**Yeast Fluctuation Session 3**

**Assigned reading to be completed before Session 3**

* This handout
* YeastFluctuationSession2Discussion.ppt, including especially the extensive notes in the Notes section under each slide

**Counting colonies**

You’ll count the red and white mutant colonies on all 120 selective agar dishes—70 dishes with individual 60-µl cultures, 50 dishes with 60-µl bulk culture samples. The instructor will give you instructions to facilitate the counting. You’ll write down the colony counts on a data entry sheet that will be provided.

**Sequencing the mutant gene in red mutant colonies**

As you’ll see, sequencing the mutant “red” gene from a sampling of the red canavanine-resistant mutant colonies on individual culture dishes provides a powerful and independent test of the pre-exposure versus post-exposure hypotheses. We will do the sequencing in two stages:

* First, in Session 3 we’ll use the polymerase chain reaction (PCR) to specifically amplify a tiny (763-bp[[1]](#footnote-1)) segment of the yeast genome (12.5 million base pairs altogether) that contains the “red” gene. The input to PCR—the “template” in PCR parlance—will be a tiny, very crude sample of genomic DNA obtained just by extracting a bit of colony. At most this template will have a million copies of the yeast genome, but in practice probably more like 10,000 or 100,000 copies; the number isn’t important, and neither is purity. The output—the “PCR product”—will be a large amount (a few µg) of nearly pure 763-bp fragment. That’s about 10 trillion copies of the fragment! So PCR amplifies one tiny 763-bp segment of the genome by a factor of at least 10 million (and probably more like 100 million or a billion), while leaving the remaining 12.5 million base pairs of the genome unamplified. You’ll learn the logic of PCR’s magic in this introduction. In Session 4 we’ll purify the PCR products in order to free them of interfering molecules.
* Second, in Session 4 we’ll submit the purified products to a DNA sequencing facility. That facility will use the chain termination method to determine the nucleotide sequence of each PCR product. The sequencing results will be returned as computer documents.

**Review of DNA structure and function**

*Structure of double-stranded DNA*

Please read the Wikipedia article on DNA at <http://en.wikipedia.org/wiki/DNA>. As you’ll learn there (or undoubtedly know already), natural double-stranded DNA molecules contain two strands that are complementary to each other. The nucleotides in each strand are strung together like beads on a string, and are held together by very strong covalent bonds. Each DNA strand has two different kinds of ends and thus a natural “direction” or polarity. The two kinds of end are called 5´ and 3´.

In contrast to the strong covalent bonds that link the nucleotides within a single strand, the two complementary strands of double-stranded DNA are held together by weak, non-covalent bonds. The double-stranded complex is nevertheless reasonably stable because there are hundreds to billions of such weak bonds in a single double-stranded molecule. The weak bonds can form only when a base in one strand is matched in a specific geometric arrangement with the complementary base in the opposite strand. The two properly-positioned bases form a non-covalent complex that is called a base pair, and are said to “base-pair” with each other. This geometry requires that the two strands lie against each other with opposite polarity and be twisted around each other to form a right-handed double-helix. The two strands in the helical complex, like the individual base-pairs, are said to “base-pair” with each other. By extension, formation of the double helix from two complementary DNA strands is called base-pairing; it’s also called “annealing” and “hybridization.” The excellent animation in the Wikipedia article shows the basic double helix structure very clearly.

Two complementary strands that are base paired with each other can be separated by “denaturing” the double helix. Denaturation breaks the non-covalent base-pairing bonds that hold the helix together, but doesn’t break the covalent bonds that keep the single strands intact. Denaturation is most often accomplished experimentally simply by heating a solution of DNA to 94–98ºC. Because heat is so often used to denature DNA, denaturation is often called “melting”—even when denaturation is not accomplished by heating. The two DNA strands that are separated by denaturation are “separated” only at the molecular level. They remain dissolved together in a single solution. If, say, a solution of a million identical double-stranded DNA molecules is denatured, the result is a solution with a million identical “plus” strands and a million identical “minus” strands all mixed together randomly in a single solution. Under denaturing conditions (e.g., at 94–98ºC), the two strands can’t base-pair with each other to make a stable double helix. If the solution is then returned to non-denaturing conditions (e.g., if the temperature is cooled to, say, 65ºC), complementary “plus” and “minus” strands can now form stable helices, and eventually the solution consists entirely of re-formed double-stranded helices.

