7.003 Applied Molecular Biology Laboratory

Spring 2022

7.003 Lab Schedule

Day 1: (Tue, 2/8)
1. Genetic screen for α-factor resistant yeast mutants
- Part 1.1: Digestion of mTn3 transposon library with NotI
- Part 1.2: Pour agarose gel
- Part 1.3: Prepare 1X TAE, 10X TE, and 10X LiAc

Day 2: (Thu, 2/10)
1. Genetic screen for α-factor resistant yeast mutants
- Part 1.4: Gel analysis of mTn3 plasmid library DNA digest
- Part 1.5: Transformation of mTn3 library into yeast

Day 3: (Tue, 2/15)
1. Genetic screen for α-factor resistant yeast mutants
- Part 1.6: Replica-plating onto αF plates
- Part 1.7: Prepare YPD liquid media

Day 4: (Thu, 2/17)
1. Genetic screen for α-factor resistant yeast mutants
- Part 1.8: Identifying αF resistant candidates
- Part 1.9: Streaking αF resistant candidates onto new plates

Day 5: (Thu, 2/24)
2. Testing the α-factor resistant mutants
- Part 2.1: Set up Shmoo Assay and analysis of results
- Part 2.2: Set up Halo Assay
- Part 2.3: Set up Mating Assay
- Part 2.4: Set up Sporulation Assay

Day 6: (Tue, 3/1)
2. Testing the α-factor resistant mutants
- Part 2.5: Analysis of Halo Assay results
- Part 2.6: Analysis of Mating Assay results
- Part 2.7: Analysis of Sporulation Assay results

Day 7: (Thu, 3/3)
3. Identifying the αF-resistant mutants by plasmid recovery
- Part 3.1: Yeast transformation of the pRSQ2 recovery plasmid

Day 8: (Tue, 3/8)
3. Identifying the αF-resistant mutants by plasmid recovery
- Part 3.2: Genomic DNA prep from pRSQ2 transformants

7.003 Lab Schedule, continued

Day 9: (Thu, 3/10)
3. Identifying the αF-resistant mutants by plasmid recovery
- Part 3.3: Complete genomic DNA prep from pRSQ2 transformants
- Part 3.4: Restriction enzyme digest of the genomic DNA
- Part 3.5: Pour agarose gel

Day 10: (Tue, 3/15)
3. Identifying the αF-resistant mutants by plasmid recovery
- Part 3.6: Gel analysis of digested genomic DNA
- Part 3.7: Ligation of the genomic DNA digest

Day 11: (Thu, 3/17)
3. Identifying the αF-resistant mutants by plasmid recovery
- Part 3.8: Purification of ligation reactions
- Part 3.9: Preparation of inverse PCR reactions
- Part 3.10: Pour agarose gel

Day 12: (Tue, 3/29)
3. Identifying the αF-resistant mutants by plasmid recovery
- Part 3.11: Gel analysis of inverse PCR products
- Part 3.12: Purification of inverse PCR products
- Part 3.13: Send out inverse PCR products for sequencing

Day 13: (Thu, 3/31)
3. Identifying the αF-resistant mutants by plasmid recovery
- Part 3.14: BLAST analysis of inverse PCR sequencing results
- Part 3.15: Re- streaking the mutant strains

Day 14: (Tue, 4/5)
4. Analyzing RNA expression of αF-resistant mutants
- Part 4.1: Isolation of total RNA from WT and mutant yeast
- Part 4.2: Pour agarose gel

Day 15: (Thu, 4/7)
4. Analyzing RNA expression of αF-resistant mutants
- Part 4.3: Gel analysis of isolated RNA
- Part 4.4: Preparation of cDNA from RNA

Day 16: (Tue, 4/12)
4. Analyzing RNA expression of αF-resistant mutants
- Part 4.5: Perform *FUS1* test PCR with cDNA
- Part 4.6: Pour agarose gel

7.003 Lab Schedule, continued

Day 17: (Thu, 4/14)
4. Analyzing RNA expression of αF-resistant mutants
- Part 4.7: Gel analysis of cDNA *FUS1* PCR test samples
- Part 4.8: Preparation of *FUS1* qPCR samples

Day 18: (Tue, 4/19)
4. Analyzing RNA expression of αF-resistant mutants
- Part 4.9: Analysis of the *FUS1* qPCR results

Day 19: (Thu, 4/21)
5. ChemE: Yeast surface display & protein engineering
- Part 5.1: Design SARS-CoV2 target protein

Day 20: (Tue, 4/26)
5. ChemE: Yeast surface display & protein engineering
- Part 5.2: Yeast display magnetic bead sorting

Day 21: (Thu, 4/28)
5. ChemE: Yeast surface display & protein engineering
- Part 5.3: Optimization of magnetic bead sorting protocol
- Part 5.4: Yeast display FACS sorting

Day 22: (Tue, 5/3)
5. ChemE: Yeast surface display & protein engineering
- Part 5.5: Analysis of magnetic bead sorting optimization results

Day 23: (Thu, 5/5)
5. ChemE: Yeast surface display & protein engineering
- Part 5.6: Clonal analysis of potential target binders

7.003 Lab Project Overview

In 7.003 (Molecular Biology Laboratory), you will learn essential molecular biological techniques and genetics methods commonly used in biology research labs. Acquiring these skills will enable you to experience first-hand how it feels to work in a molecular genetics lab and will also prepare you for UROPs and other future lab research work. This lab is designed as a series of real, hypothesis-driven experiments. At the end, you will have completed consecutive steps and techniques that in summary will lead to data and results. Importantly, not everyone in the course will generate the exact same reagents – each group's experiments will produce their own unique results for analysis and comparison.

As with all real experiments, there is no guarantee that everything you do in the lab will work. Please do not let this discourage you! The daily life of any scientist often consists of many failed experiments that need to be repeated, but even experiments that don't work can be just as valuable as ones that do (learning how to trouble-shoot experiments is almost just as important as learning the actual experimental technique itself!). The key is to carefully document your experiments so you can trust your results.

You will be studying the budding yeast *Saccharomyces cerevisiae* in 7.003. As a model organism, *S. cerevisiae* has many advantages, including its rapid generation time, ease of maintenance, and relatively low cost. Yeast is particularly useful in genetic studies because it is highly amenable to genetic manipulation – it is easy to introduce specific modifications to the yeast genome (e.g. gene insertions or deletions) due to its high rate of homologous recombination. Although *S. cerevisiae* is a single-cell organism, many of the basic biological processes studied in yeast are evolutionarily conserved in larger eukaryotes, with homologous genes functioning in similar pathways in other animals, including humans. Some of the most fundamental aspects in key processes such as mitosis, DNA replication, gene expression, and aging were first unraveled through genetic analysis of *S. cerevisiae*. For example, the 2001 Nobel Prize in Physiology and Medicine was awarded to Leland Hartwell (who was an MIT graduate student!), Timothy Hunt, and Paul Nurse for their genetic studies in the 1970's investigating cell cycle regulation in yeast. In 2016, the Nobel Prize in Physiology and Medicine was awarded to Yoshinori Ohsumi for his work over the past 25 years using yeast as a model organism to study the mechanism of autophagy, the process by which cells break down and recycle old cellular components.

In this module, you will utilize the awesome power of yeast genetics to study the yeast mating pathway while learning a variety of basic molecular genetic techniques. As a class, you will perform a genetic screen to isolate new yeast mutants with defects in the mating pathway (see Figure 1). You will characterize these mutants using various phenotypic assays and determine the identity of the mutated genes through a plasmid recovery technique. While you will be using *S. cerevisiae* as a model organism in all your experiments for this module, many of the general techniques and genetic methods you will learn about in lab and lecture are applicable to genetic studies in many different model organisms (e.g. bacteria, flies, worms, mice, and even humans!).

***Saccharomyces cerevisiae* Background Information**

S. cerevisiae is commonly referred to as "baker's yeast" or "brewer's yeast" because it is the same type of yeast used in baking bread and producing beer (the beer- and wine-making industries were actually an important reason why there was such a strong interest in yeast research when scientists initially started studying the organism over 100 years ago – in fact, a Nobel Prize was even awarded in 1907 to biochemist Eduard Buchner for his work in yeast fermentation!). Yeast is a single-cell eukaryotic organism with a cell wall that surrounds a plasma membrane (see Figure 2). *S. cerevisiae* cells are round in shape and approximately 5 – 7 μm in diameter (compared to bacteria which are usually less than 1 μm). Working with yeast in the lab has the advantage of studying a small simple unicellular organism, with the added benefit of being a eukaryote and having many similarities to humans.

***S. cerevisiae* genome**

The *S. cerevisiae* genome sequence was completed in 1996 and was the first eukaryotic genome to be sequenced. It consists of 16 chromosomes (numbered with Roman numerals from I to XVI), and the entire genome is 12.8 megabase pairs (Mb). The yeast genome contains approximately 6,000 genes. In comparison, the human genome is over 3,000 Mb and contains approximately 20,000 protein-coding genes (*what does that tell you about the genetic content and organization of the yeast genome compared to that of the human genome? How might these differences be advantageous for either yeast or humans?*). Gene splicing does occur in *S. cerevisiae*, but only a very small percentage of the gene coding sequences in the yeast genome actually contain any introns (less than 4%). The *S. cerevisiae* genome has been fully annotated and specific genomic or gene information can be searched on the free online database at: www.yeastgenome.org

***S. cerevisiae* mitotic life cycle**

Actively growing yeast cells replicate by undergoing mitosis, in which a mother cell divides to produce a daughter cell. A single round of yeast cell division (consisting of G1, S, G2, and M phase) takes approximately 90 – 120 minutes to complete, depending on the yeast strain. As its common name suggests, *S. cerevisiae* cells divide by budding – the new daughter cell initiates as a small growing bud on the mother cell, with the daughter cell's cell wall and membrane synthesized completely from new cellular material. The bud continues to grow in size throughout the cell cycle until it eventually pinches off and separates to form the new daughter cell (which will be smaller in size at first than the original mother cell). The size of the bud on a dividing cell (or whether a bud is present at all) can be easily distinguished with a simple light microscope and thus can be used to determine cell cycle stage of the yeast cell (e.g. G1, S, G2, or M phase) (see Figure 3). Note that cell division by budding is different from human (and most other eukaryotic) cells which typically divide by fission – during this type of cell division method, a mother cell grows in size and then splits down the middle to divide into two (usually) equally-sized daughter cells.

S. cerevisiae meiotic life cycle

In addition to asexual reproduction by budding, yeast can also reproduce sexually by mating. *S. cerevisiae* can exist in either a haploid state (one copy of each of the 16 chromosomes) or a diploid state (two copies of each of the 16 chromosomes). There are two different kinds of haploid yeast: mating type **a** (*MAT***a**) and mating type α (*MAT*α, or *MAT*alpha). Only yeast cells of opposite mating types can mate with each other. If both a *MAT***a** haploid yeast cell and a *MAT*α haploid yeast cell are present next to each other, then they can mate by fusing together to form a single *MAT***a**/α diploid cell containing two copies of each chromosome (one copy from the *MAT***a** parent cell and one copy from the *MAT*α parent cell).

As a diploid, yeast can continue to undergo mitotic cell division and grow indefinitely in favorable conditions, dividing every 90 – 120 minutes (same as a haploid). In unfavorable environmental conditions however (e.g. nutrient starvation), a yeast diploid will stop growing mitotically and instead undergo meiosis and sporulation. This results in the formation of four haploid spore cells originating from a single diploid cell (see Figure 4). The four spores produced from a single meiotic event are collectively called a tetrad and are encased together within a covering called the ascus (see Figure 2). Yeast spores are a specialized "survival-mode" cell type that do not divide or grow. They have a protective spore wall that helps the cell to survive until environmental conditions improve. Once conditions are favorable again (e.g. nutrients are restored), the spore will lose its protective spore coat and resume mitotic growth and cell division as a normal haploid cell. The ease with which yeast mating and sporulation can be manipulated in the lab and the fact that all four products of each yeast meiotic event are conveniently packaged together into a single tetrad is particularly helpful in constructing new yeast strains (e.g. transferring specific gene alleles from one strain to another) and performing genetic analysis (e.g. characterizing mutant phenotypes, determining if two genes are linked to each other, studying gene interactions, etc).

Yeast mating

S. cerevisiae yeast cells secrete pheromones to signal their presence to other yeast cells. *MAT***a** cells produce and secrete **a**-factor pheromone while *MAT*α cells produce and secrete α-factor pheromone. If a cell detects the pheromone from the opposite mating type (e.g. a *MAT***a** cell detects α-factor in its surroundings), then that cell now knows a suitable mating partner is present. The pheromone is detected by a specific receptor on the yeast cell surface, which then activates a kinase signaling pathway (see Figure 5) that causes the cell to arrest (i.e. stop cell division). The cell will then form a "shmoo" to initiate the mating process (see Figure 2). When a yeast cell shmoos, the yeast cell extends a cellular protusion in the direction of the detected mating pheromone until it comes in contact with another cell. If this other cell is of the opposite mating type, then the two cells can fuse together to form a diploid. For this module, you will be performing a mutant screen to try to isolate and identify yeast mutants that are no longer able to detect mating pheromone and activate the mating process.

General yeast maintenance

Yeast cells can be grown in the lab either on solid media in Petri plates or in liquid cultures. The optimal growth temperature for yeast is 30°C. A single yeast cell on a plate at 30°C usually takes two days to grow and divide enough to form a visible colony.

In this module, we will be using two different general classes of yeast growth media – YPD media and selective SC media. YPD media is a rich complete media containing yeast extract, peptone, and dextrose (another name for glucose). Yeast extract and peptone consist of processed yeast cells and digested animal proteins, respectively (i.e. they're undefined mixtures of random cellular material) and thus includes all the nutrients necessary for a yeast cell to grow and divide. Glucose is the preferred sugar carbon source for yeast (*why is that?*), but growth media can be prepared using other types of sugars for specific purposes in the lab.

SC media stands for synthetic complete media and is a more defined growth media that only contains the bare minimum of nutrients necessary for yeast to survive: a sugar carbon source (usually glucose), a nitrogen source (usually ammonium sulfate), and defined amounts of each amino acid and base. SC media can be specially prepared lacking specific amino acids or bases in order to select for yeast cells with certain phenotypes. In this module, we will be using several different kinds of selective SC media, each lacking different amino acids or bases, to select for certain yeast cells when performing yeast transformations or matings. For example, one type of media we will use is SC-Leu media, which lacks the amino acid leucine. We will use this media to allow us to differentiate between different yeast strains that can or cannot synthesize leucine on their own – yeast strains that can synthesize their own leucine would be able to grow on SC-Leu media, while yeast strains that have been modified such that they cannot synthesize their own leucine would be unable to grow on SC-Leu media.

Yeast nomenclature

Genes: *S. cerevisiae* genes are given names consisting of a three-letter designation with a number, written in italics altogether as one word with no dash or space (e.g. *STE2* or *POL4* or *CDC1*). The letters in a gene name usually refer to the mutant phenotype originally described and/or most easily observed for that gene, or it may refer to the protein/RNA product encoded by the gene or the biological process the gene is involved in. For example, yeast with a mutant *STE2* gene have a sterile mutant phenotype (they cannot mate). The *POL4* gene encodes a DNA polymerase protein, while the *CDC1* gene is involved in regulating the cell division cycle.

Genes with related properties are usually given the same three-letter name, with each gene given a different number designation. For example, there are numerous different *CDC* genes involved in regulating the cell division cycle – these genes are designated *CDC1*, *CDC2*, *CDC3*, and so on.

Alleles (i.e. genotypes): An allele is defined as one of two or more alternate forms of a particular gene. The wild-type allele of a gene is conventionally written in all uppercase italic letters, often followed by a "+" sign designation, e.g. *STE2*$^{+}$ or *CDC1*$^{+}$. For many genes, a large number of independent mutations have been identified – when referring to a mutant allele of a gene in general, it is usually written in all lowercase italic letters, often followed by a "-" designation, e.g. *ste2*$^{-}$ or *cdc1*$^{-}$. Every specific mutation for a gene has a unique allele designation that is usually written as a number or letter designation following the gene name separated by a hyphen. The Greek delta sign (Δ) is often used as well to represent mutant alleles in which all or part of the gene has been deleted from the genome. For example, the *cdc1-304* mutant allele is a specific mutant allele of the *CDC1* gene in which a histidine residue at position 323 has been mutated to an asparagine residue. The *ste2Δ20* mutant allele is a specific mutant allele of the *STE2* gene in which the first 20 amino acids of the protein have been deleted.

Phenotypes: Phenotype refers to the observable characteristics or traits of an organism. In yeast, phenotypes are designated by a non-italicized three-letter abbreviation in which the first letter of the abbreviation is capitalized. A "+" usually indicates that a yeast strain is wild-type with respect to that particular trait while a "-" usually indicates that a yeast strain is mutant or defective with respect to that trait. For example, a yeast strain that is Ste^{+} is wild-type with respect to the ste rile phenotype (i.e. a Ste^{+} yeast cell behaves normally and can mate), while a yeast strain that is Ste^{-} is mutant with respect to the ste rile phenotype (i.e. a Ste^{-} yeast cell is sterile and cannot mate). It should be noted that while a gene name often corresponds to the most prominent mutant phenotype for that gene, any given mutant can have multiple different phenotypes associated with it.

Proteins: The protein product of a gene is referred to by the corresponding gene name, written in non-italics with the first letter capitalized. For example, the proteins encoded by the *STE2* and *CDC1* genes are referred to as Ste2 and Cdc1, respectively.

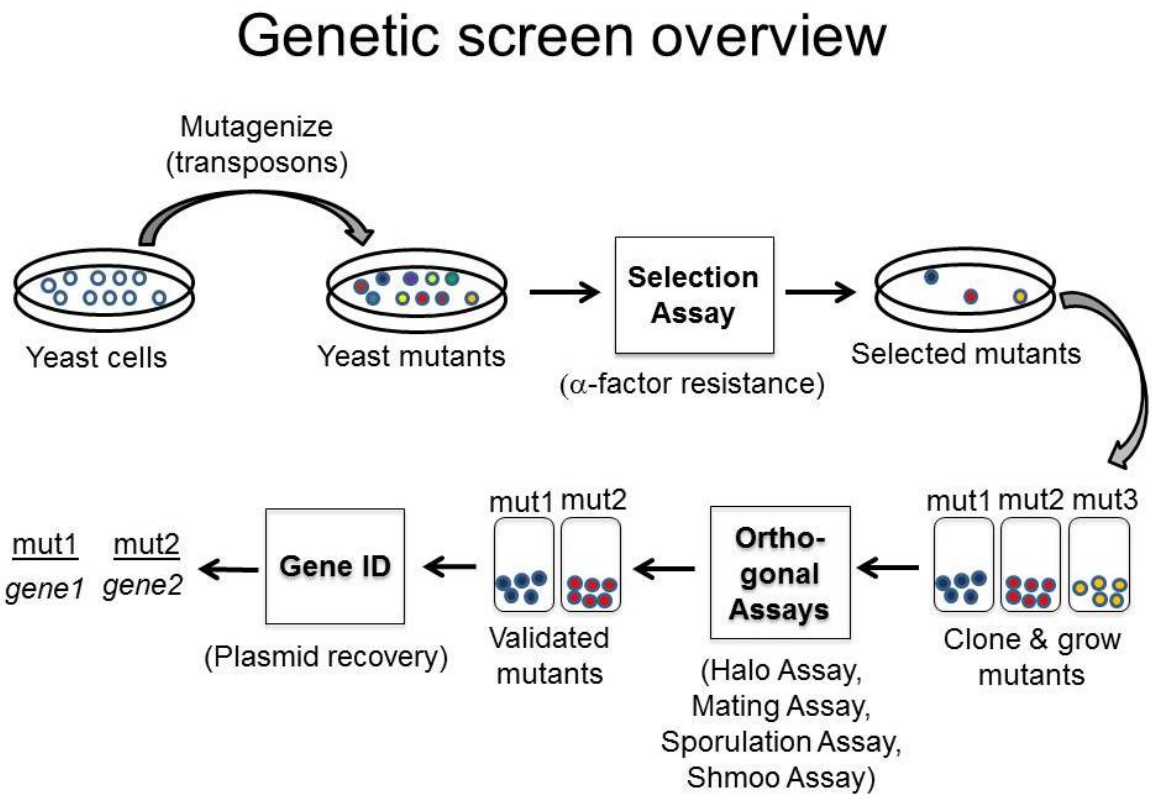


Figure 1. Overview of a forward genetics screen. (Figure adapted from Omer Yilmaz 7.02 Spring 2019 lecture slides.)

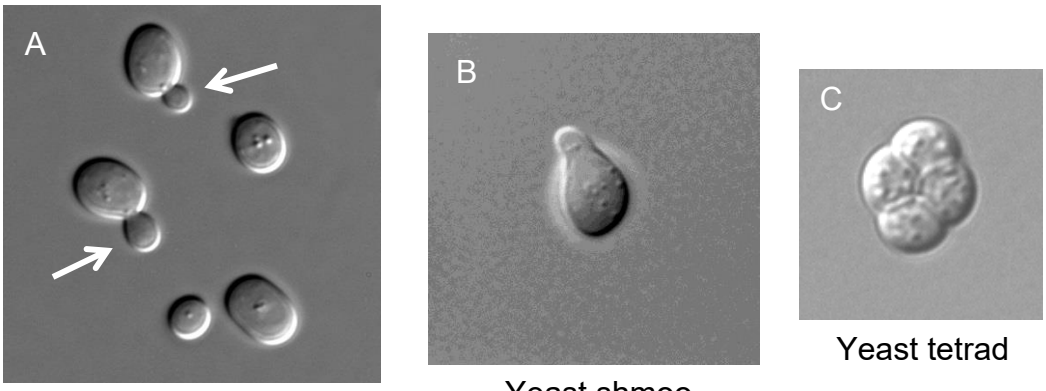


Figure 2. Microscope images of *S. cerevisiae* yeast cells in various life cycle stages. **A)** Haploid yeast cells growing mitotically. Arrows indicate buds. **B)** *MAT***a** haploid yeast cell "shmooing" in the presence of α-factor pheromone (*notice the difference between a shmoo and a yeast bud!*). **C)** Yeast tetrad consisting of four haploid spores surrounded by an ascus. Images adapted from http://en.wikipedia.org/. Public domain.

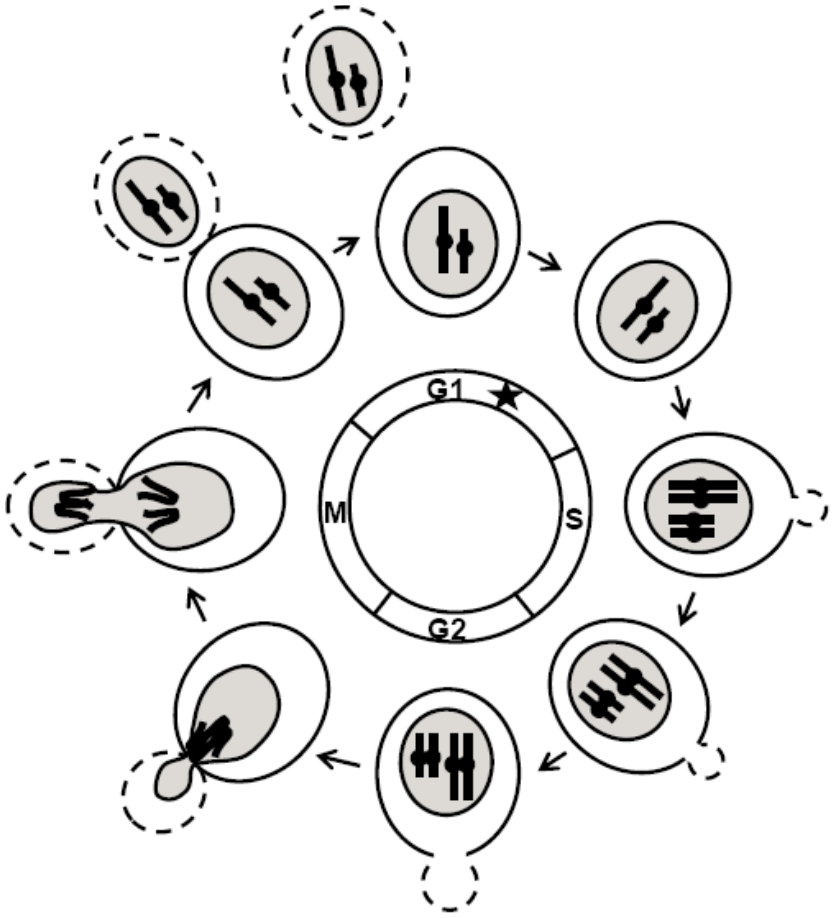


Figure 3. Diagram of the *S. cerevisiae* mitotic cell cycle. The G1 (Growth 1), S (DNA synthesis), G2 (Growth 2), and M (mitosis) phases are drawn in approximate proportion to their duration in the yeast cell cycle. The mother cell is drawn with a solid line and the budding daughter cell is drawn with a dashed line. The gray shaded material represents the yeast cell nucleus (note that for simplicity, only two out of the sixteen individual chromosomes are depicted segregating). The black star within G1 indicates the point during the cell cycle at which haploid yeast cells are arrested in growth by mating factor pheromones. Diagram and figure legend are based on a figure from Herskowitz, I. 1988. *Microbiol Rev* **52**(4): 536-553.

