INTRODUCTION

This manual complements our American Biology Teacher article entitled *The Luria-Delbrück Fluctuation Test as a Classroom Investigation in Darwinian Evolution*, by George P. Smith, Miriam Golomb, Sidney K. Billstein and Stephen Montgomery Smith; the article is available on this site. That article explains the logic of the yeast fluctuation test; this manual focuses on the practicalities of preparing labs and helping beginning biology students interpret the results and learn from them. In addition, the site includes some supplementary documents and numerous teaching documents that are made available to the students in the course of the module (names in **bold blue 14-point font**).

The 5 sessions described here are part of a larger 11-session module described in the extended article, **ExtendedArticleDescribing11SessionModule.pdf**, that is also available on this site. The 11-session module includes scientific threads that arise naturally in the context of the experiments, but that are not included in the 5-session module: simple mathematical analysis based on the Poisson distribution (given, not derived) and probability theory; and the "central dogma" of molecular biology, which underlies the genetic design of the starting yeast strain YFT1.

For there to be any hope of completing a fluctuation test in a reasonable amount of time, it is necessary for students to follow carefully planned instructions. Yet as teachers we want our students to take "ownership" of their learning. Where is there room for "discovery-based" learning? In fact, we believe that this module is rich in opportunities for student discovery. The discoveries are not to be made in the realm of physical procedure development, but rather in the realm of hypothesis development and interpretation of results. At several points in this manual, we include general suggestions for managing students' discovery process, and many more ideas emerge from reading the handout we distribute to our students (also available on this site). In the end, however, students' learning depends most on their teacher's creative use of time and resources.

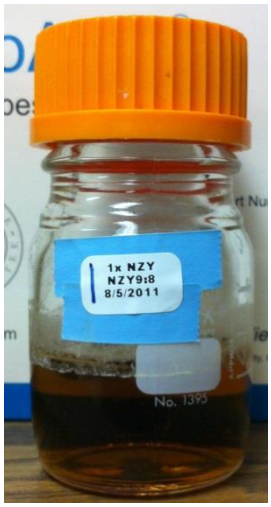
ORGANIZATION OF THIS MANUAL

This manual is organized into sections with bold, all-caps, 16-point, centered headings; subsections with bold, all-caps, 12-point, left-justified subheadings; and sub-subsections with non-bold, italic, 12-point, left-justified sub-subheadings. Those sections that include numbered steps have serially lettered headings; the step numbers within each lettered section are preceded by the section's letter. The .pdf version of the manual includes benchmarks for each section.

NOTE ON PRE-LABELING

This lab module involves hundreds of labeled tubes, petri dishes and other vessels. Students can use markers with or without tape to label their own vessels, but inevitably there will be confusions and mistakes, which can greatly complicate the students' interpretation of their results. We have elected to avoid this problem by color-coding and pre-labeling all vessels in advance, even though this admittedly compromises student "ownership" of their work in some

measure. Color-coding is achieved by use of colored vessels when available, and the labels themselves can be color coded by using colored tape or by striping with colored permanent markers (non-permanent markers are OK if the vessel isn't autoclaved).



Pre-labeling hundreds vessels by hand would be an onerous undertaking. Instead, we use Microsoft Word and an inexpensive thermal transfer label printer (such as the Zebra TLP 2824PLUS) to print up to 4 short lines of text (7.5-point Arial Black font) on white plastic 0.875 inch wide × 0.5 inch high labels with an aggressive permanent adhesive (see picture at right; labels are not applied directly to non-disposable labware since it's almost impossible to remove them). Such plastic labels tolerate both freezing and autoclaving. The 7/8 inch width is short enough that the label doesn't overlap with itself even on a 0.5-ml microtube (outer diameter 8 mm). Unfortunately, such labels are only available by custom order (our customized product number at Electronic Imaging Materials is 302791), costing about $1000 per 100,000 labels (and not much less for 10,000 labels). Obviously this investment is worthwhile only if the labels are used for other lab purposes as well. Slightly wider labels (1 inch wide × 0.5 inch high) are available in smaller quantities in stock from a number of suppliers such as Electronic Imaging Materials, but such a label overlaps slightly with itself on a 0.5-ml microtube.

A. LIQUID MEDIA

MATERIALS AND SUPPLIES

DOB medium powder (dropout base; contains mineral salts and glucose); MP Biochemicals 4025-022; use 26.7 g/liter

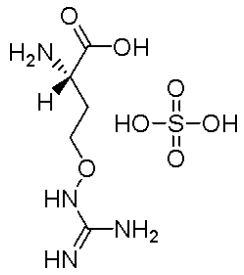
CSM-ARG powder (complete supplements mixture minus arginine); MP Biochemicals 4510-112; use 0.74 g/liter

YEP broth powder (powdered rich medium without carbon and energy source); MP Biochemicals 4004-032; use 30 g/liter; 30 grams of YEP is equivalent to 10 grams yeast extract plus 20 grams peptone; those two components can be substituted for the YEP

Adenine powder 25-gram bottle; Sigma A2786

Glycerol (any ACS grade will do)

L-canavanine sulfate (toxic arginine analogue); Sigma C9758-1G; 1 gram; molecular weight 274.25



Steriflip vacuum filtration unit; EMD-Millipore SCGP00525; available from many scientific supply companies such as Fisher

~20 1.5- or 2-ml screw-cap polypropylene microtubes; must be autoclavable

3 125- and 3 250- ml polypropylene screw-cap bottles; many brands available from many scientific supply companies such as Fisher

8 500-ml autoclavable 500-ml glass bottles; many brands available from many scientific supply companies such as Fisher

2× ARG DROPOUT MEDIUM

NOTE: This medium is used to prepare selective agar medium petri dishes; it contains 2× concentrations of all the medium ingredients except the agar and the canavanine antibiotic. Canavanine is a toxic analog of arginine. It is toxic to yeast only if there is no arginine in the medium, so that the cells have to synthesize their own arginine. Accordingly, the selective medium is arginine "dropout" medium, which contains no arginine but contains an abundance of other nutrient supplements (in the CSM, or complete supplement mixture), as well as mineral salts and glucose (in the DOB, or dropout base). Extra adenine is included in the CSM to ensure that Ade2-defective cells, which can't synthesize their own adenine, are not at a growth disadvantage. This recipe is for 8 500-ml bottles, which is enough for 2 lab sections or 1 section for 2 years. 1× Arg Dropout Medium has 25.7 g/liter DOB and 0.74 g/liter CSM-ARG powder.

A1. In a glass 4-liter beaker get ~3200 ml of deionized water stirring vigorously; into the stirring water gradually tip:

- 213.6 g DOB powder (weigh this into a beaker over a garbage can: don't weigh directly over the balance since the powder is dusty)
- 5.92 gram CSM-ARG powder

When the solid is dissolved, adjust the pH to 5.6 with 2 N NaOH on a pH meter (in our experience should take ~100 drops).

A2. Bring the volume to 4000 ml in a graduated cylinder and distribute evenly into eight 500-ml autoclave-safe glass bottles (glass is preferable to plastic so that contamination can be easily detected).

A3. Autoclave for 60 min in a small pan with water ~4/5 up the side of the bottles.

A4. When cool, screw the caps on tight; label the bottles "2× Arg dropout medium"; store at room temperature, preferably away from the light.

2× AND 1× YEPGA MEDIUM

NOTE: This is the non-selective medium. The only carbon and energy source is glycerol, which can't be fermented. That selects against mutants that have lost mitochondrial function (so-called "petite" mutants) and are therefore incapable of respiratory growth. Such mutants are very undesirable, since they can't make the red intermediate that otherwise would accumulate in Ade2-defective colonies. The medium is supplemented with extra adenine to ensure that Ade2-defective mutants are not at a growth disadvantage. The 1× medium is the liquid non-selective medium; the 2× medium is used for non-selective agar medium in petri dishes. This recipe makes enough 1× and 2× medium for 3 lab sections. 1× YEPGA has 30 g/liter YEP broth powder, 30 g/liter glycerol and 0.25 g/liter adenine.

A5. In a 1-liter beaker get ~600 ml deionized water stirring vigorously; into the stirring water weigh:

- 0.375 grams of adenine (Materials)
- 45 grams of YEP broth powder (or 15 grams yeast extract plus 30 grams peptone); weigh this into a large weigh boat over the garbage can: don't weigh directly over the balance since the powder is dusty

Continue stirring until the powder is dissolved.

A6. Into a 150-ml beaker weigh 45 grams of glycerol; pour the glycerol into the stirring medium previous step; wash the glycerol into the stirring medium with several beakers full of deionized water until you've got essentially all the glycerol in (but don't let the volume in the beaker exceed ~700 ml). When the medium is thoroughly mixed, adjust the pH to 5.6 with 10 N HCl (beware of fumes; switch to 2 N HCl instead of 10 N HCl if pH is dropping too fast to control; can back-titrate with 2 N NaOH if necessary; it may take ~1.25 ml of 10 N HCl for this amount of medium).

A7. Bring volume to 750 ml in a 1-liter graduated cylinder by adding deionized water; pour back into the beaker to mix; this is 2× medium.

A8. Use a 500-ml graduated cylinder to measure 375 ml deionized water and 375 ml of the 2× medium into a second 1-liter beaker to make 750 ml 1× medium; mix the 1× medium with a stir bar or stirring rod; distribute the 375 ml of 2× medium and the 750 ml of 1× medium into narrow-mouth polypropylene bottles (polypropylene is very preferable to glass because of drip-back when attempting to pour sterilely from glass containers) as follows:

- Pour the 2× medium evenly into 3 narrow-mouth polypropylene 125-ml bottles
- Pour the 1× medium evenly into 3 narrow-mouth polypropylene 250-ml bottles

A9. Autoclave the six bottles previous step for 45–60 min in a small pan with water about halfway up the side of the 125-ml bottles.

A10. When cool, screw the caps on tight; label the 125-ml bottles "2× YEPGA" and the 250-ml bottles "1× YEPGA," using different colored tapes; store the bottles at room temperature, preferably away from the light.

CANAVANINE STOCK SOLUTION

WARNING: Although canavanine is present in ordinary foods, especially alfalfa sprouts, it is mildly toxic at high concentrations (Akaogi et al., Role of non-protein amino acid L-canavanine in autoimmunity. Autoimmunity Reviews 5, 429–435, 2006). Take precautions when working with the powder or the 60-mg/ml stock solution:

- Wear gloves and glasses or protective eyewear
- Work over an absorbent pad and clean up spills
- Discard used labware, gloves and other unused material into chemical waste

The canavanine in the selective agar medium is so dilute (60 µg/ml) that no precautions are necessary.

A11. Weigh out the entire bottle of L-canavanine sulfate (Materials) into a tared disposable plastic 50-ml tube in a hood; record net weight (will be very close to 1 g); add sufficient water to make a 60-mg/ml solution; vortex.

A12. Filter sterilize using a Steriflip filtration unit (EMD-Millipore SCGP00525) into a fresh sterile 50-ml tube; pipette 1-ml portions of the filter-sterilized stock solution into 1.5- or 2-ml screw-cap microtubes (nominally 17 tubes); pipette any leftover solution into an additional sterile screw-cap microtube; label the tubes containing a full 1-ml portion "1 ml 60-mg/ml canavanine; toxic"; label the tube with less than 1 ml "60-mg/ml canavanine; toxic." Store tubes in a –20º deepfreeze in a closed container labeled "60-mg/ml canavanine; toxic."

B. PETRI DISHES WITH AGAR MEDIUM

NOTE: The ~140 selective dishes should be poured at least 2 weeks before Session 2, in order to allow them to dry thoroughly. Otherwise water extruded from the agar can smear the yeast colonies and undermine the experiment. The dishes will dry adequately in about 2 weeks at room temperature if arrayed upside down 1 deep, covered with air-permeable fabric or paper in order to reduce mold contamination. A few dishes are likely to become contaminated anyway; they should be removed every day or two in order to reduce contamination of neighboring dishes. The 6 non-selective medium (YEPGA) dishes can be poured about the same time as the selective dishes. A single YEPGA petri dish is needed to make the original YFT1 glycerol stocks (see below).

MATERIALS

Bacto agar

4 2000-ml polypropylene Erlenmeyer flasks; many brands available from many scientific supply companies such as Fisher

4 polypropylene beakers that will fit over the 2000-ml Erlenmeyer flasks; many brands available from many scientific supply companies such as Fisher

1 500-ml polypropylene Erlenmeyer flask; many brands available from many scientific supply companies such as Fisher

1 polypropylene beaker that will fit over the 500-ml Erlenmeyer flask; many brands available from many scientific supply companies such as Fisher

4 500-ml bottles of 2× Arg Dropout medium (step A4)

4 mictotubes with 1 ml 60-mg/ml canavanine (step A12)

1 125-ml bottle of 2× YEPGA (step A10)

~160 100-mm sterile plastic petri dishes; many brands available from many scientific supply companies such as Fisher

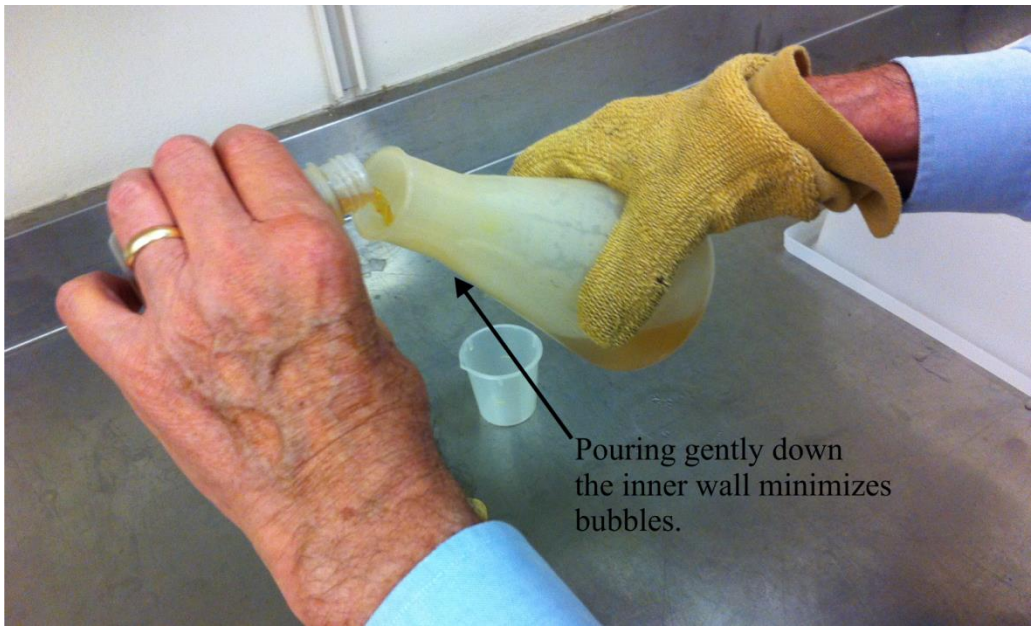
SELECTIVE PETRI DISHES CONTAINING CANAVANINE

B1. Into 4 2000-ml polypropylene Erlenmeyer flasks weigh 11 grams bacto agar; into each flask measure 500 ml deionized water; cover the flasks with polypropylene beakers; autoclave for 60 min; use immediately for step B4 without cooling.