*Representing the DNA structure*

In most cases, it is the nucleotide sequence of DNA molecules that’s of primary importance. It’s in the order of the As, Cs, Gs and Ts that its information content lies. So consider the following three representations of a simple 6-bp DNA sequence

5’-ACTTGA-3’

ACTTGA

5’-ACTTGA-3’

3’-TGAACT-5’

In the first, just one strand is represented, the other being implied in context. The 5´→3´ polarity of the strand is explicitly represented. The second representation is like the first except that polarity is understood, not explicitly represented. Unless otherwise specified, the polarity of a string of letters representing nucleotides is assumed to be written with the 5´→3´ polarity going from left to right. In the third representation, the two strands of the double helix are explicitly represented. Polarity must be explicitly represented here, since the “bottom” strand is written with the 5´→3´ going from right to left.

If we’re talking about DNA structure generically without regard to its information content, we often use an arrowed line to represent each single strand. The arrowhead end of the line is the 3´ end, while the feather end (the feathers almost never being included) is the 5´ end. The arrowed line “points” in the 5´→3´ direction. The following represent single strands and double-stranded helices, either with or without explicit designation of 5´→3´ polarity:



*DNA polymerases*

Both PCR and DNA sequencing depend critically on the properties of DNA polymerases. All known DNA polymerases have a single function: they add a new nucleotide to the 3´ end of a “primer” strand that’s base-paired with a template strand:



A simple job, but many DNA polymerases, especially those that are responsible for replicating our chromosomes, perform it extraordinarily well: they make a phenomenally small number of mistakes. A mistake would be when a polymerase adds a nucleotide that is not complementary to the opposite base on the template strand.

DNA polymerases use deoxynucleoside triphosphates (dNTPs) as the nucleotide monomers. As each new nucleotide is added to the growing primer strand, two of the phosphates are cleaved from the triphosphate, the remaining one becoming part of the growing primer strand. The bonds that link the chain of three phosphates together are high-energy bonds; it is the breaking of these high-energy bonds that provides the chemical energy that drives DNA polymerization.

When a DNA polymerase completes its one job, it can’t just go home and watch TV. That’s because the structure it thus creates is again a primed template (that’s what we call a primer strand base-paired with a template strand). So it goes ahead and adds another nucleotide. Then another. Then another. Etc. Only when it gets to the end of the template can it stop:



At that point, the enzyme has “copied” all the information in the template strand into the newly-synthesized primer strand. The DNA sequence in the primer-strand “copy” is the complement of the DNA sequence in the template-strand “original,” but the information content is the same.

Many DNA polymerases can be tricked into incorporating fake nucleotides into growing primer strands, a feature that’s exploited in DNA sequencing by the chain termination method (see below). Chain terminators are artificial nucleotides that can be added to the 3´ end of the primer strand, but that don’t themselves have an intact 3´ end that a new nucleotide can be added to. When DNA polymerase adds an artificial chain-terminating nucleotide to the 3´ end of a primer strand, therefore, all further elongation of that primer strand ceases.

