Saptasense Protocol

EibA/D Experiments

DAY 1

Make an overnight culture by doing the following:

- ☐ Using LB+CAM media, pipette 5mL into single-use plastic culture tubes
- ☐ Innoculate by picking a colony with a pipette tip from the following strains:
    - ☐ EibA - BL21
    - ☐ EibD - BL21
    - ☐ Negative control - empty BL21 (JUST LB media, NOT +CAM)
- ☐ Incubate, shaking, at 37 C

DAY 2

Grow up and induce expression using rhamnose:

- ☐ In three erlenmeyer flasks, add 49.5 mL of LB + CAM
- ☐ Add 0.5mL of each overnight culture (1-100 dilution)
- ☐ Grow at 37C with shaking until it reaches OD600 of 0.4-0.6 (mid-exponential phase)
- ☐ Split the cultures into 2 erlenmeyer flasks (need 6 total)
    - ☐ Add 1% rhamnose (w/v) to erlenmeyer flask 1
        - ☐ Amount of rhamnose = 0.01 x V, where V is the total volume of culture left
    - ☐ Add 0.05% rhamnose (w/v) to erlenmeyer flask 2
        - ☐ Amount of rhamnose = 0.0005 x V, where V is the total volume of culture left
- ☐ Incubate at 30C for 4 hours (or until reaches an OD of 1.0)

Pelleting bacteria:

- ☐ Add 1mL of the induced cells into 20 tubes.
- ☐ Centrifuge at 5000 rcf for 10 minutes

- [ ] Discard supernatant
- [ ] Wash two times with 200uL PBS
- [ ] Discard supernatant and freeze at -80C until ready for use

DAY 3

Binding GFP antibody to bacterial surface

- [ ] Prepare 2mL of 2ug/mL anti-GFP working solution - pH 7.2
    - [ ] To prepare a 2ug/mL stock solution, add 3.64 uL of 1.1mg/mL anti-GFP antibody in 1.636 mL of PBS
- [ ] Defrost cell pellets from -80 on ice
- [ ] Resuspend cell pellets in 200uL anti-GFP+Buffer solution
- [ ] Incubate for 2 hours with with shaking at 300 rpm
- [ ] Bring up in 450uL PBS
- [ ] Centrifuge at 5000rcf for 10 minutes
- [ ] Discard supernatant
- [ ] Wash two more times with 450uL PBS
- [ ] Aliquot 50uL each into 12 tubes
- [ ] Add 200uL PBS
- [ ] Centrifuge and remove supernatant

Binding GFP to antibody

- [ ] Prepare 240uL of 10uM GFP working solution - pH 7.2
    - [ ] We have a stock solution of 100uM GFP in 10uL aliquots
    - [ ] To prepare a 10uM stock solution, add 90uL ddH2O to make 100uL 10uM GFP
        - [ ] You will need 5 tubes total for this experiment, so do this separately in 5 tubes
- [ ] Combine the 5 tubes into 1 tube with a total volume of 500uL
- [ ] Perform a series of 10 2-fold dilutions to make 10uM, 5uM, … 0.01 uM (refer to the table). Each tube should have 250uL in the end.
- [ ] Resuspend cells in 50uL of GFP+buffer solution, according to the table.
- [ ] Incubate for 2 hours at room temperature with shaking at 300 rpm
- [ ] Add 200uL PBS
- [ ] Centrifuge at 5000 rcf for 10 minutes
- [ ] Discard supernatant
- [ ] Wash two more times with 200uL of PBS
- [ ] Resuspend each tube in 200uL of PBS and add to the plate

Fluorescein Calibrant and Final Measurement

- [ ] Find the fluorescein from the measurement kit. Spin down to make sure the pellet is at the bottom. Transfer 1mL of PBS to the stock reagent tube of fluorescein. Pipette and vortex. This is 10X solution with a concentration of 100uM in PBS.
- [ ] Obtain a new tube and dilute 100uL of 10X fluorescein stock into 900 uL 1X PBS to make a 1X reference working solution with a concentration of 10uM
- [ ] Transfer 100uL of PBS to A2:B12
- [ ] Transfer 200uL of fluorescein 1X solution to wells A1 and B1 of calibration plate
- [ ] Perform a series of 10 2-fold dilutions down the plate, left to right, using the 100uL of PBS in each well
- [ ] Measure plate with excitation wavelength of 488.0nm and emission filter of 530.0nm and 30.0nm bandpass

DAY 3 - TAKE 2

Background: There were several issues with the first round of the procedure. One of the biggest issues was that we started with such a small pellet, that by the end of the many washes and after dividing the small pellet into 12 even smaller pellets, there was virtually no pellet left to see. Another issue was the vast number of tubes. This next experiment is designed as a simple yes/no experiment, with no curves over different concentrations. This is meant to be a pilot experiment. Another alteration is that we start with a much larger amount of bacteria.