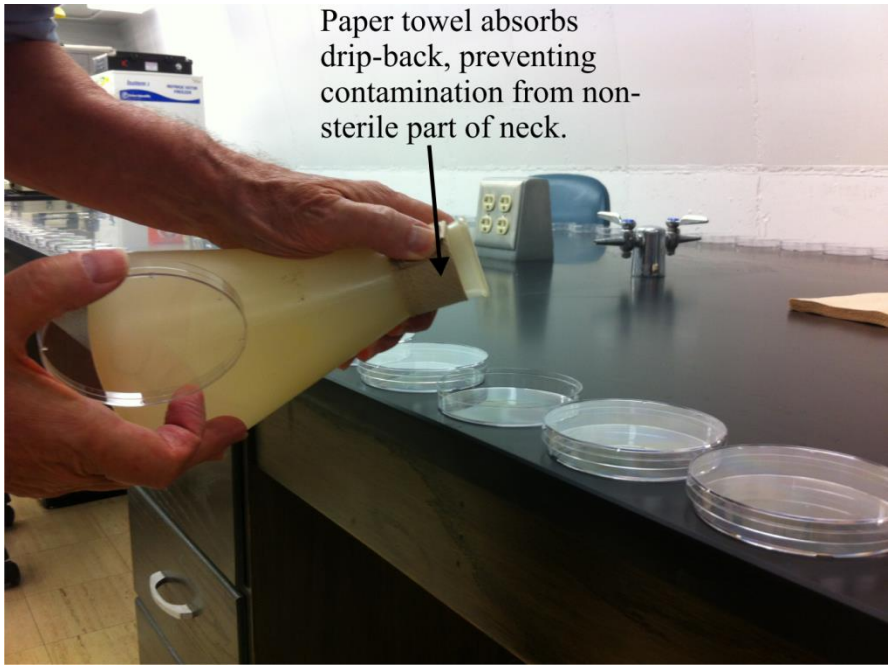
B2. Meanwhile, into each of 4 500-ml bottles of 2× Arg Dropout medium pipette the entire contents of one of the microtubes containing 1 ml 60-mg/ml canavanine (discard the microtubes into chemical waste); close caps of the 500-ml bottles and invert many times to mix the canavanine in with the rest of the medium.

B3. Set out 160 empty plastic disposable 100-mm petri dishes on a clean flat surface near the edge of the surface for easy access.

B4. As soon as the autoclaving step B1 is finished, carefully pour the contents of one of the 500-ml bottles step B2 down the inner wall of each of the 2000-ml flasks (see photo below, showing a 500-ml flask), avoiding bubbles to the extent possible; the temperature inside the flask drops from 100ºC to ~60ºC, a suitable temperature for pouring the petri dishes. Tip the flask at a shallow angle and rotate many times to mix the medium in with the molten agar. Pour the 2× medium into all four flasks before you start pouring into dishes; the agar will remain molten during the ~35 minutes required to pour all the dishes.



B5. To avoid contamination by drip-back while pouring petri dishes, fold a paper towel twice lengthwise and wrap the folded towel around the neck of the flask to absorb any drip-back, as shown below (which shows a 500-ml flask).



Working rapidly but carefully, pour the molten agar medium into the petri dishes to completely cover the surface, avoiding bubbles to the extent possible; rinse each flask with hot running water immediately after use to prevent the agar from solidifying. An experienced microbiologist can pour 40 petri dishes from a single flask, but you may end up with fewer. Be sure the lids are replaced properly on all the petri dishes. Allow the agar medium to solidify thoroughly for at

least 1 hour; then array the dishes upside down, preferably 1 deep; cover the dishes with clean fabric (we use autoclave paper) to minimize dust. Allow the dishes to dry at room temperature for 2 weeks (drying time depends on ambient humidity; see step B7); it is very important that the dishes be thoroughly dried before use by the students.

B6. Every 1 or 2 days starting on day 4, inspect the dishes for mold contamination and discard the contaminated dishes.

B7. Every day starting ~day 8, inspect a few dishes for drying, as evidenced by wavy refractive index lines visible when the light glints off the surface; once the dishes are dry, they can be wrapped in groups of 10 in Saran wrap to prevent further drying.

NOTE: The aim is to end up with at least 120 uncontaminated dishes for students to use in Session 2. If fewer uncontaminated dishes are available, have students spread fewer than 50 bulk culture sample dishes; don't reduce the number of individual culture dishes below the planned 70.

NON-SELECTIVE YEPGA PETRI DISHES

NOTE: These are poured using the same technique as explained in the previous subsection for the selective dishes, using a 500-ml flask with ¼ the weight of agar, volume of water and volume of 2× medium as for one of the 2000-ml flasks. See the pictures in the previous section for minimizing bubbles as you pour the 2× medium into the molten agar, and for wrapping the neck of the flask in a twice-folded paper towel to prevent contamination by drip-back. This recipe is more than enough for 6 petri dishes with generous amounts of agar medium; only 2 of these petri dishes are actually used, so there's plenty of back-up in case of contamination.

B8. Into a 500-ml polypropylene Erlenmeyer flask weigh 2.75 grams bacto agar; into the flask measure 125 ml deionized water; cover the flask with a polypropylene beaker; autoclave for 20 min; use immediately for step B10 without cooling.

B9. Set out 6 empty plastic disposable 100-mm petri dishes on a clean flat surface near the edge of the surface for easy access.

B10. As soon as the autoclaving step B8 is finished, carefully pour the contents of a 125-ml bottle of 2× YEPGA down the inner wall of one of the 500-ml flask, avoiding bubbles to the extent possible (see picture at step B4 of previous subsection); the temperature inside the flask drops from 100ºC to ~60ºC, a suitable temperature for pouring the petri dishes.

B11. Fold a paper towel twice lengthwise and wrap the folded towel around the neck of the flask to absorb any drip-back (see picture at step B5 in previous subsection); working rapidly but carefully, pour the molten agar medium generously into the 6 petri dishes to completely cover the surface, avoiding bubbles to the extent possible; rinse the flask with hot running water immediately after use to prevent the agar from solidifying. Be sure the lids are replaced properly on all the petri dishes. Allow the agar medium to solidify for at least 1 hour; then array the

dishes upside down, preferably 1 deep; cover the dishes with clean fabric (we use autoclave paper) to minimize dust. Allow the dishes to dry at room temperature at least 2 days.

C. GLYCEROL STOCKS OF STARTING YEAST STRAIN

MATERIALS

Vial of yeast strain YFT1; direct descendant of *Saccharomyces cerevisiae* strain SJR1921 received from Caroline Welz-Voegele in Sue Jinks-Roberston's lab at Duke University; haploid genotype *MAT***a** *ura3ΔNco lys2-1 can1-100 ade2-1 leu2-K nearARS306::SUP4-oF*. The *lys2-1*, *can1-100* and *ade2-1* mutations are ochre nonsense mutations that by themselves eliminate Lys2 function (so the cells require lysine), Can1 function (so the cells are resistant to canavanine) and Ade2 function (so the cells require adenine and accumulate a red intermediate during respiratory growth), respectively. However, all three of these ochre nonsense mutations are suppressed by the ochre suppressor tRNA encoded by the *SUP4-o* gene, so the strain does not require either lysine or adenine, does not accumulate a red intermediate, and is sensitive to canavanine. The *lys2-1* and *ura3ΔNco* mutations are not used in the yeast fluctuation test. YFT1 grows in liquid YEPGA medium, and makes white colonies on YEPGA agar medium.

1 non-selective YEPGA petri dish (step B11)

10 ml non-selective 1× YEPGA liquid medium (step A12)

50- or 125-ml culture flask

Shaker-incubator for shaking cultures at 30ºC

20 to 100 500-µl microtubes; autoclaved and dried; labeled "YFT1 glycerol tube"

Glycerol (ACS grade)

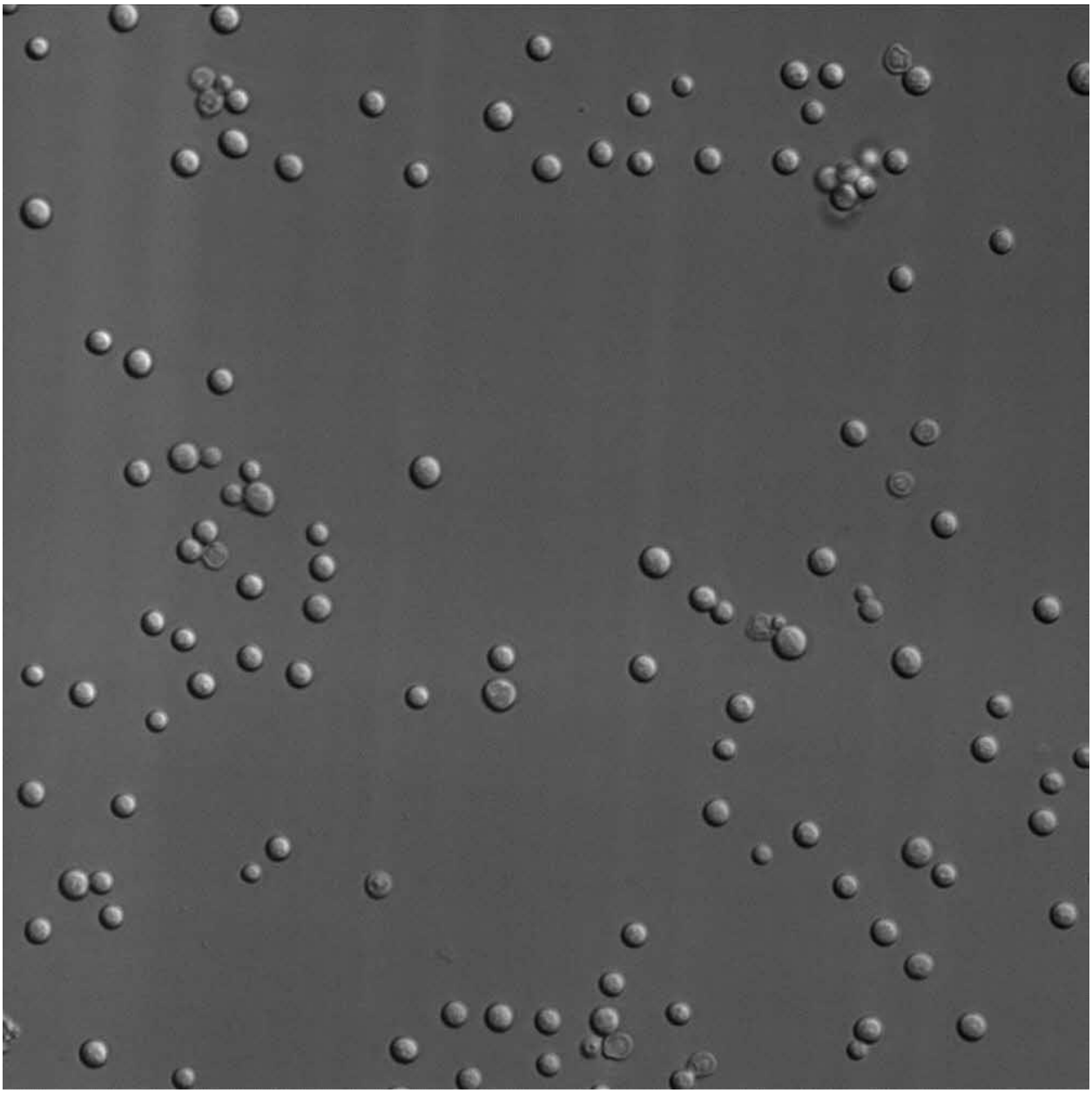
PROCEDURE

C1. Streak from the YFT1 vial onto a YEPGA petri dish; incubate dish at 30ºC for 5 days to allow colonies to develop fully. If you are not confident how to streak for isolated colonies, see http://en.wikipedia.org/wiki/Streaking_%28microbiology%29 or some other source of instruction.

C2. Use a single well-isolated white colony to inoculate 10 ml YEPGA medium in a 50- or 125-ml culture flask; shake the flask at 30ºC for 2–3 days, until the culture becomes turbid. If the culture becomes very turbid overnight it's probably contaminated with bacteria, and you'll have to start over. The picture below shows four uncontaminated cultures and one (the 4th from the left; labeled "Clone D") contaminated by bacteria. The contaminated culture is more turbid and a lighter brown than the uncontaminated cultures.

C3. Pour the culture previous step into a sterile disposable 50-ml tube to allow easy pipetting without contaminating the culture.

C4. If feasible, it is a very good idea to examine a 1/10 dilution of the culture (being careful not to contaminate the culture when making the dilution) under the microscope at 400× magnification, to confirm the absence of contaminating bacteria or other microbes. A pure culture should look like the picture below; the cells are ~2.5 µm in diameter, and some of them may have buds of various sizes (few if any small buds are visible in the cells pictured below, which are in stationary phase). Bacteria are considerably smaller, with a rod length or coccus diameter of ~1 µm; they're also much more numerous and frequently motile.



C5. Tare a small (e.g., 50-ml) Erlenmeyer flask and record tare weight; into the flask pipette ~3 ml glycerol (doesn't have to be measured accurately); re-weight the flask to get gross and net weights; divide the net weight in grams by 1.26 (the density of glycerol) to determine the volume of glycerol in ml, calling that volume "1 vol"; add 7/3 vol water, to bring the glycerol concentration to 30%; cover the flask and autoclave; cool to room temperature.

C6. In a clean work area (or better yet, in a laminar flow hood if available), sterilely pipette 50 µl of the 30% glycerol previous step into the 20 to 100 microtubes (see Materials).

C7. Sterilely pipette 50-µl portions of the culture from the 50-ml tube step C3 into each of the microtubes previous step; close caps of the tubes; vortex the tubes to mix the culture in with the

30% glycerol; store the tubes in a –80ºC freezer. Use a single tube to start the YFT1 culture each year.

INTRODUCING STUDENTS TO THE FLUCTUATION TEST

In our implementation, we don't even give students written step-by-step protocols for Sessions 1 and 2; those instructions are delivered orally during Sessions 1 and 2 themselves, as outlined in the corresponding sections below. Instead, they are assigned a PowerPoint handout **YeastFluctuationSessions1&2.ppt**, that introduces them to the two hypotheses in contention—what we call there the *pre-exposure* and *post-exposure* hypotheses—and outlines the yeast fluctuation test that they themselves will be performing. In the Notes section under each slide there are extensive notes, which are required reading. In theory, this document contains enough information for students to deduce the contrasting colony count distributions anticipated for the pre-exposure hypothesis versus the post-exposure hypothesis. In practice, however, students need gentle but persistent prodding on the part of the teacher in order to gradually come to an understanding of the fluctuation test's logic. This "prodding" takes place in numerous informal discussion periods fitted into unused blocks of time during the lab meetings.