In some primed templates, each strand acts both as primer and as template. The following primed template would be copied by two DNA polymerase molecules working in opposite directions:



*Thermophilic DNA polymerases and thermocycling*

The DNA polymerases used in PCR and sequencing are *thermophilic* enzymes obtained from bacteria or archaeae that live in very hot environments. The “extremophile” *Pyrococcus furiosus*, for example, is an archaea that grows optimally at boiling temperature. It was first isolated from a geothermally heated marine sediment. Use of such DNA polymerases permits thermocycling, in which template strands, a large excess of primer strands, the DNA polymerase and deoxynucleoside triphophate monomers are subject to dozens of three-stage temperature cycles:

* Denaturation stage: mixture is heated to 94–98ºC to denature all double helices, so that all the single strands are unpaired; ordinary (*mesophilic*) DNA polymerases, such as those in the gut bacterium *Escherichia coli*, don’t survive such temperatures, but thermophilic DNA polymerases do, especially polymerases from extremophiles like *P. furiosus*.
* Priming stage: mixture is cooled to a temperature that’s optimal for primer strands to base-pair with template strands
* Extension stage: mixture is heated to the optimal temperature for DNA polymerase to do its thing: add nucleotides to the 3´ end of template-paired primer strands until the ends of the template strands are reached

*PCR*

Please read the excellent Wikipedia article at <http://en.wikipedia.org/wiki/Polymerase_chain_reaction>. Here is the non-mutant parental sequence of part of yeast chromosome III (only one strand is shown). The 763-bp segment that will be PCR-amplified is shown in capital letters:

5’‑aggaaaaaaagaagacaaagtaaaatgtatcagcatttacaacatttgtcacgttctaaaccattgccgcttactccaaactccaaatataatggggaggctagcgtccaattagggaagacatatacagttattcaggattacgagcctagattgacagacgaaataagaatctcgctgggtgaaaaagttaaaattctggccactcataccgatggatggtgtctggtagaaaagtgtaatacacaaaagggttctattcacgtcagtgttgacgataaaagatacctcaatgaagatagaggcattgtgcctggtgactgtctccaagaatacgactgatgaaaataatattgacgttcgcatttaatctatacctataattctgtacttatatactgttccttaattgaagatttcaacatcgtttttGATGTAGGTCTTTTCACCTGGAGGTGCGGCTGGGGTACCGAAGACTAATTGAGCTTGTACGGTCCAAGACTCAGGGATTTTGCTTGGCAAAGCAGCTTTTATGTAACCATTGTAGTGTTGTAGGTGACCACCCAGGCCCATTGCCTCCAAGGCAACCCACGAGTTGATTTGAGCGGCACCAGAGGTATGGTCCGCGAAACTAGGGAATGCAGCTGCGTACGCTGGGAAGTCAGCCTTTAGCTTTTCAGTTACCTTGGGATCCGGGACCGGATAATTATTTGAAATCTCTTTTTCAATTGTATATGTGTTATGTAGTATACTCTTTCTTCAACAATTAAATACTCTCGGTAGCCAAGTTGGTTTAAGGCGCAAGACTTTAATTTATCACTACGAAATCTTGAGATCGGGCGTTCGACTCGCCCCCGGGAGATTTTTTGTTTTTTATGTCTCCATTCACTTCCCAGACTTGCAAGTTGAAATATTTCTTTCAAGAATTGGCCTCATCCCTTGCTGAAGCAGGCTCTTTTGACCGGCAGGGCTTTCTATAGCCTTAGTCACTTCGTCCCAAACTTTTTTGTGAGTTTCACCAGTCAAGATAACAGCGCGATTTGGCTGGGAGTTGAAAGCGGTGGGTGTTTCTTTAATGATGGTTTGGACGACGGATTGGATGTCGTTGATAGTAATTTCACCAGGTAACTCCGGTTTCAAAGCGTAAATAGTACGACGAGCAGTTAAAGTTTTCAAATAAGTTGCAACAGCAGACatgatattggattgtttttttgtatatatgttgatataggatacttcagtctacgaaaaaagtacaaattatgtagtcagttccttcagtatggtgtccttatatactgtagtttggacaaggtgcaaatgccaagaccctagcccgaaaagctcgaggcaccccaggatctttttctttacgtaattttcacgtaaaacgccacagtccgatttttctcgaataatcattagtaaaagcggtatactggattattgtacgataacaaggtagagctttattactaagctaagacgttcttacatcaatagtgctgttcgttattgatgttaggagaaggagcgggtctggtgaatagtgtaagcagtgtttctgaactttttcttcgtctaagtccttgtaatgtaaggtaagaatgcaagcatcttgtttgtaacgcgattgtacgttgacgttagtaagtcacaaacccaagcttaacttcttcgtgaggaaggaaagtgttgtctcctacttttttcaaattttcgaattgtatttatatttatttagtacttcttgagtttacatatccttcgtaaaaatgcaacttttgtcgaaaaacacttccaaaaaaaaataataatgaatttatgaagcatactaacgagcgagcacatcgctgaggtatcattacttcatgagataaattaagatctcctcatatgcgaatttcctgttcagtgataaacgttgattacgttattgataaaagtcttttcttctggcaaggcagttggaacaccaaagaccaattgagattgtacagtccacgcaataggaacatcttgaggcaaagcagatttgacgtagtcattatagtgttgcaaattagcccccaatcccaatagttcgagggcagtccaagactgaatttgcacagcaccggtcgtatgagcggcgcatgttgggaaagcggctgccaaggctggaaaatctctttgcagtttttcagttggtccttcatcagtgaagaa‑3’

