^{3}H-Fluconazole Sequestration Assay in 6-well biofilms
Revised by Hiram Sanchez on 1-23-17

Day 1:
1. Grow overnight cultures in YPD, 30C at 200rpm. Include WT strain.

Day 2:
1. Inoculate biofilms in 6-well plates in RPMI at 10^6 cells/ml. One full plate per strain, 2 ml per well.
2. Incubate plate at 37C to allow biofilm adherence for 1 hr.
3. Parafilm and foil plate; incubate at 37C at 50 rpm for 24 hr.

Day 3:
1. Exchange media and incubate 24 hrs as before.

Day 4:
1. Remove media from plates (48 hrs. biofilm).

2. Rinse each well gently once with 1 mL ddH20.

3. Harvest one well containing biofilm from each plate and save for normalization by dry biomass.
- Add 0.5 mL ddH20, scrape, and collect in 15 mL conical tube twice. Final volume is 1mL plus biofilm.
- Freeze and lyophilize overnight. Weigh dried biomass for normalization to WT.

4. **Pulse**: Add 0.6 mL 3H-Fluconazole at 0.5 uM in RPMI to 5 wells. Incubate 30 min at 37C, 50 rpm.

5. Remove media and save for counting (**A**).
Note for non-adherent biofilms: if cells come up in this supernatant collection, it will not affect "total biofilm" fraction because this is collected later. However, only the supernatant should be measured for this part, so be sure to let the sample settle and only collect liquid for CPM measurements.

6. **Chase**: Add 20 uM unlabeled fluconazole to all 6 wells. Incubate for 15 min at 37C, 50 rpm.

7. Remove media and discard.

8. Add 0.5 mL ddH20 to each well. Scrape with spatula and collect in a 15 mL conical tube (blue capped Falcon tubes). Add an additional 0.5 mL ddH20, scrape, and collect.

9. Save a 600 uL aliquot of this for measuring total biofilm (**B**).

10. Water-bath sonicate biofilms for 20 min. Place on ice afterwards.

11. Centrifuge 20 min at 4C, 4000 rpm.

12. Collect supernatant in a separate 15 mL conical, and save as matrix fraction (**C**). Record this volume.
- Do not pipet too close to pellet; better to have clearer matrix than contaminate with cells.
- Remove remaining supernatant and discard, do not contaminate cells with matrix material.

13. Resuspend cell pellet in 1.5 mL ddH20. Transfer to bead beating tube with ~400 uL glass beads.

14. Bead beat 3 x 1 min, cool in ice for 1 min in between.
    - Beading times can be adjusted if necessary, beat until all cell material floats at top of tube.

15. Collect sample in 15 mL conical, washing beads with 1 mL water until liquid is clear (usually 5-6 mL).

16. Centrifuge at 4C for 10 min at 4000 rpm. Collect supernatant as intracellular fraction (**D**).

17. Resuspend the pellet in 1.5 mL ddH20 and save as cell wall fraction (**E**).

18. Save all fractions at 4C until ready for counting.

Counting:

1. Each fraction will be counted in triplicate (total # tubes = # samples x 5 fractions x 3).
    - Include positive controls (0.5 uM ^{3}H Fluconazole) and negative controls (RPMI).
2. Add 3 mL scintillation fluid to each tube.
3. Add 100 uL sample to each tube. These can sit until ready for counting.
4. Cap and label with sample # and fraction letter.
5. Scintillation counter is in 4th floor core facility. Be sure to sign up on calendar. Add tubes to trays, and add the clip #11 to the first tray. Lift the machine lid and add these to the back right.
6. Select program #11 on computer (for 3H CPM) and press F2 to start. Make sure machine is operating and printing properly.

Data Analysis:

1. Type all CPM values into Excel file.
2. Determine averages and standard errors.
3. Divide values by the %WT determined from biomass normalization.
3. To determine raw CPM values: adjust values by volumes recorded for each fraction.
4. To present data as percentages of WT strain: Divide raw averages by WT averages for each fraction. These do not need to be adjusted by volume.

Rough volumes for each fraction:

A (supernatent): 0.6 mL x 5 wells.
B (total biofilm): 0.6 mL
C (matrix): 4 mL (biofilm cells take up ~1 mL of 5 mL total)
D (intracellular): 5 mL (depends on how many rinses performed after bead beating)
E (cell wall): 1.5 mL plus pellet

Note;

Radioactive H3-Flu reagent is 50 uM with a specific activity of 0.001 mCi/ml in Ethanol.
A stock reagent was prepared by adding 10 ul of H3-Flu to 990 ul of water, the final Flu concentration is 0.5uM with 7.04 x 10 ^{6} cpm. One ml of this reagent was mixed with 5 ml of RPMI and each biofilm well received a total of 600 ul ~ 8.48 x10^{5} cpm.

~ 20 ul of H3 in 1.980 ml of H_2O in 10 ml of RPMI, each well gets 0.6 ml, enough for 20 wells.