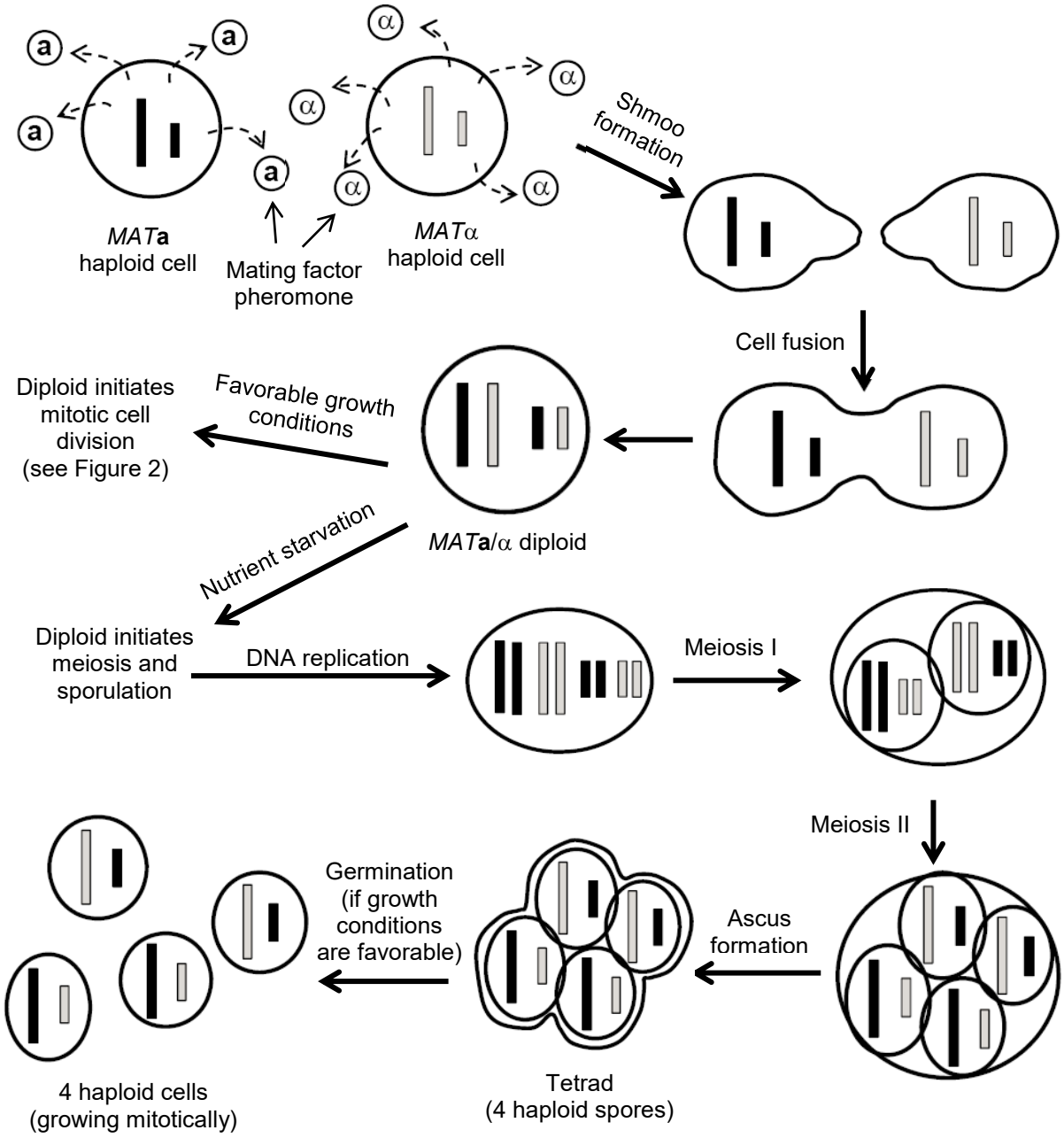


Figure 4. Diagram of *S. cerevisiae* mating and sporulation. A *MAT***a** haploid cell and a *MAT*α haploid cell secrete **a**-factor and α-factor mating pheromone, respectively, to induce shmoo formation and mating, resulting in a diploid *MAT***a**/α cell. The *MAT***a**/α diploid cell undergoes meiosis and sporulates under unfavorable growth conditions to form a tetrad. The black and grey bars within the cells represent yeast chromosomes. Note that for simplicity, only two of the sixteen yeast chromosomes are depicted.

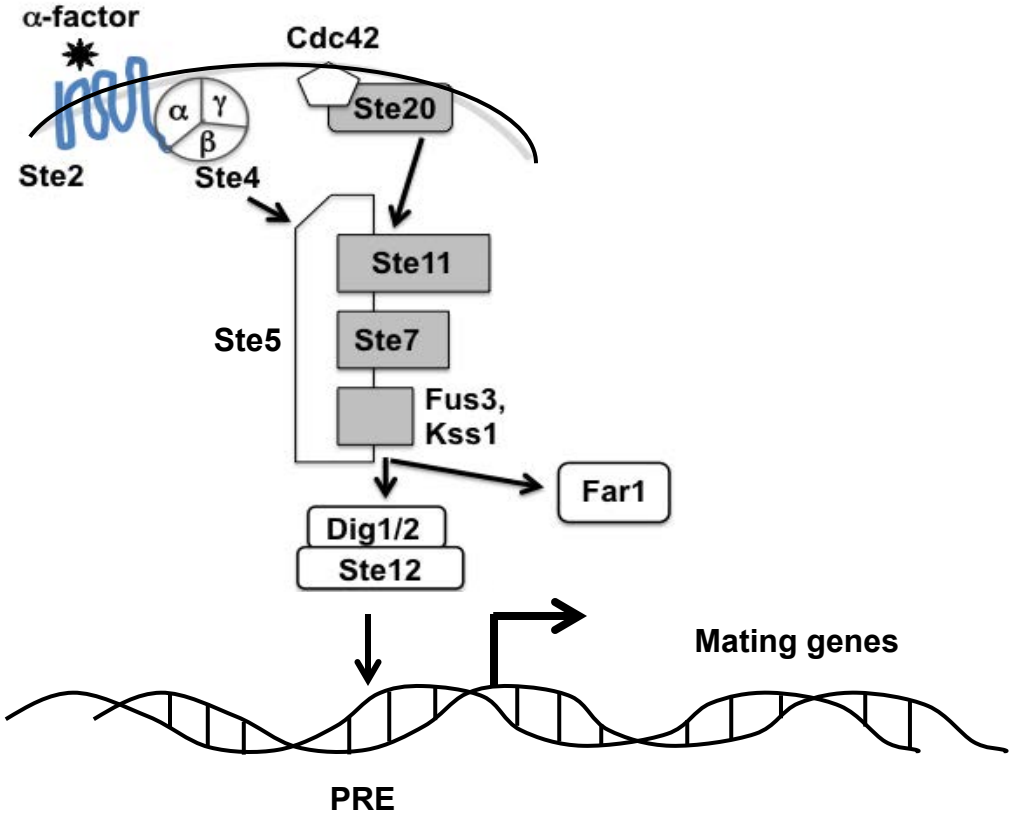


Figure 5. Diagram of the key *S. cerevisiae* mating pathway components in a *MAT***a** haploid cell. PRE: Pheromone Response Element (adapted from Bardwell (2004) Peptide 25:1465)

DAY 1 (Tue, Feb 8th)

1. Genetic screen for α-factor resistant yeast mutants

Part 1.1: Digestion of mTn3 transposon library with NotI

Background: In this class, you will be performing a forward genetics screen to isolate yeast mutants with defects in regulation of the mating pathway. You will use a transposon library to mutagenize yeast – this transposon library is a collection of millions of plasmids (circular DNA molecules) each containing a different DNA fragment of the *S. cerevisiae* genome that has been randomly disrupted by the insertion of a Tn3-derived minitransposon DNA element (mTn3) (see Figure 1 diagram on the next page). For many plasmids in the library, this transposon is inserted within the coding sequence of a yeast gene in the genome.

This library of plasmids is transformed into yeast (i.e. introduced into yeast cells). Each yeast cell will take up a different piece of plasmid DNA, and the transposon-disrupted DNA fragments from each plasmid will integrate into the yeast cell's genome at the site of the same fragment via homologous recombination. If the transposon is inserted within a gene, it will most likely disrupt that gene's normal function, potentially resulting in a yeast cell with a mutant phenotype. Because the entire *S. cerevisiae* genome sequence is represented in the transposon library, this transposon mutagenesis method will essentially allow you to screen almost every possible gene in the yeast genome for ones that when mutated can produce your mutant phenotype of interest. In 7.003, you will be specifically looking for yeast mutants that are α-factor resistant, i.e. the ability for a *MAT***a** yeast strain to grow in the presence of α-factor pheromone (*what normally happens to wild-type MAT***a** *yeast in the presence of α-factor? Why?*).

Before you transform the transposon plasmid library into yeast, today you will first digest the entire library with NotI restriction enzyme. The sequence recognized by NotI is shown below (the arrowheads represent the cleavage sites where the enzyme cuts the sequence). The yeast genomic DNA fragment on each plasmid in the transposon library is flanked by NotI restriction sites (see Figure 1 diagram on next page). Digesting the library with NotI releases a linear DNA fragment from each plasmid consisting of a portion of yeast genomic DNA (approximately 2 – 4 kb in length) disrupted by a 6-kb long mTn3 transposon insertion. These linear DNA fragments will integrate more efficiently than uncut circular plasmid DNA into the yeast genome upon transformation (*why do you think this is the case?*).

NotI Restriction Site:

5' – GCGGCCGC – 3'
3' – CGCCGGCG – 5'

DAY 1 (Tue, Feb 8th), continued

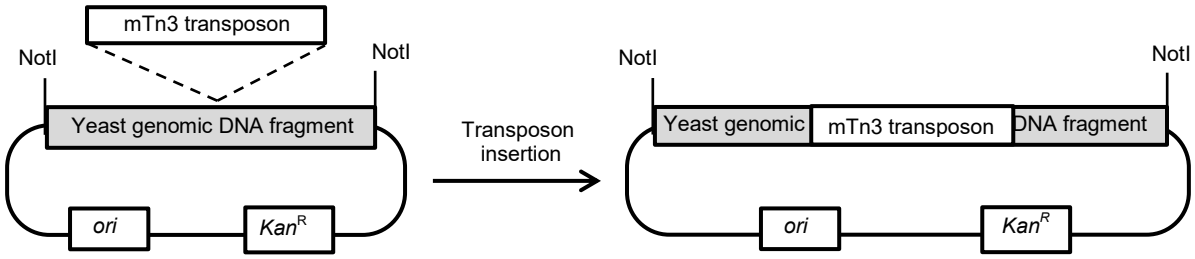


Figure 1. Yeast transposon library plasmid containing a fragment of yeast genomic DNA in which a mTn3 transposon has been randomly inserted. The plasmid backbone (the NotI-to-NotI portion containing the *ori* and *Kan*^{R} sequences) is 2.1 kb in length.

Materials: (3) different pools of mTn3 transposon-mutagenized genomic library plasmid DNA (0.1 μg/μL),
NotI-HF (High-Fidelity) restriction enzyme (10 units/μL)
10X CutSmart Buffer (commercially available from NEB)
Sterile ddH_2O

Hazardous chemicals used: None

Procedure: *Use a new tip for every solution and between tubes. Keep all reagents and tubes on ice.*

1) Label three sterile 0.5-mL Eppendorf tubes with the numbers of the three different library pools you have been given (e.g. "#21," "#22," "#23," etc.). Also write your **bench number** (e.g. "D1," "F1," "H3," etc.) and "Dig" (for "digest") on each tube.

2) Pipet 10 μL of each mTn3 genomic library pool plasmid DNA into the appropriately labeled 0.5-mL Eppendorf tube.

3) Label a 1.5-mL Eppendorf tube "Mix." Add the following reagents to this tube:

10 μL of 10X CutSmart Buffer
6 μL of NotI-HF
44 μL of sterile ddH_2O

4) Using a P200 set to ~30 μL, mix the reagents in the "Mix" tube by pipetting up and down several times (try to minimize bubble formation). Spin the "Mix" tube in the table-top micro-centrifuge for ~15 seconds at 6000 rpm (2900 x g). Make sure to balance your tubes in the centrifuge (i.e. there should always be an **even** number of tubes when spinning – either use an empty tube as a balance tube or coordinate your spin with another lab group). Note that the 0.5-mL Eppendorf tubes are too small to fit properly in the centrifuge rotor – when spinning these smaller tubes, you will need to place them inside an empty 1.5-mL Eppendorf tube before putting them in the rotor.

DAY 1 (Tue, Feb 8th), continued

5) Pipet 15 μL from the "Mix" tube into each of the three 0.5-mL tubes you labeled from Step 1 (use a new tip each time!). Pipet up and down several times to mix the contents in each tube. Try not to introduce any bubbles (*why?*).

6) Centrifuge your three digest tubes for ~15 seconds at 6000 rpm (2900 x g).

7) Give your three digest sample tubes to your TA. They will place them in a thermocycler that has been set to 37°C. Your digest tubes will incubate at this temperature for 16 hours overnight, after which they will then be frozen at -20°C until the following lab day.

Part 1.2: Pour an agarose gel

Background: On Day 2, you will analyze your NotI digest samples using gel electrophoresis. Gel electrophoresis separates DNA molecules based on their size and shape by using an electric current to run the molecules through an agarose gel. The agarose polymers in the gel form a porous sieve through which the DNA molecules travel, with smaller DNA fragments able to fit into and travel through the pores more quickly than larger DNA fragments. After running the gel, the DNA is detected using dyes such as ethidium bromide or GelGreen. These dyes intercalate between the base pairs of dsDNA and fluoresce in UV light, allowing visualization of the DNA in the gel.

Today, you will make the agarose gel that you will be using on Day 2. While it is best to pour a gel fresh right before use, you will prepare your agarose gel today in advance, just to save time on Day 2. Agarose gels can last for several weeks if stored properly.

Materials:
Agarose powder
Gel box and casting tray
10-well comb
1X TAE Buffer (made in Part 1.3)
1000X GelGreen dye (from Biotium)

Hazardous chemicals used: None (the GelGreen dye is considered non-toxic), but for safety, always wear gloves when handling agarose gels

Procedure: *Be careful when microwaving solutions that they do not boil over.*

1) Place the gel casting tray in the gel box apparatus to create a tight-fitting mold (the orange rubber piping of the tray should be against the sides of the gel box). Check that the gel tray is completely flat against the bottom of the gel box. Make sure also that the orange rubber stays within the grooves of the tray and does not buckle out (otherwise, your gel will leak out when you pour it!).

DAY 1 (Tue, Feb 8th), continued

2) Insert a 10-well comb into the slots at the top of the casting tray.

3) You will be preparing a 50-mL volume gel solution with 0.8% agarose. Calculate how much agarose powder you would need to add (check your calculations with a TA).

4) Weigh out the appropriate amount of agarose powder on a balance and add it to a 250-mL glass flask.

5) Measure 50 mL of 1X TAE Buffer (use a graduated cylinder or a Falcon tube to accurately measure the volume) and add it to the flask.

6) Crumple up 2 – 3 Kimwipes into a ball and place them in the neck of the flask as a stopper (to minimize evaporation/loss of liquid while heating). Discard any foil.

7) Microwave the agarose solution in the flask for ~2 minutes until the agarose is fully dissolved. Keep an eye on the flask the ENTIRE time and pause the microwave every ~15 seconds or so (or as soon as you notice bubbles forming) – this will prevent the solution from boiling over and overflowing the flask. Use oven mitts or rubber "lobster claw" hand protectors when handling the flask.

8) Once the agarose has completely dissolved (solution should be completely clear without any residual haziness or "wavy lines"), bring the flask back to your bench to cool.

9) Calculate how much 1000X GelGreen dye to add to your gel solution so that the final concentration of dye is 1X GelGreen (check you calculations with your TA). (Note that the starting concentration of the 7.003 stock GelGreen dye is different from the stock concentration used by most other research labs!)

10) When the melted agarose solution has cooled just enough that you can barely touch it for 1 – 2 seconds (i.e. still uncomfortably warm but not scalding to touch), pipet the appropriate amount of 1000X GelGreen dye to the solution. Swirl the flask gently to mix the GelGreen dye evenly throughout the solution.

11) Pour the agarose solution into the gel casting tray. If there are any bubbles in the gel, use a clean pipet tip or the corner of a clean Kimwipe to pop the bubbles or push them to the edge of the gel.

12) Rinse out the empty flask immediately so that any remaining gel solution will not solidify in the flask.

13) Allow the gel to solidify on your benchtop (will take ~30 minutes). During this time, you can complete Part 1.3 (preparing reagents) and/or work on ILQs.

DAY 1 (Tue, Feb 8th), continued

14) Once the gel has solidified, carefully pull the comb straight up out of the gel. Remove the gel tray from the gel box, and slide the gel out of the tray onto a piece of clean Saran wrap. Add a few drops of 1X TAE Buffer so your gel won't dry out, and then wrap the gel in the Saran wrap and/or a plastic Ziploc bag.

15) On a piece of lab tap, write the gel percentage, the date, and your bench number. Place the tape on the wrapped gel. Store the gel in the 4°C cold room until the next lab day.

Part 1.3: Prepare 1X TAE, 10X TE, and 10X LiAc

Background: Today, you will prepare several solutions that you will use during the semester. 1X TAE Buffer will be used for pouring agarose gels (including in Part 1.2 today) and for running agarose gels. 10X TE and 10X LiAc solutions will be used for yeast transformations. 10X TE will also be used for other procedures as a general buffer to maintain pH (e.g. for DNA samples). When preparing the solutions below, check all of your calculations with your TA!

Materials:
50X TAE stock (2 M Tris-HCl pH 7.2, 1 M NaOAc, 50 mM EDTA)
1 M Tris-HCl, pH 7.5
0.5 M EDTA
Lithium acetate dehydrate (molecular weight = 102 g/mol)
Deionized water (from white taps by sink)
Sterile water (from autoclaved bottles)
0.22 μm filters
10-mL syringes

Hazardous chemicals used: None

Procedure:

1X TAE Buffer (40 mM Tris-HCl pH 7.2, 20 mM NaOAc, 1 mM EDTA)

1) Obtain two 1-L glass bottles and use lab tape to label them with "1X TAE Buffer," your bench number, and the date.

2) The lab buys 50X stocks of TAE Buffer (from G Bioscience). Calculate the amount of 50X TAE stock and deionized water you would need to make 1 L of 1X TAE Buffer.

3) Use a graduated cylinder or Falcon tube to measure the appropriate volumes of 50X TAE stock and deionized water (note that TAE Buffer does not need to be sterile) and add them to one of the bottles to make 1 L of 1X TAE Buffer. Repeat this for the second bottle (you will end up with 2 L of 1X TAE Buffer total).

DAY 1 (Tue, Feb 8th), continued

4) Cap both bottles and swirl to mix the contents. You will use the 1X TAE Buffer later today to pour an agarose gel. Afterwards, the 1X TAE Buffer bottles can be stored at room temperature in your bench cupboard.

10X TE (100 mM Tris-HCl pH 7.5, 10 mM EDTA)

1) Label a 15-mL Falcon tube with "10X TE not clean."

2) The lab has stock solutions of 1 M Tris-HCl and 0.5 M EDTA. Calculate how much Tris stock, EDTA stock, and sterile water you would need to make 10 mL of 10X TE (see recipe above).

3) Add the appropriate volumes of each reagent to the "10X TE not clean" Falcon tube to make 10 mL of 10X TE. Make sure to use sterile water (e.g. from the glass bottles). Cap the tube and invert several times to mix.

4) The 10X TE solution you just prepared may not be sterile, so you will filter-sterilize it to remove any potential contaminants. Label a new 15-mL Falcon tube with "10X TE F/S" (for "filter-sterilized"), your bench number, and the date.

5) Unwrap a 0.22 μm filter and a 10-mL syringe. Connect the syringe to the filter and remove the plunger. Uncap the "10X TE F/S" tube and hold the syringe-filter in place directly over the empty tube (minimize the amount of time that the inside of the tube is left exposed to the outside air).

6) Pipet the contents of the "10X TE not clean" tube into the syringe. Insert the plunger back into the syringe and push all 10 mL of the 10X TE solution in the syringe through the filter and into the clean "F/S" tube. Cap the "F/S" tube as soon as you are done (minimize the amount of time the tube is left open to the air).

7) The 10X TE solution can be stored at room temperature in your cupboard/drawer.

8) Discard the syringe in the red sharps container. The used filter and "not clean" tube can go in the burn box.

10X LiAc (1 M LiAc)

1) Label a 15-mL Falcon tube with "10X LiAc not clean."

2) The lab has lithium acetate powder, with a molecular weight of 102 g/mol. 10X LiAc is equivalent to 1 M LiAc. Calculate how much lithium acetate powder you would need to make 10 mL of 10X LiAc.

DAY 1 (Tue, Feb 8th), continued

3) Weight out the appropriate amount of lithium acetate, and add it to the "10X LiAc not clean" tube. Add 10 mL of sterile water (e.g. from the glass bottles) to the tube. Cap the tube and invert to mix until the lithium acetate has completely dissolved.

4) The 10X LiAc solution you just prepared is not sterile, so you will filter-sterilize it to remove any potential contaminants. Label a new 15-mL Falcon tube with "10X LiAc F/S" (for "filter-sterilized"), your bench number, and the date.

5) Use a 0.22 μm filter and a 10-mL syringe to filter-sterilize the 10X LiAc solution and transfer it to the "F/S" tube (same as you did previously above with the 10X TE solution).

6) The 10X LiAc solution can be stored at room temperature in your cupboard/drawer.

7) Discard the syringe in the red sharps container. The used filter and "not clean" tube can go in the burn box.

****At the end of each lab day in the lab manual, we will provide some tips for writing up that day's Pre-Lab and Post-Lab notebook entries. It should be noted that these daily notebook tips will not necessarily cover <u>everything</u> that needs to be included in each lab notebook entry – these tips are simply reminders/hints to help you when you write up your notebooks. For a full outline of <u>everything</u> that should be included in each Pre-Lab and Post-Lab, you may refer to the general Lab Notebook Guidelines provided in the Appendices of this lab manual (these notebook guidelines are also posted on Canvas and LabArchives) – it is recommended that you refer to these general guidelines when writing up <u>every</u> single lab notebook entry.*

Day 1 Pre-Lab Notebook Tips & Reminders:

- Remember to include an overall "Course Aim" for the entire 7.003 lab project (in addition to the daily Aim for just Day 1).
- When writing your aims, you only need to describe overall what you are doing and how/why you are doing it. You do not need to list out or describe details from individual steps in the protocol.

Day 1 Post-Lab Notebook Tips & Reminders:

- You did not obtain any results for Day 1, but remember to record any deviations from the lab manual protocol and record any unique reagents that your lab group used.

DAY 2 (Thu, Feb 10th)

1. Genetic screen for α-factor resistant yeast mutants

Part 1.4: Gel analysis of the transposon-mutagenized library digestion

Background: To check whether you have successfully digested the mutagenized genomic library plasmid DNA, you will run a small sample from each of your three NotI digest reactions from Day 1 on an agarose gel. For comparison, you will also run a sample of undigested transposon library plasmid DNA.

To estimate the size of linear DNA fragments in an experimental sample, a DNA ladder containing a mixture of DNA fragments of known sizes is usually loaded in a separate lane of the gel. Thus, agarose gel electrophoresis will allow us to determine: 1) Is DNA present in our samples, and 2) If DNA is present, how many DNA fragments are there and what is the size of each fragment? *(What size DNA product(s) do you expect to see in each lane if your digest was successful? How do you think the shape of a circularized piece of DNA vs. a linearized piece of DNA may affect the way it runs through an agarose gel?)*

Materials:
(3) NotI digest reactions from Day 1
Uncut control transposon library plasmid DNA (150 ng/μL)
1-kb DNA Ladder (from NEB) in 1X Loading Dye (0.1 μg/μL, *see lab manual Appendices for the sizes of the ladder bands)*
6X Loading Dye (0.25% Bromophenol blue, 15% Ficoll)
Sterile ddH_2O
0.8% agarose gel (with 1X GelGreen in 1X TAE, poured on Day 1)
1X TAE Buffer (40 mM Tris/HCl pH 7.2, 20 mM NaOAc, 1 mM EDTA)

Hazardous chemicals used: None, but for safety, always wear gloves when handling agarose gels.

Procedure: *Use a new tip for every solution and between tubes.*

1) Label three sterile 1.5-mL Eppendorf tubes with the names of your three NotI digest reactions from Day 1 (e.g. "#21," "#22", etc). Label a fourth sterile 1.5-mL Eppendorf tube as "Uncut." Pipet 3 μL of each NotI digest reaction and of the uncut control plasmid into the appropriately labeled Eppendorf tubes. Add 6X Loading Dye and sterile water to each of your 3 μL samples such that each tube has a final volume of 12 μL total containing 1X Loading Dye. (*Check your calculations with your TA!*)

***Make sure you only add the 6X Loading Dye to the 1.5-mL tubes containing just the 3 μL samples of your digest reactions. Do NOT add the dye to the remainder of the digest reactions – **save the rest of these digest reactions** for "Part 1.5: Transformation of the digested library into yeast"

DAY 2 (Thu, Feb 10th), continued

2) Centrifuge the four Eppendorf tubes containing your gel samples for ~15 seconds at 6000 rpm (2900 x g). Remember to balance your tubes in the centrifuge.

3) Unwrap your agarose gel (from Day 1) and place it in a tray in a gel box. Add 1X TAE Buffer to the gel box so that it just covers the gel.

4) Load the first lane of the gel with 10 μL of the 1-kb DNA Ladder. Then load 12 μL each of your four prepared gel samples into the next four lanes. (Note the order of the lanes in your notebook!)

> ****Note: It's best to always load the lanes of your gel in one direction (i.e. either left to right or right to left). When loading a well, place your tip near the top of the well (try to avoid actually inserting the tip into the well as you may risk breaking the well). Add your sample slowly and make sure that it settles into the bottom of the well before pipetting the entire sample.*

5) Connect the electrodes of the power supply and the gel box in the proper orientation so that **the DNA runs towards the (red) electrode** (*why?*).

6) Turn on the power supply and turn the voltage to ~120 volts. Check that current is actually running through the gel box (look for rising bubbles in the gel box chambers) and check the running direction of the loading dye to make sure the gel orientation is correct (do not touch the gel or buffer though when the power supply is turned on!).

While your gel is running, you can do the first steps of Part 1.5 (Transformation of digested library into yeast) to prepare the necessary solutions and spin down the yeast cells, but **do not** add the transposon library DNA to the yeast cells until you have seen the results from your gel.

7) Run the gel at a constant voltage until the dye has migrated about one-half of the way down the gel (approximately 30 – 45 minutes). Turn off the power supply.

8) Wearing gloves, remove the gel tray (with the gel in it) from the gel box. Place the gel tray and gel in a white plastic box and take it to the gel doc station. The 7.003 staff will take a picture of your gel and post a JPG in the shared 7.003 Dropbox folder for you to access for your Day 2 Post-Lab (remember to properly label your gel and comment on **each** lane!).

