cap reaction tube.
   Add G-6-P.
   Add G-6-P-dh.
   Add DHQ.
   Add protein extract.

2. Mix and incubate at 30 degrees for 3 hours.

3. Add 7.5 ul 6N HCl to stop the reaction.

4. Immediately extract three times in .5 ml ethyl acetate. Shake vigorously, **but do not vortex**, then spin down for about 30 seconds in the centrifuge. Ethyl acetate is the top layer and contains the leucoanthocyanidins, which you want. Put this into another screw-cap tube.

5. Wash twice with 200 ul H_2O (H_2O forms a small bubble at the bottom – try and get as much as possible out.)

6. Evaporate ethyl acetate under vacuum at 35-45 degrees into about 100 ul (about 15 minutes?). Don't overdry.

7. For leucoanthocyanidins, add 1 ml Butanol-HCl reagent (95:5 v:v) and heat at 95% for 30 minutes. Using large heat block, high temp set to 5.

Using spectrophotometer in Nijhout lab

Take up glass cuvettes (in HPLC column drawer), samples, butanol-hcl, pipette, tips, waste bucket, and information of absorbance of whatever molecule you will be measuring.

1. Turn on spec and let warm up.

2. Turn on computer monitor and start program "UV probe".

3. Select **window-> photometric**

4. Click **connect**. It will take about 5 minutes to warm up.

5. Put 1 ml of butanol-HCl blank into each glass cuvette.

6. Open spec, put in blanks

7. Push **baseline**. For cyanidin, peak is 550. Input range 450-650 is okay.

8. **OK.**

9. **Edit -> method**. **Wavelength -> point**. **Wavelength (nm) -> 551** (for cyanidin). Push **Add**.
   If doing other substrates, add their peak absorbances.

10. **Close**

11. **Sample ID –** *enter name*. Push **enter** after each line!

12. Before running each sample, select its row in the spreadsheet.

13. To run sample, add 1 ml of sample (add butanol-hcl if needed) to the cuvette near the front of the machine (leave the cuvette in the rear of the machine as the blank).

14. **Read unknown**

15. To shut down, push **disconnect**, then quit program, turn off monitor, and turn off spec.

16. Don't accidentally leave the cuvettes full of butanol in the spec machine!