D. SESSION 1

ASSIGNED READING TO BE COMPLETED IN ADVANCE

YeastFluctuationSessions1&2.ppt. Slides from this document can also be projected during class discussion

MATERIALS

1 YFT1 glycerol tube from –80ºC freezer (step C7)

2 YEPGA petri dishes (step B11)

1 250-ml bottle of YEPGA liquid medium (step A10; check for contamination)

120 13-ml polypropylene tubes with separate polypropylene screw caps; diameter 16.5 mm; Sarstedt 60.541.021 or equivalent

120 selective petri dishes (step B7)

Individually wrapped sterile plastic inoculating loops with 1- and 10-µl loops at the two ends; e.g., Fisher 22-363-602; below is a picture of the smaller (1-µl) loop; at least 5 needed for Session 1 and another ~30 needed for Session 3

(OPTIONAL: see note step D5) Hausser-Levy hemocytometer (cell counting chamber) with double Neubauer ruling (e.g., Fisher 02-671-55A); a hemocytometer costs ~$300, and since it's used only on a single day it's best to arrange to borrow one from a research lab or hospital clinical lab.

(OPTIONAL: see note step D5) 26×20 mm glass cover slips for hemocytometer

(OPTIONAL: see notes steps D5 and D9) Microscope with 10× ocular lens and 10× and 40× objective lenses

PRIOR PREPARATION

D1. 10 days before Session 1, streak from a YFT1 glycerol tube (Materials) on a YEPGA petri dish (Materials); incubate in a 30º incubator; after 5 days at least a few well-separated white colonies should have grown to ~2 mm.

D2. 5 days before Session 1, re-streak a single colony from the streak dish previous step on another YEPGA petri dish; after 5 days well-isolated white colonies should have developed in the third streak.

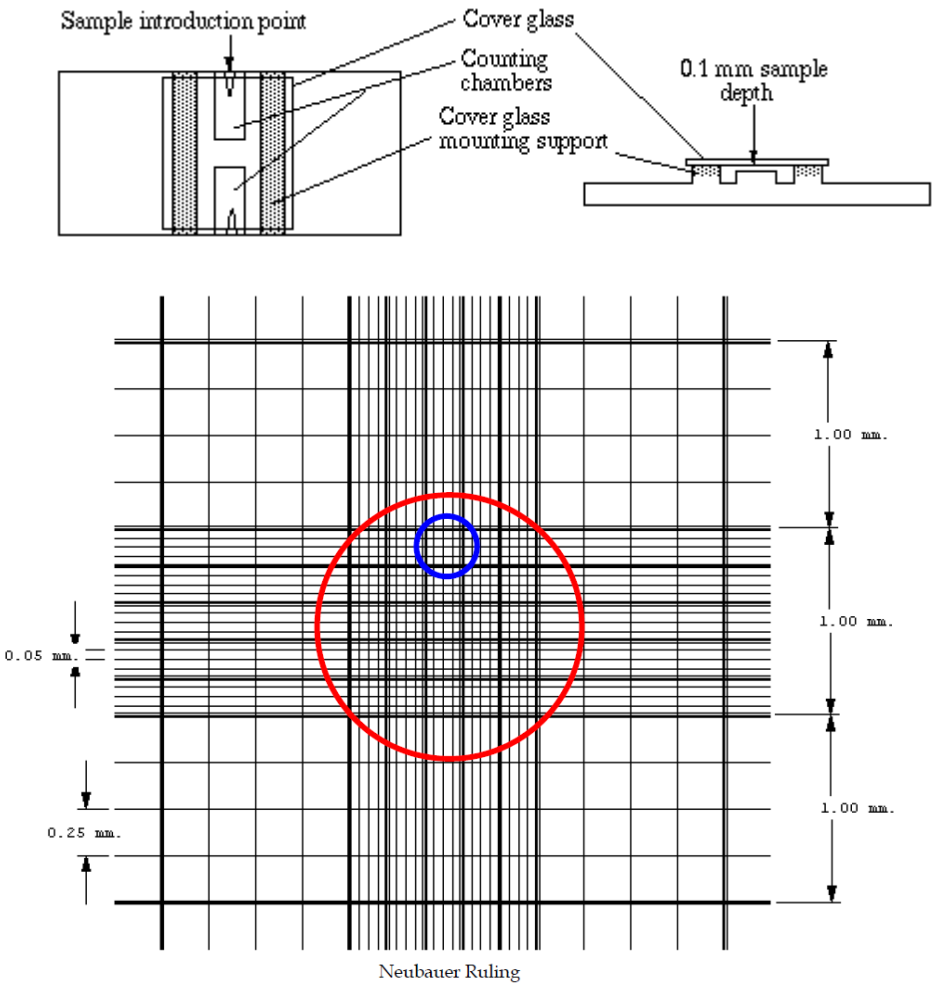
D3. Arrange 120 13-ml culture tubes (Materials) without their caps in autoclavable racks covered with aluminum foil; arrange 120 caps face down on paper towels in a covered aluminum baking pan or other suitable autoclavable container; autoclave the tubes and baking pan with the caps; when cool and dry, screw caps onto the tubes firmly but not too tight. If you elect to pre-label vessels, as suggested in the section entitled Note on Pre-Labeling above, affix labels to the tubes: 70 tubes labeled for individual 60-µl cultures, 50 tubes for 60-µl bulk culture samples. (The 50 tubes for bulk-culture samples won't be used until Session 2.)

D4. About 2 hours before Session 1, use sterile disposable plastic inoculating loops (see picture below) to pick up five well-separated yeast colonies step D2 (colonies A–E) and suspend them in 100 µl of sterile water in sterile 500-µl microtubes; vortex the microtubes vigorously in order break up clumped cells and suspend the cells evenly in the medium; allow the tubes to stand for 5 minutes to allow remaining large clumps and debris to settle while single cells remain in suspension; then pipette 70 µl of each into to a sterile 1.5-ml microtube containing 280 µl of sterile water, thus making a 1/5 dilution of the suspension; vortex the tube to thoroughly suspend the yeast cells.

D5. (NOTE: This step is complicated and requires an expensive hemocytometer (Materials). Its purpose is to determine the cell concentrations in the 1/5 dilutions previous step. This step can be safely skipped by assuming a cell density equal to the average cell density we have observed over the years in place of the actually measured cell densities in the 4th column of the table below. The exact cell densities have almost no effect on the experiment.) Pipette a 20-µl portion of each 1/5 diluted suspension previous step into a counting chamber; count the number of cells in two 0.2 mm × 0.2 mm squares (like the one circled in blue below) per suspension under a microscope, entering the results in a table like the one below (which shows typical results; if the sum of the counts is less than 61, try another colony); from those counts estimate the number of cells per ml of suspension by multiplying the sum of the two counts by 125,000, entering those estimates in a table like the example table below; calculate the volume of each 1/5 dilution to add to 22.5 ml medium to make a final dilution of 10^5 cells/ml, using the formula

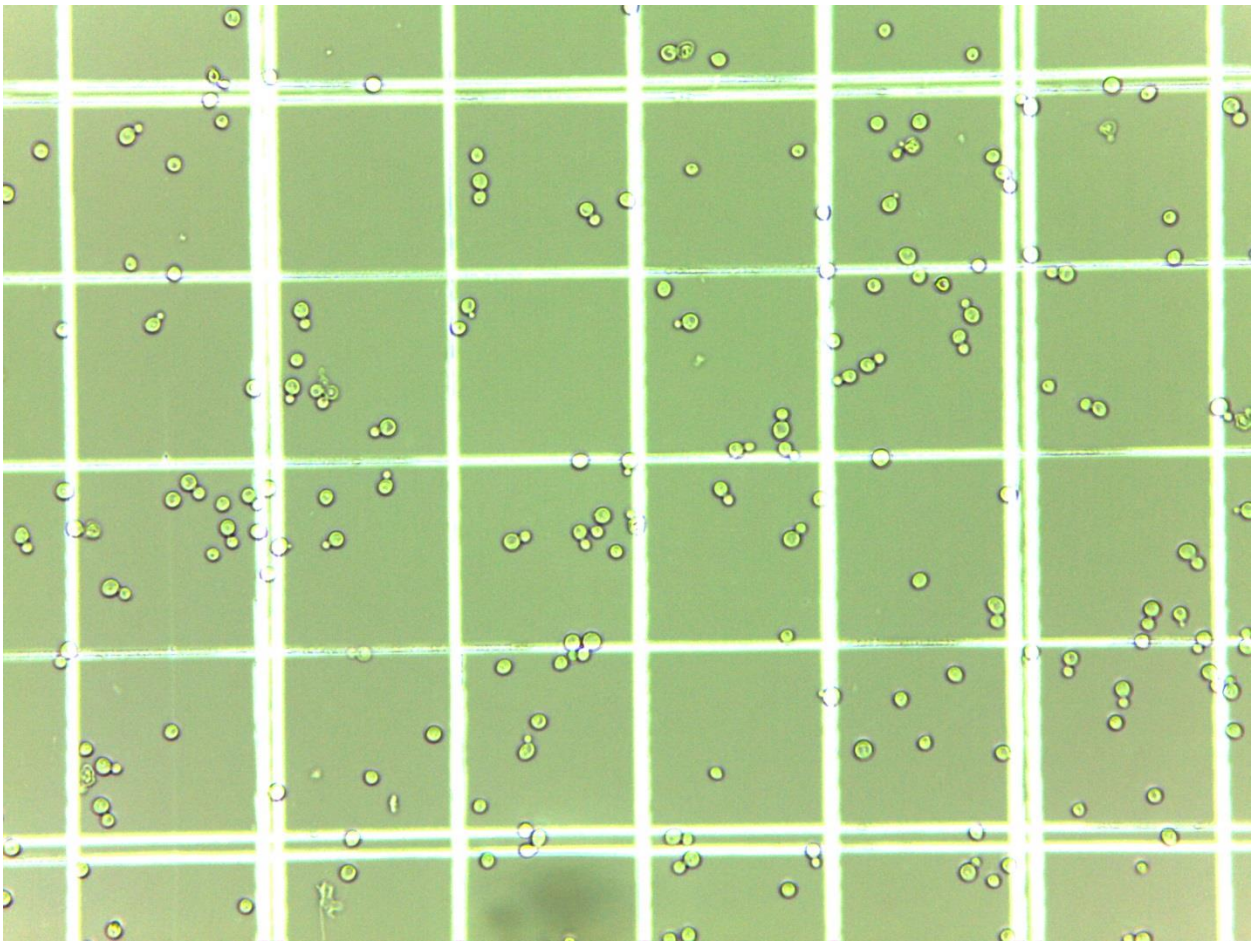
$$\text{volume (microliters)} = \frac{22500 \cdot 10^5}{(\text{cells/ml in 1/5 dilution}) - 10^5}$$

; enter that volume in the last column of the table like the example table below.



Counting chamber with Neubauer ruling; used with 26×20 mm cover glass; sample depth (space between bottom of coverslip and top of counting chamber) = 0.1 mm.

Below is a micrograph at 400× magnification showing a single complete 0.2 × 0.2 mm square bordered by double lines, containing 16 0.05 × 0.05 mm squares delineated by single lines. There are ~100 yeast cells in the 0.2 × 0.2 mm square, each with a diameter of ~2.5 µm. Several of the cells have buds, indicating that there was still some growth occurring in the colony: we include large buds (almost the size of the mother cell) but not small buds in the cell count.



Here is a table of typical cell count data.

Clone	Count in first square	Count in second square	Cell concentration (cells/ml)	Volume to add next step (µl)
A	63	60	1.538E+07	147
B	45	44	1.113E+07	205
C	55	55	1.375E+07	165
D	49	50	1.238E+07	183
E	54	54	1.350E+07	168

D6. Inoculating bulk cultures:

If you skipped the previous step: pipette 175 µl from 1/5 dilutions A–E step D4 into correspondingly labeled sterile 50-ml tubes containing 22.5 ml YEPGA medium to make ~10^5-cell/ml suspensions (the exact cell concentration not important). These are the bulk cultures.

If you did the previous step: pipette the volumes of 1/5 dilutions A–E step D4 calculated in previous step into correspondingly labeled sterile 50-ml tubes containing 22.5 ml YEPGA medium, to make 10^5-cell/ml suspensions. These are the bulk cultures.

NOTE: The reason for starting with 5 bulk cultures rather than 1 is that bulk cultures occasionally become contaminated (see step C2 above). This doesn't undermine the experiment, because the chance that any one individual 60-µl portion of a bulk culture is contaminated at this stage (before incubation) is extremely low, even if the bulk culture as a whole is contaminated. However, in Session 2 (after the cultures have been incubated for 5 days, allowing proliferation of any contaminant), at least 1 uncontaminated bulk culture is required for the bulk culture control dishes. Starting with 5 rather than 1 bulk culture ensures that at least one bulk culture remains uncontaminated.

D7. Pipette two 600-µl portions of each suspension into two sterile 1.5-ml microtubes labeled with team numbers (e.g., 10 teams with 2 students per team); students will dispense 60-µl portions from these tubes into seven individual 13-ml culture tubes.

D8. Meanwhile, pour the remaining volume in each 50-ml tube (nominally 21.3 ml, but exact volume not important) into a sterile 125-ml culture flask; secure the flasks in a 30ºC shaker-incubator in the student lab (see Materials below). These are the bulk cultures; they will be shaken at 30º for five days, along with the individual 60-µl cultures.

D9. (NOTE: This step requires an expensive hemocytometer (Materials). It and the corresponding step in the student lab can be skipped.) Set up two counting chambers for yeast colony suspensions (Microscopes 1 and 2) focused at 400× magnification. Don't fill the counting chambers until just before they're needed at step D13 below; otherwise, the suspension dries out, greatly distorting the counts.

MATERIALS PROVIDED TO STUDENTS

For each team of 2 students

- A 100-µl pipetter
- Sterile tips for the 100-µl pipetter
- Seven labeled sterile 13-ml screw-cap tubes for individual 60-µl cultures (step D3)
- A sterile 1.5-ml microtube labeled with team number, containing 600 µl of one of the ~10^5-cell/ml colony suspensions (step D7)
- Unwanted materials container (e.g., a disposable white plastic 1-liter beaker)

For class as a whole

- Testtube rack with 17-mm openings capable of accommodating 70 13-ml culture tubes
- A shaker-incubator set to 30ºC; with clips for the 5 125-ml culture flasks step D8 and provisions for mounting a testtube rack on the platform. Shaking will be at 250 rpm.
- (NOTE: This microscope is optional: see note at step D9) Light microscope for examining yeast cells under 400× magnification. We use a Leica DM750 microscope which allows the image to be visualized on a computer and thus projected to a screen or large-format flat-screen monitor. But two ordinary teaching microscope work fine.

DURING SESSION 1

During this session, each team of 2 students will sterilely dispense 60-µl portions of the 10^5-cell/ml bulk cultures into 7 13-ml culture tubes. These are the individual 60-µl cultures that are the core of the fluctuation test. The tubes will be put into a testtube rack, which will be mounted in the shaker-incubator.

D10. Review (or explain for the first time) use of pipetters and maintaining sterile technique.

D11. Review the steps for dispensing the 60-µl portions of the 10^5-cell/ml bulk cultures:

- Remove the caps of the 13-ml culture tubes and place them upside-down on the bench
- Vortex the 1.5-ml microtube containing the 10^5-cell/ml bulk culture
- Carefully pipette 60-µl portions of the vortexed bulk culture into each culture tube, inserting only the sterile tip, not the non-sterile stem of the pipetter, into the tube; try to aim the tip so that the 60-µl droplet falls to the bottom of the tube; if any liquid remains on the outer surface of the tip, touch the tip to the inner wall of the tube to ensure that the entire 60 µl is delivered into the tube. A single pipette tip can be used for all 7 transfers, but if the tip is contaminated it must be discarded and replaced with a fresh sterile tip.
- Carefully screw the cap tightly on each tube without contaminating it. It's important that the cap be screwed on tight to prevent evaporation during the 5 day incubation to follow.
- Give each tube a sharp snap of the wrist to drive the liquid to the bottom.