9) Check your gel results with your TA or instructor. If you have successfully digested the transposon library plasmid, you may continue with the rest of Part 1.5 (yeast transformation). If any of your NotI digest reactions did **not** work, you will have to obtain back-up samples from the 7.003 staff before you can proceed with the rest of Part 1.5.

DAY 2 (Thu, Feb 10th), continued

11) The bromophenol blue dye in the Loading Dye serves two main purposes. It makes it easier to see your sample when loading it on the gel, and it allows you to easily track the progress of your DNA samples (without needing a UV light) as they are running through the gel so that you don't run the gel for too short or too long a time (*why is this important?*). Bromophenol blue runs at a constant rate through a gel depending on the agarose density and buffer used – at approximate what DNA fragment size does the bromophenol blue dye run in a 0.8% agarose gel in TAE Buffer? **Include your answer in your Day 2 Post-Lab!**

Part 1.5: Transformation of mTn3 library into yeast

Background: Yeast cells can efficiently take up both circular plasmid DNA and linear DNA fragments via the transformation protocol outlined in this section, which uses lithium acetate, polyethylene glycol (PEG), and single-stranded carrier DNA. While plasmids can replicate autonomously in yeast (provided the plasmid contains the proper replication initiation sequence), linear DNA fragments induce recombination via the double-strand break repair pathway. Unlike in most other organisms, recombination in yeast occurs almost exclusively by homologous recombination – the free ends of the transformed linear DNA fragments direct integration into specific chromosomal loci at sites of homology. This method can be used to design genetic screens as outlined in this module or to make specific gene deletions or mutations in the yeast genome.

Today, you will transform the three different NotI-digested transposon library pools into the "wild-type" yeast strain PPY295. The genotype of the yeast strain PPY295 is shown below. *(PPY295 is a haploid strain. What might be the advantages or disadvantages of using a haploid strain in a mutagenesis study as opposed to a diploid strain?)*

PPY295 genotype: *MAT***a** *cry1 his4 leu2 lys2 trp1 tyr1 ura3 cyh2 SUP4-3 bar1-1*

The NotI digestion of the transposon library plasmids generated linear DNA fragments which contain the mTn3 transposon insert (see Figure 1 on the next page). The mTn3 transposon insert consists of a *lacZ* bacteria reporter gene, a wild-type yeast $LEU2^+$ gene, and the bacteria ampicillin resistance gene (Amp^R), all flanked by terminal repeats (labeled as "TR" and shown in black in Figure 1). The transposon itself is flanked by yeast genomic DNA sequences (shown in gray in Figure 1) at each end. These yeast genomic DNA sequences will promote homologous recombination and integration of the NotI-digested DNA fragments into areas of matching homology in the yeast genome (also shown in gray) upon transformation into the PPY295 yeast cell.

Yeast cells in which the NotI-digested fragment successfully integrated into the yeast genome are selected for on SC-Leu plates. These plates contain all amino acids and nutrients required for yeast to grow except for leucine. The PPY295 yeast strain has a recessive *leu2Δ* mutation – the wild-type $LEU2^+$ gene in its genome is deleted. This

DAY 2 (Thu, Feb 10th), continued

gene encodes an enzyme required for the biosynthesis of leucine, so without it, PPY295 yeast cannot synthesize leucine on its own and thus would be unable to grow on SC-Leu media (they can't make their own leucine and wouldn't find any external leucine in the SC-Leu media). The wild-type copy of the *LEU2*$^+$ gene from the transposon, however, can compensate for the PPY295 recessive *leu2Δ* mutation and allow the yeast to synthesize its own leucine. In this manner, only PPY295 yeast cells in which the mTn3 transposon successfully integrated into the genome will be able to grow on SC-Leu plates after transformation.

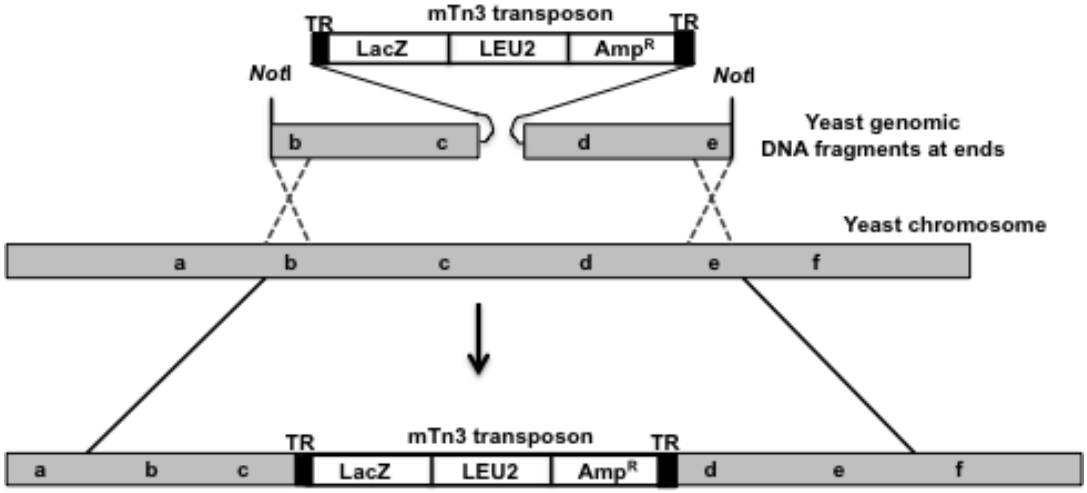


Figure 2. Integration of the NotI-digested linear mTn3-mutagenized yeast transposon library DNA fragment by homologous recombination. Lowercase letters represent the homologous regions between the yeast genomic DNA fragments at the ends of the transposon insert and the yeast chromosome. Final chromosomal configuration after the integration is shown at the bottom.

Materials:
- Liquid culture of yeast strain PPY295 in logarithmic growth phase in YAPD media (YPD media + 30 mg/L adenine)
- (3) NotI-digested transposon library plasmid DNA samples (from Day 1)
- YCplac111 plasmid (50 ng/μL, see plasmid map in the Appendices at the end of the lab manual)
- Sterile ddH_2O
- 10X LiAc solution (1 M LiAc)
- 10X TE solution (100 mM Tris pH 7.5, 10 mM EDTA)
- 50% PEG solution
- Salmon sperm DNA (10 μg/μL)
- (8) SC-Leu plates (single green stripe)

Hazardous chemicals used: None

Procedure: *Use a new tip for every solution and between tubes. Use the specially provided barrier P1000 tips whenever you are pipetting the PEG solution (this solution is very viscous and can easily get into and clog up the P1000 Pipetman barrel otherwise).*

DAY 2 (Thu, Feb 10th), continued

Growing the yeast culture to logarithmic growth phase

(Note: The 7.003 staff has done this step for you)

1) The day before the transformation, inoculate a single colony of PPY295 yeast into 5 mL YAPD media in a test tube. Place the tube in a rolling drum at 30°C overnight.

2) The morning of the transformation, dilute the culture to a final concentration of 5 x 10^6 cells/mL in 72 mL YAPD at 9 AM. The doubling time for this yeast strain is 2 hours, so the cells should divide twice by 1 PM and should be in actively dividing log phase at class time. *(What do you expect the cell density to be at 1 PM?)*.

3) Right before the start of class, spin the cell culture in the Sorvall floor centrifuge at 6000 rpm (5900 x g) at 4°C for 10 minutes to pellet the yeast cells. Carefully discard the supernatant. Resuspend the yeast cell pellet in a total volume of 12 mL YAPD media. The resuspended cells are now ready to use for the transformation. (*What is the approximate final cell density of the resuspended cells? Why do you think we went to the trouble of performing Step 3 and spinning down/resuspending the cells to this final cell density, instead of simply letting the culture from Step 2 continue growing until it reached the final desired density?*)

Yeast transformation *(You start here!)*

4) **You start here!** Label a sterile 1.5-mL Eppendorf tube with "1X Li/TE." Calculate how much 10X LiAc, 10X TE, and sterile water you would need to make 1.5 mL of 1X Li/TE solution (*check your calculations with your TA!*).

1X Li/TE = 0.1 M LiAc, 10 mM Tris-HCl pH 7.5, 1 mM EDTA

5) Add the appropriate volumes of reagents into the "1X Li/TE" Eppendorf tube to make 1.5 mL 1X Li/TE solution (use sterile water!). Invert the tube several times to mix well.

6) Label **four** new sterile 1.5-mL Eppendorf tubes with "PPY295" and your **bench number**.

7) Vortex and/or invert the Falcon tube containing the PPY295 yeast cell culture to ensure that the cells are uniformly dispersed. Pipet 1.5 mL of the PPY295 yeast cell culture into each of the **four** Eppendorf tubes you labeled in Step 6.

8) Pellet the cells in the **four** tubes by spinning them at 13,000 rpm for 30 sec. Using a P1000, carefully remove just the supernatant liquid and discard it in a plastic waste beaker. Be careful not to disturb the yeast cell pellet when removing the supernatant.

9) Pipet another 1.5 mL of the PPY295 yeast cell culture into each of the **four** Eppendorf tubes on top of the cell pellets from Step 8.

DAY 2 (Thu Sept 16th), continued

10) Repeat Step 8 (spin tubes to pellet cells and discard supernatant).

11) Resuspend the cells in each of the four tubes with 500 μL sterile water. Combine the cells from two of the tubes together into the same Eppendorf tube. Combine the cells from the remaining two tubes together into a separate Eppendorf tube. You should now have **two** tubes of yeast cells, each resuspended in a total volume of approximately 1 mL water.

12) Spin the **two** tubes at 13,000 rpm for 30 sec. Discard the supernatant as before.

13) Resuspend the yeast cells in each of the **two** tubes with 600 μL 1X Li/TE Solution. Keep these yeast cell/Li/TE mixtures on ice until you are ready to use them.

14) You will be setting up **five** separate transformation reactions. Label three 1.5-mL Eppendorf tubes with the pool numbers of your three different NotI-digested library samples (e.g. "#21," "#22," "#23"). Label two more 1.5-mL tubes with "No DNA" and "YCplac111." Also write your **bench number** on each of the five tubes.

15) Pipet 20 μL of salmon sperm DNA into each of the **five** labeled empty Eppendorf tubes from Step 14.

16) Into the three tubes labeled with library plasmid pool numbers, pipet 20 μL of the corresponding NotI digest reaction of the mTn3 library plasmid DNA (*how many μg of DNA was added to each reaction?*).

17) Pipet 20 μL sterile water into the "No DNA" tube and 20 μL YCplac111 plasmid DNA into the "YCplac111" tube (*how many μg of DNA was added to each reaction?)*.

18) Transfer 200 μL PPY295 yeast cell/Li/TE mixture into each of the **five** transformation reaction tubes.

19) Place the **five** tubes in a tube rack in the 30°C warm room for 20 minutes (make sure the tubes and/or rack are labeled with your bench number!).

20) While the cells are incubating, calculate how much 50% PEG, 10X LiAc, 10X TE, and sterile water you will need to make 7.5 mL of 1X PEG/Li/TE solution. (*Check your calculations with your TA!*)

1X PEG/Li/TE = 40% PEG, 0.1 M LiAc, 10 mM Tris-HCl pH 7.5, 1 mM EDTA

21) Label a 15-mL Falcon tube with "1X PEG/Li/TE." Add the appropriate amount of reagents into this tube to make 7.5 mL of 1X PEG/Li/TE solution (use the barrier P1000 tips when pipetting the PEG!). Invert the tube until the contents are **thoroughly mixed**

DAY 2 (Thu, Feb 10th), continued

(note that the PEG is very viscous – it will take **a lot** of inversions to make sure the Li/TE liquid gets thoroughly mixed with the PEG).

22) After the 20-minute incubation, add 1.2 mL 1X PEG/Li/TE solution into each of the **five** tubes (use the barrier P1000 tips when pipetting PEG). Place your **five** Eppendorf tubes on the rotator in the 30°C warm room to incubate for 30 minutes (remember to turn the rotator back on after adding/removing tubes – it is very important to keep the tubes mixed during this incubation!).

23) During the incubation, label **eight** SC-Leu plates with your **bench number,** the date, and the type of plate. Label two of the plates as "No DNA" and "YCplac111." For each of your three library pool transformation reactions, label two plates "A" and "B" (e.g. label them "#21-A" and "#21-B," "#22-A" and "#22-B," etc). While waiting for this incubation to finish, you can also work on ILQs.

24) After the 30-minute incubation, place the **five** Eppendorf tubes in a foam float and place the float in a 42°C water bath for 40 minutes to heat-shock the cells. Every five minutes or so during this step, invert the tubes to resuspend the cells that will have settled to the bottom of the tube. While waiting for this incubation to finish, you can continue working on ILQs.

25) After the 40-minute incubation, pellet the cells by centrifuging the five tubes at 10,000 rpm for 2 minutes. Make sure to balance your tubes in the centrifuge.

26) Carefully pipet out and discard the PEG solution supernatant without disrupting the cell pellet (remember to use the barrier P1000 tips for the PEG!).

27) Resuspend the cell pellets in each tube with 300 μl sterile water.

28) Use plastic spreaders to plate half of the yeast cell suspension (~150 μL) onto each of the corresponding two plates labeled "A" and "B" for the library pool transformations. Plate 150 μL of the "No DNA" and "YCplac111" yeast suspensions onto the appropriate plates. Use a new sterile plastic spreader for each plate.

29) When the plates are dry, tape them together (make sure they're labeled with your bench number!) and place them upside-down in the 30°C warm room to incubate for three days. After three days, the 7.003 teaching staff will store the plates in the 4°C cold room until the next lab session.

DAY 2 (Thu, Feb 10th), continued

Day 2 Pre-Lab Notebook Tips & Reminders:

- Include Aims for all parts (Part 1.4 & 1.5). To save space, you can combine the Aims from different protocol parts into a single section. Remember, keep the Aims short (five sentences or less!)

Day 2 Post-Lab Notebook Tips & Reminders:

- Be sure to label the gel photo such that it is clear what is loaded in each lane and what the sizes of all ladder bands are.

- Remember to state where the bromophenol blue dye runs on the gel.

- You should discuss the results of each lane of your gel. If certain lanes had similar overall results or purposes, you may comment on those lanes together as a group.

    - What was the expected overall result for each lane and why? (Did you expect to see DNA or not, and if so, what size DNA fragments?)

    - What can you conclude overall from your gel based on whether or not you saw the expected results for each lane (think back to what your original Aim was for running this gel in the first place!)? Explain your reasoning. If you did not get the expected results, what might be a possible reason to explain the difference?

DAY 3 (Tue, Feb 15th)

1. Genetic screen for α-factor resistant yeast mutants

Part 1.6: Replica plating onto αF plates

Background: Today, you will transfer colonies from each of your transformation plates from Day 2 (i.e. the mTn3-mutagenized yeast library you generated), onto new plates containing α-factor (SC-Leu + αF) to screen for α-factor resistant mutants. Individual colonies can be transferred from one plate to another (e.g. using toothpicks), but today instead you will be using a simple, rapid method called "replica plating" to efficiently transfer hundreds of colonies all at once.

For replica plating, a cotton fabric with a velvety surface called velveteen is used as a mediator to transfer yeast colonies from one plate to up to ten plates. The velveteen is secured on a cylindrical block, called a "replica-plating block" with a ring as shown in Figure 1. Then the master plate is pressed against the velveteen to transfer and make a replica of all of the cells on the velveteen. Up to ten new plates containing different selective media (if desired) can be sequentially pressed onto the velveteen and grown at various conditions (if desired).

Figure 1. A simple replica-plating apparatus. Image taken from the Scienceware Replica-Plating Tool instruction manual (www.belart.com).

Materials:
(6) mutagenized mTn3 library transformation plates from Day 2
(6) SC-Leu plates (single green stripe)
(6) SC-Leu + αF (10^{-5} M) plates (single green & single orange stripes)
(6) intermediate throw-away plates (labeled with "X")
Replica plating block and ring
Sterile velveteens packed in aluminum foil

Hazardous chemicals used: None

Procedure: *During replica plating do not touch the velvety part of the velveteen when handling them (why not?). If you touch it by mistake, get a fresh velveteen. If you are unsure about what to do at any point, ask a TA or an instructor.*

DAY 3 (Tue, Feb 15th), continued

1) Count and record the number of colonies on **each** transformation plate (including all control plates). You can use a Sharpie to mark off colonies on the plate as you count them to prevent from counting the same colony twice.

2) Label the six SC-Leu and six SC-Leu + αF plates with your **bench number**, the date, the type of plate and with the names of your six library pool transformation plates from Day 2 (*e.g.* "#21-A," "#22-B," etc). Lay all similarly labeled SC-Leu, SC-Leu + αF and transformation plates in order so you do not get confused when you are replica-plating. Mark each plate so you will recognize the plate orientation after replica-plating, e.g. mark the top of each plate with a small line at the "12 o'clock" location.

3) Take the replica-plating block. Lift off and put aside the replica-plating ring. Open the aluminum foil pack and position a sterile velveteen carefully on top of the block (velvety-side facing up). Try not to touch the velvety surface of the fabric. This surface needs to stay sterile as it will bind and transfer yeast cells. Now place the replica-plating ring around the velveteen squarely on the block to generate a pulled, flat surface. Make sure that the ring does not touch the top surface of the velveteen.

4) Take your first transformation plate. Place the plate gently on the velveteen all at once. Lightly press or pat the plate first from the North to South direction until the entire plate has had contact with the cloth. Repeat going from East to West. Note that cells from all of the colonies on your transformation plate have now been transferred onto the velveteen, regardless of whether you can see them or not on the fabric.

5) Now carefully remove your original transformation plate by lifting it straight up without smudging the colonies onto the velveteen. Remove that velveteen and place it in the wash bins by the sink (this first velveteen has too many cells on it for you to use).

6) Prepare a fresh velveteen on the block as described above. Place your first transformation plate (from which you have just transferred some colonies) again onto the new velveteen. Lightly press the plate onto the cloth first from the North to South direction until the entire plate has had contact with the cloth as before. Repeat the same procedure going from East to West. Carefully remove the plate while keeping the velveteen on the block (be careful not to smudge any of the colonies on the velveteen!).

7) Now take a throw-away plate (marked with an 'X'). Place it on the velveteen with the colonies. Press lightly as described in the step above. You may discard the throw-away plate into the burn box (this throw-away plate is simply used to remove excess cells from the velveteen). **Keep the velveteen on the block.**

8) Take the SC-Leu plate labeled to match the original transformation plate. Place it gently on the velveteen to transfer the colonies onto the plate as mentioned above. Try to match the orientation of the "12 o'clock" marking. Put this SC-Leu plate aside. **Keep the velveteen on the block.**

DAY 3 (Tue, Feb 15th), continued

9) Now take the corresponding labeled SC-Leu + αF plate. Place it gently on the velveteen to transfer the rest of the colonies onto the plate. Put this plate aside.

10) Remove the used velveteen from the block and place in the wash bins by the sink.

11) Repeat Steps 3 – 10 for each of your six transposon library transformation plates.

12) When you have transferred all of the transformation colonies onto appropriate plates, tape your six SC-Leu and six SC-Leu + αF plates together (label them with your bench number!) and incubate them upside-down in the 30°C room to grow overnight. The 7.003 teaching staff will then store the plates at 4°C until the next lab session.

13) From your transformation results (colony counts), calculate the transformation efficiency of your library transformation and the YCplac111 control transformation using the formula below (**include this calculation in your Day 3 Post-Lab**!):

$$\text{Transformation efficiency} = \frac{\text{Number of transformants (colonies) on a plate}}{\text{Total } \mu\text{g of DNA used to transform the cells}}$$

The units of transformation efficiency are cfu (colony forming unit) / μg DNA. (Note: If you had a lawn on your YCplac111 plate (too many colonies to count), assume you had 3000 colonies.) For your library transformation, calculate the average number of colonies from all six of your library transformation plates and use that average number to calculate the library transformation efficiency.

Remember to comment on **each** plate in your Day 3 Post-Lab (e.g. what were the expected overall results for each plate? What can you conclude based on whether or not you got the expected results for each plate?). Also compare and comment on the transformation efficiencies of your transposon library pools versus the YCplac111 plasmid – what may account for any differences in transformation efficiency?

Part 1.7: Prepare YPD liquid media

Background: Today, you will prepare YPD liquid media to use for growing yeast cells throughout the semester. The media will then be subjected to high temperature in an autoclave to sterilize it and kill off any contaminating microbes. When preparing the media, check all of your calculations with your TA!

Materials:
Yeast extract (powder)
Peptone (powder)
Deionized water (dH2O, white taps at sink)
40% glucose (liquid)
100X Adenine (3 mg/mL, liquid)

DAY 3 (Tue, Feb 15th), continued

Hazardous chemicals used: None

Procedure:

1) The standard recipe for 1 L of YPD media is:

10 g yeast extract (yeast cells that have been broken open)
20 g peptone (partially hydrolyzed proteins)
20 g glucose (or dextrose)
1 L dH_2O

You will not need a full liter of YPD, so to save on reagents, you will only make 200 mL of YPD. Calculate how much yeast extract, peptone, 40% glucose, and dH_2O you would need. *(Note that the lab only has available glucose in liquid form at 40% stock concentration so you will need to calculate what volume of that stock to add.)*

2) Use lab tape to label a 500-mL glass bottle with "YEP" (for Yeast Extract Peptone), your bench number, and the date.

3) Weigh out the appropriate amount of yeast extract and peptone and transfer it to the glass bottle (use a funnel if needed to minimize messes!).

4) Use a graduated cylinder to measure out the appropriate volume of dH_2O (use the white taps at the sink) and add it to the bottle. Swirl the bottle gently to mix the contents, but it is not necessary for the powder to dissolve (it will melt in the autoclave).

5) **Loosely** cap the bottle (*why?*) and place a piece of autoclave tape on the cap. Bring your YEP bottle to Bench B/C, and the 7.003 staff will collect and autoclave the media.

6) After the YEP media has been autoclaved, add the appropriate volume of 40% glucose to the autoclaved media (use sterile technique!). Add a "D" to the bottle label so that it reads "YEPD" to indicate that the glucose (dextrose) has been added. (*Why do you think the glucose is not added until <u>after</u> the media has been autoclaved?*)

7) Add the appropriate amount of 100X adenine solution to your YEPD media such that the final concentration of adenine in the media will be 1X (or 30 mg/L). Use sterile technique! Change the "E" in the label to an "A" so that it reads "YAPD" to indicate that adenine has been added. (Adenine is not necessary for YPD media but it can enhance the efficiency of some experiments, like yeast transformations.)

8) Once the YPD media has cooled sufficiently, you can tighten the cap on the bottle and store it at room temperature in your cupboard/drawer.

DAY 3 (Tue, Feb 15th), continued

Day 3 Pre-Lab Notebook Tips & Reminders:

- What are your Aims for Parts 1.6 and 1.7?

Day 3 Post-Lab Notebook Tips & Reminders:

- Record colony counts for all transformation plates. Keep in mind that "zero colonies" is still a valid result!

- Remember to calculate transformation efficiencies for both your YCplac111 plate and your library transformation plates.

    - How do the transformation efficiencies compare between the YCplac111 plasmid and the transposon libraries? What might be a potential reason for any differences?

- You should comment on each transformation plate (if certain plates have similar overall results or purposes though, you may comment on those plates together as a group if you want).

    - What was the expected overall result for each plate and why?

    - What can you conclude from each plate based on whether or not you got the expected results? If you did not get the expected result for certain plates, what might be a potential reason why to explain the difference?

DAY 4 (Thu, Feb 17th)

1. Genetic screen for α-factor resistant yeast mutants

Part 1.8: Identifying αF-resistant candidates

Background: On Day 3, you replica-plated your transposon library transformation plates to SC-Leu + αF plates. Today, you will observe your replica-plates to determine if any transformants are αF-resistant – these colonies potentially contain a transposon insertion mutation in a gene involved in regulating the mating pathway.

Materials: (6) SC-Leu and (6) SC-Leu + αF replica plates from Day 3

Hazardous chemicals used: None

Procedure:

1) Count and record the number of αF-resistant colonies on all six of your SC-Leu + αF plates. When trying to determine if a colony is actually αF-resistant or not, carefully compare the SC-Leu + αF replica plate with its corresponding SC-Leu replica plate. This will help you distinguish between a genuine αF-resistant colony that actually grew after being replica-plated versus clumps of non-αF-resistant cells that may be visible on the SC-Leu + αF plate simply because there was an excess number of cells transferred to the plate during the replica-plating process. It also helps to compare a potential αF-resistant candidate with the surrounding colonies on the plate – a genuine αF-resistant colony should be noticeably larger or denser (and more "colony-like" instead of "clump-like") than its immediate neighbors on the plate.

2) Select six different αF-resistant colonies to use for further study and mark them as "Mut1" through "Mut6" on the plates so you know which one is which. Record which library pool each of your six different mutants came from (*why might this be important?*). If possible, try to select two separate αF-resistant candidates from each of the three library pools you transformed. Confirm your choices with your TA or instructor. If you do not have six different αF-resistant colonies available on your replica-plates, check with an instructor to receive a back-up strain.

Part 1.9: Streaking αF resistant candidates onto new plates

Background: In genetics, it is important to work with a genetically pure culture consisting of cells that originate from a single clone. Pure cultures yield consistent and reliable data. Today, you will streak the six αF-resistant colonies you selected from Part 1.8 onto fresh SC-Leu + αF plates in order to generate single pure colonies. This

DAY 4 (Thu, Feb 17th), continued

procedure will not only give you pure cultures to continue our analysis but will also help you re-confirm the αF-resistant phenotype of the mutant colonies.

Materials: (6) SC-Leu + αF replica plates with the six αF resistant candidates (Mut1 – Mut6) selected in Part 1.8
(6) fresh SC-Leu + αF (10^{-5} M) plates (single green & orange stripe)
Sterile toothpicks

Hazardous chemicals used: None

Procedure: *Use a new sterile toothpick between sequences of streaks and between colonies. Make sure that you are using the blunt rounded end of the toothpick to streak so as not to puncture the surface of the agar.*

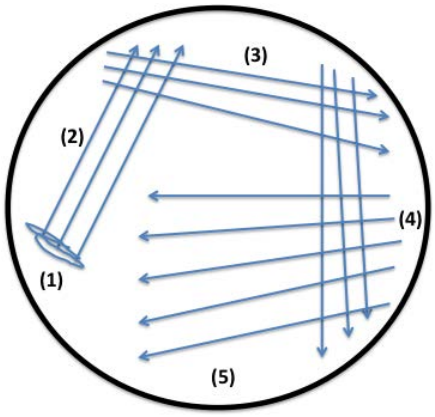


Figure 1. A simple streaking procedure to get single colonies on a plate.

