Bi 421 Advanced Molecular Genetics Laboratory   Winter 2016

Instructors: Dr. Eric Selker [355D Streisinger; phone: 541-346-5193; email: selker@uoregon.edu];
Dr. Tish Wiles (355 Streisinger; phone: 541-346-5197; email: tishw@uoregon.edu]
Grad. TA: Austin Harvey [275 Streisinger; lab phone: 541-346-2886; cell: 630-429-8589 (for emergencies) email: aharvey@uoregon.edu]
Undergrad. TA: Collin Hickman [365 Streisinger; lab phone: 541-346-5197; cell: 541-977-7415 (for emergencies); email: collinh@uoregon.edu]

Laboratory: Room 33, Klamath Hall

Class meetings: one hour lecture/discussion and 8+ hours lab/week during class hours (M, W, F 3-6 PM); ~3 hours/week additional time outside of class; we hope to allow students 24/7 card-access to the room but for significant non-class time in lab, please email GTF to notify us of the activity/timing.

Grading: lab work/notebook (30%), quizzes (20%), final report (40%), effort/other (10%).

General structure of course
-students will work mostly independently but for convenience can "collaborate" with a partner for some procedures
-students will keep individual lab notebooks, which will be examined periodically (~weekly); carbon copy notebooks should be purchased before first meeting (Duckstore).

OVERALL PLAN OF QUARTER: 1. select for mutants that de-repress antibiotic genes *hph* and *nat-1*, which encode resistance to hygromycin ("hyg") and nourseothricin ("nor"), respectively, when they are expressed. In the starting strain these genes are repressed by histone H3K27 methylation; 2. confirm that they do so (spot tests); 3 test if they also de-repress an H3K27me-repressed gene that we didn't select for (by RT-PCR); 4. test if the mutations go through in sexual crosses; 5. perform complementation tests to see if mutations are in known genes (encoding PRC2 components); 6. determine the approximate chromosomal position of mutation by RFLP mapping.

Please see endnotes for some additional information that will be important for the work; other information/protocols will be provided; schedule is necessarily approximate.

Brief week-by-week schedule (tentative):
- Week 1: Mutagenesis
- Week 2: Confirm mutants with spot tests, set up crosses
- Week 3: RNA extraction and quantitation
- Week 4: RT-PCR
- Week 5: Isolation of progeny from crosses
- Week 6: Mating type-tests and forced hets (*Sad-1* cross), DNA preps (Mauriceville cross)
- Week 7: Examine DNA on gel; PCR pooled progeny DNA (Mauriceville cross), complementation spot tests (*Sad-1* cross)
- Week 8: RFLP mapping
- Week 9: Catch up, follow up, continue mapping
- Week 10: Catch up, follow up, organize strains and notebooks

More detailed plan:

Week 1
Discussion: Scientific background; laboratory orientation; use of lab notebooks; sterile technique; making solutions and media; use of microscope for determining cell density; planning; resources.
Laboratory session 1: Make media[i] for mutagenesis[ii] and filter-beaker to harvest conidia[iii]; plan mutagenesis and platings to generate mutants (these plates will later get antibiotic(s) to select mutants) and to determine survival (these will be non-selective so we'll want to plate many fewer spores).
Laboratory session 2: Harvest and count conidia[iv] of strain with antibiotic markers in H3K27m regions (N6279 *NCU05173::hph, NCU07152::nat-1, his-3*, *mat a*; note that this strain requires supplementation with histidine) using a hemocytometer[1]; UV-mutagenesis (plates to select mutants and others to calculate survivorship); plan & prepare materials.
Laboratory session 3: Add selective top-agar[v] 18 hours after plating (you will need to come into the lab at an unscheduled time to do this, but it won't take long); plan & prepare materials (e.g. make cross plates; for spot tests make three types of FGS plates supplemented with: 1. his; 2. his, nor; or 3. his, hyg; why?).