D12. Have students load their tubes in the testtube rack; secure the rack in the shaker-incubator; start incubation at 30ºC, shaking at 250 rpm.

D13. (NOTE: This step is optional: see note step D9.) Just before needed, vortex one of the 1/5 dilutions step D5 and use 20 µl of it to fill a counting chamber for each microscope; focus on a single 0.2 × 0.2 mm square at 400× magnification. If the microscope has a digital output, the image can be projected onto a screen or flat-screen monitor to the class as a whole. Otherwise, students can look at the yeast cells directly in a microscope. This will give them an idea of what yeast cells look like under the microscope. If time permits, each student can count the number of cells in a different 0.2 × 0.2 mm square, writing down his or her count. The counts can be analyzed to see if they conform approximately to the expectations of random sampling error, with the cell counts clustering about their mean. (More exactly, according to the Poisson distribution, the mean count should be approximately equal to the variance in counts.)

SESSION 1 DISCUSSION

There should be plenty of additional time in Session 1 to introduce the basic elements of the yeast fluctuation test:

- Introduction to the intellectual background of the fluctuation test; Lamarck versus Darwin in the realm of microbial resistance mutations
- Introduction to the yeast *Saccharomyces cerevisiae*, a eukaryotic microbe

- Introduction to the antibiotic canavanine[1]
- Outline of the experiment

The slides in the assigned reading, **YeastFluctuationSessions1&2.ppt**, can be used to facilitate the discussion.

E. SESSION 2

MATERIALS

At least 120 uncontaminated selective petri dishes (step B7)

50 empty labeled sterile culture tubes for the 60-µl bulk-culture samples (step D3)

70 labeled sterile culture tubes in 30ºC shaker-incubator, with the individual 60-µl cultures from Session 1 (step D12)

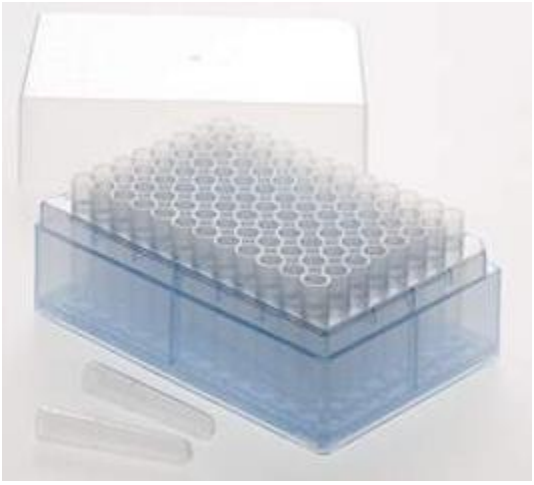
5 ~21-ml bulk cultures in 125-ml culture flasks in 30ºC shaker incubator from Session 1 (step D8)

Sterile 1.5-ml microtubes

4-mm glass beads for spreading petri dishes; e.g., Fisher 11-312B; at least 1000 beads

[1] We don't explain how canavanine poisons cells, or how resistance arises. But these things are not hard for students to understand. Canavanine is an analog of arginine; significant incorporation of the analog into proteins kills the cell. The *CAN1* gene encodes an arginine permease that is required for high-level import of canavanine. In medium containing canavanine but no arginine, canavanine is imported to toxic levels as long as the permease is functional. Severe loss-of-function mutations in the *CAN1* gene eliminate or greatly reduce permease function, sparing the cell from canavanine toxicity. Yeast strain YFT1 is prototrophic for arginine, so it can grow on the arginine-free (but canavanine-containing) selective medium.

At least 150 capless autoclavable minitubes in 10 covered autoclavable racks; e.g., Fisher 14-222-206 (tubes are purchased pre-racked and non-sterile; the tubes from all 10 racks are poured into a clean bag, and 15 tubes are put back in each rack)



PRIOR PREPARATION

E1. Put 15 minitubes in each of 10 minitube racks (Materials); into each tube count 6 4-mm glass beads (Materials); autoclave; 6 sterile glass beads will be used to spread each of the 120 selective petri dishes. (NOTE: Alternatively, each team can be supplied with a sterile tube filled with beads; ~6 beads can be tapped out of the sterile tube onto each petri dish to be spread. This is how experienced microbiologists use the beads. However, inexperienced students are liable to accidentally pour all or most of a tube's beads onto a dish. It's safer to pre-dispense 6 beads into minitubes, so that such accidents can't occur.)

E2. Check the selective petri dishes (Materials) for contamination on the day of Session 2; label 70 uncontaminated dishes for the 70 individual 60-µl cultures; label 50 uncontaminated dishes for the 50 60-µl bulk-culture samples; if there are fewer than 120 uncontaminated dishes, label fewer bulk-culture sample dishes, leaving the number of individual culture dishes at 70. It's a good idea to color-code the dishes to distinguish those containing individual 60-µl cultures from those containing 60-µl bulk-culture samples.

E3. Check the 5 bulk cultures for contamination as in step C2; pour an uncontaminated bulk culture into a sterile 50-ml tube for easy pipetting; examine a 1/10 dilution of the chosen bulk culture under the microscope at 400× magnification to confirm the absence of contamination (see step C4); if contamination is evident, choose another bulk culture.

E4. Vortex the chosen bulk culture previous step to suspend cells uniformly; pipette 600 µl of the culture into each of 10 sterile 1.5-ml microtubes, one for each team.

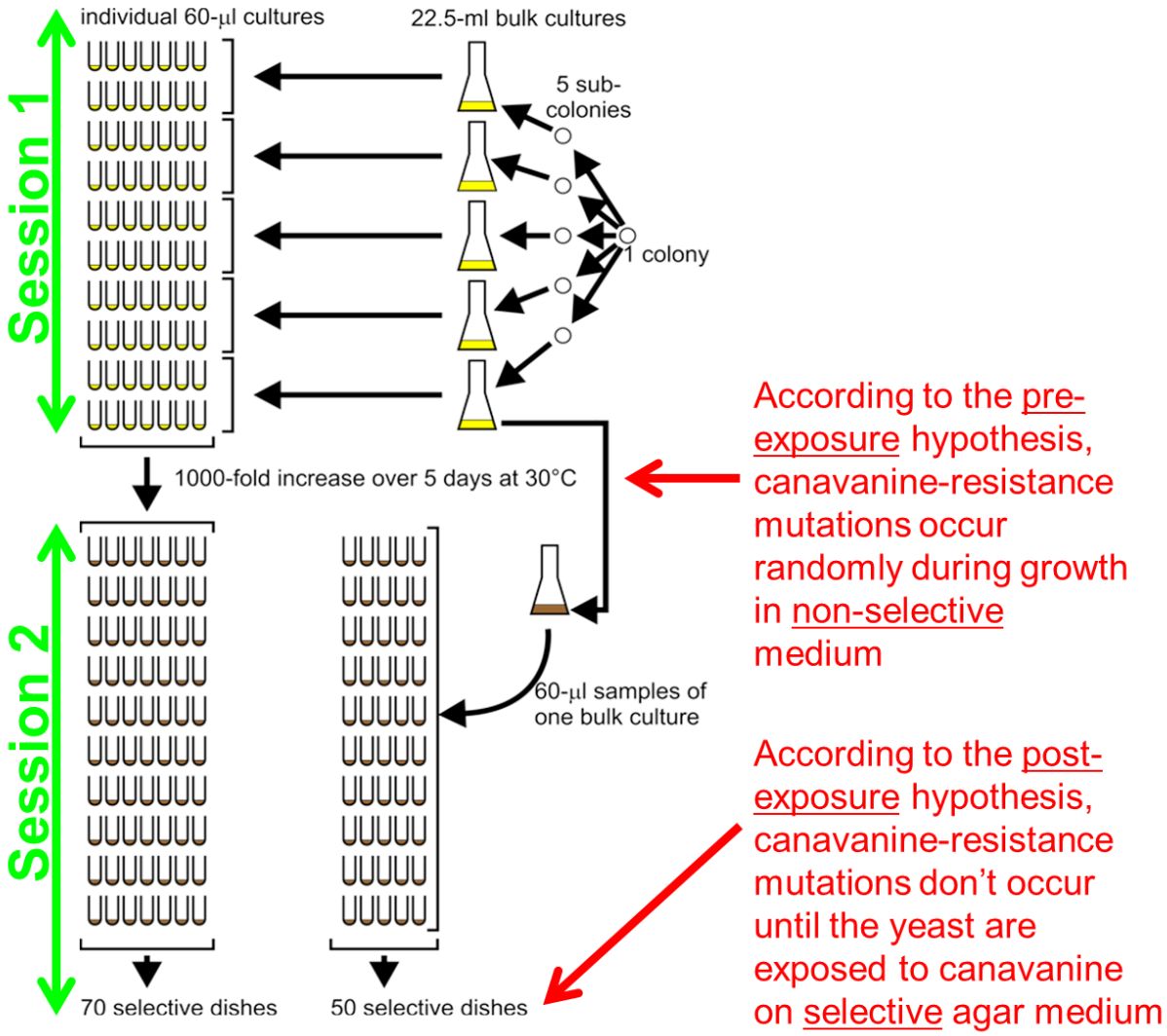
MATERIALS PROVIDED TO STUDENTS

For each team of 2 students

- A 100-µl pipetter
- Sterile tips for the 100-µl pipetter
- A 1000-ml pipetter
- Sterile tips for 1000-µl pipetter
- 12 labeled selective petri dishes, 7 for individual 60-µl cultures, 5 for 60-µl bulk-culture samples
- 5 empty labeled sterile culture tubes for the 60-µl bulk-culture samples (Materials)
- Wide-mouth bottle containing sterile water (should be easy to pipette from sterilely without contaminating inside with non-sterile pipetter stem)
- Sterile rack with 15 capless minitubes containing 6 4-mm glass beads each (step E1)
- Petri dish top or bottom for discarding used glass beads (they will be washed and dried for re-use)
- Unwanted materials container (e.g., a disposable white plastic 1-liter beaker)

DURING SESSION 2

During this session, students will dispense 60-µl portions of a single bulk culture into 50 13-ml culture tubes like the ones with the individual 60-µl cultures they set up in Session 1. They will then spread all 70 individual 60-µl cultures and all 50 60-µl bulk-culture samples on Petri dishes containing selective agar medium with canavanine. The 120 dishes will be incubated at 30ºC for a week to allow canavanine-resistant colonies to develop and for red color to accumulate. Below is a diagram showing the lineages leading from the original YFT1 colony from step D2 to the 120 selective dishes. This should occupy about half of the session; the other half can be used for classroom discussion of the anticipated results (see SESSION 2 DISCUSSION below).



E5. Have each team retrieve the 7 13-ml culture tubes with its individual 60-µl cultures (Materials).

E6. Explain how to spread cultures on selective agar dishes:

- Remove the caps from all 12 13-ml screw-cap tubes (the 7 tubes with the individual cultures step E5; the 5 empty culture tubes from step D3) and discard them in the Unwanted Materials beaker
- Vortex the 1.5-ml microtube with the bulk-culture sample (step E4), and open the cap. Without delay, use the 100-µl pipetter to sterilely pipette 60 µl of the bulk culture into each of the 5 empty 13-ml bulk culture tubes; try to deliver the drop of culture to the bottom of the tube by holding the pipetter vertically (however, don't worry if the drop ends up on the wall); the same pipette tip can be used for all 5 tubes.

- Use the 1000-µl pipetter to sterilely pipette 160 µl of sterile water into all 13 13-ml tubes; if students are careful they can use the same pipette tip for all 12 additions; however, if the pipette tip accidentally touches the inside of a 13-ml tube, they should discard it and get a new tip before continuing to fill tubes. (NOTE: The reason for increasing the volume from 60 to 220 µl is to make it easier to spread the cultures on petri dishes at the next step.)
- Spread the contents of each of the 12 13-ml tubes on the selective petri dishes as follows, being sure that tubes with individual 60-µl cultures and tubes with 60-µl bulk culture samples are delivered to the corresponding selective petri dishes:
    - Vortex the 13-ml tube
    - Pour the contents of the tube onto the dish, thumping the heel of your hand onto the bench to knock out all the liquid onto the agar surface; discard tube in Unwanted Materials beaker
    - Tap the 6 glass beads from one of the microtubes (in the blue 96-place rack) onto the agar surface of the petri dish
    - Rock the dish vigorously so that the glass beads roll over the entire surface of the agar
    - Tip the dish so that all the beads roll down to one corner
    - Tap the used beads into the empty petri dish bottom or top for used beads (we wash the beads and re-autoclave them for further use; see step E8)

E7. Allow dishes to sit at room temperature for the remainder of the session, so that the liquid is absorbed by the agar.

SESSION 2 DISCUSSION AND READING ASSIGNMENT

Students have now completed the steps of the fluctuation test, but won't acquire the results of their experiment—the colony counts—until Session 3 a week later. We use the remaining time in Session 2 (about an hour) for a classroom discussion of the results anticipated according to the pre-exposure hypothesis on the one hand and the post-exposure hypothesis on the other. We provide a PowerPoint document, **YeastFluctuationSession2Discussion.ppt**, to help guide the discussion. The PowerPoint document is also assigned as required reading in anticipation of Session 3. There are extensive notes in the Notes section under each slide; those notes are intended to reinforce what students hopefully learned from the discussion, and are an essential part of the required reading.

We try to shepherd students gently toward productive discussion, but without forcing the "correct" opinion on them. It is extremely rare for students to be able to come up with a fully coherent account on their own, but it's not at all uncommon for key elements of the logic to emerge from the class discussion. These discussions continue after the colony count data become available, in the hope that a full understanding eventually emerges.

AFTER SESSION 2

E8. Collect the used glass beads from the petri dish tops and bottoms; the beads are thoroughly washed with deionized water in a strainer and dried for re-use.

E9. Put the dishes upside down in a 30ºC incubator for 1 week.

E10. Create a master spreadsheet in which colony counts will be recorded. We provide an exemplar spreadsheet, **ExemplarColonyCountMasterSpreadsheet.xls**.

E11. Every day starting on the 4th day, dishes should be examined for contamination. Remove contaminated dishes; count the number of red and white colonies on the contaminated dishes, and record the counts in the master spreadsheet. Then discard the contaminated dishes so they don't contaminate the remaining dishes in the incubator. Typically, ~10–20 percent of the dishes ultimately become contaminated, but in very few cases does the contamination interfere with colony counting.

IN ANTICIPATION OF SESSION 3

In Session 3, students will be scoring the results of the basic fluctuation test: the distribution of the red colony counts on the bulk culture sample dishes versus the individual culture dishes.

In Session 3, students also start the colony sequencing part of the module, which will continue in Sessions 4 and 5. Colony sequencing brings into play fundamental principles of molecular biology, including the structure of DNA, the action of DNA polymerases, PCR and DNA sequencing. In our implementation, we use the lab to reinforce students' understanding of these principles, which are also covered in the lecture part of beginning biology courses.

Students are required to read a long handout, **YeastFluctuationLab3.docx**, in advance of Session 3. The handout covers the basic molecular biology outlined in the previous paragraph, and includes a step-by-step protocol for Session 3.