1) Label **six** fresh SC-Leu + αF plates with "Mut 1" – "Mut 6" and with your bench number and the date.

2) Using the rounded end of a toothpick, pick the Mut1 colony from the SC-Leu + αF replica plate. Gently and liberally spread the rest of the cell contents from the toothpick onto a small area of the newly-labeled "Mut1" SC-Leu + αF plate to generate streak (1) as shown in Figure 1 above. Hold the toothpick at an angle and try to move the toothpick over the agar surface without digging into it.

3) With a new sterile toothpick, drag a toothpick to make three additional lines starting from streak (1) to generate the new discontinuous lines for streak group (2). Repeat for streak groups (3) – (5), using a new toothpick for each new streak group. Make sure that each new streak overlaps with a previous streak line to ensure transfer of cells from the streaked area over to the new areas.

4) Repeat Steps 2 – 3 until all six SC-Leu + αF plates have each been streaked with a different αF-resistant candidate (Mut1 – Mut6). Tape the six plates together and incubate them upside-down in the 30°C warm room for three days until single colonies are grown. The 7.003 staff will then store the plates at 4°C until the next lab session.

5) In preparation for Day 5 next week, you will prepare tubes of YPD liquid media to use later for a Shmoo Assay and a Halo Assay. Obtain 24 glass test tubes. Label eight of them with "PPY295 O/N," "ste11Δ O/N," and "Mut1 O/N" – "Mut6 O/N" and with your bench number. When labeling the tubes, please place a small piece of lab tape on the plastic blue caps (not on the glass tubes) and write on the lab tape – please do not write

DAY 4 (Thu, Feb 17th), continued

directly on the caps or glass tubes. Using sterile technique, pipet 3 mL of YPD media into each tube.

6) Label eight more glass tubes with "PPY295 log," "ste11Δ log," and "Mut1 log" – "Mut6 log" and with your bench number. Using sterile technique, pipet 4 mL of YPD media into each tube.

7) Label the remaining eight glass tubes with "PPY295 αF," "ste11Δ αF," and "Mut1 αF" – "Mut6 αF" and with your bench number. Do not add any YPD to these tubes.

8) Altogether, you should have labeled 24 new test tubes (eight "O/N" tubes and eight "log" tubes with YPD liquid media in them and eight "αF" tubes with no YPD). Leave these 24 tubes with your TA – the teaching staff will save them for setting up overnight and log-phase cultures of your group's samples for the Shmoo Assay and Halo Assay on Day 5.

Day 4 Pre-Lab Notebook Tips & Reminders:

- Remember to include Aims for Parts 1.8 and 1.9.

Day 4 Post-Lab Notebook Tips & Reminders:

- Record the number of αF-resistant colonies on each replica-plate.
- Record which library pool each of your final selected six αF-resistant mutants originated from (*why do you think this might be important to know?*).

DAY 5 (Thu, Feb 24th)

2. Testing the α-factor resistant mutants

Part 2.1: Set up Shmoo assay and analysis of results

Background: Your first functional test to check your αF-resistant mutants for false positive candidates is a shmoo assay. When two yeast cells from opposite mating types are close to each other, their secreted mating pheromones bind corresponding receptors on the surface of cells. Upon binding pheromone, the receptors undergo conformational changes and activate the downstream MAP kinase-signaling pathway, which then causes cell cycle arrest in G1 phase and induces cytoskeletal changes required for mating/fusion. Yeast cells of opposite mating types "find" each other by growing projections called "shmoos" (inspired from Al Capp's cartoon character "The Shmoo") towards the source of the pheromone gradient (see Figure 1 below). Cell cycle arrest and shmoo formation can be artificially induced in the laboratory by exposing yeast cells to synthetically produced pheromones. By treating your αF-resistant mutant cells with α-factor and observing the cells under a microscope to look for shmoo formation, you can directly observe whether or not your mutant cells are able to sense and respond properly to a mating pheromone. You will also observe PPY295 and *ste11Δ* cells, along with untreated samples of each strain as controls (*what is the purpose of each of these controls?*). The results of this assay will be used to exclude false positives from further analysis.

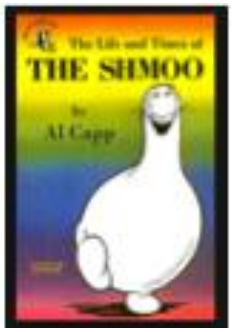


Figure 1. Yeast cells align their shmoos toward the pheromone gradient http://www.umassmed.edu/celldynamics/) (left image). Al Capp's cartoon character "The Shmoo" (right image).

Materials:
(6) YPD liquid cultures of α-factor-resistant mutants (Mut1 – Mut6) in logarithmic growth phase (labeled "log")
(2) YPD liquid cultures of PPY295 and *ste11Δ* in logarithmic growth phase (labeled "log")
α-factor stock solution (10^{-3} M)
(8) glass test tubes (labeled "αF" for Mut1 – Mu6, PPY295, and *ste11Δ*)
Phase contrast microscope
Glass slides and cover slips

DAY 5 (Thu, Feb 24th), continued

Hazardous chemicals used: None

Procedure: *Use a new pipet tip or glass pipette for every solution/culture and between tubes to avoid cross-contamination of samples.*

Preparing liquid cultures of yeast strains in logarithmic growth phase

(Note: The 7.003 staff has done this step for you)

1) The day before the Shmoo Assay, prepare overnight cultures by inoculating a single colony from each of the **six** αF-resistant colony plates (Mut1 – Mut6, streaked on Day 4) into 3 mL YPD liquid culture in test tubes. Also inoculate a single colony of PPY295 WT yeast and a single colony of the *ste11Δ* deletion strain each into 3 mL YPD liquid culture. (Note: these will be the "O/N" tubes you labeled from Day 4.)

2) Place the tubes on the roller drum in the 30°C warm room to grow overnight.

3) The next morning (the day of the Shmoo Assay), dilute 200 μL of each overnight cell culture in 4 mL YPD at 10:00 AM (these will be the "log" tubes you labeled from Day 4). Allow the cells to grow for another 3 hours (until around 1:00 PM), at which point they should reach log phase.

Shmoo assay *(You start here!)*

4) **You start here!** Obtain the eight tubes of log-phase liquid cultures of your samples ("log" tubes prepared by the 7.003 staff), as well as the eight empty "αF" tubes you labeled from Day 4. **Make sure you are using the "log" tubes here (and not the "O/N" tubes)!!**

5) Pipet 2 mL of liquid culture from each "log" tube into its corresponding "αF" tube. All sixteen tubes should now contain 2 mL of log-phase liquid culture.

6) Add 20 μL of α-factor stock solution to each of the eight "αF" tubes (*what is the final concentration of α-factor in these eight cultures?*).

7) Incubate all sixteen tubes (eight untreated "log" tubes and eight αF-treated "αF" tubes) in the roller drum in the 30°C room for at least two hours (this is how long it takes before the α-factor will produce a visible effect). During this wait time, you can set up the Halo, Mating, and Sporulation Assays (Parts 2.2 – 2.4) and/or work on ILQs.

8) After the two-hour incubation, retrieve your sixteen tubes. Label eight glass slides with the names of your eight strain samples. For each slide, label one end as "untreat" and the other end as "αF."

DAY 5 (Thu, Feb 24th), continued

9) Briefly vortex the untreated PPY295 "log" tube to mix the cells and transfer 5 μL to one end of the appropriately-labeled glass slide. Gently lay a cover slip over the cells. Repeat the same procedure on the other end of the same slide with the αF-treated PPY295 cells from the "αF" tube.

10) Repeat Step 9 for all of your strains.

11) Place the glass slide containing PPY295 cells on the microscope stage and position the slide so you are looking at the "untreated" side using the 10X objective (*what phase contrast setting should the condenser turret be turned to for this objective – 0, Ph1, Ph2, or Ph3?*). CAREFULLY bring the stage to the highest position at the closest point to the objective (look at the objective/stage from the "outside" while doing this (*why?*)). Then, while looking through the eyepiece, slowly move the stage back down until the yeast cells come into focus on the slide.

12) When the cells are in focus, change the objective to 20X, and subsequently to 40X (*what phase contrast setting should you use for each objective?*).

13) Find a good "countable" field of cells (ideally between 25 – 50 cells total, with the cells evenly spread out in the field of view for easier counting). Count and record the total number of shmoos and total number of cells that are not shmooing in that entire field (*why is it important to count all cells you see in the entire field?*). Ideally, you will have counted at least 30 cells total in that first field. If there were less than 30 total cells, scan the slide to find an appropriate second field, and count the total number of shmoos and non-shmoo cells in that entire second field. Continue counting cells in different fields as needed until you have counted over 30 total cells.

14) Repeat Step 13 for the αF-treated PPY295 cells on the slide.

15) Repeat Steps 11 – 14 for the rest of your shmoo assay samples and record your cell counts (and any relevant observations) for each of the strains.

16) Calculate the shmoo formation rate for each strain.

Shmoo formation rate = (Total # shmoos) / (Total # cells counted)

(*What did you observe for your control strains? Did they behave as expected? Did your mutant strains form shmoos or not? What conclusions can you make about your mutant strains?* **Include all your observations, counts, and conclusions in your Day 5 Post-Lab!**)

Note: Day 5 continues on the next page!

DAY 5 (Thu, Feb 24th), continued

Part 2.2: Set up Halo assay

Background: The second functional test to eliminate false positive candidates is a halo assay, which will measure the level of response from your α-factor resistant mutants to α-factor. Haploid yeast cells stop dividing when exposed to pheromones from the opposite mating type. The sensitivity of each strain to different concentrations of pheromone can be quantitatively measured by performing a halo assay. For a halo assay, yeast cells grown in liquid culture are mixed with melted top agar and poured onto a plate to produce a uniform lawn. Varying concentrations of a pheromone (e.g. α-factor) are applied onto filter discs. These discs are then placed on the plate, and the plate is incubated at 30°C to allow the cells to grow. If cells respond to the pheromone on the discs, then there will be a zone of growth inhibition around the discs in the shape of a halo, whereas the cells elsewhere on the plate outside the diffusion zone of the pheromone will grow normally.

Materials:
(8) YPD plates (single blue stripe)
(6) αF-resistant candidates (Mut1 – Mut6) grown overnight in liquid culture
WT *MAT***a** strain PPY295 grown overnight in liquid culture
ste11Δ deletion strain grown overnight in liquid culture (see lab manual Appendices for strain genotype)
(8) test tubes each containing 5 mL of 0.5% top agar in water bath or heat block at 55°C
α-factor (10^{-3} M)
Sterile ddH_2O
Glass vial filled with sterile filter discs
(1) empty sterile Petri dish

Hazardous chemicals used: None

Procedure: *Be very careful when handling the tubes of top agar – they will be hot!*

Preparing liquid cultures of yeast strains
(Note: The 7.003 staff has done this step for you)

1) The day before the halo assay, inoculate a single colony from each of the **six** αF-resistant colony plates (Mut1 – Mut6, streaked on Day 4) into 3 mL YPD liquid culture in test culture tubes. Also inoculate a single colony of PPY295 WT yeast and a single colony of the *ste11Δ* deletion strain each into 3 mL YPD liquid culture. (Note: These are the "O/N" tubes you labeled from Day 4).

2) Place the eight "O/N" tubes on the roller drum in the 30°C warm room overnight.

DAY 5 (Thu, Feb 24th), continued

Halo assay *(You start here!)*

3) **You start here!** Label **six** YPD plates with the names of your six αF resistant candidates (Mut1 – Mut6) and **two** YPD plates as PPY295 and *ste11Δ*. Label all plates with the date, type of plate and your **bench number**.

4) Vortex the glass tubes containing the overnight cell culture to ensure that the cells are uniformly dispersed. **Make sure you are using the "O/N" tubes (and not the "log" tubes)!!**

5) Take out one tube of top agar from the 55°C water bath/heat block and quickly transfer the tube to a rack to bring back to your bench (the tubes will be very hot – be careful not to burn yourself!).

6) Gently touch the bottom part of the tube containing the warm melted agar. If you can just barely touch it without immediately burning your hands, the top agar is sufficiently cool enough to use. If the tube is still too scalding hot to touch, wait a little and then check again.

7) Without giving the top agar a chance to solidify, pipet 10 μl of cells from one of your cell cultures into the top agar tube and carefully vortex the tube to mix the cells with the agar (make sure that the tube's cap is held firmly closed while vortexing.)

8) Gently pour the warm agar mixed with cells onto the appropriately labeled YPD plate, making sure that the agar is evenly spread throughout the plate. Let it sit undisturbed on your benchtop to solidify.

9) Repeat Steps 3 - 8 for each of your cultures. You should end up with eight YPD plates with top agar each containing a different strain. Let all your plates sit to solidify on your bench for 15 – 30 minutes. During this time, you can prepare the α-factor serial dilutions (Steps 10 – 11 below) or start Parts 2.3 – 2.4 (Setting up Mating Assay and Sporulation Assay).

10) You will need to make serial dilutions of α-factor to obtain different concentrations for using in the halo assay. The tube of α-factor provided will be at a concentration of 10^{-3} M. Label two 1.5-mL Eppendorf tubes "10^{-4} M" and "10^{-5} M" (*what is an advantage of using serial dilutions to make these two different concentrations of α-factor versus making each concentration up independently?*). Determine how much α-factor and how much sterile water you should add to make 100 μL volume of 10^{-4} M α-factor and 100 μL volume of 10^{-5} M α-factor using serial dilutions. (*Check your calculations with your TA.)*

11) Based on your Step 10 calculations, use serial dilutions to make 100 μL of 10^{-4} M α-factor and 100 μL of 10^{-5} M α-factor in the appropriately labeled Eppendorf tubes.

DAY 5 (Thu, Feb 24th), continued

12) Once the top agar has solidified on all eight YPD plates, use a Sharpie to divide each plate into four equal sections labeled "Water," "10^{-3} M α-F," "10^{-4} M α-F" and "10^{-5} M α-F."

13) Carefully pour ~32 sterile filter discs into an empty open Petri dish. Sterilize a pair of tweezers by briefly passing them through a Bunsen burner flame. Use the sterile tweezers to separate the discs such that each disc is lying flat on the dish without touching any other discs. Always use sterile tweezers to handle the discs – do not touch them with your fingers (or anything else non-sterile!).

14) Pipet 10 μl of water onto a filter disc. Sterilize the tweezers in the Bunsen burner as before. Using the sterile tweezers, carefully pick up the disc and place it in the designated "Water" section on one of your labeled YPD plates. Repeat for all of the plates, remembering to sterilize your tweezers between each transfer.

15) Pipet 10 μl of the 10^{-5} M α-F onto a filter disc. Sterilize the tweezers and carefully pick up the disc and place it in the designated "10^{-5} M α-F" section on one of your labeled YPD plates. Repeat for all of the plates, remembering to sterilize your tweezers between each transfer.

16) Repeat the same procedure with 10^{-4} M and then 10^{-3} M α-F for all your plates. In the end, you should have eight YPD plates each with four different filter discs on them.

17) Tape all eight plates together (label them with your bench number!) and incubate them upside-down in the 30°C warm room for two days. The 7.003 staff will then store the plates at 4°C until the next lab session.

Part 2.3: Set up Mating assay

Background: In this part, you will perform a mating assay as the third functional test on your α-factor resistant mutants to eliminate false positive mutants. In this assay, you will test the ability of your α-factor resistant mutants to mate with cells of the opposite mating type. In order to distinguish unmated haploid cells from the successfully mated diploids, a simple complementation method will be used. The haploid cells of the opposite mating types used today each have mutations in different auxotrophic genes. For example, the genotype of the *MAT***a** strain PPY295 carries a mutated copy of the *his4* gene, which encodes for an enzyme in the histidine biosynthesis pathway. Therefore, PPY295 is a histidine auxotroph and cannot grow in the absence of histidine. The *MAT*α strain PPY144 is another histidine auxotroph but it has a mutated *his7* gene (and therefore a functional *HIS4*$^+$ gene). If these two strains of opposite mating types mate, once they are fused, the newly formed diploid will now have one functional copy each of the *HIS4*$^+$ gene (coming from PPY144) and the *HIS7*$^+$ gene (coming from PPY295) and therefore will be able to grow on a SC-His plate (whereas each individual

DAY 5 (Thu, Feb 24th), continued

original haploid parent strain would not be able to grow on SC-His). You will mix strains of opposite mating types and monitor their growth on SC-His plates to test for mating (see lab manual Appendices for genotypes of all strains used).

Materials: (6) αF-resistant candidates (Mut1 – Mut6, streaked from Day 4)
PPY295 *MAT***a** wild-type yeast strain
PPY144 *MAT*α wild-type yeast strain
*MAT***a** *ste2Δ* mutant strain
(1) SC-His plate (single yellow stripe)
YPD liquid media
Sterile toothpicks

Hazardous chemicals used: None

Procedure: *Use a new sterile toothpick between colonies. Use the blunt rounded end of the toothpick when spreading cells so as not to puncture the agar.*

1) Label **eleven** 1.5-mL Eppendorf tubes as shown in the table below**:**

	Tube Label	Strains mixed
1	PPY295	PPY295 + PPY295
2	PPY144	PPY144 + PPY144
3	PPY295 + PPY144	PPY295 + PPY144
4	ste2Δ	*ste2Δ* + *ste2Δ*
5	ste2Δ + PPY144	*ste2Δ* + PPY144
6	Mut1 +PPY144	Mut1 + PPY144
7	Mut2 + PPY144	Mut2 + PPY144
8	Mut3 + PPY144	Mut3 + PPY144
9	Mut4 + PPY144	Mut4 + PPY144
10	Mut5 + PPY144	Mut5 + PPY144
11	Mut6 + PPY144	Mut6 + PPY144

2) Pipet 20 μL YPD into each of the eleven Eppendorf tubes (use sterile technique!).

3) To prepare the "PPY295" tube, take a sterile toothpick and pick **ten colonies** from the PPY295 plate. Mix it into the YPD in the tube labeled "PPY295." Pipet the solution up and down a few times to mix.

4) Repeat Step 3 for the "PPY144" and "*ste2Δ*" tubes, using colonies from either the PPY144 plate or the *ste2Δ* plate, respectively. Use a new sterile toothpick each time.

5) For the tube labeled "PPY295 + PPY144," take a sterile toothpick and pick **five colonies** from the PPY295 plate. Mix it into the YPD. Then with a new sterile toothpick pick **five colonies** from the PPY144 plate. Mix it into the YPD in the same "PPY295 + PPY144" tube. Pipet the solution up and down a few times to mix.

DAY 5 (Thu, Feb 24th), continued

6) Repeat Step 5 for the rest of your tubes ("*ste2Δ* + PPY144" and the six αF-resistant tubes). For each of these tubes, mix **five** colonies of each of the two different yeast strains being tested (e.g. five colonies of *ste2Δ* mixed with five colonies of PPY144, etc). Remember to use a new sterile toothpick each time!

7) Label the bottom of the SC-His plate with your **bench number**, the date, and the type of plate. Draw a grid to divide the plate into eleven sections. Label the eleven sections in the grid with the names of the eleven mating tubes you have prepared.

8) Mix the contents of each tube and pipet 5 μL from each tube onto each corresponding grid location on the SC-His plate. Gently spread each mating sample with a sterile toothpick until the liquid is absorbed (use a new toothpick for each sample and make sure that none of the different samples accidentally touch each other).

9) Incubate your SC-His plate in the 34°C incubator (at the front of the lab) for two days. The 7.003 staff will then store the plates at 4°C until the next lab session.

Part 2.4: Set up Sporulation assay

Background: Your final functional test on your α-factor resistant mutants is a sporulation assay to eliminate false positive diploid cells from your screen. You are going to test if your α-factor resistant mutants are able to undergo sporulation. Upon encountering nitrogen starvation in the presence of a poor carbon source, diploid yeast cells undergo meiosis and produce four products of meiotic division called "spores" or "asci" packaged together as a tetrad within a thick spore cell wall called an "ascus sac" (see figure in *S. cerevisiae* Background Information in lab manual introduction).

During transposon mutagenesis of yeast cells, some *MAT***a** yeast cells we used in our screen may have switched mating type to the α mating type (all yeast cells (haploid and diploid) possess both mating cassettes in their genome and through a simple chromosomal rearrangement, are able to switch between mating types). Any newly switched MATα cells would then mate with one of the surrounding *MAT***a** cells to form a diploid. The newly formed diploid yeast cells would grow normally in the presence of α-factor because diploids are unresponsive to the presence of either mating pheromone (*why?*) and would therefore be isolated as a potential positive candidate in our mutagenesis screen. The sporulation assay will help us to identify such false positives.

Materials: (6) αF-resistant candidates (Mut1 – Mut6, streaked from Day 4)
Haploid wild-type PPY295 yeast strain
Diploid wild-type PPY917 yeast strain (see lab manual Appendices for strain genotype)

DAY 5 (Thu, Feb 24th), continued

(1) Sporulation plate (1% Potassium acetate + 30 μg/mL adenine, 20 μg/mL histidine, 60 μg/mL leucine, 30 μg/mL lysine, 40 μg/mL tryptophan, 20 μg/mL uracil) (single pink stripe)

Procedure: *Use a new sterile toothpick between colonies. Use the blunt rounded end of the toothpick to streak so as not to puncture the surface of the agar.*

1) Label the bottom of the sporulation plate with your bench number, the date, and the type of plate. Draw a grid to divide the plate into eight sections. Label the eight sections with the names of your six α-factor resistant candidates ("Mut1," "Mut2," etc) and the two control strains (PPY295 and PPY917).

2) Using the rounded end of a clean toothpick, pick two colonies from the Mut1 plate. Gently drag the toothpick on the grid section corresponding to Mut1 on the sporulation plate to make a small ½-inch long patch of cells. Hold the toothpick at an angle and try to move the toothpick on the surface of the agar plate without digging into it. If needed, use the toothpick to spread the cells around to create an even distribution within the patch.

3) Repeat Step 2 for all six α-factor resistant candidates (Mut1 – Mut6) and for the two control strains. Give the sporulation plate to your TA (make sure the plate is labeled with your bench number!). The teaching staff will incubate the plate at room temperature until next week (sporulation takes approximately five days at least at room temperature).

4) When you have finished setting up the Mating and Sporulation Assays, return the original SC-Leu + αF plates streaked with your six αF-resistant candidates (Mut1 – Mut6) to your TA for the teaching staff to store – we will still need these streaked strain plates to use for later lab days.

DAY 5 (Thu, Feb 24th), continued

Day 5 Pre-Lab Notebook Tips & Reminders:

- What orthogonal assays are you setting up today and what is the purpose of each one? Note that each orthogonal assay has a related but distinct purpose from the other assays.

Day 5 Post-Lab Notebook Tips & Reminders:

- Record the results from restreaking your αF-resistant mutants onto fresh plates.

- Include both the Shmoo Assay cell counts and the calculated shmoo formation rates.

- For the Shmoo Assay, discuss the results for each control sample/strain/condition (similar samples can be discussed together as a group if you want).
    - What was the expected result for each control? Why did you expect that result?
    - What can you conclude from each control based on whether or not you got the expected results? If you did not get the expected result for your controls, what might be a potential reason why to explain the difference?

- For the Shmoo Assay, discuss the results for each of your six αF-resistant mutants (similar samples can be discussed together as a group if you want).
    - What were the results of each mutant strain? How did they compare with your controls, if applicable?
    - What can you conclude about each mutant strain based on the result for each assay? Explain your reasoning.

DAY 6 (Tue, Mar 1st)

2. Testing the α-factor resistant mutants

Part 2.5: Analysis of the halo assay results

Background: Today, you will analyze the results of the halo assay you set up from Day 5. This assay provides another (more quantitative) measure of α-factor responsiveness of your α-factor resistant mutants. You will use the results of this assay to exclude false positives from further analysis.

Materials: (8) YPD halo assay plates from Day 5

Procedure:

1) Use a ruler to measure the halo diameter in mm for each of the four filter discs on all eight of your YPD halo assay plates. Make two measurements, at 90° angles, and take the average for the diameter of the halo. Record your results in your notebook. If applicable, make a note if the halo intensity and/or diameters differ significantly between any strains.

2) When writing your Day 6 Post-Lab, remember to include the plate photos (properly labeled!) in your notebook entry. Be sure to comment on **each** plate and include all your observations and conclusions in your Day 6 Post-Lab. (*What did you observe for your control strains? Did they behave as expected? What conclusions can you make about the α-factor responsiveness of your mutants based on your halo assay results?)*

3) Using Excel (or similar software), make a graph of the average halo diameter (e.g. in mm, etc) on the y-axis and of the α-factor concentration used (e.g. in M, mM, μM, etc.) on the x-axis for each of the eight strains you analyzed. You may find it helpful to use a log-scale for the x-axis. **Include this graph in your Day 6 Post-Lab.**

Part 2.6: Analysis of the mating assay results

Background: Today, you will analyze the results of the mating assay you set up from Day 5. This assay tests the mating ability of your α-factor resistant mutants. You will use the results of this assay to exclude false positives from further analysis.

Materials: (1) SC-His mating assay plate from Day 5

Procedure:

DAY 6 (Tue, Mar 1st)

1) Record in your lab notebook whether you see growth or not for each sample on the mating assay plate. If applicable, make a note if the amount of growth differs between different mating combinations.

2) When turning in your Day 6 Post-Lab, remember to include the plate photo (properly labeled!). Be sure to comment on **each** mating sample and include all your observations and conclusions in your Day 6 Post-Lab. (*What does growth on the SC–His plate indicate? What did you observe for your control mating samples? Did they behave as expected? Do you expect to see growth for your* α*-factor resistant colonies on an SC–His plate when mixed with PPY144 cells?Why or why not?*)

Part 2.7: Analysis of the sporulation assay results

Background: Today, you will analyze the results of the sporulation assay you set up on Day 5. This test will enable you to decide if any of the α-factor resistant mutants you have isolated are diploids. You will use this information then to exclude the false positive diploids from further analysis.