Binding GFP antibody to bacterial surface

- [ ] Prepare 2mL of 2ug/mL anti-GFP working solution - pH 7.2
    - [ ] To prepare a 2ug/mL stock solution, add 3.64 uL of 1.1mg/mL anti-GFP antibody in 1.636 mL of 1xPBS
- [ ] Defrost 2 cell tubes containing cell pellets of each type:
    - [ ] 2 tubes EibA 1%
    - [ ] 2 tubes EibA 0.05%
    - [ ] 2 tubes EibD 1%
    - [ ] 2 tubes BL21 control
- [ ] Resuspend in 100uL anti-GFP+buffer solution
- [ ] Combine the identical tubes to have a total of 4 tubes
- [ ] Incubate 2 hours with shaking at 300 rpm
- [ ] Bring up volume with 400uL PBS
- [ ] Centrifuge 5000xg for 10 min
- [ ] Discard supernatant, resuspend in 600uL PBS

- [ ] Centrifuge 500x for 10 min
- [ ] Repeat 2 more times

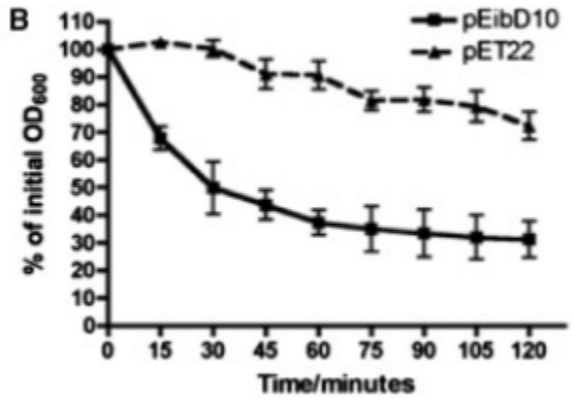
Binding GFP to antibody

- [ ] Prepare 200uL of 10uM GFP working solution - pH 7.2
    - [ ] We have a stock solution of 100uM GFP in 10uL aliquots
    - [ ] To prepare a 10uM stock solution, add 90uL PBS to make 100uL 10uM GFP
    - [ ] (We will need 2 tubes total)
- [ ] Add 50uL of 10uM GFP working solution to each tube
- [ ] Incubate for 2 hours with shaking at 300 rpm
- [ ] Bring up volume with 550uL PBS
- [ ] Centrifuge 500x for 10 min
- [ ] Discard supernatant, resuspend in 600uL PBS
- [ ] Centrifuge 500x for 10 min
- [ ] Repeat 2 more times, the final time bringing up in 100uL PBS
- [ ] Image samples

Examining Agglutination Assay

Background: Up to this point, we have confirmed expression and functionality of EibD via fluorescence microscopy with an anti-GFP antibody, GFP, and NucBlue staining. We have also found that EibD causes auto-agglutination via self-self interactions, forming "zipper-like" structures between the individual bacterial cell walls. These self-self interactions are so strong that they can induce cell lysis if forcefully ripped apart. The purpose of this experiment is to test whether these interactions can be out-competed by introducing an IgG antibody and antigen. In this first pilot experiment, we will be testing this by measuring OD600s over a time course. Measuring OD600s will tell us the relative amount of turbid, solution-suspended cells.

This has been done to quantitatively analyze agglutination in EibD-expressing bacteria previously. (see here)



Materials:
0.05% and 1% rhamnose-induced EibD expressing BL21 bacteria
Spectrophotometer (to measure ODs)
Antibody of choice
Antigen of choice

Procedure:

1. Briefly defrost three of each of the following aliquots of cells from the -80 freezer:
    a. EibA (1%)
    b. EibA (0.05%)
    c. EibD (1%)
    d. EibD (0.05%)
    e. BL21 (1%)
2. Resuspend 2 tubes of each in 200uL 2ug/mL antibody solution. Incubate for at least 2 hours at room temperature, gently rocking.

3. Meanwhile, resuspend the remaining tube of each in 1mL of 1x PBS. Measure the OD600 at the following timepoints starting immediately after resuspension:
    a. 0min
    b. 0.5min
    c. 1min
    d. 2min
    e. 4min
    f. 6min
    g. 10min
    h. 15min
    i. 20min
    j. 40min
    k. 60min
4. After 2 hours incubation with the antibody, centrifuge at 5000xg for 5 min at 25 C.
5. Remove supernatant and resuspend in 200uL PBS.
6. Repeat the centrifugation step.
7. Remove supernatant.
8. Resuspend one of each of the tubes in 40uL of antigen solution. Incubate for at least 2 hours at room temperature.
9. Resuspend the remaining tube of each kind in 1mL PBS. Immediately measure the OD600 at the time points previously indicated.
10. After 2 hours of incubation with antigen, bring up the volume by adding 200uL PBS. Centrifuge at 5000xg for 5 min at 25 C.
11. Remove supernatant and resuspend in 200uL PBS.
12. Repeat the centrifugation step.
13. Remove supernatant.
14. Resuspend in 1mL PBS. Immediately measure the OD600 at the time points previously indicated.