F. SESSION 3

NOTE ON SEQUENCING STRATEGY

Colony sequencing begins with the PCR reactions in Session 3. However, because the intellectual focus of Session 3 is on colony counts rather than sequencing, we delay explaining the sequencing strategy until the SEQUENCING STRATEGY section just before the instructions for Session 4 (Section G. SESSION 4).

MATERIALS

Selective petri dishes from step E9

Flat toothpicks with a non-pointed end for counting colonies

Individually wrapped sterile plastic inoculating loops with 1- and 10-µl loops at the two ends (at least 30 required); e.g., Fisher 22-363-602; below is a picture of the smaller (1-µl) loop

200-µl thin-walled PCR tubes with domed caps in 3 colors (e.g., Fisher 14-230-206 through 14-230-208; red, blue and green, respectively); the colors correspond to the petri dishes chosen at step F1

500-µl and 1.5-ml microtubes in same 3 colors as the PCR tubes

24 adaptors for centrifuging 200-µl PCR tubes in microfuges

Phusion Hot Start II High-Fidelity DNA polymerase; Fisher F-549S; supplied with 5× Phusion HF Reaction Buffer; both buffer and enzyme kept at –20ºC

10-mM dNTP mix; e.g., Promega #PRU1515; pH should be between 5 and 9

Forward PCR primer 5´-`gatgtaggtcttttcacctggagg`-3´; 1-mM stock in 200 mM Tris.HCl pH 8.3; stored frozen at –20ºC

Reverse PCR primer 5´-`gtctgctgttgcaacttatttg`-3´; 1-mM stock in 200 mM Tris.HCl pH 8.3; stored frozen at –20ºC

Thermocycler for PCR, with heated lid; able to accommodate at least 24 200-µl PCR tubes with domed caps

PRIOR PREPARATION

F1. From among the remaining uncontaminated <u>individual culture</u> petri dishes (not the bulk culture sample dishes) from step E9, choose 10 dishes for colony sequencing as follows

- 2 uncontaminated "jackpot" dishes, called "red" and "blue," with at least 10 well-separated red colonies (the number of white colonies doesn't matter, as long as each well-separated red colony is well separated from white colonies as well as other red colonies); 8 red colonies will be sequenced from each of these 2 dishes; assign 1 or 2 of these red colonies to individual students; the assigned red colonies from the "red" dish are numbered R1–R8; the assigned red colonies from the "blue" dish are numbered B1–B8. Materials from clones R1–R8 will be processed in red tubes; materials from clones B1–B8 will be processed in blue tubes.
- 8 uncontaminated dishes, called "green," with fewer red colonies, but with at least 2 well-separated red colonies (the number of white colonies doesn't matter, as long as each well-separated red colony is well separated from white colonies as well as other red colonies); a single red colony will be sequenced from each of these 8 dishes; assign 1 or 2 of these 8

dishes to individual students; the assigned red colonies from the "green" dishes are numbered G1–G8. Materials from clones G1–G8 will be processed in green tubes.

Count the red and white colonies on these 10 petri dishes without disturbing the colonies; record the colony counts and student-colony assignments in the master colony count spreadsheet created at step E10. Set these dishes aside so they won't be included among the dishes that the students count.

F2. (OPTIONAL: See NOTE ON PRE-LABELING at beginning of this document). Pre-label color-coded tubes as follows:

- 2 sets of 24 color-coded PCR tubes labeled R1–R8, B1–B8 and G1–G8 (for steps F3 and F13)
- 1 set of 24 color-coded 500-µl microtubes labeled R1–R8, B1–B8 and G1–G8 (for step F4)
- 1 set of 24 color-coded 1.5-ml microtubes labeled R1–R8, B1–B8 and G1–G8, unpurified PCR product (for step F15)

F3. Fill one set of color-coded labeled PCR tubes previous step with 30 µl of 0.2% SDS (for step F9)

F4. Fill the color-coded labeled 500-µl microtubes step F2 with 90 µl water (for step F12)

F5. In a 4-ml glass vial or other suitable vessel, prepare the PCR premix (except for the enzyme, which will be added just before needed):

- 1.8 ml water
- 500 µl 5× Phusion HF Reaction Buffer (Materials)
- 50 µl 10-mM dNTP mix (Materials)
- 1.25 µl 1-mM forward PCR primer (Materials)
- 1.25 µl 1-mM reverse PCR primer (Materials)

Keep on ice until needed at step F14.

F6. Put 24 adaptors for 200-µl PCR tubes in microfuge(s)

F7. Set up thermocycler with two programs:

- Lysis program (for step F10)
  - 4 minutes at 90ºC (SDS lyses yeast cells, releasing DNA)
  - Indefinite soak at 4ºC (lysate kept at refrigerator temperature until the tubes are removed)
- PCR program (for step F14):
  - 98°C for 30 sec (initial "melting" = denaturation of DNA)
  - 35 cycles of
    - 10 sec at 98°C ("melting" = denaturation)

        - 30 sec at 61°C ("annealing": primers base-pair with template strands)
        - 20 sec at 72°C (polymerizing: optimum temperature for DNA polymerase)
    - 5 min extension at 72ºC (any incomplete primer strands are completed)
    - Indefinite soak at 4ºC (final product kept at refrigerator temperature until the tubes are removed)

MATERIALS PROVIDED TO STUDENTS

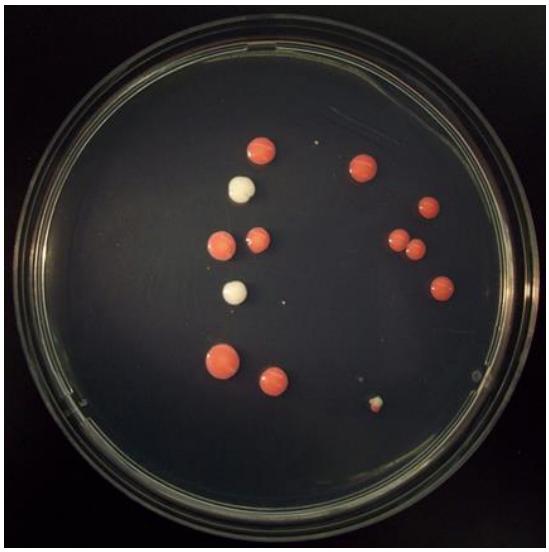
- Selective petri dishes from step E9 (a few will have already been counted and either discarded because they were contaminated or set aside for sequencing at step F1)
- Flat toothpicks (with one blunt, unsharpened end) for counting colonies; need not be sterile
- Data sheets in which students will record their colony counts; colony counts from steps E11 and F1 will already be entered in the data sheets; we provide an exemplar set of data entry sheets: **ExemplarColonyCountDataEntrySheets.docx**.
- Color-coded, labeled 200-µl PCR tubes, each containing 30 µl 0.2% SDS (step F3)
- Color-coded, labeled 500-µl microtubes, each containing 90 µl water (step F4)
- Color-coded, labeled empty 200-µl PCR tubes for the PCR amplifications (step F2)
- Color-coded, labeled empty 1.5-ml microtubes for storing the final PCR products (step F2; these will be filled by the instructor after the lab, not by the students)
- Sterile, disposable white plastic inoculating loops; individually wrapped (Materials)
- 10-µl pipetters
- Tips for 10-µl pipetters
- Unwanted Materials beakers for discarding used inoculating loops, tubes and tips
- Microcentrifuge(s) with adaptors for centrifuging 200-µl PCR tubes (step F6)
- The pre-mixed PCR ingredients from step F5 (the enzyme will be added at step F14)
- Thermocycler with Lysis and PCR programs (step F7)

DURING SESSION 3

Colony counts

F8. Students count the red colonies on their petri dishes, using the blunt end of a toothpick to obliterate each colony as it's counted. Count as red only those colonies that are large and definitely red (see photo below, which shows 10 red colonies); all small colonies and faint pinkish colonies should be counted as white, not red. The definitely red colonies will be relatively uniform in size and easy to count, while the white colonies will often differ greatly in size and be difficult to count unambiguously. Record the red colony counts in the colony count data entry sheet provided (see MATERIALS PROVIDED TO STUDENTS), being careful to get the dish numbers correct. Then count and record the white colonies in the same manner, again being careful to get the dish numbers correct. Don't worry about the size variation in the white colonies. It's the red colonies that are important in the experiment; it's OK if a few of your white colony counts are only approximate because there are so many white colonies of widely varying sizes on those dishes. Some of the spaces will already be filled in because the dishes have already been counted and either discarded because they were contaminated or set aside for colony sequencing. The colony count data entry sheets will be collective so the results from the

entire class can be entered in the master colony count spreadsheet, which will be distributed to all the students.



Colony PCR

F9. Each student will be assigned a particular petri dish and a particular number of clones from each dish. These are the clones whose genes will be sequenced. Use the **small** (1-µl) loop of a plastic inoculating loop (Materials) to pick up a bit of red colony (about this much: ●), avoiding picking up any of the agar (if you do accidentally pick up a significant amount of agar, discard the inoculating loop and try with another colony); immerse the loop in the 30 µl of SDS detergent solution in a properly color-coded 200-µl PCR tube (step F3); if the colony material sticks to the loop, vortex the tube with the loop held immersed in it; once the colony material is dislodged from the loop, discard the loop in an Unwanted Material beaker and close the cap of the PCR tube; vortex the PCR tube vigorously until the cells in the colony are completely suspended.

F10. Put the PCR tubes in the Thermocycler; run the Lysis program (step F7), which will heat the tubes to 90º for 4 min.

F11. Using the special adaptors (step F6), microfuge the heated PCR tubes for 5 min to pellet insoluble material.

F12. Use the 10-µl pipetter with a 10-µl pipette tip to draw up 10 µl of supernatant from each centrifuged PCR tube, being very careful to avoid letting the pipette tip reach the pellet; deliver the 10 µl of supernatant into a properly labeled and color-coded 500-µl microtube step F4 (already contains 90 µl water); close the cap of the microtube; vortex it to mix the supernatant in with the water. After removing 5-µl portions of the supernatants for PCR amplifications next step, the 500-µl microtubes will be stored in the deepfreeze as a backup.

F13. Pipette 5 µl of each supernatant into the corresponding empty labeled color-coded 200-µl PCR tube step F2, using a different tip for each tube.

F14. When all 24 PCR tubes are ready, 25 µl enzyme will be added to the premix step F5, the premix will be vortexed gently to mix its contents, and a 95-µl portion of the resulting PCR ingredient premix will be pipetted into each PCR tube previous step, changing tips with each addition; close the caps of the PCR tubes securely; vortex the PCR tubes; microfuge briefly as at step F11; put the tubes in the thermocycler and run the PCR program (step F7), which will commence the repeated temperature cycles:

- Initial heating subprogram: 98°C for 30 sec (initial "melting" = denaturation of DNA)
- Links to cycling subprogram: 35 cycles of
  - 10 sec 98°C melting (denaturation)
  - 30 sec 61°C annealing (primers base-pair with template strands)
  - 20 sec 72°C polymerizing (optimum temperature for DNA polymerase)
- Links to extension subprogram: 5 min at 72ºC (any incomplete primer strands are completed)
- Links to soak subprogram: indefinite time at 4ºC (final product kept at refrigerator temperature until the tubes are removed)

Here is a list of the ingredients and their final concentrations in the PCR reaction mixture:

Component	Final concentration
Yeast gDNA from one of the red colonies	~0.1–10 fM yeast genomes
Forward PCR primer	0.5 µM
Reverse PCR primer	0.5 µM
dNTPs	200 µM each
Reaction buffer to supply required salts and keep pH optimal for the DNA polymerase (supplied as 5× concentrate)	1× concentration
Thermophilic DNA polymerase from an extremophile archaea	20 enzyme units/ml

After the thermal cycles, we expect a final amplified PCR product concentration of ~50 nM (25 µg/ml)—at least a 5 million fold increase compared to the starting gDNA concentration.

DISCUSSION

The lab manipulations take up about 1–1.5 hour of Session 3. The remaining time can be used to start discussion of colony count data (next section).

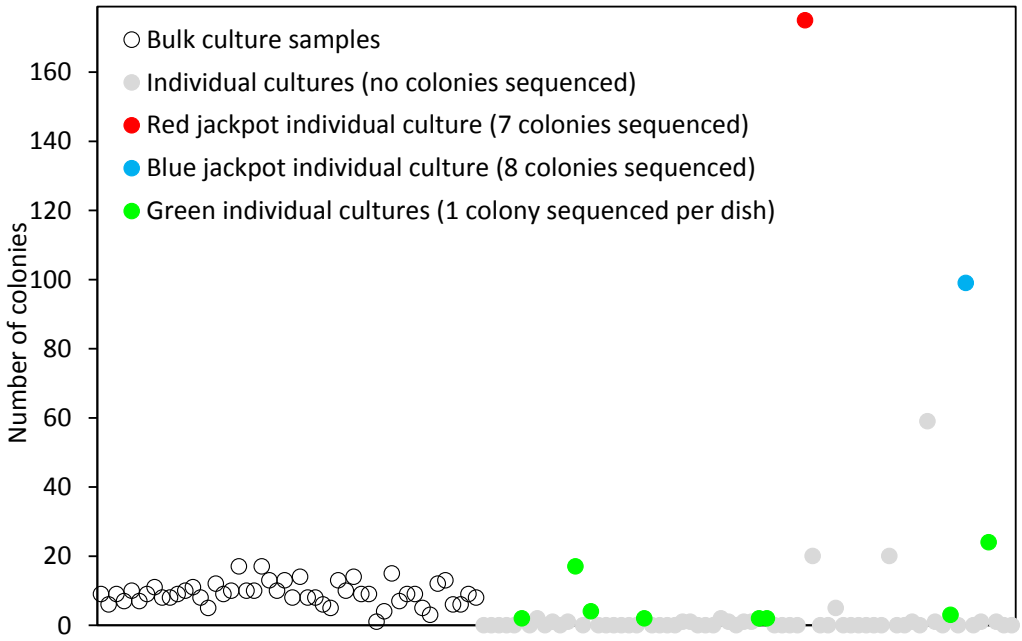
AFTER SESSION 3

F15. When the temperature cycles previous step are complete after the lab, the instructor transfers the PCR reaction mixtures (the unpurified PCR products) to the corresponding color-coded labeled 1.5-ml microtubes step F2; these will be stored in the freezer for processing in Session 4.

F16. Enter the students' colony count data from the data entry sheets step F8 into the master colony count spreadsheet, thus completing the spreadsheet. In our lab course, the completed spreadsheet is distributed to the students, who are required to create a scattergram of the colony counts (see next Section) themselves as a problem set assignment. Alternatively, the instructor can create the scattergram. We proved a completed spreadsheet exemplar, **ExemplarColonyCountMasterSpreadsheetCompleted.xls**, that includes a scattergram. (The completed spreadsheet also includes a table of Poisson statistics.)

