PBIO/BIOL3250L: The Dynamic Genome
Fall 2008

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Syllabus Fall 2008

Dr Susan Wessler, Dr Jim Burnette, Dr Nathan Hancock

Eunyoung Cho, TA

Tuesday – Thursday 12:30 – 4:45

Course website: http://www.dynamicgenome.org/classes/

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Attendance: We require 100% attendance and class participation. Any missed lab will be difficult to make up. If you know you will be absent for any class, make arrangements in advance with the instructor. Discuss unplanned absences immediately upon returning to class.

Class participation is a major part of a lab course. You are expected to be prepared for each day, participate in all discussions, and ask a lot of questions. Twenty-five percent of your grade is based on class participation.

For your safety, you must wear closed toe shoes (no flip-flops or sandals). Long shorts are permitted. Long hair should be pulled back away from the face for all labs. Goggles and gloves will be provided.

Grading:

Class Participation (includes attendance)	25%
Take home Mid-term	15%
Project proposal	10%
Quizzes and Notebook checks	10%
Take home Assignments	25%
Final Paper	15%
	100%

Yeast Growth Curve

Purpose: Many experiments using the model organism yeast require liquid cultures where the cells are growing exponentially (called log phase growth). You use a liquid culture when you need large numbers of cells. A colony usually has about ten million cells in it. For transformation you need billions of cells. Yeast cells are metabolically most active (and happy) during log phase growth. Yeast cells typically divide in 2 hours, but the doubling time may differ in the strains used in your experiments. In this first laboratory of the fall semester you will take culture samples at 3 time points, determine the optical density, and plate the yeast to determine viable cell count. With this information you and the instructors will be able to accurately predict when a culture is in log phase without periodic measurements. By plotting OD vs. cell number on a graph, you will be able in the future to determine cell number simply by reading the OD – rather than by plating and waiting the 2 days for yeast cells to grow into visible colonies.

Materials:

YPD plates (9/group) (what does YPD stand for?)
Yeast cultures
Sterile water
Sterile glass tubes
Sterile glass beads
10 ml sterile pipettes
Pump or bulb for pipettes
UV/Vis spectrophotometer
Plastic cuvettes

NanoDrop Spec

For each time point:

1. Label a set of glass tubes 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and pipette 9 mL of sterile water into each.

2. Label YPD (agar) plates with your initials, date, time point, and dilution for the 10^{-2}, 10^{-3}, 10^{-4}

3. Pipette 1 mL of yeast culture into tube 10^{-1} containing 9 mL of sterile water. This is the first ten-fold dilution.

4. Vortex. Place on ice!

5. For Blank: Put 3 mL of sterile H2O into a cuvette. Blank instrument.

6. Pipette 3 mL of the 10^{-1} dilution into a clean cuvette. Read and record O.D.

7. Transfer 1 ml of the 10^{-1} dilution into the 10^{-2} dilution tube. Vortex.

8. Transfer 1 ml of the 10^{-2} dilution into the 10^{-3} dilution tube. Vortex.

9. Transfer 1 ml of the 10^{-3} dilution into the 10^{-4} dilution tube. Vortex.

10. Plate 100 µl of each dilution onto the appropriate plate. Use glass beads to spread the culture.

11. Incubate at 30°C for 2 days.

Draw a chart like this following in your notebook :

Time	O.D. (10 fold dilution)	Counts (dilution)		
		10^{-2}	10^{-3}	10^{-4}

Day 2 after incubation:

Calculating cell number from your plates:

Colonies on all plates are counted and recorded. If there are Too Many To Count then 'TMTC' is recorded. You can divide the plate into sectors and count the number of colonies in a sector. This is done by drawing pie-shaped sectors with a sharpie on the bottom of the plate. For example if you divide a plate into quarters you would count one quarter. In your notes you would record the value as 'count X 4' to indicate you only counted $\frac{1}{4}$ of the colonies. If you counted 225 in a quadrant you would record 225x4. This method is usually not desirable as it introduces error.

For calculating cell number you use the plate that has between 50 and 150 colonies on it. Plates with fewer colonies are not used because they are more likely to lead to sampling error.