The PCR primers will be about 20 bases long. The rightward-pointing primer will be called the “forward” primer; the leftward-pointing primer will be called the “reverse” primer. If you understand PCR, you’ll be able to write out the 20-base sequences of the forward and reverse primers, including their 5´→3´ polarity. (HINT: The reverse primer’s 5´ end begins 5´‑GTCTGCT…)

*DNA sequencing by the chain-termination method*

Please read the Chain-termination methods section of the Wikipedia article at <http://en.wikipedia.org/wiki/DNA_sequencing>. The DNA Core will sequence your samples using the Dye-terminator sequencing method, with forward and reverse primers that prime within the 763-bp PCR product (it’s not important to know the locations of the priming sites). They will return colored traces like the one pictured in the Wikipedia figure titled “View of the start of an example dye-terminator read.”

*Choosing colonies to sequence*

We will choose the 24 clones to be sequenced very carefully in order to increase our depth of understanding of the fluctuation test. Here’s a table of the dishes chosen (NOTE TO INSTRUCTOR: Fill in this table in light of the choices made at step F1 of InstuctorManual.docx.):

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Dish ID | Red colonies | White colonies | Red colony ID | Student sequencer |
|  |  |  | R1 |  |
| R2 |  |
| R3 |  |
| R4 |  |
| R5 |  |
| R6 |  |
| R7 |  |
| R8 |  |
|  |  |  | B1 |  |
| B2 |  |
| B3 |  |
| B4 |  |
| B5 |  |
| B6 |  |
| B7 |  |
| B8 |  |
|  |  |  | G1 |  |
|  |  |  | G2 |  |
|  |  |  | G3 |  |
|  |  |  | G4 |  |
|  |  |  | G5 |  |
|  |  |  | G6 |  |
|  |  |  | G7 |  |
|  |  |  | G8 |  |