Materials: (1) Sporulation assay plate from Day 5
Sterile water
Phase contrast microscope
Glass slides and cover slips

Hazardous chemicals used: None

Procedure:

1) Label **four** glass slides on both ends of the slide with the **eight** strain designations you patched onto the sporulation plate on Day 5.

2) Pipet 5 μL water onto the end of the glass slide labeled “PPY917.” With one sterile toothpick, use two separate strokes to scoop up yeast from the patch on the sporulation plate corresponding to PPY917 you generated on Day 5. Try not to puncture and carry over agar to the glass slide. Dip the toothpick in the water drop on the slide and mix the cells into the water. Place the cover slip gently on top.

3) Place the glass slide under the microscope with the 10X objective in place (*what phase contrast setting should you use for this objective?*). CAREFULLY bring the stage to the highest position at the closest point to the objective. Then slowly move the stage down to bring the cells on the slide into focus.

DAY 6 (Tue, Mar 1st)

4) When you find the cells, change the objective to 20X and subsequently to 40X (*what phase contrast settings should you use for each objective?*).

5) Find a good "countable" field of cells (ideally between 25 – 50 cells total, with the cells evenly spread out in the field of view for easier counting). Count and record the total number of tetrads and total number of non-tetrad cells you see in that entire field (*why is it important to count all cells you see in the entire field?*). Ideally, you will have counted at least 30 cells total in that first field. If there were less than 30 total cells, scan the slide to find a second representative field and count the total number of tetrads and total number of non-tetrad cells in that entire second field. Continue counting cells in different fields as needed until you have counted over 30 total cells.

6) Repeat the microscopic examination for all of your strains in the Sporulation Assay, and write down your cell counts and observations for each of the strains.

7) Calculate the sporulation rate for each strain.

Sporulation rate = (total # tetrads) / (total # cells counted)

(*What did you observe for your control strains? Did they behave as expected? Did you observe any mutant strains which sporulated? What would you do if one of your α-factor resistant mutants sporulated? Explain your reasoning.* **Include all your observations, counts, and conclusions in your Day 6 Post-Lab!***)*

8) Based on the results for all four orthogonal assays (Shmoo, Halo, Mating, and Sporulation assays), select the three best confirmed αF-resistant mutants (out of your original six candidates) that you will use for further study in Days 7 – 13. Check your choices with your TA or an instructor.

9) In preparation for Day 7, you will prepare and label tubes of YAPD media today. Obtain three glass test tubes and label them with the names of your three mutants you selected in Step 8, "O/N," and with your bench number. Using sterile technique, pipet 2.5 mL of YAPD media into each of these three tubes.

10) Obtain another three glass test tubes and label them with the names of your three mutants, "log," and with your bench number. Using sterile technique, pipet 10 mL of YAPD media into each of these three tubes.

11) Give all six tubes of YAPD media to your TA – these tubes will be saved until the next day, when they will be used for setting up overnight cultures and log phase cultures of your samples for the Day 7 transformation procedure.

DAY 6 (Tue, Mar 1st)

Day 6 Pre-Lab Notebook Tips & Reminders:

- Remember to describe Aims for all parts (Parts 2.5 – 2.7)

Day 6 Post-Lab Notebook Tips & Reminders:

- Include both the halo measurement data and the photos of the actual halo plates (properly labeled!)

- Include a properly labeled (computer-generated) graph plotting the α-factor concentration vs. average halo diameter.

- Include both recorded observations from your mating plate and the photo of the actual mating plate (properly labeled!)

- Include both the Sporulation Assay cell counts and the calculated sporulation rates.

- For each orthogonal assay, discuss the results for each control sample/plate/strain/condition (similar samples can be discussed together as a group if you want).
    - What was the expected result for each control? Why did you expect that result?
    - What can you conclude from each control based on whether or not you got the expected results? If you did not get the expected result for your controls, what might be a potential reason why to explain the difference?

- For each orthogonal assay, discuss the results for each of your six αF-resistant mutants (similar samples can be discussed together as a group if you want).
    - What were the results of each mutant strain? How did they compare with your controls, if applicable?
    - What can you conclude about each mutant strain based on the result for each assay? Explain your reasoning.

- What were the three “best” mutants that you picked? Why did you pick them?

DAY 7 (Thu, Mar 3rd)

3. Identifying the αF-resistant mutants by plasmid recovery

Part 3.1: Yeast transformation of the recovery plasmid

Background: One of the key advantages of performing a transposon mutagenesis screen is that it is usually easier to identify the mutated gene in any isolated mutant yeast because the gene is marked via the transposon insertion. (*Note that this is not the case for a traditional mutagenesis screen where one screens for spontaneous genomic mutations – how might one go about identifying the mutated gene from a traditional mutagenesis screen?*) In 7.003, you will use a plasmid recovery technique to identify the mutated gene in your α-factor-resistant mutants. This technique involves introducing a "recovery plasmid" (containing an *Amp*^{R} selective marker and origin of replication) into the transposon insertion site in the genome of your α-factor-resistant mutants. A linear fragment of yeast genomic DNA containing the transposon insertion site is isolated from your mutant and can be ligated to form a circular plasmid. This plasmid can then either be transformed into bacteria or used as a PCR template to isolate the yeast genomic DNA at the transposon insertion site. The recovered yeast genomic DNA can then be sequenced to determine in what gene the transposon was inserted. The recovery plasmid you will use is the pRSQ2 plasmid, shown below.

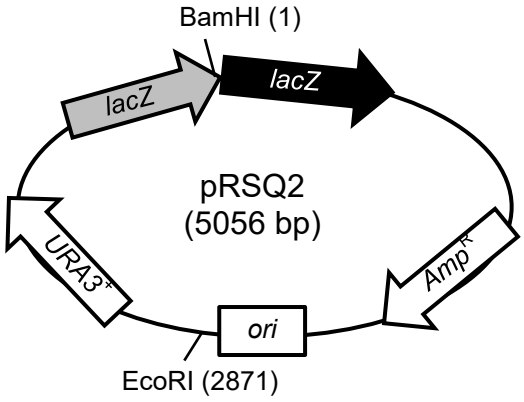


Diagram of pRSQ2 recovery plasmid. Black solid arrow corresponds to bases 26 – 638 of the *lacZ* coding sequence. Gray solid arrow corresponds to bases 2348 – 2970 of the *lacZ* coding sequence. Relevant restriction sites (with relative locations in basepairs) are shown.

Today, you will transform pRSQ2 recovery plasmid that has been linearized with BamHI restriction enzyme into your α-factor-resistant mutant strains. The linearized pRSQ2 plasmid will integrate into the genome of your mutant strains at the transposon insertion site. (*Where specifically in the transposon will the BamHI-digested pRSQ2 plasmid integrate in the genome of your α-factor resistant mutants? What will that region of the yeast genome look like after the recovery plasmid has integrated?*)

Materials: (3) YAPD liquid cultures of your confirmed α-factor-resistant mutants in logarithmic growth phase
pRSQ2 plasmid linearized with BamHI (175 ng/μL)
pCUG plasmid (175 ng/μL – for plasmid map, see lab manual Appendices)
10X LiAc solution (1 M LiAc)

DAY 7 (Thu, Mar 3rd), continued

10X TE solution (100 mM Tris pH 7.5, 10 mM EDTA)
50% PEG solution
Salmon sperm DNA (10 μg/μL)
Sterile ddH_2O
(5) SC-Ura-Leu plates (single black stripe and single green stripe)

Hazardous chemicals used: None

Procedure: *Use a new tip for every solution and between tubes. Use the specially provided barrier P1000 tips when pipetting PEG solution.*

Growing the yeast cultures to logarithmic growth phase

(*Note: The 7.003 staff has done this step for you)*

1) The day before the transformation, inoculate a single colony of each of the three confirmed αF-resistant mutants (streaked on Day 4) in 2.5 mL YAPD media in test tubes. Place the tubes on the roller drum at 30°C overnight (~16 hours).

2) The next morning (the day of the transformation), dilute each cell culture to a final concentration of 5 x 10^6 cells/mL in 10 mL YAPD at 9:00 AM. Allow the cells to grow for another 4 hours (until around 1:00 PM), at which point they should reach log phase.

Yeast transformation (*You start here!*)

3) **You start here!** Label a sterile 1.5-mL Eppendorf tube with "1X Li/TE." Calculate how much 10X LiAc, 10X TE, and sterile water you would need to make 1.2 mL of 1X Li/TE solution (*check your calculations with your TA!*).

1X Li/TE = 0.1 M LiAc, 10 mM Tris-HCl pH 7.5, 1 mM EDTA

4) Add the appropriate volumes of reagents into the "1X Li/TE" Falcon tube to make 1.2 mL of 1X Li/TE solution (use sterile water!). Invert the tube several times to mix well.

5) Label three separate sterile 1.5-mL Eppendorf tubes all with the names of your three confirmed α-factor-resistant mutants you selected from Day 6 (e.g. "Mut2," "Mut4," etc) and with "pRSQ2." Pick any one of your three confirmed mutants and label two additional sterile 1.5-mL Eppendorf tubes with that mutant name (e.g. if you selected Mut2, Mut4, and Mut6 as your three confirmed mutants, you can label two additional tubes with "Mut2"). Label one of these additional tubes with "pCUG" and the other additional tube with "No DNA." Write your **bench number** on all five tubes. You will spin down the yeast cells and set up the transformation reactions in these five tubes.

DAY 7 (Thu, Mar 3rd), continued

6) Pipet 1.5 mL of each log phase α-factor-resistant mutant culture into the appropriate labeled Eppendorf tube (note that for one of your mutant cell cultures, you will be pipetting 1.5 mL of culture into each of three separate tubes). Make sure the yeast cultures are fully resuspended (e.g. by vortexing or pipetting up and down) before you pipet them.

7) Pellet the cells in the Eppendorf tubes by spinning the cells in a centrifuge at 13,000 rpm for 30 seconds (remember to balance your tubes!). Discard the supernatant by pipetting it into a waste beaker.

8) Repeat Steps 6 – 7. *(Make sure you add the correct log phase culture to the proper tube to avoid cross-contamination of your samples!)* Each Eppendorf tube should now contain a cell pellet representing 3 mL of cell culture.

9) Resuspend the cells in each tube with 500 μL sterile water. Pellet the cells by spinning them at 13,000 rpm for 30 seconds. Discard the supernatant as before.

10) Resuspend the cells in each Eppendorf tube in 200 μL 1X Li/TE solution. You will now set up the transformation reactions in your five tubes.

11) Pipet 20 μL of salmon sperm DNA into each of the five Eppendorf tubes.

12) Pipet 5.5 μL of the BamHI-linearized pRSQ2 plasmid into the three Eppendorf tubes labeled "pRSQ2."

13) Pipet 5.5 μL of pCUG plasmid into the "pCUG" tube. Pipet 5.5 μL of sterile water into the "No DNA" tube.

14) Put the five Eppendorf tubes with your yeast transformations in a tube rack in the 30°C warm room for 20 minutes. During this incubation (and the following incubations), you can prepare 1X PEG solution (see Steps 15 – 16 below) and/or work on ILQs.

15) Calculate how much 50% PEG, 10X LiAc, 10X TE, and sterile water you will need to make 8 mL of 1X PEG/Li/TE solution. (*Check your calculations with your TA!*)

1X PEG/Li/TE = 40% PEG, 0.1 M LiAc, 10 mM Tris-HCl pH 7.5, 1 mM EDTA

16) Label a 15-mL Falcon tube with "1X PEG/Li/TE." Add the appropriate amount of reagents into this tube to make 8 mL of 1X PEG/Li/TE solution (use the barrier P1000 tips when pipetting the PEG!). Invert the tube until the contents are thoroughly mixed.

17) After the 15-minute incubation, add 1.2 mL 1X PEG solution to each of your five transformation tubes (use the barrier P1000 tips!). Place the five transformation tubes on the rotator in the 30°C warm room and allow them to incubate for 30 minutes (*make sure you turn the rotator on so that your cells will be mixed during the incubation!*).

DAY 7 (Thu, Mar 3rd), continued

18) After the 30-minute incubation, place your five transformation tubes in a foam float in a 42°C water bath for 40 minutes. Every five minutes or so during this heat shock step, gently invert the tubes to resuspend the cells.

19) Label five SC-Ura-Leu plates with the names of your five transformation reactions. Label all five plates with your **bench number**. (*Why are we using plates lacking both uracil and leucine?*)

20) After the 40-minute heat shock step, pellet the cells by spinning the transformation tubes at 10,000 rpm for 2 minutes. Use a P1000 to carefully discard the supernatant into an appropriate waste beaker (use the barrier P1000 tips!).

21) Resuspend the cells in each tube in 200 μL sterile water.

22) Using sterile glass beads, plate all 200 μL of each yeast transformation onto the appropriately labeled SC-Ura-Leu plate. Use fresh glass beads for each transformation you plate. When the plates are dry, tape all five plates together (label them with your bench number!) and place the plates upside-down in the 30°C warm room to incubate for three days. The 7.003 staff will then store the plates at 4°C until the next lab day.

23) In preparation for Day 8 next week, you will be preparing and labeling tubes of YPD media today. Obtain three glass test tubes and label them with the names of your three confirmed αF-resistant mutants. Also label each tube with your bench number. (When labeling the tubes, please remember to place the tape label on the blue caps, not on the glass tubes.) Using sterile technique, pipet 5 mL of YPD media into each tube. Leave these tubes with your TA for the teaching staff to store – these tubes will be saved until next week, when they will be used for setting up overnight cultures for your group's samples for the genomic DNA prep procedure for Day 8.

Day 7 Pre-Lab Notebook Tips & Reminders:

- What is your Aim for Part 3.1?

Day 7 Post-Lab Notebook Tips & Reminders:

- There is no Post-Lab for Day 7 because you did not obtain any results today, but you should make a note of any deviations you made from the lab manual protocols for your own records.

DAY 8 (Tue, Mar 8th)

3. Identifying the αF-resistant mutants by plasmid recovery

Part 3.2: Genomic DNA prep from αF-resistant candidates

Background: Today, you will start the procedure for isolating yeast genomic DNA (gDNA) from your three confirmed αF-resistant mutants (which you transformed with the pRSQ2 recovery plasmid on Day 7). This isolated gDNA will include the portion of the yeast genome containing the transposon insertion site from each of your three candidates. Due to the length of this procedure, you will only perform the first half of the gDNA isolation today will complete the second half of the protocol on Day 9.

Yeast cells are surrounded by a sturdy cell wall that must be broken down first before you can access and purify any of the contents within the yeast cell. The yeast cell wall can be degraded either mechanically (e.g. via strong agitation in the presence of glass beads) or enzymatically. In 7.003, you will use lyticase to enzymatically break down the yeast cell wall when purifying yeast gDNA. Lyticase is the collective name for a combination of enzymes purified from *Arthrobacter luteus* bacteria and includes a β-1,3-glucanase enzyme and various proteases capable of digesting the glucan polysaccharides and proteins in the yeast cell wall.

Once the yeast cells have been lysed open, the nucleic acids (gDNA and RNA) are isolated from the proteins and cell wall/membrane debris by differentially causing the proteins to precipitate while the nucleic acids remain in solution. This is achieved using potassium acetate (KAc) salt with SDS (sodium dodecyl sulfate) detergent (*how do these reagents cause proteins to precipitate?*). Precipitated proteins and cell debris are then separated from the soluble nucleic acids via centrifugation. The nucleic acid fraction will then be saved until the next lab day for the rest of the gDNA isolation procedure.

Materials: (3) overnight yeast cultures of αF-resistant candidates that have been transformed with the pRSQ2 recovery plasmid
Lyticase (150 μg/mL, in 0.1 M EDTA pH 7.5, 14 mM β-mercaptoethanol)
Genomic Prep Mix (0.5 M Tris-base, 0.25 M EDTA pH 8.5, 2.5% SDS)
5 M potassium acetate (KAc)
100% ethanol

Hazardous chemicals used: Ethanol, isopropanol, β-mercaptoethanol

Procedure: *Use a new tip for every solution and between tubes. Make sure your tubes are balanced in the centrifuge for all spins!*

DAY 8 (Tue, Mar 8th), continued

Preparation of yeast overnight cultures

(*Note: The 7.003 staff has done this step for you)*

1) The day before the gDNA prep procedure, inoculate a single colony from the SC-Ura-Leu pRSQ2-transformed plates (from Part 3.1) for each of your three confirmed αF-resistant mutants into 5-mL YPD media in test tubes.

2) Place the test tubes on the roller drum at 30°C overnight to allow the cultures to grow to saturation.

Yeast genomic DNA prep (*You start here!*)

3) **You start here!** Label three 1.5-mL Eppendorf tubes with the names of your three αF-resistant mutants (e.g. "Mut1," "Mut4," etc) and your **bench number**.

4) Pipet 1 mL of each saturated αF-resistant mutant overnight culture into the appropriately labeled Eppendorf tube. Make sure the overnight cultures are fully resuspended before you pipet them to ensure the cells are uniformly dispersed.

5) Spin the three Eppendorf tubes of cells in a centrifuge at 13000 rpm for 30 seconds to pellet the cells. Carefully remove and discard the supernatant into an appropriate waste beaker without disturbing the cell pellet.

6) Repeat Steps 4 – 5. (*Make sure you add the correct overnight culture to the proper Eppendorf tube to avoid cross-contamination of your samples!*) Each Eppendorf tube should now contain a cell pellet representing 2 mL of cell culture.

7) Resuspend each cell pellet in 250 μL Lyticase. Place the three Eppendorf tubes in a foam float in the **37°C water bath** for 30 minutes. *(What is the purpose of the Lyticase and why are you incubating at 37°C for this step?)* While waiting for this incubation (and for subsequent incubations), you can count your transformation plate colonies (Step 13) and/or work on ILQs.

8) After the Lyticase incubation, add 50 μL of Genome Prep Mix to each tube. Gently invert the tubes several times to mix. Place the three Eppendorf tubes in a foam float in the **65°C water bath** for 30 minutes. *(Note: What is the purpose of the SDS present in the Genome Prep Mix and why are you incubating at 65°C for this step?)*

9) After the 65°C incubation, add 63 μL of 5 M KAc to each tube. Gently invert the tubes several times to mix. Incubate the three Eppendorf tubes on ice for 30 minutes. *(Note: What happened to the contents of the tubes after you mixed in the KAc? What is the purpose of the KAc and why are you incubating on ice for this step?)*

DAY 8 (Tue, Mar 8th), continued

10) Spin the Eppendorf tubes in a centrifuge at 13000 rpm for 10 minutes. During the spin, label three new sterile Eppendorf tubes with the names of your αF-resistant mutants. Write "N.A." (for "nucleic acids") and your **bench number** on each tube.

11) After the 10-minute spin, **save the supernatant** – transfer the supernatant from each tube to the new "N.A." tubes you labeled in Step 10. The pellets left in the old tubes can be discarded at this point (*what cellular components are present in the supernatant and what cellular components were left in the pellet?).*

12) Add 720 μL 100% ethanol to the contents of the three "N.A." tubes (*what is the final concentration of ethanol in each tube?*). Gently invert the tubes several times to mix. Give the three "N.A." tubes to your TA – they will be incubated at -20°C until the next lab session. *(Note: What happened to the contents of the tubes after you mixed in the ethanol? What has occurred?)*

13) Count and record the number of colonies on each of your SC-Ura-Leu transformation plates from Part 3.1. After you have finished counting, please save the three SC-Ura-Leu transformation plates of your αF-resistant mutants and leave them with your TA. Remember to comment briefly on the results of **each** of your plates in your Day 8 Post-Lab. (*Note: How does your pCUG transformation plate compare with the transformation plates for your αF-resistant mutants? What might be a potential reason for any differences?*)

Day 8 Pre-Lab Notebook Tips & Reminders:

- What are your Aims for Part 3.2?

Day 8 Post-Lab Notebook Tips & Reminders:

- Record colony counts for <u>all</u> transformation plates. Remember to comment on <u>each</u> transformation plate in terms of expected vs. observed results and what you can conclude from each (similar plates/samples may be discussed together as a group if you want).

DAY 9 (Thu, Mar 10th)

3. Identifying the αF-resistant mutants by plasmid recovery

Part 3.3: Complete genomic DNA prep from αF-resistant candidates

Background: Today, you will finish the procedure you began on Day 8 to isolate yeast genomic DNA (gDNA) from your αF-resistant mutants. On Day 8, you ended with saving the nucleic acid fraction from your mutant cell lysates, which you then incubated in ethanol at -20°C overnight. Alcohols like ethanol or isopropanol cause nucleic acids to precipitate (*how?*), allowing you to save the precipitated DNA/RNA pellet after centrifugation while removing any impurities (e.g. excess salt, carbohydrates, etc) in the supernatant. RNA is removed from the DNA/RNA nucleic acid fraction using RNase A enzyme to specifically degrade all RNA molecules while leaving DNA molecules intact. Once the gDNA has been sufficiently purified, the DNA pellet can be resolubilized in a suitable buffer (e.g. TE Buffer or plain water).

Materials:
(3) αF-resistant mutant nucleic acid samples in ethanol ("N.A." tubes from Day 8)
100% isopropanol
70% ethanol
RNase A (1 mg/mL in 1X TE buffer)
10X TE Buffer (10 mM Tris-HCl, 1 mM EDTA)
Sterile ddH_2O

Hazardous chemicals used: Ethanol, isopropanol

Procedure: *Use a new tip for every solution and between tubes. Make sure your tubes are balanced in the centrifuge for all spins!*

1) Spin your three "N.A." tubes at 13000 rpm for 5 minutes. **Save the pellet** – using a P1000, carefully remove and discard the supernatant in an appropriate waste beaker, making sure not to disrupt or accidentally suck up the white pellet at the bottom of the tubes (*what cellular components are present in the pellet right now?*).

2) Add 130 μL RNase A to each tube. Place the tubes in a foam float **in the 37°C waterbath** for 30 minutes to dissolve and resuspend the pellets (*what is the purpose of the RNase A and why are you incubating at 37°C for this step?*). During this 30-minute 37°C incubation, briefly vortex the tubes every 5 minutes to help resuspend and dissolve the pellet. While waiting for this incubation, you can pour an agarose gel (Part 3.5) to use for the next lab day and/or work on ILQs.

3) At the end of the RNase 30-minute incubation, add 130 μL 100% isopropanol to each tube. Gently invert the tubes several times to mix. Incubate the tubes at room temperature for 10 minutes (*what is the purpose of the isopropanol and why are you incubating at room temperature for this step?*).

DAY 9 (Thu, Mar 10th), continued

4) Spin the tubes at 13000 rpm for 5 minutes. **Save the pellet** – using a P1000, carefully remove and discard the supernatant into a waste beaker, making sure not to disrupt the white pellet at the bottom of the tubes.

5) Add 500 μL 70% ethanol to each tube to wash the pellets. Repeat Step 4.

6) Use a P200 to remove the last traces of ethanol from each tube (be careful not to disrupt the pellet!). Leave the tubes open and place them upside-down on a clean Kimwipe on your benchtop to air-dry for 5 minutes.

7) Calculate how much 10X TE solution and sterile dH_2O you need to make 200 μL of 1X TE Buffer (*check your calculations with your TA!*). Label a 1.5-mL Eppendorf tube with "1X TE" and prepare 200 μL of 1X TE Buffer accordingly.

8) Add 40 μL 1X TE to each tube and place them in a foam float **in the 37°C waterbath** for 10 minutes (or until the pellet has completely resuspended and dissolved). During this 10-minute incubation, gently flick the bottom of the tubes every few minutes to help resuspend the pellets. Make sure the pellet gets completely dissolved, with no residual pellet "chunks" left in your final genomic DNA samples.

9) Spin the tubes at 13000 rpm for 1 minute to precipitate any undissolved chunks, which may interfere with spectrophotometric measurement. During the spin, label three new sterile 1.5-mL Eppendorf tubes with the names of your αF-resistant mutants. Write "GD" (for genomic DNA) and your **bench number** on each tube.

10) After the 1-minute spin, **save the supernatant** – transfer the supernatant from each "N.A." tube to the new "GD" tubes you labeled in Step 9.

11) Use the spectrophotometer and the microcuvette to measure the DNA concentration in each of your three "GD" gDNA samples (see lab manual Appendices for complete instructions on how to use the specs and microcuvettes). (*What should you use as a blank solution when measuring the concentration of your gDNA samples? Check your answer with your TA!*)

(Note: If the DNA concentration of one of your samples is too high for the spec to measure, you will have to prepare a 1:5 dilution of that sample in 1X TE Buffer (e.g. make ~10 μL of a 1:5 dilution of your gDNA sample in 1X TE Buffer) and measure that diluted sample.)

12) Record the final DNA concentration and A_{260}/A_{280} ratio (*what is the significance of this ratio?*) for each of your gDNA samples in your notebook (remember to account for any dilutions you may have made for any of your samples). Your gDNA is now ready to use for Part 3.4 ("Restriction enzyme digest of the genomic DNA") of the plasmid recovery procedure (*Day 9 continues on the next page!*).

DAY 9 (Thu, Mar 10th), continued

Part 3.4: Restriction enzyme digest of the genomic DNA

Background: In Parts 3.2 – 3.3, you isolated the yeast genomic DNA from your three confirmed αF-resistant mutants that had been transformed with the pRSQ2 recovery plasmid. For the subsequent steps of the plasmid recovery technique, you will need to separate and release a fragment of DNA containing a portion of the recovery plasmid inserted at the transposon site from the rest of the yeast gDNA. This will be accomplished by digesting the gDNA samples with EcoRI restriction enzyme (see restriction site below). Any EcoRI restriction sites present within the entire yeast genome will be cut, including any EcoRI sites within the transposon sequence, the recovery plasmid sequence, and the yeast genomic region surrounding the transposon insertion site. *(After the EcoRI digestion, what will the DNA fragment(s) containing any transposon and/or recovery plasmid sequences look like?)*

EcoRI Restriction Site:

5' – G▼AATTC – 3'
3' – CTTAA▲G – 5'

Materials: (3) yeast genomic DNA samples from your three αF-resistant mutants ("GD" tubes from Part 3.3)
EcoRI-HF (High-Fidelity) restriction enzyme (20 U/μL)
10X CutSmart Buffer (commercially available from NEB)
Sterile ddH_2O

Hazardous chemicals used: None

Procedure: *Use a new tip for every solution and between tubes.*

1) Label three 0.5-mL Eppendorf tubes with the names of your three αF-resistant mutants. Write "Dig" and your **bench number** on each tube as well.