Once you have determined the number of colonies per plate the rest is easy. Let's say you counted 130 colonies on the 10^{-4} plate. First, you must take into account the fact that you plated 100 μl of the 10 ml in the test tube. So the final dilution for the plate is 10^{-5}. The viable count in number of cells per milliliter is 130×10^5 or 1.3×10^7 cells/ml. For yeast this is mid-log growth.

Data Analysis:

You want to correlate the cell count with O.D. To do this you will need to: 1. Determine the cell number in the culture at each time point from the plates. 2. Draw a graph or use Excel to plot O.D. vs Time vs Cell number. You should fully label your axes.

Please read the whole paper. Pay special attention to the first 2 results sections "Yeast Transposition Assay" and "Excision of Osmar5NA" and to figures 1, 2 and 3. This is the introduction to the experiment you will start on Thursday.

For those of you who have not read a research paper before, here is a very brief summary of the purpose of each section:

Abstract – a short summary of the paper that is written like a mini-paper. The first sentence or two provides an introduction and rationale, the next part summarizes the most important results and the abstract ends with the most important conclusion. Believe it or not, the abstract is usually written last. Can you think of reasons why?

Introduction – this section provides enough background so that the reader can understand the Results section and answers the following questions – what is the rationale for this series of experiments and what "hole" in our knowledge does it fill? The introduction is usually written next to last (before the abstract) and it may include a figure but usually does not.

Results – this section is usually a narrative of the experimental results and includes the data figures. It is important to know what should be in this section (results) and what should not (background and discussion). This section is written first – usually starting with the figures and then writing the text that tells the "story of the results." The vast majority of time on the project is summarized in the results section.

Discussion – to some (like Dr. Wessler) this is the most enjoyable part to write as it is the only place where one can be less "rigorous" and perhaps more visionary. To most others, it is the hardest section to write. The idea is to reach conclusions based on your data. The best discussions are syntheses of conclusions supported by several experimental results. The worst discussions largely ignore the data and discuss issues only distantly related to the study done. The discussion is written after the Results.

Sometimes Results and Discussions are combined into one section. I can tell you when and why this happens.

The Materials and Methods (sometimes just called Methods) are just that. Usually very boring to read, but necessary if one really wants to know what was done and to repeat it. One tough call is determining what should be left out of the Results and put into the M and M section.

Please come with questions written down about research methods you don't understand and/or terms you need defined. Also draw parallels between the yeast experiments and the Arabidopsis Osmar experiment you did this summer.

In the first experiment you will transform yeast DG2523 with two plasmids. On plasmid ZMar20_MITE0 was inserted into the ADE2 gene making it mutant. On another plasmid the ZMar20 transposase was cloned so that it will be expressed by yeast only when the yeast are grown on galactose. There are two versions of this clone pAG415-ZMar20Tpase and pAG425-ZMar20Tpase. The pAG415 is a low copy vector and pAG425 is a high copy vector. We know the excision frequency with the low copy vector. Your experiment is to determine if you get higher excision rates with the high copy vector compared to the low copy vector.

Yeast Transformation Protocol

Materials:
DG2523 Log-phase Yeast culture at 0.5-0.8 O.D.
100 mM Li Acetate
Sterile Water
10 mg/ml Salmon sperm DNA (pre-warmed).
PEG Buffer
Plasmids:
- pWL89A reporter with Mariner in ADE2
- pAG415-ZMar20Tpase low copy plasmid
- pAG425-ZMar20Tpase high copy plasmid
- pAG415ccdb no transposase gene.

Plates:
- DOB(dex) CM -ura, -leu (4/group)

Water bath 42°C.
Ice
Vortex
Sterile 50 ml Falcon tubes
Clinical Centrifuge

Day 1: A liquid culture of DG2523 will be started by the instructors.