DISCUSSION OF COLONY COUNT DATA

The expectations for the colony counts on the pre- and post-exposure hypotheses are explained in the American Biology Teacher article. Below is a graph of the actual red colony counts from one year (white colonies are also counted, but those counts aren't analyzed in the class). Open circles show the counts from the 50 bulk culture sample dishes. As expected on both pre- and post-exposure hypotheses, they cluster around their mean. The red, blue, green and gray filled circles show the counts from the 70 individual culture dishes. As expected on the pre-exposure hypothesis, but not on the post-exposure hypothesis, the counts are very scattered rather than being clustered about their mean.[2]

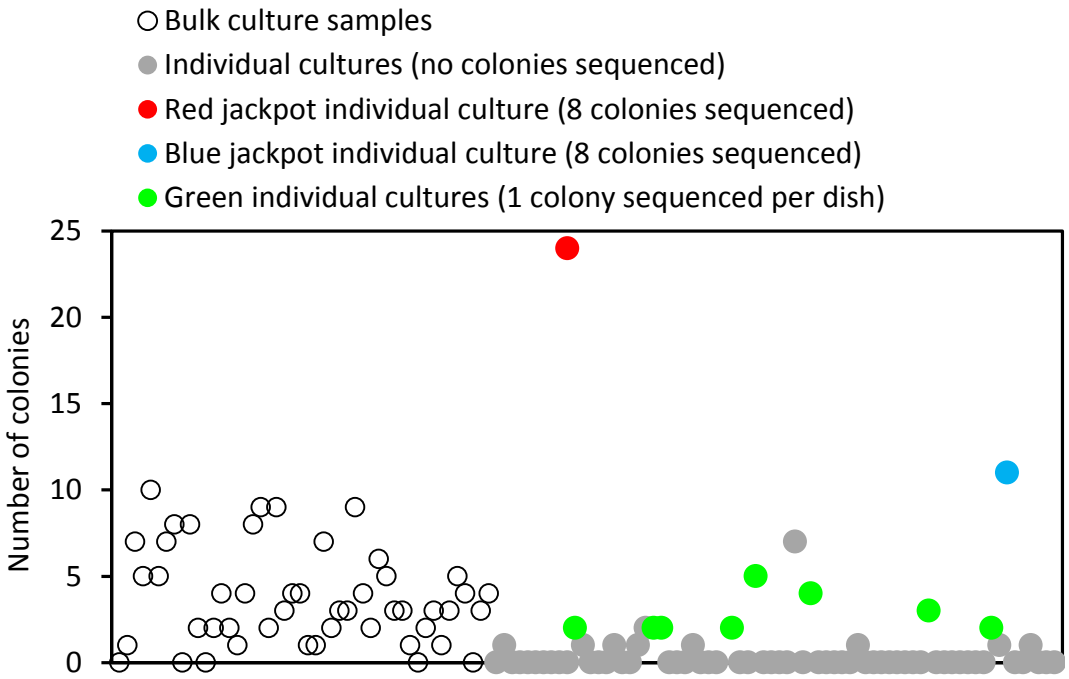


The red and blue circles in the scattergram above correspond to the "red" and "blue" jackpot dishes (step F1 in Session 3); 8 red colonies were sequenced from each of these dishes. The 8 green circles correspond to the 8 "green" dishes (step F1 in Session 3); 1 red colony was

[2] The contrasting results can be described more succinctly in statistical terms. The bulk culture sample dishes had a mean colony count of 9.18 and a colony count variance of 10.63; the two statistics are close to each other, as predicted for both pre- and post-exposure hypotheses by the Poisson distribution (see next section). For the individual culture dishes, in contrast, the variance (609) was 95 times larger than the mean (6.40), a result that is entirely expected on the pre-exposure hypothesis but entirely unexpected on the post-exposure hypothesis.

sequenced from each of these dishes. The sequencing procedure continues in Session 4, and the resulting sequences are discussed in Session 5.

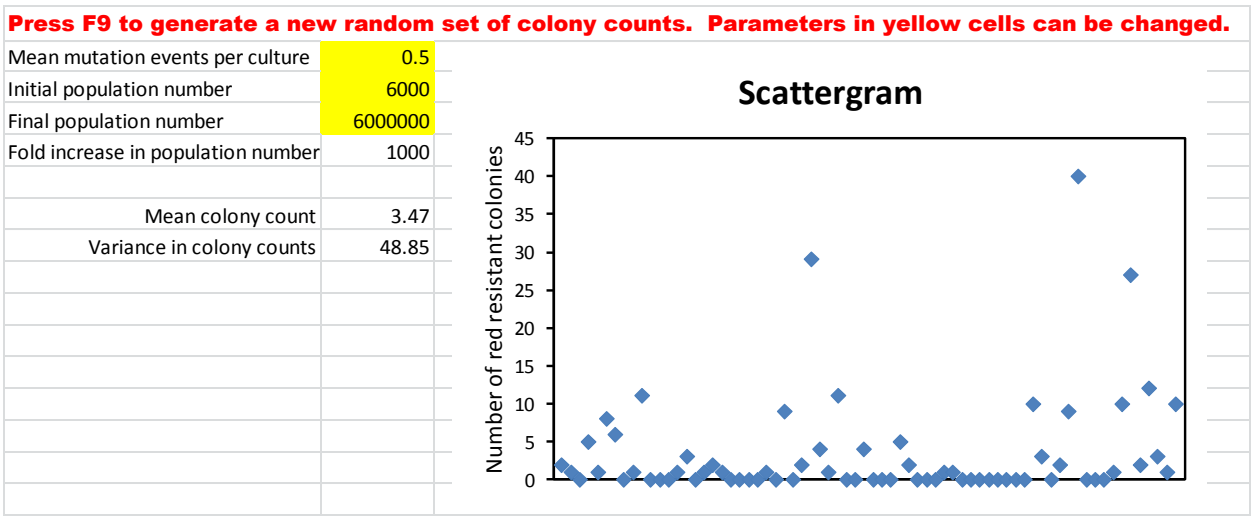
It should be emphasized that according to the pre-exposure hypothesis, canavanine resistance mutation events occur randomly, both with respect to number per individual culture and with respect to time during growth of those individual cultures in non-selective medium. Accordingly, we must also anticipate year-to-year variation in numerical data. We certainly can't expect colony counts to be superimposable from year to year. In one year, for example, the bulk culture itself turned out by chance to be a jackpot culture, so that the bulk-culture sample colony counts were clustered around 300 rather than 9. In another year, whose results are graphed below, the mean red colony counts were low (3.75 for the bulk culture samples; 1.03 for the individual cultures), and there were no individual jackpot dishes with dramatically high colony counts, so that the variance in individual colony counts was only 10 times higher than the mean.



Despite the numerical differences in colony counts from year to year, those aspects of the colony count distributions that are salient in distinguishing between the two competing hypotheses are consistent. In particular, the bulk culture sample colony counts are clustered about a mean (though that mean and degree of clustering may vary greatly from year to year), while the individual culture colony counts are very scattered, with many dishes showing no colonies and a few dishes showing many colonies. In no year have the results contradicted this summary.

In order to give instructors (and possibly students) an idea of the year-to-year variation to be expected, we provide an Excel document, **FluctuationSimulation.xlsx**, that simulates the fluctuation test according to the pre-exposure hypothesis. It uses a random number generator to simulate random mutation in each of the 70 individual cultures, assuming fixed values of the salient parameters (the initial and final number of cells per culture, and the mean number of mutation events per culture; numbers highlighted in yellow). The colony counts are displayed as

a scattergram. Each time the recalculate key F9 is pressed, a new independent simulation is launched. Below is a screen shot of the simulator.



POISSON DISTRIBUTION—AN OPTIONAL MATHEMATICAL ENRICHMENT

Our yeast fluctuation test module is taught in the context of a freshman biology lab that seeks to integrate simple mathematical analysis into the beginning natural science curriculum. No mathematics background beyond high school algebra is assumed.

The Poisson distribution is a recurring theme in the course, enriching students' understanding of both the yeast fluctuation module and of other modules at multiple stages. The Poisson distribution is not derived in the course, but the general conditions under which it applies are spelled out and its key characteristics are described in a short "thumbnail" document **PoissonDistribution.docx**.

At several points in the yeast fluctuation module where numbers (e.g., colony counts) are said qualitatively to "cluster about their mean," the Poisson distribution allows substitution of a much more specific numerical description: the variance in the numbers is close to their mean. Here is a list of components of the fluctuation test investigation in which the Poisson distribution is particularly salient:

- Optional step D13 (counting yeast cells in a hemocytometer under the microscope)
- Red colony counts (previous section):
  - According to the post-exposure hypothesis, the variance in the colony counts should approximate the mean for both bulk culture sample and individual culture dishes
  - According to the pre-exposure hypothesis, the variance in the colony counts should approximate the mean for the bulk culture sample dishes, but should be much larger than the mean for the individual culture dishes

- Assuming the pre-exposure hypothesis, the number of individual culture dishes with no colonies allows construction of a likelihood function for the mean number of mutation events per culture is $\overline{m}$. The likelihood function gauges the numerical degree of support the observed number of dishes with no colonies lends to various possible values of $\overline{m}$.

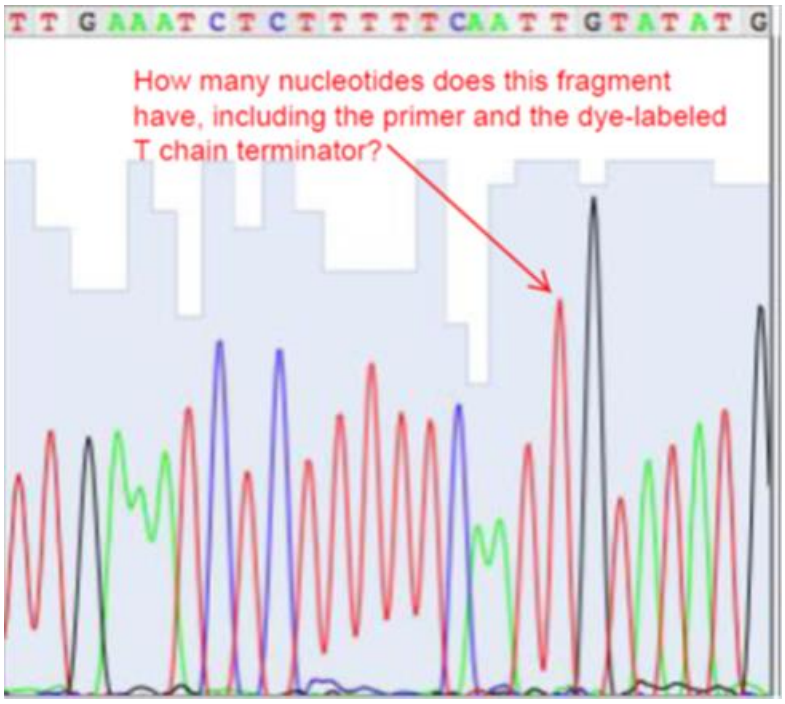
The Poisson distribution also provides a simple derivation of Beer's law, describing the absorption or scattering of light in spectrophotometry, which frequently figures in beginning biology experiments.

SEQUENCING STRATEGY

In Session 3 above, students prepared crude genomic DNA from individual red colonies, and used forward and reverse PCR primers to amplify a 763-bp segment of yeast chromosome III containing the 89 bp "red" gene. In Session 4 below, students will purify the PCR product and mix portions of the purified product separately with forward and reverse sequencing primers for submission to a DNA sequencing facility.

Shown below is the sequence of one strand (the "plus" strand) of the 763-bp PCR product from the nonmutant, canavanine-sensitive parental strain YFT1. The 89-bp red gene sequence is shown in bold red letters. The forward and reverse PCR primers correspond to the bold magenta and cyan letters, respectively. The forward and reverse sequencing primers correspond to the bold blue and green letters, respectively. (The T highlighted in black will be explained below.)

5′-**GATGTAGGTCTTTTCACCTGGAGG**TGCGGCTGGGGTACCGAAGACTAATTGAGC
TTGTACGGTCCAAGACTCAGGGATTTTGCTTGGCAAAGCAGCTTTTATGTAACCATT
GTAGTGTTGTAGGTGACCACCCAGGCCCATTGCCTCCAAGGCAACCCACGAGTTGAT
TTGAGCGGCACCAGAGGTATGGTCCGCGAAACTA**GGGAATGCAGCTGCGTACGC**TGG
GAAGTCAGCCTTTAGCTTTTCAGTTACCTTGGGATCCGGGACCGGATAATTATTTGA
AATCTCTTTTTCAATTGTATATGTGTTATGTAGTATACTCTTTCTTCAACAATTAAA
TA**CTCTCGGTAGCCAAGTTGGTTTAAGGCGCAAGACTTTAATTTATCACTACGAAAT**
CTTGAGATCGGGCGTTCGACTCGCCCCCGGGAGATTTTTTGTTTTTTATGTCTCCAT
TCACTTCCCAGACTTGCAAGTTGAAATATTTCTTTCAAGAATTGGCCTCATCCCTTG
CTGAAGCAGGCTCTTTTGA**CCGGCAGGGCTTTCTATAGCC**TTAGTCACTTCGTCCCA
AACTTTTTTGTGAGTTTCACCAGTCAAGATAACAGCGCGATTTGGCTGGGAGTTGAA
AGCGGTGGGTGTTTCTTTAATGATGGTTTGGACGACGGATTGGATGTCGTTGATAGT
AATTTCACCAGGTAACTCCGGTTTCAAAGCGTAAATAGTACGACGAGCAGTTAAAGT
TTT**CAAATAAGTTGCAACAGCAGAC**-3′



To the right is shown part of one of the sequencing documents returned by the DNA sequencing facility (the question in red and the T highlighted in black above will be explained in the next paragraph). On the bottom are shown the four false-colored traces corresponding to the fluorescent signals from the four bases A (green), G (black), C (blue) and T (red). Along the top is the nucleotide sequence deduced from the traces by a base-calling algorithm. It is obvious by inspecting the traces themselves that the algorithm has called the correct base at each position. Sometimes, however, the algorithm makes perfectly obvious errors that can easily be corrected by visual inspection of the traces. Making those corrections is called "editing." The corrections can be made directly in any program for visualizing the sequencing data, and the resulting edited sequences can be exported in many formats. Editing the sequences can significantly improve the success of sequence analysis, as explained in the footnote at step H1 below.

In our full 11-session implementation of the fluctuation test module, there is a lecture on the logic of DNA sequence analysis, and students are assigned a problem set that tests their understanding of that logic. Here is an example of such a problem set question, referring to the picture above:

> The accompanying picture shows part of one of your edited sequencing documents (it doesn't matter which one). Which sequencing primer, forward or reverse, was used to obtain this sequence? What is the exact length in nucleotides of the fragment at the indicated red peak, including the primer and the dye-labeled T terminator? Explain your reasoning briefly.

By comparing the sequence along the top of the picture with the known sequence of the PCR product, it's clear that the former is anti-complementary to (i.e., the "same" as) the latter, implying that the primer was the blue forward sequencing primer; so that's the answer to the first part of the question. It's also clear that the indicated red peak corresponds to the T highlighted in black in the PCR product sequence. The answer to the second part of the question is therefore 96, since that's the number of letters from the G at the beginning of the blue forward sequencing primer through the black-highlighted T.