2) You will be using 10 μg of genomic DNA for each of the three digest reactions you will be setting up today. Based on the DNA concentration you measured for each of your genomic DNA samples in Part 3.3, calculate the volume needed to obtain 10 μg of DNA for each of your three genomic DNA samples. Check your calculations with your TA before proceeding.

3) Set up the digest reactions in each the three "Dig" tubes as follows:

__ μL of yeast genomic DNA (volume added to be calculated in Step 2)
1 μL EcoRI-HF restriction enzyme
4 μL 10X CutSmart Buffer

DAY 9 (Thu, Mar 10th), continued

4) Add enough sterile water to each of your three "Dig" tubes such that the final total volume of the digest reaction in each tube is 40 μL. (Note that the amount of water you add to each tube will vary depending on how much volume of genomic DNA was added. **Be sure to include all your calculations in your Day 9 Post-Lab!**)

5) Using a P200 set to ~20 μL, mix the reagents in each tube by gently pipetting up and down several times (try to minimize bubble formation though). Remember to use a new pipet tip for each tube!

6) Spin the tubes for ~15 seconds at 6000 rpm to collect all the contents together at the bottom of the tube (balance your tubes!).

7) Give your three digest reaction tubes to your TA. They will incubate them in a thermocycler set to 37°C overnight (16 hours), after which the 7.003 staff will freeze the tubes at -20°C until the next lab session.

8) **Save the leftover genomic DNA samples in your three "GD" tubes!** Give these three "GD" tubes also to your TA for the teaching staff to store at -20°C – you will need these again during the next lab session.

Part 3.5: Pour an agarose gel

Background: Today, you will pour an agarose gel which will be used on Day 10 for analyzing your lab group's EcoRI gDNA digest samples.

Materials:
Agarose powder
Gel box and casting tray
10-well comb
1X TAE Buffer (40 mM Tris-HCl pH 7.2, 20 mM NaOAc, 1 mM EDTA)
1000X GelGreen dye (from Biotium)

Hazardous chemicals used: None (the GelGreen dye is considered non-toxic), but for safety, always wear gloves when handling agarose gels

Procedure: *Be careful when microwaving solutions that they do not boil over.*

1) Follow the instructions from Part 1.2 on Day 1 to pour a 0.8% agarose gel with 10 wells. When the gel has solidified, label and store the gel properly in Saran wrap and/or a Ziploc bag in the 4°C cold room as previously described.

DAY 9 (Thu, Mar 10th), continued

Day 9 Pre-Lab Notebook Tips & Reminders:

- Include Aims for all parts (Parts 3.3 – 3.5).

Day 9 Post-Lab Notebook Tips & Reminders:

- Record all spectrophotometer measurements/results.
- Remember to include the calculations for setting up your digest reactions.
- How successful do you think your overall genomic DNA prep was, based on your spectrophotometer results? What can you conclude from the A_{260}/A_{280} ratios?

DAY 10 (Tue, Mar 15th)

3. Identifying the αF-resistant mutants by plasmid recovery

Part 3.6: Gel analysis of the digested genomic DNA

Background: To verify that the genomic DNA from your α-factor-resistant mutants was successfully digested, you will run your EcoRI digest reactions on an agarose gel to analyze the sizes of any DNA fragments in your samples. You will use a sample of uncut genomic DNA for comparison in your gel analysis.

Materials:
(3) EcoRI-digested yeast gDNA samples (from Part 3.4, labeled "Dig")
(3) uncut yeast gDNA samples (from Part 3.3, labeled "GD")
0.8% agarose gel (with 1X GelGreen in 1X TAE, poured on Day 10)
1X TAE Buffer (40 mM Tris-HCl pH 7.2, 20 mM NaOAc, 1 mM EDTA)
6X Loading Dye (0.25% Bromophenol blue, 15% Ficoll)
1-kb DNA Ladder (NEB) in 1X Loading Dye (0.1 μg/μL) (see lab manual Appendices for sizes of ladder bands)
Sterile ddH_2O
Gel electrophoresis apparatus and power supply

Hazardous chemicals used: None, but for safety, always wear gloves when handling agarose gels.

Procedure: *Use a new tip for every solution and between tubes.*

1) Label three sterile 1.5-mL Eppendorf tubes with the names of your three αF-resistant mutants and "EcoRI." Label another three 1.5-mL Eppendorf tubes with the names of your three mutants and "uncut."

2) Transfer 10 μL of each EcoRI-digested yeast genomic DNA sample (from the "Dig" tubes) into the appropriate tube labeled in Step 1. **Set aside and save the remainder of each EcoRI-digested genomic DNA sample** – you will need the rest for setting up ligation reactions later in Part 3.7!

3) Transfer 2 μL of each uncut yeast genomic DNA sample (from the "GD" tubes) into the appropriate tube labeled in Step 1.

4) Add 6X Loading Dye and sterile water to each of your "EcoRI" and "uncut" gel sample tubes such that each tube has a final volume of 12 μL total containing 1X Loading Dye. (*Check your calculations with your TA!*)

***Only add the 6X Loading Dye to the "EcoRI" and "uncut" labeled 1.5-mL tubes containing the samples for the gel. Do NOT add the dye to the remainder of the

DAY 10 (Tue, Mar 15th), continued

digest reactions (the "Dig" tubes) – **save the remainders of these digest reactions** for Part 3.7 for setting up the ligations!

5) Centrifuge the six gel sample tubes for ~15 seconds at 6000 rpm (2900 x g).

6) Unwrap your agarose gel (from Day 10) and place it in a tray in a gel box. Add 1X TAE Buffer to the gel box so that it just covers the gel.

7) Load the first lane of the gel with 10 μL of the 1-kb DNA Ladder. Then load 12 μL each of your six prepared samples into the next six lanes. (Note the order of the lanes in your notebook!)

8) Connect the electrodes of the power supply and gel box in the proper orientation so **the DNA runs towards the (red) electrode**. Turn on the power supply and turn the voltage to ~100 volts. Check current is actually running through the gel box and check the running direction of the loading dye to make sure the gel orientation is correct.

9) Run the gel at a constant voltage until the dye has migrated about two-thirds down the gel (approximately 45 – 60 minutes). Turn off the power supply.

10) Wearing gloves, remove the gel tray (with the gel in it) from the gel box. Place the gel tray and gel in a white plastic box and take it to the gel doc station.

11) Check your gel results with your TA or instructor before setting up the ligation reactions in Part 3.7 – if your genomic DNA was not properly digested, you may need to obtain a back-up sample from the 7.003 staff before proceeding.

Part 3.7: Ligation of the genomic DNA digest

Background: Following EcoRI digestion of the yeast gDNA from your αF-resistant mutants, you have a collection of linear fragments of gDNA (including a linear fragment of gDNA containing a portion of the pRSQ2 recovery plasmid integrated at the transposon insertion site). You will now use T4 DNA Ligase to ligate these linear genomic fragments into circularized DNA so that they can be used as PCR templates for the next step in the plasmid recovery procedure (*why do you think we need to circularize the DNA before performing the PCR step?*).

For this particular ligation step, it should be noted that *intra*molecular ligation reactions are preferred over *inter*molecular ligation reactions – ideally, we want individual linear

DAY 10 (Tue, Mar 15th), continued

genomic fragments to re-circularize (i.e. one DNA fragment per re-ligated circle), instead of having multiple linear fragments ligated together into a circle. *(Why do you think an intramolecular ligation is better for the plasmid recovery procedure than an intermolecular ligation? What ligation reaction conditions might favor an intramolecular ligation over an intermolecular ligation?)*

Materials: (3) EcoRI-digested yeast gDNA samples from your αF-resistant mutants (from Part 3.4, labeled "Dig")
T4 DNA ligase (400 U/μL)
10X T4 DNA Ligase Buffer (commercially available from NEB)
100 mM ATP
Sterile ddH_2O

Hazardous chemicals used: None

Procedure: *Use a new tip for every solution and between tubes.*

1) Label three sterile 0.5-mL Eppendorf tubes with the names of your three α-factor-resistant mutants. Write "Lig" and your **bench number** on each tube.

2) Set up the ligation reactions in each of the three "Lig" tubes as follows:

25 μL EcoRI-digested yeast gDNA ("Dig" tubes from Part 3.4)
1 μL T4 DNA Ligase
2.5 μL ATP *(what is the purpose of the ATP?)*
25 μL 10X T4 DNA Ligase Buffer
196.5 μL sterile water
Total: 250 μL

3) Mix by pipetting the reagents up and down several times – try to minimize bubbles. Spin the tubes for ~15 seconds at 6000 rpm.

4) Give your three ligation reaction to your TA. They will incubate them in a thermocycler set to 16°C overnight (16 hours), after which the tubes will be frozen at -20°C until the next lab session.

DAY 10 (Tue, Mar 15th), continued

Day 10 Pre-Lab Notebook Tips & Reminders:

- Remember to include Aims for Parts 3.6 and 3.7 today.

Day 10 Post-Lab Notebook Tips & Reminders:

- Be sure to label the gel photo such that it's clear what is loaded in each lane and what the sizes of all ladder bands are.

- You should discuss each lane of your gel (lanes/samples with similar overall results can be discussed together as a group if you want).

    - What was the expected overall result for each lane? Why?

    - What can you conclude based on whether or not you saw the expected result for each lane? Explain your reasoning. If you did not get the expected results, what might be a possible reason to explain the difference?

DAY 11 (Thu, Mar 17th)

3. Identifying the αF-resistant mutants by plasmid recovery

Part 3.8: Purification of ligation reactions

Background: The ligation reactions containing your αF-resistant mutant genomic DNA currently have a relatively large total volume (250 μL). Before you can perform an inverse PCR reaction from these ligation reactions, you will first need to concentrate your ligation samples such that they are in a smaller total volume more amenable for the procedure. Furthermore, the DNA ligase, salt, ATP and other ligation buffer ingredients must be removed from the sample because they may interfere with any subsequent experiments. These goals will be accomplished by using DNA purification kits such as the E.Z.N.A. Cycle Pure Kit (Omega). Here, the sample being purified is first mixed with the high-salt CP Buffer to dehydrate the DNA. The mixture is passed over a silica column where the dehydrated DNA is able to bind the column whereas impurities pass through the column and are removed. Salts are then removed from the sample by washing the DNA in the column with the ethanol-based DNA Wash Buffer. Finally, DNA is eluted off the column under low salt conditions using an Elution Buffer amenable to use in the next inverse PCR step and in a smaller volume to concentrate the sample.

Materials: (3) ligation reactions of your αF-resistant mutants (from Part 3.7)
CP Buffer
DNA Wash Buffer
Elution Buffer
Sterile dH_2O
(3) HiBind DNA spin columns (blue) and collection tubes

Hazardous chemicals used: Guanidine hydrochloride (CP), isopropanol (CP), ethanol (DNA Wash)

Procedure: *This protocol is taken from the E.Z.N.A. Cycle Pure manual (Omega, 2012). Perform this procedure at* ***room temperature*** *(why?). Use a new tip for every solution and between tubes. When adding solutions to the spin columns, be careful not to pierce the column silica membrane with your pipet tip.*

1) Label three sterile 1.5-mL Eppendorf tubes with the names of your three αF-resistant mutants. Write "Lig" and your **bench number** on each tube.

2) Transfer the entire contents of your ligation reactions (from the small 0.5-mL tubes from Part 3.7) into the corresponding 1.5-mL tubes you labeled in Step 1.

3) Add five volumes of CP Buffer to each tube (*check your calculations with your TA!*). Mix each tube well by inverting them five times.

DAY 11 (Thu, Mar 17th), continued

4) Label the three blue HiBind spin columns and their 2-mL collection tubes with the names of your three αF-resistant mutants and with your bench number (write on the frosted surfaces). Place the spin columns inside the collection tubes.

5) Transfer 750 μL of the Lig/CP Buffer mixture in each tube into the corresponding spin column.

6) Centrifuge the spin columns at 13000 rpm (14000 x g) for 1 minute. Discard the flow-through into a waste beaker and place the columns back into the collection tubes.

7) Repeat Steps 5 and 6 until all of the Lig/CP Buffer mixture has passed through the spin column.

8) Add 700 μL of DNA Wash Buffer to each column.

9) Centrifuge the spin columns at 13000 rpm (14000 x g) for 1 minute. Discard the flow-through in the waste beaker as before.

10) Centrifuge the spin columns again at 13000 rpm (14000 x g) for 2 minutes. (*Note: This step removes any residual ethanol that may inhibit subsequent experiments.*)

11) Label three sterile 1.5-mL Eppendorf tubes with the names of your three αF-resistant mutants. Write "PL" (for Purified Ligations) and your bench number on each tube. Remove the spin columns from the collection tubes and place each column into the appropriate labeled Eppendorf tube. The collection tubes can now be discarded.

12) Add 30 μL of Elution Buffer to each column. Pipette the buffer directly onto the center of the column membrane, but be careful not to touch the membrane with your pipet tip.

13) Let the columns sit undisturbed (with the Elution Buffer in them) for 2 minutes.

14) Centrifuge the Eppendorf tubes with the columns in them for 1 minute at 13000 rpm (14000 x g). Discard the spin columns and keep the "PL" Eppendorf tubes which should now each contain ~30 μL of your purified ligation reactions for your three mutants. Use these purified ligations to set up the inverse PCR samples in Part 3.9 below.

Part 3.9: Preparation of the inverse PCR reactions

Background: Your ligation reactions contain yeast genomic fragments from your αF-resistant mutants that have been self-ligated to form a large collection of many different circular DNA plasmids. One of these plasmids consists of a portion of the original pRSQ2 recovery plasmid integrated in the transposon insertion site (*what does this*

DAY 11 (Thu, Mar 17th), continued

particular circularized piece of DNA look like?). You now need to isolate just this piece of DNA containing the transposon insertion site from the rest of the circularized genomic fragments so that you can sequence it to identify the mutated gene(s) in your αF-resistant mutants. You will perform an inverse PCR reaction to specifically amplify this desired region by using two primers complementary to two regions in the pRSQ2 plasmid. "Primer lacZ" binds to +268 to +250 in pRSQ2, and "Primer ori" binds to +2771 to +2789 in pRSQ2 (see primer sequences below). *(What would be the orientation of these primers so they could amplify the region that contains the genomic DNA? What would your final PCR product look like?)*

Primer lacZ: 5' – gta acc gtg cat ctg cca g – 3'

Primer ori: 5' – cca tga tta cgc caa gct c 3'

Materials:
(3) purified ligation reaction tubes ("PL" tubes from Part 3.8)
pRSQ2 plasmid DNA (~5 kb, 33 ng/μL, see plasmid map in lab manual Appendices)
2X Phusion High-Fidelity Master Mix (from NEB)
Primer lacZ stock (100 μM)
Primer ori stock (100 μM)
Sterile ddH_2O
(3) 0.5-mL PCR tubes (blue)

Hazardous chemicals used: None

Procedure: *Keep all reagents and tubes on ice at all times. Make sure you use a new tip for every solution and between tubes.*

1) The stock tubes of primers are too concentrated, so you will need to dilute them before you can use them in your PCR samples. Calculate how much Primer lacZ stock, Primer ori stock, and sterile water you would need to make a 40 μL solution containing both primers each at a final concentration of 10 μM. *Check your calculations with a TA!*

2) Label a 1.5-mL Eppendorf tube as "Primers" along with your bench number. Follow your calculations from Step 1 to make a 40 μL solution with both primers each at a final concentration of 10 μM.

3) Mix the contents of the "Primers" tube by gently pipetting up and down several times. Spin the tube for ~15 seconds at 6000 rpm (2900 x g) and leave it on ice. When you set up your PCR samples, make sure you use this diluted "Primers" Eppendorf tube when adding the primers (and not the original stock primer tubes).

DAY 11 (Thu, Mar 17th), continued

4) Obtain five sterile **blue** 0.5-mL PCR tubes (*what do you think is the difference between the blue 0.5-mL PCR tubes and the clear regular Eppendorf tubes?*). Label three tubes with the names of your three αF-resistant mutants. Label one tube with "pRSQ2" and another tube with "No DNA." Write your bench number on all five tubes.

5) Set up the five inverse PCR reactions as follows (*what is the purpose of each tube?*):

	Three αF-resistant mutants tubes	**"pRSQ2" tube**	**"No DNA" tube**
	25 μL 2X Phusion Master Mix	25 μL 2X Phusion Master Mix	25 μL 2X Phusion Master Mix
	5 μL primers (from the "Primers" Epp tube)	5 μL primers (from the "Primers" Epp tube)	5 μL primers (from the "Primers" Epp tube)
	3 μL from the corresponding "PL" ligation reaction tube	3 μL pRSQ2 template DNA	-
	17 μL sterile dH_2O	17 μL sterile dH_2O	20 μL sterile dH_2O
Total Volume	50 μL	50 μL	50 μL

6) Mix the reagents in each tube by gently pipetting up and down several times (try not to introduce any bubbles). Spin all three tubes for ~15 seconds at 6000 rpm (2900 x g).

7) Make sure your five tubes are tightly capped and give them to your TA. They will place them in the thermocycler to run the following PCR program (*what is the purpose of each thermocycler step?*):

1) 95°C for 2 minute
2) 95°C for 30 seconds
3) 60°C for 30 seconds
4) 72°C for 90 seconds
5) Repeat Steps (2) through (4) 29 more times
6) 72°C for 10 minutes
7) 4°C indefinitely until the next lab session

8) Save your leftover purified ligation reactions (three "PL" tubes) and also give them to your TA – these will be stored at -20°C until the next lab session when they will be needed again.

******Note: Day 11 continues on the next page!******

DAY 11 (Thu, Mar 17th), continued

Part 3.10: Pour an agarose gel

Background: You will pour an agarose gel today, which will be used on Day 12 for analyzing your inverse PCR samples.

Materials: Agarose powder
Gel box and casting tray
10-well comb
1X TAE Buffer (40 mM Tris-HCl pH 7.2, 20 mM NaOAc, 1 mM EDTA)
1000X GelGreen dye (from Biotium)

Hazardous chemicals used: None (the GelGreen dye is considered non-toxic), but for safety, always wear gloves when handling agarose gels

Procedure: *Be careful when microwaving solutions that they do not boil over.*

1) Follow the instructions from Part 1.2 on Day 1 to pour a 0.8% agarose gel with 10 wells. When the gel has solidified, label and store the gel properly in Saran wrap and/or a Ziploc bag in the 4^{o}C cold room as previously described.

Day 11 Pre-Lab Notebook Tips & Reminders:

- Remember to include Aims for all parts (Parts 3.8 – 3.10)

Day 11 Post-Lab Notebook Tips & Reminders:

- There is no Post-Lab for Day 11 because you did not obtain any results today, but you should make a note of any deviations you made from the lab manual protocols for your own records.

DAY 12 (Tue, Mar 29th)

3. Identifying the αF-resistant mutants by plasmid recovery

Part 3.11: Gel analysis of inverse PCR products

Background: The inverse PCR procedure should amplify the yeast genomic DNA region where the transposon integrated into the genome. Eventually, we will sequence this region to determine the exact site of the transposon integration. First though, we will check if the region of interest has been amplified at all by performing agarose gel electrophoresis on our inverse PCR products to confirm if new copies of DNA were synthesized.

Materials: (5) inverse PCR reactions (from Part 3.9 on Day 11)
(3) purified ligation reactions ("PL" tubes from Part 3.8 on Day 11)
0.8% agarose with 1X GelGreen in 1X TAE (poured from Day 11)
1X TAE Buffer (40 mM Tris-HCl pH 7.2, 20 mM NaOAc, 1 mM EDTA)
6X Loading Dye (0.25% Bromophenol blue, 15% Ficoll)
1-kb DNA Ladder (NEB) in 1X Loading Dye (0.1 μg/μL)
(see Appendices for the sizes of the 1-kb DNA ladder fragments)
Sterile ddH_2O
Gel electrophoresis apparatus and power supply

Hazardous chemicals used: None, but for safety, always wear gloves when handling agarose gels.

Procedure: *Use a new tip for every solution and between tubes.*

1) Label five 1.5-mL Eppendorf tubes with the names of your five inverse PCR reactions and with "PCR." Pipet 5 μL of each PCR reaction into the appropriately labeled Eppendorf tube. Also label three 1.5-mL Eppendorf tubes with the names of your three αF-resistant mutants and with "Lig" – pipet 3 μL of each purified ligation reaction (from the "PL" tubes) into the appropriate "Lig" tube. These are your eight gel samples you will be running on an agarose gel.

2) Add 6X Loading Dye and sterile water to each of your eight gel samples such that each tube has a final volume of 12 μL total containing 1X Loading Dye. (*Check your calculations with your TA!*)

> ***Make sure you only add the 6X Loading Dye to the 1.5-mL tubes containing just the 5 μL or 3 μL aliquots of your samples (i.e. the gel sample Eppendorf tubes you labeled from Step 1). Do NOT add the dye to the remainder of the PCR reactions – **save the remainders of these PCR reactions for "Part 3.12: Purification of inverse PCR products."**

DAY 12 (Tue, Mar 29th), continued

3) Centrifuge the eight Eppendorf tubes containing your gel samples + Loading Dye for ~15 seconds at 6000 rpm (2900 x g).

4) Unwrap your agarose gel (from Day 11) and place it in a tray in a gel box. Add 1X TAE Buffer to the gel box so that it just covers the gel.

5) Load the first lane of the gel with 10 μL of the 1-kb DNA Ladder. Then load each of your prepared gel samples into the next eight lanes. (Note the order of the lanes in your notebook!)

6) Connect the electrodes of the power supply and the gel box in the proper orientation so that the DNA runs towards the **positive (red)** electrode.

7) Turn on the power supply and turn the voltage to ~100 volts. Check that the current is actually running through the gel box and check the running direction of the loading dye to make sure the gel orientation is correct. While your gel is running, you can proceed to "Part 3.12: Purification of inverse PCR mutagenesis products."

8) Run the gel at a constant voltage until the dye has migrated about two-thirds of the way down the gel (approximately 45 – 60 minutes). Turn off the power supply.

9) Wearing gloves, remove the entire gel tray (with the gel in it) from the gel box. Place the gel tray and gel in a white plastic box and take it to the gel doc system.

Part 3.12: Purification of inverse PCR products

Background: Following the inverse PCR reaction, the DNA polymerase, leftover primers, and other PCR reagents must be removed from the sample because they may interfere with the subsequent sequencing reaction. As done before, their removal can be achieved using DNA purification kits such as the E.Z.N.A.® Cycle Pure Kit (Omega).

Materials: (3) Inverse PCR reactions of your αF-resistant mutants (from Part 3.9)
CP Buffer
DNA Wash Buffer
Elution Buffer
(3) HiBind® DNA spin columns (blue) and collection tubes

Hazardous chemicals used: Guanidine hydrochloride (CP), isopropanol (CP), ethanol (DNA Wash)

DAY 12 (Tue, Mar 29th), continued

Procedure: *This protocol is taken from the E.Z.N.A.® Cycle Pure manual (Omega, 2012). Perform this procedure at* ***room temperature****. Use a new tip for every solution and between tubes. When adding solutions to the spin columns, be careful not to pierce the column silica membrane with your pipet tip.*

1) You will only be purifying the PCR reactions in the three tubes corresponding to your three αF-resistant mutants. You can discard the "pRSQ2" and "No DNA" PCR tubes at this time. Add five volumes of CP Buffer to each of the remaining PCR reactions in your three αF-resistant mutant PCR tubes (*check your calculations with your TA first!*) . Mix each tube by inverting them five times.

2) Label the three blue HiBind® spin columns and their 2-mL collection tubes with the names of your three αF-resistant mutants and with your bench number (write on the frosted surfaces). Place the spin columns inside the collection tubes.

3) Transfer all of the PCR/CP Buffer mixture in each tube into the corresponding spin column.

4) Centrifuge the spin columns at 13000 rpm (14000 x g) for 1 minute. Discard the flow-through into a waste beaker and place the columns back into the collection tubes.

5) Add 700 μL of DNA Wash Buffer to each column.

6) Centrifuge the spin columns at 13000 rpm (14000 x g) for 1 minute. Discard the flow-through in the waste beaker as before.

7) Centrifuge the spin columns again at 13000 rpm (14000 x g) for 2 minutes. (*Note: This step is critical to remove any residual ethanol that may inhibit subsequent experiments.*)

8) Label three sterile 1.5-mL Eppendorf tubes with the names of your three αF-resistant mutants. Also write "PIP" (for Purified Inverse PCR) and your bench number on each tube. Remove the spin columns from the collection tubes and place each column into the appropriate labeled Eppendorf tube. The collection tubes can now be discarded.

9) Add 30 μL of Elution Buffer to each column. Pipette the Elution Buffer directly onto the center of the column membrane, but be careful not to touch the membrane with your pipet tip.

10) Let the columns sit undisturbed (with the Elution Buffer in them) for 2 minutes.

11) Centrifuge the Eppendorf tubes with the columns in them for 1 minute at 13000 rpm (14000 x g). Discard the spin columns and keep the Eppendorf tubes which should now

DAY 12 (Tue, Mar 29th), continued

contain ~30 μL of your purified PCR reactions for your three αF-resistant mutants. Place these tubes on ice.

12) Check on your agarose gel from Part 3.11 if you have not done so yet – if it's ready, you can view your gel on the gel doc station and have a picture taken.

Part 3.13: Sending inverse PCR products out for sequencing

Background: The αF-resistant phenotype of your mutant strains is (presumably) caused by the mTn3 transposon inserting in the yeast genome within a gene's coding sequence (or a gene's regulatory elements) and thus disrupting the normal function of that gene. The disrupted gene most likely is involved somehow in α-factor detection and/or the mating response pathway. The identity of this gene can be determined by sequencing the "junction site" where the transposon integrated into the gene coding sequence.