Transformations:
1. Water negative control
2. pWL89A + pAG415 empty
3. pWL89A + pAG415-ZMar20Tpase
4. pWL89A + pAG425-ZMar20Tpase

Day 2:
Instructors will provide a log phase liquid culture of DG2523 at 0.5 OD.
1. Centrifuge a 25 ml culture in a Falcon tube at speed 5 for 5 min.
2. Pour off super and re-suspend in 20 ml sterile H_2O.
3. Spin at speed 5 for 5 min.
4. Pour off super. Re-suspend in 0.5 ml 100 mM LiOAC.
5. Transfer to a sterile 1.5 ml tube.
6. Pellet cells for 20 secs, 7,000 rpm.
7. Pipette off super. Resuspend in 225 µl 100 mM LiOAC.
8. Label four 1.5 ml tubes with the name of the vectors.
9. Add 5.8 µl of carrier DNA to each transformation tube.
10. Add 1.0 µl of vector(s) to the correct tube.
11. Add 50 µl of yeast solution to each transformation. Mix the yeast before pipetting.
12. Vortex briefly on speed 6 of the vortexer.
13. Add 400 µl PEG buffer.
14. Vortex briefly on speed 6 of the vortexer.
15. Heat shock at 42°C for 45 minutes. Label plates.
16. Pellet cells 20 secs at 7,000 rpm.
17. Completely pipette off super and add 100 µl sterile H2O.
18. Plate. Incubate 30°C for several days.

Experiment 1: Does increasing the copy number of the ZMar20 transposase result in an increase in the excision rate of ZMar20NA?

Yeast strain DG2523

DG2523 is a strain of yeast obtained from the lab of David Garfinkel (Center for Cancer Research, Maryland). The strain contains the following mutant alleles:*ura3*, *his3*, *trp1*, *leu2*, and *ade2*. For this experiment we are only concerned with *ura3*, *leu2*, and *ade2*. On YPD (rich) media, this strain grows well because the media contains all nutrients needed by the cells. DG2523 colonies will turn red due to a block in the adenine biosynthesis pathway caused by the *ade2* mutation. This strain will not grow on defined media (where the nutrients uracil, adenine and leucine are omitted) because it cannot make a supply of the three nutrients. If we provide a functional allele (e.g. on a plasmid), the strain will grow on defined media without adding uracil, adenine, or leucine. For example, if we transform the strain with a plasmid containing *URA3* (note the capital letters – this denotes a functional gene) it will grow on defined media that lacks uracil (but includes adenine and leucine, get it?). The *URA3* gene on the plasmid is said to <u>complement</u> the *ura3* mutant allele on the yeast chromosome. (also note the spelling of complement with an 'e.')

Plasmid pZMar20NA2 (Figure 1)

This is called the 'reporter' plasmid because it reports when transposition occurs. The plasmid contains two yeast genes, *URA3* and *ADE2*, as well as the yeast centromere *CEN/ARS4* (discussed below). The parent plasmid pWL89a (obtained from Dr. Cliff Weil, Purdue University) complements the *ura3* and *ade2* mutant alleles of DG2523 and permits the strain to grow without added uracil and adenine in the medium. To construct the plasmid pZMar20NA2, the parent plasmid (pWL89A) was digested with a restriction enzyme that cut only in the middle of the ADE2 gene and the ZMar20NA2 element was ligated (inserted) into the *ADE2* gene (by Yujun Han). This made *ADE2* nonfunctional.

You might be wondering where the ZMar20NA2 element came from. It did not come from the maize genome. Rather, it was constructed by Yujun Han by deleting most of the autonomous element ZMar20 and leaving only ~100 bp from each end of the element. When transformed into yeast,

pZMar20NA2 will complement the *ura3* mutant allele but not the *ade2* allele. Yeast has a tendency to get rid of plasmids that it does not need. So, to select for and maintain pZMar20NA2 in DG2523 you must always grow strains containing this plasmid on media lacking uracil. This forces the strain to make its own uracil and thus ensures that only yeast with the plasmid will grow. The yeast colonies on plates will be red and may even make liquid cultures turn red.

As mentioned above, pZMar20NA2 is called a 'reporter' plasmid because it will 'report' excision events of ZMar20NA2. Under the right conditions (see below) ZMar20NA2 can excise from the *ADE2* gene and the gene will re-gain function, and thus complement the *ade2* mutation in DG2523. If you grow the yeast on media lacking adenine, only the cells in which ZMar20NA2 excised and restored *ADE2* function will grow into white colonies. Each colony will represent an independent excision event and you can analyze the sequence of the *ADE2* gene from each colony for footprints.