Week 2
Discussion: Sexual crosses; complementation testing; meiotic silencing.
Laboratory session 1: Score plates and "pick" (transfer) 20 potential mutants on to hyg slants, grow 3-5 days at 32C. Consider what control strains you'll want for upcoming experiments (spot tests) and make sure you have them.
Laboratory session 2: If potential mutant slants have grown enough, suspend in water and spot test on FGS, his, FGS his, nor and FGS his, hyg (include positive and negative controls; save conidial suspensions at 4C). Inoculate 10 mating plates with Mauriceville strain (N51) and 10 mating plates with N3756 (*Sad-1*, *his-3*, mat A).
Laboratory session 3: Check spot tests and determine which strains might be "real" mutants. Pick 10 of these mutants to move forward - use these to fertilize your cross plates from last time. Also grow new slant of each of these strains for storage/future use (label carefully). Plan for next week – prepare any plates, media, solutions you may need.

Week 3
Discussion: Heterokaryon incompatibility; RFLP mapping; RNA isolation.
Laboratory session 1: Plan RNA extraction and RT *Need to come in tomorrow afternoon/evening to inoculate cultures for RNA – include controls.
Laboratory session 2: RNA extraction
Laboratory session 3: Quantify RNA, run on gel to determine quality, perform DNAseI treatment and RT reaction
Materials: Vogels 1.5% sucrose + his, culture tubes, Buchner filter flasks, whatman paper, scale, glass beads, bead beater, filter tips (or autoclaved), acid phenol:chloroform, NETS buffer, chloroform:IAA, 100% Et-OH, 70% Et-OH, DEPC-H2O, DNAseI, Qubit and Qubit reagents, agarose gel and running buffer/equipment, RT kit

Week 4
Discussion: RT-PCR etc.
Laboratory session 1: Plan PCR – what primers do you need, what controls need to be included; what is your reaction?
Laboratory session 2: PCR – known K27me2/3 regulated gene (NCU08570) and actin

---

Laboratory session 3: Run gel and analyze PCR data, plan for next week
Materials: Primers, WT and set-7 cDNA, primers, polymerase, buffers, dNTPs, agarose gel and running buffer/equipment; Pre-lab prep – 2000 his, hyg(?) slants

Week 5
Laboratory session 1: Make plates to germinate spores from crosses (FGS his, nor and FGS his, hyg), make 'fluffy' plates for determining mating type
Laboratory session 2: Select one mutant that seems to have lost K27me2/3 for RFLP mapping (using progeny of cross with Mauriceville) germinate spores and plate onto selective medium (FGS his, hyg) and germinate spores (his, nor, hyg) for all crosses with *Sad-1, his-3* strain to use for determining mating type and complementation testing
Laboratory session 3: Pick 40 progeny of the Mauriceville cross and 10 progeny for each *Sad-1* cross to check mating type
Materials: Plates, media, fluffy strains, slants; Pre-lab prep – 200 min slants

Week 6
Discussion: Bulked Segregant Analysis; Mating Type tests; DNA isolation
Laboratory session 1: Prepare conidial suspensions (*Sad-1* progeny) and perform mating type spot tests and inoculate cultures for DNA preps (Mauriceville progeny).
Laboratory session 2: Examine mating type test plates. Harvest culture for DNA and lyophilize tissue. Make buffers needed for DNA preps.
Laboratory session 3: Examine mating type test plates. Perform DNA isolation protocol through step 9 (TCA-ethanol precipitation). Set up forced heterokaryons with known K27me2/3 mutant strains in *trp-2* background.
Materials: Vogels sucrose + his, Salt detergent, TCA-Et-OH, NH_4OAc/RNaseA, 8:1 Isoproanol: NH_4OAc, 70% Et-OH

Week 7
Discussion: Complementation testing
Laboratory session 1: Finish DNA isolations; examine 5ul aliquot on a gel, make plates for spot tests
Laboratory session 2: Spot tests forced heterokaryons and controls on min, hyg and nor
Laboratory session 3: Set up PCR on pooled progeny of Mauriceville cross and controls, take pictures of spo tests of forced heterokaryons
Materials: Plates, media, polymerase, buffers, dNTPs, primers

Week 8:
Discussion: RFLP mapping review
Laboratory session 1: Start restriction digests and gel electrophoresis to score linkage
Laboratory session 2 + 3: Continue RFLP mapping
Materials: restriction enzymes, agarose gel and running buffer/equipment

Week 9 and 10:
Catch up, follow-up, continue mapping, organize strains into PRC2 complementation groups or potentially novel mutants; Discussion.