G. SESSION 4

READING ASSIGNMENT to be completed before Session 4

YeastFluctuationSession4Discussion.ppt
YeastFluctuationSession4.docx

MATERIALS

NOTE: We use agarose gel electrophoresis to check that the PCR amplifications (Session 3) and PCR purifications (Session 4) yield the expected DNA product in about the expected amount. These "check-gels" aren't strictly required, but are advisable to avoid paying fees for sequencing non-existent samples. Gel electrophoresis is also a core technology in life science; learning about it should arguably be a part of every biology major's education. Electrophoresis requires one or more agarose gel electrophoresis units (total capacity 24 samples, with at least 1 extra well for DNA size markers in each rank of wells). We use an Owl D2 wide gel electrophoresis unit (Fisher OWD2BP; gel volume 75 ml; total travel distance from wells to end of gel 9.5 cm) with a 30-well comb (wells easily accommodate 14-µl samples); this allows all 24 samples plus size markers to be electrophoresed side by side.

(Required for gel electrophoresis) Ordinary DNA grade agarose (e.g., Fisher BP164-100)

(Required for gel electrophoresis) 50× TAE bottle (Fisher BP1332-500); 1× composition is 40 mM Tris-acetate, 1 mM EDTA, pH 8.3 ±0.1

(Required for gel electrophoresis) 7×LoDye loading buffer [dissolve 30 mg bromophenol blue in 30 ml 250 mM EDTA disodium salt, pH adjusted to 8.3 with solid Tris base; add 90 grams of glycerol by weight; mix thoroughly]. Any other electrophoresis loading buffer is OK, as long as it's added to sample to give the correct final concentration

QIAquick PCR purification kit (Qiagen #28104) with at least:

- 24 lavender spin columns in their 2-ml collection tubes
- 15 ml Buffer PB (binding buffer)
- 20 ml Buffer PE (washing buffer)
- 5 ml Buffer EB (elution buffer: 10 mM Tris.HCl pH 8.5)

1 M acetic acid, pH adjusted to 4.75 with NaOH

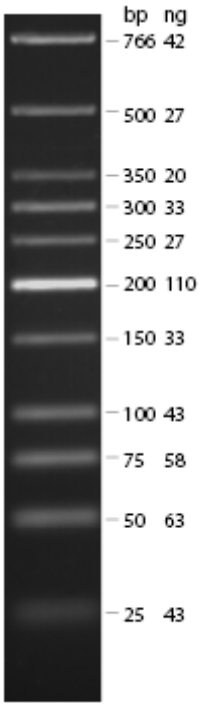
10 mM phenol red, pH adjusted to ~7 with dilute NaOH (a 1/100 dilution should have a rosé color)

Sequencing primers: 1-mM solutions in 200 mM Tris.HCl pH 8.3, stored at –20ºC

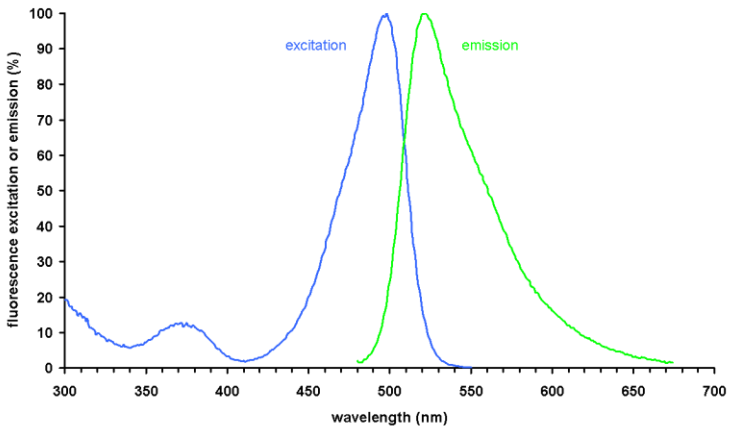
Forward sequencing primer: 5´-gggaatgcagctgcgtacgc-3´
Reverse sequencing primer: 5´-ggctatagaaagccctgccgg-3´

Unpurified PCR products step F15

(Required for gel electrophoresis) Low Molecular Weight DNA Ladder (New England Biolabs N3233L) 500 µg/ml; the band pattern is shown below. Other DNA size markers can be used, but this one is particularly convenient in that the largest marker, 766 bp, is almost exactly the same size as the intended PCR product, 763 bp.



(Required for gel electrophoresis) SYBR Green fluorescent DNA stain; 10,000× stock solution (e.g., Lonza # 50512). When bound to DNA, this cyanine fluorescent dye is maximally excited at 497 nm, and emits maximally at 520 nm (see spectra below).



The DNA bands stained with SYBR Green can be visualized on a standard 300-nm UVA transilluminator, but as a matter of student safety we use a visible light transilluminator with a blue low-pass excitation filter, in conjunction with an orange high-pass emission filter on the camera or goggles to visualize the stained gel (e.g., Dark Reader DR46B from Clare Chemical). No UV safety goggles are required.

ADVANCED PREPARATION

G1. Label 24 lavender spin columns in their 2-ml collection tubes (from QIAquick PCR purification kit; Qiagen #28104) to correspond to the colonies chosen in step F1 above; color-code the labels with blue, red or green vertical stripes to correspond to color-coded tubes:

Colony # [# = R1–R8, B1–B8, or G1–G8]
Spin column

Affix the labels to the outer collection tubes, not to the columns themselves.

G2. Label 24 color-coded 1.5-ml microtubes for the purified PCR products to the colonies chosen in step F1

Colony # [# = R1–R8, B1–B8, or G1–G8]
Purified PCR

G3. Label two sets of 24 color-coded 500-µl microtubes for electrophoresis samples to the colonies chosen in step F1

Colony # [# = R1–R8, B1–B8, or G1–G8]
electro

In a 1.5-ml microtube premix 960 µl water and 180 µl 7×LoDye loading buffer (Materials); pipette 19 µl of this premix into each labeled 500-µl microtube.

G4. Label and fill tubes with buffers from Qiagen PCR purification kit:

- 12 lavender 1.5-ml microtubes containing 1.1 ml Buffer PB

Qiagen
Buffer PB
(binding
buffer)

- 12 yellow 2.2-ml microtubes containing 1.6 ml Buffer PE

Qiagen
Buffer PE
(washing
buffer)

- 12 orange 500-µl microtubes containing 250 µl Buffer EB

Qiagen
Buffer EB
(elution
buffer)

G5. Label 48 1.5-ml microtubes for sequence submission, to correspond to the colonies chosen in step F1 as follows:

Colony # [# = R1–R8, B1–B8, or G1–G8]
Forward [*or* Reverse]
primer

In two 500-µl microtubes labeled Forward and Reverse make 3-µM dilutions of the forward and reverse sequencing primers by mixing:

- 330 µl water
- 3 µl 10 mM phenol red (Materials; final concentration 100 µM)
- 1 µl 1-M acetic acid pH 4.75 with NaOH (Materials; final concentration 3 mM)
- 1 µl 1-mM forward or reverse sequencing primer (Materials; final concentration 3 µM)

Pipette 8 µl into the 24 forward or reverse sequencing tubes. The purpose of the phenol red pH indicator is explained at step G13; at the slightly acidic pH of these primer solutions, it is pale yellow.

G6. Pour a 30-well 1.2% agarose gel as follows (the amounts in this step are for the Owl D2 wide gel electrophoresis unit specified in Materials; other electrophoresis units may require different amounts):

- In a 1000-ml vessel make 1000 ml 1× TAE by mixing 980 ml water and 20 ml 50× TAE (Materials); after use in this session, discard remaining buffer.
- Weigh out 0.9 grams of agarose and tip it into a glass 250-ml Erlenmeyer flask.
- Measure in 75 ml 1× TAE and add a magnetic stir bar.
- Weigh the flask and record the initial gross weight.
- Stretch a small piece of Saran wrap tightly over the mouth of the flask and use a needle to punch a single small hole in the middle; heat to boiling with constant stirring on a hot plate until the agarose is completely dissolved; during this step and the next, set up a minigel casting tray with a 30-well comb
- Put a lead donut on the flask and cool it to 50ºC in the 50º water bath with occasional swirling.
- Swirl the flask again to ensure that the still-molten agarose solution is uniform; remove the Saran wrap; dry off the outside and inside neck of the flask; re-weigh the flask to determine the final gross weight; add sufficient extra water to bring the gross weight back up to the initial gross weight recorded above; swirl to thoroughly mix the water in with the molten agar.
- Using a second magnetic stir bar to hold back the stir bar in the flask, pour the molten gel into the Owl D2 wide gel casting tray with 30-well comb; allow the agarose to gel thoroughly.
- Fill the buffer chamber with 1× TAE to cover the platform
- Pour 1× TAE onto the gel; carefully remove the comb (the buffer will act as lubricant)
- Loosen the casting tray; transfer the UV transparent tray from the casting tray to the buffer chamber
- Add additional 1× TAE as necessary to cover the gel, including the wells

G7. In a 500-µl microtube make low molecular weight DNA ladder sample by mixing:

- 144 µl water
- 26 µl of 7×LoDye loading buffer
- 10 µl (5 µg) NEB Low Molecular Weight DNA Ladder (Materials)

G8. Pipette 2 µl of each of the unpurified PCR products step F15 into the corresponding tubes of one set of 500-µl Ep electrophoresis sample tubes step G1, vortexing to mix.

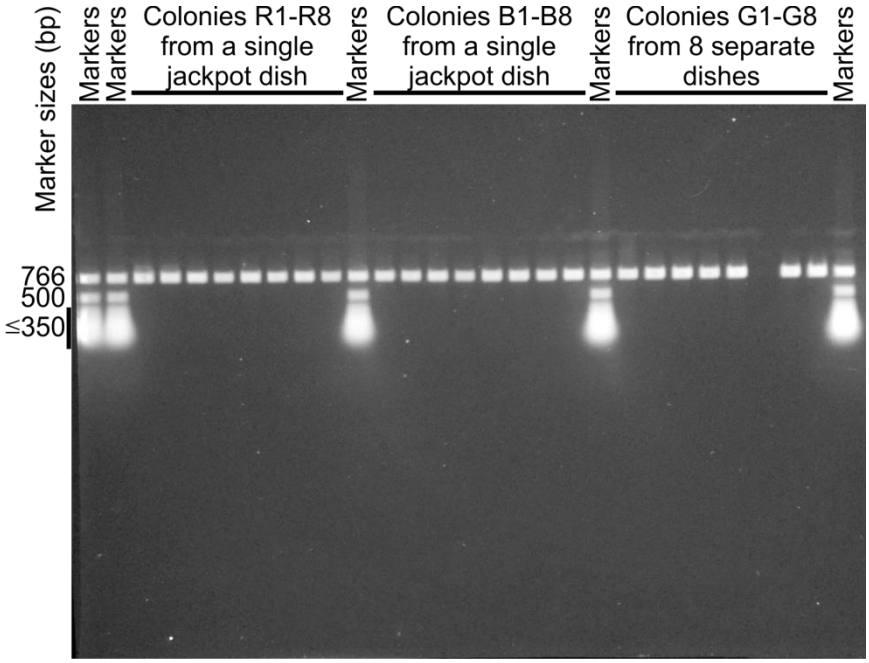
G9. Load gel step G6 with 14-µl portions of DNA markers step G4 and of the electrophoresis samples previous step in the following pattern (other patterns will be necessary as appropriate for gel electrophoresis units with fewer wells):

Ladder step G7
Ladder step G7
Colony R1 previous step
Colony R2 previous step
Colony R3 previous step
Colony R4 previous step
Colony R5 previous step
Colony R6 previous step
Colony R7 previous step
Colony R8 previous step
Ladder step G7
Colony B1 previous step
Colony B2 previous step
Colony B3 previous step
Colony B4 previous step → direction of migration
Colony B5 previous step
Colony B6 previous step
Colony B7 previous step
Colony B8 previous step
Ladder step G7
Colony G1 previous step
Colony G2 previous step
Colony G3 previous step
Colony G4 previous step
Colony G5 previous step
Colony G6 previous step
Colony G7 previous step
Colony G8 previous step
Ladder step G7
(none)

Connect the power supply and run the gels at 120 volts for 30 min.

G10. Transfer the gel to a staining dish; add enough water to allow the gel to move when the dish is jiggled; add 5–10 µl SYBR Green stock solution (Materials; exact volume not important); rock the gel at least 1 hour at room temperature in the dark; wash in water; photodocument; keep submerged in water away from the light for use as a demonstration at step G15.

A typical gel is shown below. The resolution is poor: the 500-bp DNA marker is resolved from the 350-bp and smaller DNA markers, but the latter are not individually discernible. Nevertheless, the resolution is sufficient to determine whether or not the intended 763-bp PCR product is present.[3] In this particular gel, no PCR product is evident from colony G6. This doesn't necessarily result from a procedural failure; it's also plausible that the "red" gene mutation in this mutant was a deletion that removed one or both PCR priming sites. Preparations giving no discernable PCR product are not submitted for sequencing. The other 23 colonies all gave excellent PCR yields. The 766-bp DNA ladder marker has a theoretical amount of 32.7 ng. The 763-bp PCR bands, from 1.33 µl of PCR product, approximately match that marker band in intensity. That means that the concentration of the PCR product is ~25 ng/µl, and that the entire 100-µl PCR product samples have ~2.5 µg 763-bp product.



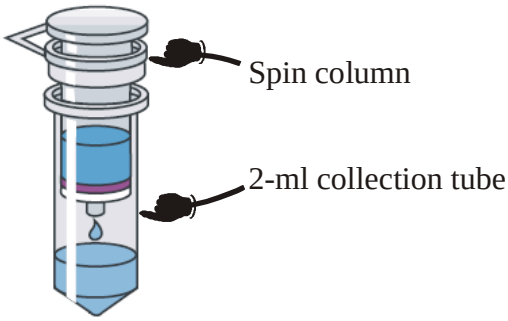
G11. (OPTIONAL In our implementation, students load and run a second agarose gel with the purified PCR products they prepare in Session 4. This second "check-gel" not only checks that their PCR purifications were successful, but also gives them hands-on experience with a core technique in molecular biology. The second check-gel will be stained and photodocumented by the instructor, but students will see the stained first check-gel in Session 4.) In preparation for Session 4, pour another 1.2% agarose gel as in step G6.

MATERIALS PROVIDED FOR STUDENTS

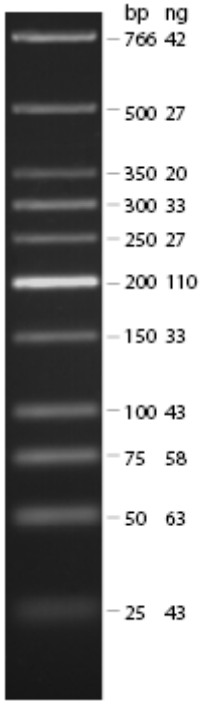
- Unpurified PCR products step F15
- Qiagen Buffer PB, step G4 (yellow-colored binding buffer; 12 lavender 1.5-ml microtubes)

[3] Much better resolution can be achieved by electrophoresis in 1.7% Metaphor agarose in 1× TAE for 90 min at 120 volts. But Metaphor agarose is expensive, and Metaphor agarose gels are much more fragile than ordinary agarose gels. We do not consider the added expense and inconvenience worthwhile.