Plasmid pAG415-ZMar20-Transposase and pAG415-ZMar20-Transposase (Figures 2 and 3)

Now you might be wondering about the other plasmid containing the transposase (ZMar20-tpase). Using bioinformatics and TATE to analyze maize genomic sequence, Yujun found a promising autonomous *Mariner* element and he named it ZMar20. To test whether the transposase was active, Yujun used PCR to isolate a DNA fragment containing just the transposase gene from the maize genome and ligated it into the parent plasmid pAG415 so he could express the transposase in yeast. pAG415 and pAG425 are two of a series of vectors created for expressing genes under controlled conditions. Read Chapter 14 pages 318-321 of *Life* to review transcriptional regulation. For your experiment, the transposase gene will only be expressed (turned on) when we want excision to take place. If it were to be expressed all the time, we would never recover excision events. Can you figure out why? In addition, it is also possible that expressing a lot of transposase will make the yeast cells sick and die. Can you figure out why? To regulate its expression the transposase gene was fused to a GAL1 promoter. This promoter was isolated from the *GAL1* gene of yeast and is active only when yeast is growing on media containing galactose. On YPD or other dextrose containing media, the promoter is off and the transposase

gene is not expressed. On the galactose plates the GAL1 promoter is turned on and the transposase gene is expressed.

The pAG series of plasmids contains the *LEU2* gene for selection and maintenance of the plasmid. (The AG stands for the proprietary name Advanced Gateway.) The *LEU2* gene on the plasmid complements the *leu2* mutation in the genome of DG2523 and allows the cells to grow on defined media lacking leucine. So to maintain this plasmid in a strain, the strain must be always be grown on media lacking leucine.

Finally, you might be wondering why we are using two different sources of transposases for this experiment. Plasmids pAG415 and pAG425 differ in the way they replicate and are segregated during division. Yeast cells have two mechanisms for replication and segregation. One is the system used for the chromosomes. In this system, an Autonomous Replicating Sequence (*ARS*) directs DNA replication on a chromosome so that each chromosome is replicated only once. Then to segregate the duplicated chromosome the centromere sequence (*CEN*) is used for attachment of the chromosomes to the spindle. Review pages 187-192 in *Life* on DNA replication and segregation. A plasmid that contains *CEN* and *ARS* (called *CEN/ARS*) essentially behaves as a circular chromosome and is maintained by the cell in 1-5 copies per cell (called low-copy). In lab jargon we say that the parent plasmid pAG415 is a low-copy plasmid (as is all derivatives of this plasmid made in the lab). The second mechanism of replication and segregation is based on a natural plasmid of yeast called the 2 micron circle. The 2 micron circle has a sequence that directs its replication to 50-100 copies per cell. This is considered high-copy and the parent plasmid pAG425 is called a high copy vector. Segregation of the 2 micron based vectors is thought to be passive and independent of the spindle.

To repeat something that was mentioned above - because the plasmids are independent of the chromosome, they are not efficiently maintained by the cell. In rich media the cells do not need the genes on the plasmids and thus the plasmids can be 'lost' at a high frequency. In addition, during the rapid division of log-phase growth some daughter cells will not receive any plasmid. If growth is in YPD, the daughter lacking the plasmid will have a growth advantage and its descendants will take over the culture. Strains with plasmids must be maintained under selective pressure to keep the plasmids.

So, in media lacking uracil, a daughter that does not receive a copy of pWL89A-ZMar20NA will not grow because it cannot make its own uracil.

Previous data collected by Yujun and Nathan have shown that excision of a *Mariner* element from *ADE2* occurs about 1 in every million cells (1 event in $1x10^6$ cells) (stated another way - a frequency of $1x10^{-6}$ events/cell) when the transposase is encoded by a low copy (pAG415) vector. You would need to plate ten million cells to recover 10 excision events. One hypothesis for this low excision frequency is that there is very low expression of the transposase gene. To test this, Nathan made the pAG425-ZMar20-Transposase plasmid so that there will be between 50 and 100 copies of the transposase gene in each cell. This should increase the expression of transposase compared to the pAG415 plasmid. Will this increase in gene expression result in a higher transposition frequency? This is the question you will answer over the next week.

To determine the excision frequency you will follow a 4 day protocol.