Your inverse PCR product will be sequenced by Quintara Biosciences using the Sanger sequencing method. In this method, template DNA (e.g. dsDNA like your PCR products) is mixed with **one** primer, dNTPs, a limiting amount of fluorescently-labeled dideoxy NTPs (ddNTPs with a different fluorescent dye conjugated to each type of ddNTP), and DNA Polymerase. During the sequencing reaction, the primer binds the template DNA, and DNA Polymerase extends the primer by incorporating dNTPs that match the template DNA. Occasionally, however, extension of the new DNA strand is prematurely terminated when a fluorescent ddNTP is incorporated (*why does incorporation of a ddNTP terminate DNA chain extension?*). At the end of the sequencing reaction, various sizes of fluorescently-labeled DNA fragments are synthesized (note that there are many copies of each different-sized DNA fragment). This mixture of DNA fragments is then separated using acrylamide gel electrophoresis, and the ddNTP at the 3' end of each DNA fragment in the gel is distinguished using a fluorescent detector. The final readout is a chromatic trace containing a series of peaks corresponding to the order of the nucleotides in the template DNA you are sequencing.

The plasmid recovery technique isolated and PCR-amplified a region containing the transposon junction site (fused to part of the pRSQ2 recovery plasmid sequence), which you will send to Quintara Biosciences for sequencing (*why didn't we just directly sequence the transposon junction site from the genomic DNA of our mutants? Why did we bother doing the plasmid recovery technique to ligate and amplify the transposon junction site on a plasmid?*). For a sequencing primer, you will use the M13 (-40) primer: 5'–GTT TTC CCA GTC ACG AC–3'. This primer is homologous to part of the *lacZ* coding sequence (from base position +57 to +41). The primer anneals to the sense strand of the *lacZ* coding sequence (i.e. the M13 (-40) primer is a "reverse" primer with respect to *lacZ* – it is complementary to the *lacZ* sense strand and has the same sequence as the *lacZ* anti-sense strand). *(Where will the M13 (-40) primer anneal*

DAY 12 (Tue, Mar 29th), continued

on your inverse PCR product sample? How far away is the M13 primer annealing site from the transposon junction site?)

Normally, when sending samples to be sequenced, you would include both the DNA template to be sequenced and your specific sequencing primer. Your particular primer (M13(-40) sequencing primer), however, is a primer that is commonly enough used and so is readily available at the Quintara Biosciences facility. You will therefore just be sending your purified PCR DNA products to Quintara, and the M13(-40) sequencing primer will be added later by the technicians at Quintara at the sequencing facility.

Materials: (3) purified inverse PCR products from your three αF-resistant mutants ("PIP" tubes from Part 3.12)
M13 (-40) sequencing primer (5 μM, will be provided at QuintaraBio)
Sterile ddH_2O
0.2-mL 8-tube strips

Hazardous chemicals used: None

Procedure: *Use a new tip for every solution and between tubes.*

1) Check your gel results with a TA or instructor before setting up your sequencing samples – if your inverse PCR did not work properly, you may need to obtain a back-up sample to use for your sequencing reactions.

2) Use the spectrophotometer and microcuvette to measure the DNA concentration in each of your "PIP" samples (see lab manual Appendices for complete instructions on how to use the specs and microcuvettes). (*What should you use as a blank solution when measuring the concentration of your "PIP" DNA samples? Check your answer with your TA!*). Record the final DNA concentration and A_{260}/A_{280} ratio for each of your "PIP" PCR samples in your notebook.

3) The Quintara sequencing facility requires that PCR DNA templates be submitted at a final concentration of 20 ng/μL. For each of your three mutant samples, calculate how much "PIP" PCR product and how much sterile water you need to mix to end up with 12 μL total volume of diluted "PIP" PCR product at 20 ng/μL final concentration. **Check your calculations with your TA and include this calculation in your Day 12 Post-Lab**!

4) Label three 0.5-mL Eppendorf tubes with "dil" (for "dilute") and the names of your three αF-resistant mutants. Dilute each "PIP" PCR product in sterile water to a final concentration of 20 ng/μL in a final total volume of 12 μL according to your calculations from Step 3. Mix the contents of each tube by gently pipetting up and down several times and spin them for ~15 seconds at 6000 rpm (2900 x g). **When setting up your sequencing samples in Step 8, remember to use these diluted PCR samples!**

DAY 12 (Tue, Mar 29th), continued

5) The class sequencing samples will be sent to Quintara Biosciences in small 8-tube strips, numbered sequentially (1, 2, 3, 4, etc). Each lab group will be assigned three class sample numbers (e.g. Bench D1 may be assigned class sample numbers 1 – 3, Bench D3 may be assigned class sample numbers 4 – 6, etc). Check with your TA to find out which three class sample numbers have been assigned to your lab group.

6) Decide which of your three mutant PCR samples (e.g. Mut2, Mut5, etc) will be associated with each class sample number you were assigned. Record this information in your notebook (you will need it for your Day 12 Post-Lab!). For instance, if you were analyzing mutants Mut2, Mut5, and Mut6, and your assigned three class numbers were 13, 14, and 15, then you might decide 13 = Mut2, 14 = Mut5, and 15 = Mut6.

7) When ready, ask the teaching staff for the tip boxes containing the 8-tube strips. Each strip consists of 8 tubes connected together, so multiple groups will be sharing the same strip. Do not remove the tube strips from the tip box they are sitting in (to minimize accidental tube/sample-switching). Find the tube strip with the tubes labeled with your three assigned class sample numbers. (Note that for some groups, their three sample numbers may be split between two different tube strips.)

8) Add 10 μL of each **diluted** "PIP" PCR product into its corresponding tube in the strip. Make sure you match each mutant sample with its appropriate class sample number tube in the strip as you decided in Step 6 (e.g. add Mut2 to Tube 13, add Mut5 to Tube 14, etc). If you accidentally add the wrong sample into the wrong tube, let a TA or instructor know at once so we can prepare and label a new 8-tube strip to use.

9) When you have finished adding your diluted "PIP" PCR samples, close the lid on the tip box rack (to minimize contaminating the samples). Once all samples have been added by all lab groups, they will be collected by the 7.003 staff to be sent off to the sequencing facility.

10) Please save your remaining purified inverse PCR product samples (the undiluted "PIP" tubes) and your remaining purified ligation samples (the "PL" tubes) and give to your TA – we will store them in case for future use.

DAY 12 (Tue, Mar 29th), continued

Day 12 Pre-Lab Notebook Tips & Reminders:

- Remember to include Aims for all parts (Parts 3.11 – 3.13)

Day 12 Post-Lab Notebook Tips & Reminders:

- Be sure to label the gel photo such that it's clear what is loaded in each lane and what the sizes of all ladder bands are.

- You should discuss each lane of your gel (lanes/samples with similar overall results can be discussed together as a group if you want).

    - What was the expected overall result for each lane? Why?

    - What can you conclude based on whether or not you saw the expected result for each lane? Explain your reasoning. If you did not get the expected results, what might be a possible reason to explain the difference?

- Record all spectrophotometer measurement/results and all DNA dilution calculations.

- Record which class sequencing sample numbers were assigned to your lab group and which mutant PCR was associated with each class number.

DAY 13 (Thu, Mar 31st)

3. Identifying the αF-resistant mutants by plasmid recovery

Part 3.14: Analysis of the inverse PCR sequencing results

Background: Today, you will interpret the sequencing results from your three inverse PCR reactions from Day 12 and determine the yeast genomic location where the mTn3 transposon has integrated to cause the αF-resistant mutant phenotype. You will use the NCBI BLAST website. BLAST (Basic Local Alignment Search Tool) is an online database that allows one to search any annotated genomes (e.g. the human genome or the yeast genome, etc.) for a particular query sequence of interest.

Note that two different versions are provided for each individual sequencing result: one version with NNN base-calls and one version without NNN base-calls. The "with NNNs" version has an "N" at each base position in the sequencing result where the QuintaraBio analysis software was unable to make an accurate base-call (i.e. it wasn't confident enough to declare a particular base for that position above a certain stringency threshold). The "no NNNs" version replaces each "N" in the sequencing result with the most likely base predicted for that position, regardless of how confident that prediction is. You may find it useful to analyze both the "with NNNs" and the "no NNNs" versions for each of your three sample sequencing results.

Materials: (3) sets of sequencing data for your three mutants

Procedure:

1) A Day 12 Gene ID BLAST Worksheet has been posted on Canvas. Use the worksheet as a guide to first determine the location of the first EcoRI restriction site in each PCR sequence (*why is this important?*) and then to search on the NCBI BLAST website to identify the mutated gene at the transposon insertion site.

2) Confirm your sequencing results with your TA – we will be compiling the sequencing results from all the lab groups and will be posting this class data for you to discuss later in your SciComm paper Discussion section.

Part 3.15: Re-streaking mutant strains

Background: You will re-streak the mutant strains for which you have positively identified the mutated gene, onto fresh new plates. This will ensure fresh colonies of your mutant strains to use for future experiments.

DAY 13 (Thu, Mar 31st), continued

Materials: SC-Leu + αF plates with identified αF-resistant mutants (streaked from Day 4)
Fresh SC-Leu + αF (10^{-5} M) plates (single green & orange stripe)

Procedure: *Use a new sterile toothpick between sequences of streaks and colonies.*

1) Label fresh SC- Leu + αF plates with your mutant strain names, along with your bench number, the date, and the identified gene name for that corresponding mutant.

2) Re-streak each mutant strain onto the newly labeled plates, using the streaking technique as described in Part 1.9 on Day 4.

3) Incubate the plates upside-down in the 30°C warm room for two – three days. The 7.003 staff will then store the plates at 4°C until they are needed again.

4) On the next lab day (Day 14), you will be isolating RNA from one of your mutant strains to analyze its gene expression during α-factor treatment. In preparation for Day 14, you will be preparing and labeling tubes of YPD media today. Pick one of your three αF-resistant mutants – you will be isolating RNA from this mutant to analyze its gene expression during α-factor treatment in the subsequent lab days. The mutant you pick can be a mutant for which you successfully identified the mutated gene or a mutant which is still unidentified – either option is fine. Note that if you choose an unidentified mutant, then you should do Steps 1 – 3 for that mutant to restreak it to a fresh SC-Leu + αF plate.

5) Obtain six glass test tubes. Label three of them with "PPY295" and the other three with the name of the mutant you picked in Step 4. Label the three PPY295 tubes with "O/N," "untreat," and "αF." Label the three mutant tubes the same as well. Label all six tubes with your bench number. Add 5 mL YPD liquid media to each of the six test tubes (use sterile technique!). Give the tubes to your TA – these tubes will be saved to use later for setting up overnight cultures and for diluting the cultures on the morning of Day 14 for the RNA isolation procedure.

DAY 13 (Thu, Mar 31st), continued

Day 13 Pre-Lab Notebook Tips & Reminders:

- Remember to include Aims for all parts (Parts 3.14 – 3.15).

Day 13 Post-Lab Notebook Tips & Reminders:

- Record and discuss your results for all three genes identified. If you were unable to identify a gene for some of your mutants, you may use the back-up sample sequences provided on Canvas so that you have three genes total to discuss.
    - What is known about each gene identified in terms of its function or properties (e.g. what processes is it involved in, where is it localized, what other proteins does it interact with, etc)?
    - How might each gene have been isolated in your screen? Try to come up with a specific molecular mechanism.

DAY 14 (Tue, Apr 5th)

4. Analyzing RNA expression of αF-resistant mutants

Part 4.1: Isolation of total RNA from WT and mutant yeast

Background: Today, you will isolate total RNA from WT PPY295 yeast and your αF-resistant mutant yeast that have been treated with or without α-factor pheromone. The RNA can then be later analyzed to see if there are any changes in gene expression after exposure to the mating pheromone (e.g. is transcription of mating-specific genes increased in either the WT or mutant yeast in the presence of α-factor pheromone?).

To isolate the RNA from yeast cells, you will use the RNeasy Mini Kit (from Qiagen), which utilizes several principles similar to the Omega commercial DNA purification kits. After yeast cells are lysed open (e.g. either via enzymatic lysis or mechanical lysis), the cell lysate is passed through a silica membrane column in the presence of ethanol and high salts. These conditions allow the RNA molecules to selectively bind the silica membrane while other molecules pass through and are washed away. A DNase I treatment step also helps to remove contaminating genomic DNA. The final RNA sample can be eluted from the column with water, which hydrates the RNA and releases it from the silica membrane.

The RNA isolation protocol requires starting with a specific number of yeast cells, so you must first determine the concentration of the yeast liquid cultures you are using. You will do this using a spectrophotometer to measure the optical density (OD) of each yeast culture. The number of yeast cells in a culture will affect how much light is scattered when light is passed through the sample. The more yeast cells present, the greater the light scattering, resulting in a higher OD reading. Yeast cultures are usually measured using light at wavelength 600 nm to obtain an OD_{600} reading. For yeast, the standard OD conversion is OD_{600} = 1.0 is equivalent to a cell concentration of 1 x 10^7 cells/mL.

Materials:
- WT PPY295 yeast liquid cultures, untreated ("UT") and treated with α-factor ("αF")
- αF-resistant mutant yeast liquid cultures, untreated ("UT") and treated with α-factor ("αF")
- YPD liquid media
- Y1BL Buffer (from Qiagen, 1 M sorbitol, 0.1 M EDTA pH 7.4, 0.1% BME, 1 U/μL lyticase)
- RLT Buffer + 1% β-mercaptoethanol (from Qiagen)
- 100% ethanol
- RW1 Buffer (from Qiagen)
- DNase I (340 Kunitz Units/mL in RDD Buffer, from Qiagen)
- RPE Buffer (from Qiagen)
- RNase-free H_2O

DAY 14 (Tue, Apr 5th), continued

Homogenizer columns (green, from Omega) and collection tubes
RNeasy spin columns (pink, from Qiagen) and collection tubes
Spectrophotometer
Cuvettes (in Styrofoam boxes in lab drawers)
Microcuvette (for measuring RNA concentration

Hazardous chemicals used: β-mercaptoethanol (in Y1BL and RLT Buffers), ethanol (in RPE Buffers), guanidinium chloride (in RLTand RW1 Buffers)

Procedure: *Make sure you use a new tip for every solution and between tubes. Perform the RNA isolation steps at room temperature, unless noted otherwise.*

Very important RNA note: *RNA molecules are very delicate and easily degraded by RNase enzymes (which are very hardy enzymes that are everywhere – all over the bench tops, on the equipment, all over your hands, everywhere!). When working with RNA, try to work as cleanly as possible. Always wear fresh gloves when handling anything and wipe down your bench top surface and Pipetmen with RNaseZap or 70% ethanol before starting your experiments. Only use (clean!) tips or tubes that have been specially designated for RNA work.*

Treatment of yeast liquid cultures with a-factor

(*Note: The 7.003 staff has done this step for you*)

1) The day before the RNA isolation, inoculate a single colony of PPY295 and your αF-resistant mutant yeast each into 5 mL YPD media in a test tube. Incubate the tubes on a rolling drum at 30°C overnight.

2) The next morning (day of RNA isolation) at 10 AM, dilute each overnight culture 1:30 into two fresh tubes containing 5 mL YPD (167 μL of O/N culture into 5 mL YPD). Note that you will end up with four tubes total (two diluted tubes of PPY295, and two diluted tubes of the αF-resistant mutant). Continuing growing these four tubes of diluted cultures at 30°C for another 3 hours.

3) At ~1:00 PM, treat one of the diluted PPY295 cultures and one of the αF-resistant mutant diluted cultures each with 10^{-5} M α-factor ("αF" tubes). Leave the other tube of each strain untreated ("UT" tubes). Continue growing all four tubes at 30°C for another hour.

RNA isolation from yeast cells (*you start here!*)

4) **You start here!** Label four 1.5-mL Eppendorf tubes with the names of your four sample cultures (either PPY295 or Mut, and either "UT" or "αF") and with "1:5." In each tube, make a 1:5 dilution of the appropriate yeast cell culture. The final volume of the 1:5 dilution should be 1 mL and use YPD liquid media to dilute each culture (*check your*

DAY 14 (Tue, Apr 5th), continued

calculations with your TA and use sterile technique when pipetting your YPD media!). Invert the tube or flick the bottom of the tube several times to make sure each dilution is thoroughly mixed.

5) Label five cuvettes with "Blank" and the names of your four cultures (use the cuvettes in the Styrofoam boxes in your bench drawers). Add 600 μL of blank solution into the "Blank" cuvette and 600 μL of each 1:5 diluted yeast culture into the appropriately labeled cuvette (make sure each culture is well-mixed before pipetting!). (*What should you use as a blank? Check your answer with your TA!*)

6) Use the spectrophotometer to measure the OD_{600} for each of your four cultures (see lab manual Appendices for complete instructions on how to use the specs to measure light at a single wavelength). Record your results.

7) Calculate the cell concentration of the original (undiluted) yeast cultures for each of your four samples, based on your OD_{600} reading. Use the standard conversion of OD_{600} = 1.0 being equal to 1 x 10^7 cells/mL. Make sure to take the 1:5 dilution into account in your final calculations! **Check you calculations with your TA and include your calculations in your Day 14 Post-Lab.**

8) For the RNA isolation protocol, you will need exactly 2 x 10^7 yeast cells total for each sample. Based on the cell concentration you determined in Step 7, calculate what volume (in mL) you would need of each of your four samples to get exactly 2 x 10^7 cells of each cell culture. **Check you calculations with your TA and include your calculations in your Day 14 Post-Lab.**

9) Label four 1.5-mL Eppendorf tubes with the names of your four sample cultures.

10) Into each Eppendorf tube, pipet the volume of the appropriate cell culture such that there are exactly 2 x 10^7 cells total in each tube (see your calculations from Step 8). Use the original undiluted culture from the glass test tubes (make sure the culture is well-resuspended before your pipet it). Note: If the volume of cells needed is greater than 1.5 mL (i.e. it won't fit in the Eppendorf tube all at once), then add only half of that volume to the tube for now.

11) Spin the four Eppendorf tubes for 30 sec at 13,000 rpm. Carefully remove and discard the supernatant from each tube into a waste beaker labeled "YPD Waste" (do not disrupt the cell pellet!). You should now have a cell pellet (containing 2 x 10^7 yeast cells) at the bottom of each tube.

Note: If in Step 10, you only added half of the required volume of cells, you can now repeat Steps 10 – 11 to add the remaining half of the required volume of cells, such that you will eventually end up with 2 x 10^7 cells total in a pellet in the bottom of each tube.

DAY 14 (Tue, Apr 5th), continued

12) Resuspend the cell pellet in each tube in 100 μL Y1BL Buffer. Pipet up and down multiple times to make sure the cell pellet is fully resuspended (no clumps).

13) Incubate both tubes on the rotator in the 30°C warm room for 30 minutes (*what is occurring to the yeast cells during this step?*). During this incubation, you can pour an agarose gel (see Part 4.2) and/or work on ILQs.

14) After the 30-minute incubation is done, add 350 μL of RLT Buffer to each tube. Vortex the tubes briefly to thoroughly mix the contents (make sure the tubes are tightly capped!).

15) Place four Homogenizer Spin Columns (the **green** columns) in the round-bottom collection tubes. Label the collection tubes with the names of your four samples. Pipet the entire contents of each "Cells" tube into the appropriate Homogenizer Spin Column (be careful not to pierce the white membrane of the spin column with your pipet tip).

16) Spin the four Homogenizer Spin Columns (in the collection tubes) for 2 minutes at 13,000 rpm. The yeast cells will lyse as they pass through the column (due to high pressure disrupting the phospholipid interactions of the cell membrane), and the homogenized lysate will be collected in the collection tube.

17) Label four new 1.5-mL Eppendorf tubes with the names of your four samples and with "HL" (HL = homogenized lysate). Transfer the homogenized lysate in each collection tube to its appropriately labeled Eppendorf tube. Discard the green Homogenizer Columns.

18) Add 250 μL of ethanol to each of your four "HL" tubes. Invert the tubes several times to mix thoroughly.

19) Label four RNeasy Spin Columns (**pink**) and round-bottom collection tubes with the names of your four samples. Place the RNeasy Spin Columns in the appropriately labeled round-bottom collection tubes.

20) Transfer the entire contents of each "HL" tube into the corresponding labeled RNeasy Spin Column. Be careful not to pierce the white membrane at the bottom of the spin column with your pipet tip when adding solutions to the column.

21) Centrifuge the spin columns for 15 seconds at 13,000 rpm. Discard the flow-through into a new waste beaker labeled "Qiagen Waste" and replace the spin column back into the appropriate collection tube. (*Where is the RNA now? What types of molecules were discarded in the flow-through waste?*)

22) Add 350 μL of RW1 Buffer to each spin column. Let the columns sit (with the buffer in them) for 3 minutes undisturbed on your bench top at room temperature.

DAY 14 (Tue, Apr 5th), continued

23) Centrifuge the spin columns for 15 seconds at 13,000 rpm and discard the flow-through in your Qiagen waste beaker as before.

24) Add 80 μL of DNase I to each spin column. Pipette the DNase solution directly onto the center of the column membrane, but be careful not to touch or pierce the membrane with your pipet tip.

25) Let the spin columns sit (with the DNase in them) for 15 minutes undisturbed on your bench top at room temperature.

26) After the 15-minute incubation is done, add 350 μL of RW1 Buffer to each spin column. Centrifuge the spin columns for 15 seconds at 13,000 rpm and discard the flow-through in the Qiagen waste beaker as before. (*Where is the RNA now? What types of molecules were discarded in the flow-through waste?*)

27) Add 500 μL of RPE Buffer to each spin column. Centrifuge the spin columns for 15 seconds at 13,000 rpm and discard the flow-through in the Qiagen waste beaker.

28) Add 500 μL of RPE Buffer again to each spin column. Centrifuge the spin columns for **2 minutes** at 13,000 rpm (note difference in time!). Discard the flow-through in the Qiagen waste beaker as before.

29) Place each spin column in a new clean round-bottom collection tube (make sure the pink spin columns themselves are properly labeled so you know which spin column contains which sample!).

30) Centrifuge the spin columns (in the new collection tubes) for **1 minute** at 13,000 rpm (note difference in time!). This step removes residual ethanol from RPE Buffer (*why is this important?*).

31) Label four 1.5-mL Eppendorf tubes with the names of your four samples. Label all four tubes with "RNA" and your bench number. Remove the pink spin columns from their round-bottom collection tubes and place each column into the appropriately labeled Eppendorf tube. All round-bottom collection tubes can now be discarded.

32) Add 50 μL of RNase-free dH_2O to each spin column. Pipette the RNase-free H_2O directly onto the center of the column membrane, but be careful not to touch or pierce the membrane with your pipet tip.

33) Let the spin columns sit (with the RNase-free H_2O in them) undisturbed on your bench top at room temperature for 2 minutes.

DAY 14 (Tue, Apr 5th), continued

34) Centrifuge the Eppendorf tubes with the spin columns in them for **1 minute** at 13,000 rpm. The RNA has now eluted from the columns and collected at the bottom of the Eppendorf tubes.

35) To ensure all the RNA has fully eluted from the column, you will repeat the elution process. Pipet up the ~50 μL of RNA sample collected at the bottom of the Eppendorf tube and add it back to the **same** corresponding spin column (e.g. pipet the "PPY295 UT RNA" sample and add it back to the "PPY295 UT" RNeasy Spin Column). Place the spin column back in to the corresponding Eppendorf tube.

36) Repeat Step 34 for the second elution round. Your final RNA sample will now be collected at the bottom of the Eppendorf tubes. The pink spin columns can now be discarded. Cap the Eppendorf tubes and leave the RNA samples on ice from now on as you continue with the next steps.

37) Use the spectrophotometer and microcuvette to measure the RNA concentration of each of your four RNA samples (see lab manual Appendices for instructions on using the specs and microcuvettes). Record the RNA concentration and A_{260}/A_{280} ratio for each sample. (*What should you use as a blank? Check your answer with your TA!*)

38) Give your four RNA samples to your TA – the samples will be stored at -20°C until the next lab session.

Part 4.2: Pour an agarose gel

Background: You will pour an agarose gel today, which will be used on Day 15 for analyzing your RNA samples.

Materials: Agarose powder
Gel box and casting tray
10-well comb
1X TAE Buffer (40 mM Tris-HCl pH 7.2, 20 mM NaOAc, 1 mM EDTA)
1000X GelGreen dye (from Biotium)

Hazardous chemicals used: None (the GelGreen dye is considered non-toxic), but for safety, always wear gloves when handling agarose gels

Procedure: *Be careful when microwaving solutions that they do not boil over.*

1) Follow the instructions from Part 1.2 on Day 1 to pour a 0.8% agarose gel with 10 wells. When the gel has solidified, label and store the gel properly in Saran wrap and/or a Ziploc bag in the 4°C cold room to save until the next lab day.

DAY 14 (Tue, Apr 5th), continued

Day 14 Pre-Lab Notebook Tips & Reminders:

- Remember to include Aims for all parts (Parts 4.1 – 4.2).

Day 14 Post-Lab Notebook Tips & Reminders:

- Include all your spectrophotometer OD_{600} readings and calculations for determining cell concentration and volume of cell cultures to use.

- Include all spectrophotometer RNA concentration measurements/results.

- How successful do you think your overall RNA prep was, based on your spectrophotometer results? What can you conclude from the A_{260}/A_{280} ratios?

DAY 15 (Thu, Apr 7th)

4. Analyzing RNA expression of αF-resistant mutants

Part 4.3: Gel analysis of isolated RNA

Background: To check the quality and yield of your RNA samples from Day 14, you will perform gel electrophoresis. RNA molecules will run and separate on an agarose gel by size in a similar manner to DNA molecules, and they can be visualized using similar dyes like ethidium bromide and GelGreen (*ethidium bromide and GelGreen dyes typically work by intercalating between bases in double-stranded nucleic acid molecules – why do you think you can still visualize single-stranded RNA molecules with these same dyes?*).

While you likely would not be able to detect any mRNA molecules from your total RNA prep on an agarose gel (*why not?*), you may be able to detect ribosomal RNA (rRNA) or transfer RNA (tRNA) (*why?*). Yeast have four different RNAs that are part of the ribosome: 5S, 5.8S, 18S, and 25S RNA. The 5S and 5.8S RNAs (and tRNAs) are much shorter molecules (less than <200 nucleotides long) and often are not isolated much in the original total RNA prep to begin with (shorter RNA molecules do not bind the purification spin column membrane as well). The longer 18S and 25S RNA molecules (~1800 nt and ~3400 nt in length, respectively) would have been isolated though in your original RNA prep and may be detected on a gel.