ENDNOTES:

[i] To grow Neurospora, you just need to supplement a diluted “salts” solution (e.g. regular “Vogel’s” or modified versions for crossing) and add sugar. Regarding the sugar, typically 1.5% sucrose is used if you don’t need colonies but if you need cells to grow colonially (e.g. for counting viable cells or for looking for mutants), instead you use a combination of fructose (0.05%), glucose (0.05%) and sorbose (2%). We make a 10X concentration stock "10X") of these and add this after autoclaving since the sugars can caramelize if heated with the salts. To make 100 mls of "10X FGS" dissolve 0.5 g fructose, 0.5 g glucose and 10 g sorbose in water and adjust to 100 ml. (Note that sorbose is very expensive so just take what you need.) Always check what extra supplements strains require, e.g. histidine or tryptophan, both of which are stored as "50X" stocks. We normally use supplements at “1X”. For histidine and tryptophan, 1X is defined as 1.0 mg/ml and 0.2 mg/ml, respectively. For solid medium (plates, slants, and "conidia flasks"), we include 1.5% agar (not agarose!).

[ii] Consider that for each “treatment” (untreated and three UV treatments, e.g. 0, 3, 6, 9 seconds) you should plate about 10,000 conidia/plate on four plates for selection of mutants. But you don’t include the selective agent (hygromycin) right away because this would probably kill nearly all the cells, including your mutants (why?). So, instead, 18 hours after the treatments, 10 ml of hygromycin-containing agar medium (use 3X hygromycin because it will be diluted by the bottom agar, which would be ~20 ml; note hygromycin is toxic to humans and is very expensive; be careful!). To calculate total viability, you will want to put a smaller number of conidia on plates that won’t receive hygromycin. A 1/50 dilution of what you put on the other selection plates (i.e. 400 rather than 10,000 cells) might be good. Thus you will need at least 20 plates, which you should be able to make from 0.5 liters of medium (use 20 ml/plate). Be sure to wrap plates in foil immediately after UV treatment to avoid photo-activated repair; the red safe light in the dark room is OK; it will not support photoreactivation. Thus, for your UV-mutagenesis, in preparation for the hygromycin selection, plate on non-selective medium containing Vogel’s, 1X FGS (0.5 mg/ml fructose, 0.5 mg/ml glucose, 20 mg/ml sorbose); 1X histidine (0.5 mg/ml) solidified with 1.5% agar. This is the “BOTTOM AGAR”. Use 20 ml per plate. 18 hours later, you should add 10 ml of “TOP AGAR” which has the same composition as the bottom agar except that it will contain “3X” hygromcyin when you need to select for mutants and will only have 1.0% agar. When you just want to know how many viable cells you have you don’t want to select for hygromcyin and therefore will not include this drug in the top agar.

[iii] Use rubber band or tape to attach double layer of cheesecloth to top of small (~150 ml) beaker making a funnel shape by pushing cheesecloth in middle; cover with foil and autoclave.

[iv] Examine the hemocytometer under the compound microscope (use total magnification of 100X or less) and note that in the middle there are a set of 25 squares (5x5), each divided into 16 smaller squares. If you were to count all the spores in the 25 squares, this would correspond to 10^{-4} ml. But you don’t really need to count all the area. We usually count the 4 corners and the middle square and then multiply by 5×10^4 to get the concentration of spores in the suspension. Note that you will need to dilute your stock so that you get a reasonable number of spores to count. It’s sometimes necessary to do serial dilutions. Typically, dense conidial suspensions contain 10^8-10^9/ml. You might want to first try counting conidia in a 100 X dilution (dilute 10 μl of your conidial suspension into 1 ml) but it there are too many conidia to easily count, you might then want to do another 10 X dilution (e.g. mix 100 μl of the diluted conidia with 900 μl water). If you were to count conidia in this second dilution and found a total of 40 conidia in 5 of the 25 big squares, you’d calculate the concentration in your original stock to be: $40 \times 5 \times 10^4 \times 10^2$ (first dilution) $\times 10^1$ (2nd dilution) = 2×10^9 conidia/ml.

[v] We suggest simultaneous selection with nor and hyg but would be happy to consider possible variations on the envisioned project