- Unwanted Materials beakers for discarding pipette tips and other used labware
- Qiagen spin columns in their 2-ml collection tubes step G1 (24 columns with color-coded labels)



- Microcentrifuges
- Qiagen Buffer PE, step G4 (washing buffer; 12 yellow 2.2-ml microtubes)
- Tubes for the final purified PCR products, step G2 (24 empty color-coded 1.5-ml microtubes)
- Qiagen Buffer EB, step G4 (elution buffer; 12 orange 500-µl microtubes)
- Tubes for electrophoresis samples, step G3 (24 color-coded 500-µl microtubes, each containing 19 µl loading dye)
- A 500-µl microtube containing at least 80 µl DNA markers of known sizes and amounts, step G7 (size range 766 to 25 bp; nominally 389 ng total DNA per 14-µl sample, including 32.7 ng of the 766-bp marker)



- A gel electrophoresis unit, with a 1.2% agarose gel with 30 wells, step G11 (or alternative units if necessary)
- A DC power supply capable of delivering 120 volts
- A staining tray for the agarose gel
- SYBR Green stock solution (Materials)
- A rocker for rocking the gel during staining
- The stained gel from step G10
- A visible-light transilluminator and emission filter for visualizing DNA bands stained with SYBR Green (Materials)
- Micotubes containing 8 µl of 3-µM forward or reverse sequencing primer, step G5 (48 1.5-ml microtubes; contents are pale yellow)

DURING SESSION 4

G12. Each student will purify each of his or her assigned unpurified PCR product(s), step F15, on a QIAquick spin column as follows:

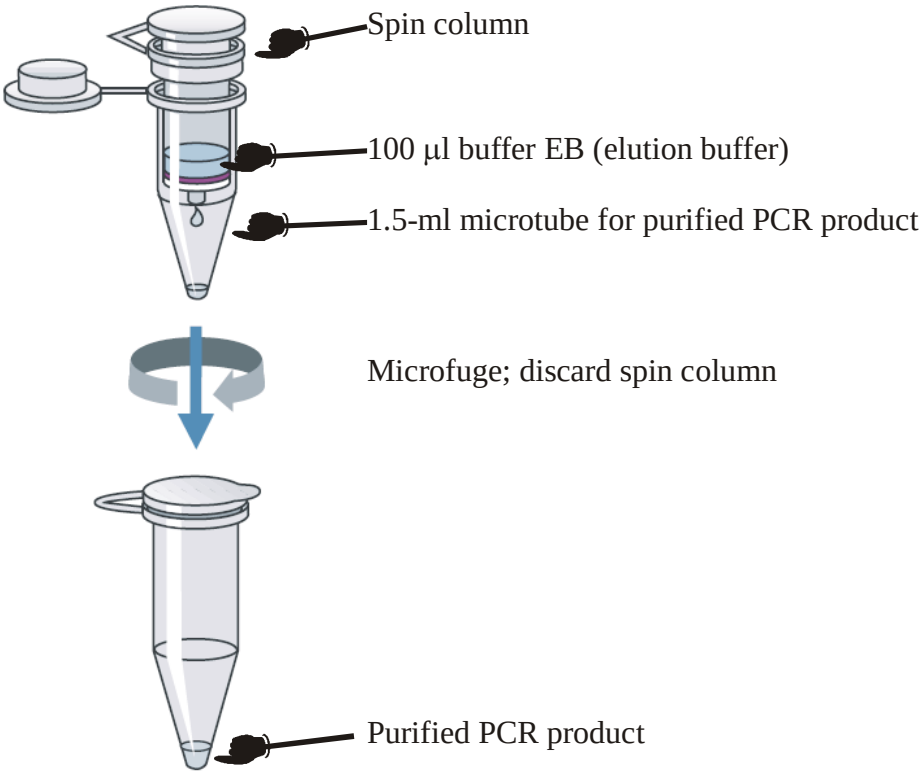
- Into the 1.5-ml microtube with the unpurified PCR product in 100 µl pipette 500 µl QIAGEN buffer PB (yellow solution in lavender 1.5-ml microtube; one tube per student); the color should remain yellow (you may not be able to see the color very well through the colored microtube walls though)
- Close the cap of the microtube and vortex it to mix the contents
- Pipette all 600 µl of the diluted PCR product into the correspondingly labeled spin column
- Microfuge the spin column for 1 min
- Discard the flow-through from the collection tube; shake out any excess flow-through; replace the column in the collection tube

  NOTE: When diluted in the PB binding buffer, the PCR product DNA binds to a filter at the bottom of the spin column, while other components of the PCR reaction mixture, including the enzyme, the unused primers, unused dNTPs, and buffer components, pass through the filter.

- Into the spin column pipette 750 µl QIAGEN buffer PE (in yellow 2.2-ml microtube)
- Microfuge the spin column for 1 min
- Discard the flow-through from the collection tube; shake out any excess flow-through; replace the column in the collection tube
- Microfuge again for 1 min to drive out all residual PE
- Shake out any residual flow-through

  NOTE: The previous five substeps wash the filter at the bottom of the spin column to remove residual unbound components, while leaving the PCR product DNA bound to the filter; the extra centrifugation to remove residual PE wash buffer is important, since any residual wash buffer can interfere with subsequent use of the purified PCR product (including sequencing).

- Wipe off the outside of the now-empty spin column and place it into the correspond empty purified PCR product 1.5-ml microtube, step G2; discard the 2-ml collection tube
- Pipette 100 µl QIAGEN buffer EB (in yellow 500-µl microtube; EB is an elution buffer that releases the PCR product DNA from the filter at the bottom of the spin column)
- Allow to stand for 1 min
- Microfuge 1 min
- Discard the labeled spin column



After use in the next step, the 1.5-ml microtubes containing the purified PCR products will be stored in the deepfreeze as backups.

G13. Pipette portions of the purified PCR product from the previous step into three microtubes as follows:

- 8 µl into the corresponding special 1.5-ml microtube for sequencing with "forward" primer (labeled on cap; already contains 8 µl of 3-µM primer); contents of tube should change color from pale yellow to red as the slightly alkaline elution buffer neutralizes the acid in the 3-µM primer solution
- 8 µl into the corresponding special 1.5-ml microtube for sequencing with "reverse" primer (labeled on cap; already contains 8 µl of 3-µM primer); the phenol red pH indicator in the tube should change color from pale yellow to red as the slightly alkaline elution buffer neutralizes the acid in the 3-µM primer solution
- 2 µl into the corresponding 500-µl microtube for electrophoresis sample (already contains 19 µl blue loading dye)

Vortex all three microtubes; the 1.5-ml microtubes will be submitted to a DNA sequencing facility for sequencing (the sequencing data will be edited by the instructor and analyzed in Session 5); meanwhile, the 500-µl microtubes with the electrophoresis samples will be used at step G14.

G14. Load the 1.2% agarose gel with the purified PCR product electrophoresis samples (previous step) and the DNA size markers, and run the gel, as in step G9.

G15. During the gel electrophoresis previous step, students view the stained gel step G10 on the transilluminator; students can photodocument the stained gel on their smartphones if time permits.

G16. When electrophoretic run step 14 is finished, stain the gel as in step G10. The stained gel will be photodocumented at step G17 after Session 4.

DISCUSSION

There is generally some unused time during Session 4. We use this time to ask students to anticipate the sequence results (see the *American Biology Teacher* article); the slides in their assigned reading **YeastFluctuationSession4Discussion.pptx** can be used to guide the discussion. Although the time might also be used to discuss gel electrophoresis, we have chosen to leave that subject to independent reading (in our full 11-session implementation of the yeast fluctuation test module, there is also a problem set question on the subject).

AFTER SESSION 4

G17. Photodocument the stained check gel step G16.

G18. Submit the sequencing samples step G13 for sequencing (samples from colonies that don't give a strong PCR product band on the check gel previous step are not submitted).

H. SESSION 5

NOTE: Bioinformatics has become a central component of life sciences research, and the yeast fluctuation test well illustrates its power. Without the paradigmatic bioinformatic tools deployed in Session 5, analyzing the colony red gene sequences would be far more difficult. Nevertheless, even in our full 11-session implementation of the yeast fluctuation test, we do not ask students to carry out the necessary bioinformatic procedures on their own. Instead, those procedures are carried out by the instructor as described in steps H1–H3 below, and introduced to students as a class demonstration rather than a hands-on student exercise. The demonstration consumes about half of Session 5. The remaining half is then available to discuss the bearing of the sequence information on the evolutionary question at issue in the fluctuation test.

BIOINFORMATIC ANALYSIS CARRIED OUT BY INSTRUCTOR

H1. When the sequence data from step G18 become available, edit the files in preparation for Session 5 (see previous Section SEQUENCING STRATEGY).[4] Export the edited sequences as text documents for input into alignment utilities such as Clustal-omega. Shown below are the two complementary sequences from a single colony in standard FASTA format. In that format, the ">" signals the start of a new sequence, and the characters following the ">" up to the next white space (space or return) are interpreted as the name of the sequence. All characters following that first white space up to the next ">" are interpreted as the nucleotide sequence itself; any white spaces within those characters are ignored.

```
>B2Forward
gttaccttgggatccGGGAcCGGataaTTatTtGaaatctttttttcaattGTAtATGTGTTAtGTAGTAT
ACTctttcTtcaACAATTAAATACTCTCtGTAGCCAAGTtGGTttAaGGCGCAagactttaaTttATCACT
ACGAAATCTtGAGATCGGGCGTTCGACTCGCCCCCGGGagatttttttGTTTTTTAtGTCTCCaTtCaCttC
CCAGACTTGCaAGTTGAAATATTTCTTTCAAGAATTGGCCTCATCCCTTGCTGAAGCAGGCTCTTTTGACC
GGCAGGGCTTTCTATAGCCTTAGTCACTTCGTCCCAAACTTTTTTGTGAGTTTCACCAGTCAAGATAACAG
CGCGATTTGGCTGGGAGTTGAAAGCGGTGGGTGTTTCTTTAATGATGGTTTGGACGACGGATTGGATGTCG
TTGATAGTAATTTCACCAGGTAACTCCGGTTTCAAAGCGTAAATAGTACGACGAGCAGTTAAAGTTTTCAA
ATAagtt
>B2Reverse
TTcTTgAaAgaaatATTTCaACTTGCAAgtctGGGAAGTGAAtGGAGACatAaAAAACAAAAAATCtCCCG
GGGGCGAGTCGAACGCCCGATCTCAAGAtTTCGTAGTGATAAATTAAAGTCTTGCGCCTTAAACCAACttG
GCTACAGAGAGTATTTAATtGTTGAAGAAAGAGTATACTACATAACACATATACAATtGAAAAAGAGATTT
CAAATAATTATCCGGTCCCGGATCCCAAGGTAACTGAAAAGCTAAAGGCTGACTTCCCAGCGTACGCAGCT
GCATTCCCTAGTTTCGCGGACCATACCTCTGGTGCCGCTCAAATCAACTCGTGGGTTGCCTTGGAGGCAAT
GGGCCTGGGTGGTCACCTACAACACTACAATGGTTACATAAAAGCTGCTTTGCCAAGCAAAATCCCTGAGT
CTTGGACCGTACAAGCTCAATTAGTCTTCGGTACCCCAGCCGCACCTCCagGTGAa
```

The lower-case letters within these sequences are positions where the algorithmic base calls had to be edited. Some of those edits lay within the 89-bp red gene; failure to make those corrections would have seriously complicated the interpretation of the sequence data.

H2. Use the forward and reverse sequences for each colony to create a single consensus sequence (called the "contig") in the forward orientation. A number of free online sequence assembly utilities are available for this purpose: for example, EGassembler at http://egassembler.hgc.jp/cgi-bin/eassembler4.cgi. Here is the consensus contig that emerges from the two complementary sequences above; it is shown below in FASTA format, and is named after the colony, B2, from which it derives. There are no discrepancies in the forward and reverse input sequences where they overlap. This will almost always be true if the input sequences are edited. In order to simplify interpretation of the sequence data, try to resolve any discrepancies that do arise.

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[4] Editing is essential, since the routine base-calling algorithm makes occasional errors that are obvious by visual inspection of the four false-colored output traces. Since the aim of sequencing is to identify a single mutational change in each colony's red gene, and since there are only two reads per colony, ambiguities arising from these obvious base-calling errors can seriously undermine the experiment. In our full 11-session implementation, the students edit at least some of their own sequence data files. This is an excellent opportunity to reinforce their understanding of the logic of DNA sequencing. However, editing by itself requires an entire session for inexperienced learners. An experienced instructor can edit the 48 sequences in about 2 hours.

```
>B2
TTCACCTGGAGGTGCGGCTGGGGTACCGAAGACTAATTGAGCTTGTACGGTCCAAGACTCAGGGATTTTGC
TTGGCAAAGCAGCTTTTATGTAACCATTGTAGTGTTGTAGGTGACCACCCAGGCCCATTGCCTCCAAGGCA
ACCCACGAGTTGATTTGAGCGGCACCAGAGGTATGGTCCGCGAAACTAGGGAATGCAGCTGCGTACGCTGG
GAAGTCAGCCTTTAGCTTTTCAGTTACCTTGGGATCCGGGACCGGATAATTATTTGAAATCTCTTTTTCAA
TTGTATATGTGTTATGTAGTATACTCTTTCTTCAACAATTAAATACTCTCTGTAGCCAAGTTGGTTTAAGG
CGCAAGACTTTAATTTATCACTACGAAATCTTGAGATCGGGCGTTCGACTCGCCCCCGGGAGATTTTTTGT
TTTTTATGTCTCCATTCACTTCCCAGACTTGCAAGTTGAAATATTTCTTTCAAGAATTGGCCTCATCCCTT
GCTGAAGCAGGCTCTTTTGACCGGCAGGGCTTTCTATAGCCTTAGTCACTTCGTCCCAAACTTTTTTGTGA
GTTTCACCAGTCAAGATAACAGCGCGATTTGGCTGGGAGTTGAAAGCGGTGGGTGTTTCTTTAATGATGGT
TTGGACGACGGATTGGATGTCGTTGATAGTAATTTCACCAGGTAACTCCGGTTTCAAAGCGTAAATAGTAC
GACGAGCAGTTAAAGTTTTCAAATAAGTT
```

The result of this step will be 24 consensus sequences from the 24 red colonies plus the non-mutant parental sequence. All sequences must be in FASTA format and in the forward orientation.

H3. Submit the 25 FASTA consensus sequences from the previous step to the ClustalW2 Multiple Sequence Alignment program at http://www.ebi.ac.uk/Tools/msa/clustalw2/. Export the results into a text processing program such as Microsoft Word. Highlight each position where a colony sequence differs from the nonmutant parental sequence. These highlighted positions will lie (almost) entirely within the 89-nucleotide "red" gene. The reason that the mutations cluster in this segment is that only by inactivating the red gene can a red canavanine-resistant mutant result.

DEMONSTRATION OF BIOINFORMATIC ANALYSIS IN SESSION 5

The bioinformatics processes in steps H1–H3 above are presented as demonstrations during the first hour or so of Session 5. Use of a sequence editing program in step H1, and of online analytical programs in steps H2 and H3, are illustrated in the session, but the full results are already available so that they can be presented to the class in effective form.

DISCUSSION AND WRAP-UP

The final slide of **YeastFluctuationSession5.pptx** can be used to guide discussion of the sequencing results. That document is also distributed to all the students as required reading. There are extensive notes in the Notes section under each slide.

We assign an essay (including a graded draft) as homework following the module, as described in **YeastFluctuationEssay.docx**.