Day 1. Grow a single colony in 5 ml of liquid media to high cell numbers. A colony of yeast on a plate contains around one million cells ($1x10^6$). This is not enough yeast for the assay. In liquid culture we can grow yeast to around $5x10^7$ cells/ml. how many yeast will you have in a 5 ml culture? These yeast cells must be grown in medium that maintains the selection for the two plasmids: pWL89A-ZMar20NA and pAG415 or pAG425. What nutrients should this medium lack? Why? This medium will contain dextrose (also called glucose) but will not have galactose. Will the transposase gene be expressed during this growth period?

Day 2. The cells will be washed with 5 ml dH_2O. Washing the cells removes the old medium and, most importantly, removes any unused dextrose from the cells. The cells are pelleted in a centrifuge, the old medium taken off and 5 ml of dH_2O is added. After thoroughly re-suspending the cells you will take a 100µl sample for a viable count.

Day 3. The cells are concentrated from 5 ml to 1 ml by pelleting the cells in a centrifuge and resuspending in 1 ml of dH_2O.

Day 4. All of the cells are plated on one large plate. The medium in this plate has only galactose as a carbon source. Besides lacking the same nutrients as in Day 1, these plates also lack adenine. What must occur in cells that grow into colonies on this plate? Why is adenine missing from the plate? What will be the color of the colonies? The colonies will take at least a week to develop.

You must write the protocol in your notebook. No cut-and-pasting.

Materials:
10 ml sterile glass pipettes
Disposable plastic culture tubes
Sterile blue cap culture tubes.
Liquid Media:
DOB(dex) CM-ura,-leu
Plates:
DOB(gal) CM-ade, -ura, -leu (9/group) (large plates)
YPD (9/group)
Sterile water
Sterile loops

Assays (each done in triplicate):
(Numbering from transformation).
2. pWL89A-ZMar20NA2 + pAG425 empty
3. pWL89A-ZMar20NA2 + pAG415-Zmar20-TPase
4. pWL89A-ZMar20NA2 + pAG425-Zmar20-TPase

Day 1: Tuesday, August 26: Starting Cultures
1. Label 10 blue cap glass tubes (e.g. 2-a, 2-b, 2-c, 3-a, 3-b, etc.).
2. Put 5 ml of DOB(dex) CM-ura, -leu into each tube.
3. Inoculate each tube by picking 1 pink colony with a loop from the appropriate plate. Leave the 10^{th} tube with no yeast.
4. Incubate 2 days at 30°C in the spinner.

For Day 2: You need to answer the questions and design the dilution strategy before coming to class. We will check notebooks at the

beginning of class. You must be very familiar with this protocol before doing it.

Day 2: Thursday, August 28: Plating for excision.

1. Label DOB(gal) CM-ade, -ura, -leu large Plates.
2. Label 9 plastic culture tubes. Pour yeast culture from glass tubes to plastic ones.
3. Label 9 glass tubes. Put 9.9 ml sterile water in each.

Go to Sue's lab on 4th floor.

4. Pellet cells speed 5 for 5 min.
5. Pour of super carefully. Re-suspend in 5 ml Sterile Water.
6. Transfer 100 µl of each culture to the appropriate dilution tube.
7. Pellet cells speed 5 for 5 min.
8. Pour of super carefully.

Return to 1506 lab.

9. Re-suspend in 1 ml sterile water.
10. Plate all 1 ml on the correct plate. **Do not discard glass beads yet.**
11. Keep plates '**right-side up**' for 1- 2 hours in 30°C incubator.
12. Finish dilution series to 10^{-5}. Plate 100 µl of the 10^{-5} dilution only. How will you set up the dilution series? Remember the first dilution was 0.1 ml in 9.9 ml of water. What magnitude is this first dilution? How many colonies will you expect on the 10^{-5} plate if the starting cultures had $7x10^6$ cells/ml?
13. Pour off the glass beads from the large plates. Return to the incubator **up-side down** for one week.

Day3: Tuesday, September 2: Counting the viable count colonies.

Count and record the number of colonies on each plate. Design a table in your notebook for recording the data.

Day 4: Thursday, September 4: Collecting the data.

Count and record the number of colonies on each plate. Design a table in your notebook for recording the data.

Photograph plates.