**Instructions**

*Material for teams*

* Selective petri dishes from Session 2 (some will have already been counted and either discarded because they were contaminated or set aside for sequencing as explained below)
* Flat toothpicks for counting colonies (for step 1 below)
* Table for entering colony counts (for step 1 below)
* Color-coded, labeled 200-µl PCR tubes, each containing 30 µl 0.2% SDS detergent (for step 2 below)
* Color-coded, labeled 500-µl microtubes, each containing 90 µl water (for step 5 below)
* Color-coded, labeled empty 200-µl PCR tubes for the PCR amplifications (for step 6 below)
* Color-coded, labeled empty 1.5-ml microtubes for storing the final PCR products (for step 8 below, which will be carried out by the instructor after the lab)
* Sterile, disposable white plastic inoculating loops; individually wrapped (for step 2 below)
* 10-µl pipetters (for step 5 below)
* Tips for 10-µl pipetters (for step 5 below)
* Unwanted Materials beakers for discarding used toothpicks, inoculating loops, tubes and tips (for many steps)
* Microcentrifuge with adaptors for centrifuging 200-µl PCR tubes (for step 4 below)
* Premix with PCR ingredients (the thermophilic DNA polymerase will be added just before use at step 7 below)
* A thermocycler (for steps 3 and 7 below)

*Colony counts*

1. Count the red colonies on your petri dishes, using the toothpick method that will be demonstrated to you. Count as red only those colonies that are large and definitely red; 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. Write the red colony counts in the proper column of the table provided, being careful to get the dish numbers correct; some of the spaces will already be filled in because the dishes have already been counted and either discarded or set aside, as explained above. Then count the white colonies in the same manner. Unlike the red colonies, the white colonies may differ in size and be difficult to count unambiguously. Don’t worry about problems counting 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. ; Write the white colony counts in the proper column of the table provided, again being careful to get the dish numbers correct. At the end of the lab, each team will hand in its colony count table so the results from the entire class can be entered in a single master spreadsheet to be distributed to all the students. The results will be discussed in detail in Session 4.

*Processing colonies for sequencing*

2. 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 to pick up a bit of red colony (about this much:), avoiding picking up any of the agar (if you do accidentally pick up agar, discard the inoculating loop and try with another colony); immerse the loop in the 30 µl of detergent solution in a properly color-coded 200-µl PCR tube; 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 and close the cap of the PCR tube; vortex the PCR tube vigorously until the cells in the colony are completely suspended.

3. Put the PCR tubes in the Thermocycler; run Program 10, which will heat the tubes to 90º for 4 min.

4. Using the special adaptors (see *Materials* above), microfuge the heated PCR tubes for 5 min to pellet insoluble material.

5. 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 (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.

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

7. When all 24 PCR tubes are ready, the enzyme will be added to the 4-ml glass vial step 4, the vial 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, changing tips with each addition; close the caps of the PCR tubes securely; vortex the PCR tubes; microfuge briefly as at step 9; put the tubes in the thermocycler and run Program 7, which will commence the repeated temperature cycles:

* Program 7: 98°C for 30 sec (initial “melting” = denaturation of DNA)
* Links to Program 8: 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 Program 9: 5 min extension at 72ºC (any incomplete primer strands are completed)
* Links to Program 5: indefinite soak 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 ~100 nM—at least a 10 million fold increase compared to the starting gDNA concentration.

8. When the temperature cycles are complete a few hours after the lab, the PCR reaction mixtures will be transferred to the corresponding color-coded labeled 1.5-ml microtubes; these will be stored in the deepfreeze for processing in Session 4.

**Discussion of logic of fluctuation test**

Discussion will be continued from Session 2 as time permits. To prepare for this discussion you must have read YeastFluctuationSession2Discussion.ppt in advance, including especially the extensive notes in the Notes section under each slide.

1. “bp” is the abbreviation for base pair (= nucleotide pair). Normal, double-stranded DNA contains two complementary strands with the same number of bases each. So it’s logical to gauge the length (= size) of a DNA molecule in terms of it number of base pairs. Somewhat illogically, the bp abbreviation is extended to mean just base when we’re considering the length (size) of a single-stranded DNA molecule. Kb or Kbp means kilobase (pair), Mb or Mbp means megabase (pair), Gb or Gbp means gigabase (pair), etc., with no distinction made between single- and double-stranded DNA. The human genome has about 3 Gb of DNA; the yeast genome has 12.5 Mb (= 0.0125 Gb). [↑](#footnote-ref-1)