Materials: (4) RNA samples (WT UT, WT αF, Mut UT, and Mut αF, from Day 14)
0.8% agarose with 1X GelGreen in 1X TAE (poured from Day 14)
1X TAE Buffer (40 mM Tris-HCl pH 7.2, 20 mM NaOAc, 1 mM EDTA)
6X Loading Dye (0.25% Bromophenol blue, 15% Ficoll)
1-kb DNA Ladder (NEB) in 1X Loading Dye (0.1 μg/μL)
(see Appendices for the sizes of the 1-kb DNA ladder fragments)
RNase-free ddH_2O

Hazardous chemicals used: None, but wear gloves when handling gels

Procedure: *Change tips between each sample/tube to avoid cross-contamination. You are working with RNA, so remember to use proper clean technique when handling RNA samples (e.g. use RNaseZap to clean surfaces and Pipetman, use RNase-free tips/tubes, etc).*

1) Label four 1.5-mL Eppendorf tubes with the names of your four RNA samples and with "Gel." Pipet 5 μL of each RNA sample into the appropriately labeled "Gel" Eppendorf tube. **Save the rest of your RNA samples** and leave them on ice**.**

DAY 15 (Thu, Apr 7th), continued

2) Add an appropriate amount of 6X Loading Dye and RNase-free water to each of your four gel samples to prepare them for running on a gel. (*Check your calculations with your TA!*)

> ***Make sure you only add the 6X Loading Dye to the 1.5-mL "Gel" tubes containing just the 5 μL aliquots of your samples (i.e. the "Gel" sample Eppendorf tubes you labeled from Step 1). Do NOT add the dye to the remainder of the RNA samples – **save the remainders of these RNA samples for Part 4.4: Preparation of cDNA!**

3) Centrifuge the four Eppendorf tubes containing your gel samples + Loading Dye for ~15 seconds at 6000 rpm (2900 x g).

4) Unwrap your agarose gel (from Day 14) and place it in a tray in a gel box. Add 1X TAE Buffer to the gel box so that it just covers the gel.

5) Load the first lane of the gel with 10 μL of the 1-kb DNA Ladder. Then load each of your prepared gel samples into the next four lanes. (Note the order of the lanes in your notebook!)

6) Connect the electrodes of the power supply and the gel box in the proper orientation so that the DNA runs towards the **positive (red)** electrode.

7) Turn on the power supply and turn the voltage to ~100 volts. Check that the current is actually running through the gel box and check the running direction of the loading dye to make sure the gel orientation is correct. **While the gel is running, you can proceed to "Part 4.4: Preparation of cDNA from RNA" and/or work on lLQs.**

8) Run the gel at a constant voltage until the dye has migrated about two-thirds down the gel (approximately 45 – 60 minutes). Turn off the power supply.

9) Wearing gloves, remove the entire gel tray (with the gel in it) from the gel box. Place the gel tray and gel in a white plastic box and take it to the gel doc system. Based on your gel results, what can you conclude about the yield and integrity of your RNA samples?

10) Check your gel results with your TA or instructor – depending on your RNA sample quality, you may have to use a back-up RNA sample for the following experiments.

(Note: Day 15 continues on the next page!)

DAY 15 (Thu, Apr 7th), continued

Part 4.4: Preparation of cDNA from RNA

Background: The quantitative PCR (qPCR) method you will be using to analyze gene expression in your cells requires that you first create complementary DNA (cDNA) from your RNA samples. This is done using reverse transcriptase enzymes (RNA-directed DNA polymerases), which can synthesize a strand of DNA complementary to an RNA template (e.g. any mRNA molecules in your sample). The cDNA made in this reaction can then be used for cloning purposes or gene expression assays (e.g. qPCR to analyze single gene expression or RNA-Seq to analyze whole genome expression).

You will be using the M-MuLV reverse transcriptase enzyme, which originated from the Moloney Murine Leukemia Virus (*many reverse transcriptase enzymes used in labs come from viruses – why do you think this is so?*). In addition to being able to synthesize DNA from an RNA template, the M-MuLV enzyme also has RNase H activity. This allows it to digest and remove the RNA molecule from the DNA-RNA hybrid after cDNA synthesis.

Like other DNA polymerases, the M-MuLV reverse transcriptase requires a primer bound to a single-stranded template to initiate DNA synthesis. For your cDNA synthesis reaction, you will be using an Oligo-d$(T)_{23}$VN primer (an "anchored" Oligo-d(T) primer), which has the sequence: 5' – $(T)_{23}$VN – 3'. ("V" represents any A, G, or C, while "N" represents any base.) The 23 thymidines will bind and recognize the poly(A) tail on any mRNA molecule (so that cDNA synthesis will be specifically from mRNA while still allowing many different gene targets to be studied from the same cDNA synthesis reaction). The "VN" sequence at the 3' end of the primer forces the primer to bind at the very beginning (5' end) of the poly(A) tail on the mRNA (closest to the actual open reading frame on the mRNA). This ensures cDNA synthesis of the actual coding sequence region of the gene (and not just amplification of poly(A) tail sequence).

Materials:
(4) RNA samples (WT UT, WT αF, Mut UT, and Mut αF, from Day 14)
2X M-MuLV Reaction Mix (from NEB Protoscript First Strand cDNA Synthesis Kit)
10X M-MuLV Enzyme Mix (from NEB Protoscript First Strand cDNA Synthesis Kit)
Oligo-d$(T)_{23}$VN stock (50 μM)
RNase-free dH_2O

Hazardous chemicals used: None

DAY 15 (Thu, Apr 7th), continued

Procedure: *Change tips between each sample/tube to avoid cross-contamination. You are still working with RNA, so remember to use proper clean technique when handling RNA samples (e.g. use RNaseZap to clean surfaces and Pipetman, use RNase-free tips/tubes, etc).*

1) Based on your RNA concentration measurements from Day 14, calculate what volume you would need to obtain 500 ng of RNA for each of your four samples. **Check your calculations with your TA and include them in your Day 15 Post-Lab.** Note that this volume needs to be less than 6 μL for each of your samples – if any of your RNA sample's concentration is too low for this, check with your TA on how to proceed.

2) Label four blue 0.5-mL PCR tubes with the names of your four RNA samples, "cDNA," and your bench number. Label another four blue 0.5-mL PCR tubes with the names of your four RNA samples, "-RT," and your bench number.

3) Add 500 ng of RNA from your Day 14 samples to the appropriate PCR tube labeled from Step 2, based on your Step 1 calculations. (Note: Save the remaining RNA samples and give them to your TA to store at -20°C until they are needed again.)

4) Add 2 μL of Oligo-d$(T)_{23}$VN to each of your eight tubes.

5) Add enough RNase-free water to each of your eight tubes such that the final volume in each tube is 8 μL (**check your calculations with your TA and include them in your Day 15 Post-Lab**).

6) Cap the eight tubes tightly and incubate them in a thermocycler set to 70°C for 5 minutes. Note that the thermocycler will be set to 70°C indefinitely, so you will have to time this 5-minute incubation for yourself (*what is the purpose of this incubation step?*).

7) After the 70°C incubation, incubate the eight tubes on ice for 5 minutes.

8) Add 10 μL of 2X M-MuLV **Reaction Mix** just to the four "cDNA" tubes.

9) Add 2 μL of 10X M-MuLV **Enzyme Mix** just to the four "cDNA" tubes.

10) Add 12 μL of RNase-free water just to the four "-RT" tubes (*what is the purpose of these four tubes?*).

11) Cap all eight tubes tightly and incubate them in a thermocycler set to 42°C for 1 hour. Note that the thermocycler will be set to 42°C indefinitely, so you will have to time this 1-hour incubation for yourself (*what is the purpose of this incubation step?*). During this 1-hour incubation, you can work on ILQs.

DAY 15 (Thu, Apr 7th), continued

12) After the 42°C incubation, incubate all eight tubes in a thermocycler set to 80°C for 5 minutes. Note that the thermocycler will be set to 80°C indefinitely, so you will have to time this 5-minute incubation for yourself (*what is the purpose of this incubation step?*).

13) After the 80°C incubation, add 30 μL of sterile dH2O to each of your eight tubes. Your cDNA samples are now complete. Give them to your TA to store at -20°C until the next lab session.

Day 15 Pre-Lab Notebook Tips & Reminders:

- Remember to include Aims for all parts (Parts 4.3 – 4.4).

Day 15 Post-Lab Notebook Tips & Reminders:

- Be sure to label the gel photo such that it's clear what is loaded in each lane and what the sizes of all ladder bands are.
- You should discuss each lane of your gel (lanes/samples with similar overall results can be discussed together as a group if you want).
    - What was the expected overall result for each lane? Why?
    - What can you conclude based on whether or not you saw the expected result for each lane? Explain your reasoning. If you did not get the expected results, what might be a possible reason to explain the difference?
- Remember to include the calculations for setting up your cDNA samples.

DAY 16 (Tue, Apr 12th)

4. Analyzing RNA expression of αF-resistant mutants

Part 4.5: Perform *FUS1* test PCR with cDNA

Background: To test the quality of your cDNA samples and to check for any potential genomic DNA contamination of your original RNA preps, you will perform a test PCR using your cDNA samples as a template. You will use primers that amplify a region of the *FUS1* yeast gene. *FUS1* encodes a membrane protein that localizes to the end of a yeast shmoo and is involved in cell fusion between *MAT***a** and *MAT*α cells during mating. Expression of the *FUS1* gene is regulated by the Ste12 transcription factor. (*Under what conditions do you expect FUS1 expression to be turned on? How do you think this may vary in your αF-resistant mutant? Why?*)

The two PCR primers you are using have the following sequences and bind at the following locations within the *FUS1* coding sequence (+1 is equivalent to the very first nucleotide in the *FUS1* coding sequence).

FUS1-For primer = 5' – gcg tcc aat tag gga aga ca – 3' (binds +1301 to +1320 of *FUS1*)
FUS1-Rev primer = 5' – aac ttt ttc acc cag cga ga – 3' (binds +1389 to +1370 of *FUS1*)

(*Do these primers bind near the beginning, middle, or end of the FUS1 coding sequence? Why do you think that particular region of FUS1 was chosen for this PCR?*)

Materials:
(4) "cDNA" samples (WT UT, WT αF, Mut UT, and Mut αF, from Part 4.4)
(4) "–RT" samples (WT UT, WT αF, Mut UT, and Mut αF, from Part 4.4)
(1) yeast genomic DNA ("GD") sample (from Part 3.3)
2X Phusion Master Mix (from NEB)
FUS1-For primer stock (100 μM)
FUS1-Rev primer stock (100 μM)
Sterile ddH_2O

Hazardous chemicals used: None

Procedure: *Change tips between each sample/tube to avoid cross-contamination.*

1) The stock tubes of primers are at too high of a concentration for your PCR, so you will need to dilute them first. Calculate how much FUS1-For primer stock, FUS1-Rev primer stock, and sterile water you would need to make a 20 μL solution containing both primers each at a final concentration of 10 μM. (*Check your calculations with a TA!)*

DAY 16 (Tue, Apr 12th), continued

2) Label a 1.5-mL Eppendorf tube as "Primers" along with your bench number. Follow your calculations from Step 1 to make a 20 μL solution with both primers each at a final concentration of 10 μM.

3) Mix the contents of the "Primers" tube by gently pipetting up and down several times. Spin the tube for ~15 seconds at 6000 rpm (2900 x g) and leave it on ice. When you set up your PCR samples, make sure you use this diluted "Primers" Eppendorf tube when adding the primers (and not the original stock primer tubes).

4) The genomic DNA sample is also at too high of a concentration to use for a PCR template. Label a 1.5-mL Eppendorf tube with the genomic DNA sample name (i.e. what mutant it came from), "GD 1:10," and your bench number. Make a 1:10 dilution of your genomic DNA sample with sterile water in this tube in a final total volume of 20 μL.

5) Label 10 blue 0.5-mL PCR tubes "PCR 1" – "PCR 10," along with your bench number and the date. Add 12.5 μL of 2X Phusion Master Mix and 1.25 μL of primers (from the diluted "Primers" tube) to each of the 10 PCR tubes.

6) Add the following amounts of DNA template and sterile ddH_2O to each PCR tube (*what is the purpose of PCR #9 and #10?)*:

PCR	DNA template	Sterile ddH_2O
1	2 μL of PPY295 WT UT cDNA	9.25 μL ddH_2O
2	2 μL of PPY295 WT aF cDNA	9.25 μL ddH_2O
3	2 μL of Mutant UT cDNA	9.25 μL ddH_2O
4	2 μL of Mutant aF cDNA	9.25 μL ddH_2O
5	2 μL of PPY295 WT UT –RT	9.25 μL ddH_2O
6	2 μL of PPY295 WT aF –RT	9.25 μL ddH_2O
7	2 μL of Mutant UT –RT	9.25 μL ddH_2O
8	2 μL of Mutant aF –RT	9.25 μL ddH_2O
9	2 μL of diluted genomic DNA (from "GD 1:10" tube)	9.25 μL ddH_2O
10	None	11.25 μL ddH_2O

7) Mix the reagents in each PCR tube by gently pipetting up and down several times (use a new tip for each tube and minimize bubble formation). Spin all ten tubes for ~15 seconds at 6000 rpm (2900 x g).

DAY 16 (Tue, Apr 12th), continued

8) Make sure all ten PCR tubes are tightly capped and leave them in the ice bucket at the end of the bench for the teaching staff to collect. They will place them in the thermocycler to run the following PCR program:

1) 95°C for 2 min
2) 95°C for 30 sec
3) 60°C for 20 sec
4) 72°C for 30 sec
5) Repeat Steps 2 – 4 35 cycles
6) 72°C for 2 min
7) 4°C indefinitely to store until the next lab session

9) Save your leftover "cDNA" and "-RT" samples, as well as the "GD" samples – give these to your TA to store at -20°C until the next lab session.

Part 4.6: Pour an agarose gel

Background: You will pour an agarose gel today, which will be used on Day 17 for analyzing your cDNA PCR test samples.

Materials: Agarose powder
Gel box and casting tray
12-well comb
1X TAE Buffer (40 mM Tris-HCl pH 7.2, 20 mM NaOAc, 1 mM EDTA)
1000X GelGreen dye (from Biotium)

Hazardous chemicals used: None (the GelGreen dye is considered non-toxic), but for safety, always wear gloves when handling agarose gels

Procedure: *Be careful when microwaving solutions that they do not boil over.*

1) Follow the general instructions from Part 1.2 on Day 1 to pour a **2% agarose gel with 12 wells.** Note the difference in agarose percentage and gel well numbers – check your calculations with your TA before pouring the gel!!. (*Why do you think you will be using an agarose gel with this different percentage of agarose?*) When the gel has solidified, label and store the gel properly in Saran wrap and/or a Ziploc bag in the 4°C cold room until the next lab day.

DAY 16 (Tue, Apr 12th), continued

Day 16 Pre-Lab Notebook Tips & Reminders:

- Remember to include Aims for all parts (Parts 4.5 – 4.6).

Day 16 Post-Lab Notebook Tips & Reminders:

- There is no Post-Lab for Day 16 since you did not get any data, but you should make a note of any deviations you made from the lab manual protocols for your own records.

DAY 17 (Thu, Apr 14th)

4. Analyzing RNA expression of αF-resistant mutants

Part 4.7: Gel analysis of cDNA *FUS1* PCR test samples

Background: Today, you will analyze your cDNA *FUS1* PCR samples using gel electrophoresis. By running your samples on an agarose gel, you will be able to determine what size PCR product(s) were amplified (if any) in each of your PCR samples and thus assess the quality of your cDNA samples (and of your original RNA samples) and the specificity of your *FUS1* PCR primers. (*For which PCR samples do you expect to see PCR products? Why or why not? What length do you expect any PCR products to be?)*

Materials: (10) cDNA PCR samples (PCR #1 – #10, from Day 16)
2% agarose with 1X GelGreen in 1X TAE (poured from Day 16)
1X TAE Buffer (40 mM Tris-HCl pH 7.2, 20 mM NaOAc, 1 mM EDTA)
6X Loading Dye (0.25% Bromophenol blue, 15% Ficoll)
100-bp DNA Ladder (NEB) in 1X Loading Dye (0.1 μg/μL)
(see Appendices for the sizes of the 100-bp DNA ladder fragments)
RNase-free ddH_2O

Hazardous chemicals used: None, but wear gloves when handling gels

Procedure: *Change tips between each sample/tube to avoid cross-contamination.*

1) Label ten 1.5-mL Eppendorf tubes with "PCR Gel 1" – "PCR Gel 10." Pipet 10 μL of each PCR sample into the appropriately labeled "Gel" Eppendorf tube.

2) Add an appropriate amount of 6X Loading Dye and RNase-free water to each of your 10 gel samples to prepare them for running on a gel. (*Check your calculations with your TA!*)

3) Centrifuge the ten Eppendorf tubes containing your gel samples + Loading Dye for ~15 seconds at 6000 rpm (2900 x g).

4) Unwrap your agarose gel (from Day 16) and place it in a tray in a gel box. Add 1X TAE Buffer to the gel box so that it just covers the gel.

5) Load the first lane of the gel with 10 μL of the 100-bp DNA Ladder. Then load each of your prepared gel samples into the next ten lanes. (Note the order of the lanes in your notebook!)

DAY 17 (Thu, Apr 14th), continued

6) Connect the electrodes of the power supply and the gel box in the proper orientation so that the DNA runs towards the **positive (red)** electrode.

7) Turn on the power supply and turn the voltage to ~100 volts. Check that the current is actually running through the gel box and check the running direction of the loading dye to make sure the gel orientation is correct. **While your gel is running, you can proceed to "Part 4.8: Preparation of *FUS1* qPCR samples."**

8) Run the gel at a constant voltage until the dye has migrated about halfway to two-thirds down the gel (approximately 45 – 60 minutes). Turn off the power supply.

9) Wearing gloves, remove the entire gel tray (with the gel in it) from the gel box. Place the gel tray and gel in a white plastic box and take it to the gel doc system. Based on your gel results, what can you conclude about the quality of your cDNA samples (and the original RNA samples)? What can you conclude about the specificity of the *FUS1* primers?

Part 4.8: Preparation of *FUS1* qPCR samples

Background: Today, you will prepare quantitative PCR (qPCR) using your cDNA samples as PCR templates. This method allows you to analyze the gene expression levels in the RNA isolated from your samples (e.g. WT vs. mutant strain, untreated vs. αF-treated). You will again use the same *FUS1* primers from before to look specifically at *FUS1* gene expression. You will also use primers that amplify a portion of the *ACT1* gene. *ACT1* is a "housekeeping" gene that encodes actin, an essential structural component of the yeast cell cytoskeleton (*why do you think these ACT1 primer samples are included in your qPCR experiment along with the FUS1 primer samples?*).

Most conventional PCR (such as the inverse PCR you performed on Day 11) are "endpoint" PCR reactions, which uses or analyzes PCR products only at the end of the reaction when it has reached saturation in the "plateau" phase of PCR (see Figure 1). The goal for endpoint PCR reactions is to amplify a particular region of DNA, whether it is to directly use those DNA copies (e.g. for cloning or sequencing) or for analytical purposes (e.g. to see if a particular gene allele is present in a cell). In such instances, it is sufficient to only use PCR products from the plateau phase, since one is mainly concerned just with obtaining as many copies of the desired DNA as possible (and not with directly comparing amounts of PCR product produced between different reactions). While it is sometimes possible to infer differences in starting template amounts from endpoint PCR reactions, this typically is not the case (e.g. in Figure 1 below, even though the two different PCR reactions depicted have different starting template amounts, they both end with similar amounts of PCR product in their plateau phases).

DAY 17 (Thu, Apr 14th), continued

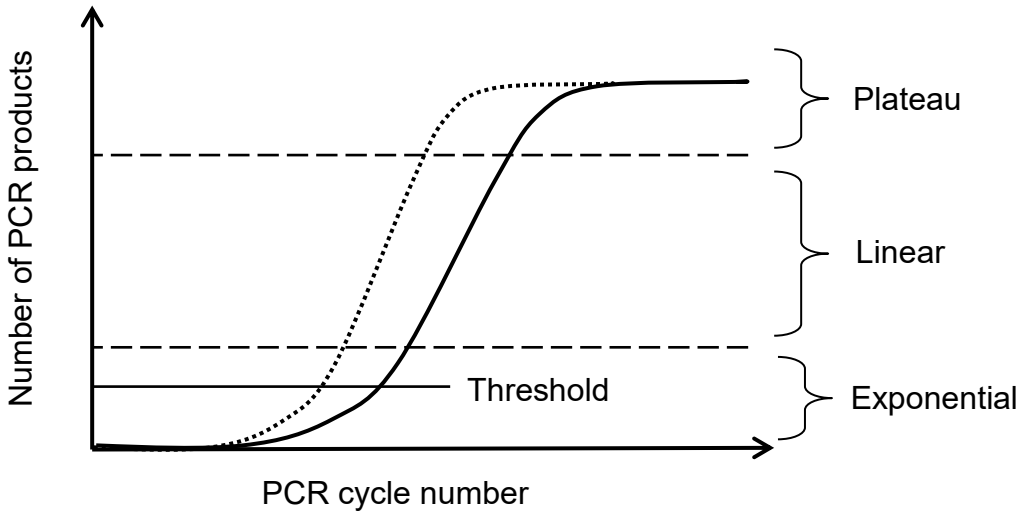


Figure 1. Phases of PCR. During the beginning cycles (when the PCR reagents are in excess), PCR products are created exponentially at close to 100% efficiency (amount of product being made doubles every cycle). As the PCR progresses, the reaction enters a linear phase, where the number of PCR products still increase every cycle but the overall PCR efficiency decreases as reagents become more limiting. Finally, the PCR enters the plateau phase, where PCR amplification ceases as the reagents become exhausted or lose efficiency. (*Which PCR reaction depicted in the figure had a greater amount of starting template, the dotted line or solid line reaction? Why?*)

Quantitative PCR differs from endpoint PCR because it is designed to detect how much PCR product is actually being made with each cycle. (This is why qPCR is also referred to as "real-time" PCR because you are following PCR product formation in real-time.) qPCR allows you to measure relative differences in starting template amounts because a reaction with less starting template would require more cycles before it can produce enough PCR product to be detected above a certain threshold (see Figure 1). This arbitrary threshold is usually set so that you are analyzing the PCR reaction while it is still in its exponential phase (when PCR products are actually still doubling each cycle). For your experiment, qPCR will let you know if there were any differences in cDNA starting template (specifically *FUS1* cDNA starting template) between your samples, which in turn will tell you if there were any differences in original mRNA expression (e.g. was there more *FUS1* mRNA present in one strain or condition than another?).

qPCR shares the usual components of regular endpoint PCR (e.g. DNA template, primers, DNA polymerase, and dNTPs), but it also utilizes additional reagents to help it detect the amount of PCR product being made in real-time. Various qPCR methods have been developed that use different types of fluorescent dyes or probes. Some methods use dyes that generally detect any double-stranded (ds) DNA products while some use probes that specifically will detect formation of particular DNA sequence. In your 7.003 qPCR experiment, you will be using the DNA-binding dye, SYBR Green, which fluoresces when bound to dsDNA (and not when bound to ssDNA or when unbound). Higher SYBR Green fluorescence levels detected in a PCR cycle will

DAY 17 (Thu, Apr 14th), continued

correspond to greater amounts of dsDNA (e.g. PCR product) present in the reaction at that cycle. Note that because SYBR Green binds nonspecifically to all dsDNA, this can complicate the qPCR analysis, since unwanted dsDNA products like primer dimers or amplification products from misannealed primers will also be detected by SYBR Green dye. An advantage of SYBR Green dye though over sequence-specific probes in qPCR is that SYBR Green reagents are much cheaper to use and don't require designing/creating specific probes for each gene or DNA region being analyzed.

qPCR reactions are usually performed in 96-well plates and run in specialized PCR machines equipped with detectors that can measure the fluorescence levels in each well after each PCR cycle. Due to the high cost of such machines, the 7.003 lab does not have its own qPCR machine, so you will instead use a shared qPCR machine (the Roche Light Cycler 480) from the MIT BioMicro Center core facility. Today, you will set up a 96-well plate using your cDNA template with *FUS1* and *ACT1* primers. Later, the 7.003 teaching staff will add the other qPCR reagents for you and send the plate to the BioMicro Center to be processed once one of their qPCR machines is available to use.

Materials:
(4) "cDNA" samples (WT UT, WT αF, Mut UT, and Mut αF, from Part 4.4)
(4) "–RT" samples (WT UT, WT αF, Mut UT, and Mut αF, from Part 4.4)
FUS1-For primer stock (100 μM)
FUS1-Rev primer stock (100 μM)
ACT1-For primer stock (100 μM)
ACT1-Rev primer stock (100 μM)
2X KAPA SYBR Fast qPCR Master Mix (from Roche)
96-well plate

Hazardous chemicals used: None

Procedure: *Change tips between each sample/tube to avoid cross-contamination. To keep track of what wells you've already added reagents to when setting up the 96-well plate, it is helpful to use a brand new box of P20 tips so that you can match up each tip used with the corresponding well position in the 96-well plate.*

1) The stock tubes of *FUS1* and *ACT1* primers are at too high of a concentration for your qPCR, so you will need to dilute them first. Calculate how much Forward primer stock, Reverse primer stock, and sterile water you would need to make a 500 μL solution containing Forward + Reverse primers each at a final concentration of 500 nM. (*Check your calculations with a TA!)*

2) Label two 1.5-mL Eppendorf tubes, one with "FUS1 primers 500 nM" and the other with "ACT1 primers 500 nM" (along with your bench number). Follow your calculations from Step 1 to make a 500 μL solution of both *FUS1* primers at a final concentration of 500 nM and a 500 μL solution of both *ACT1* primers at a final concentration of 500 nM.

DAY 17 (Thu, Apr 14th), continued

3) Mix the contents of each diluted primers tube by gently pipetting up and down several times (change tips between tubes!). Spin the tubes for ~15 seconds at 6000 rpm (2900 x g) and leave them on ice. When you set up your qPCR plate, make sure you use the diluted primers tubes (and not the original stock primer tubes).

4) You will be testing qPCR reactions with 18 different combinations of DNA template and primers. Some of the reactions will be performed in triplicate and some reactions will be performed only in duplicate. (Note that ideally, all reactions would be done in triplicate, but due to space constraints on the qPCR plates, you will only do duplicates for some reactions.) In total, you will be setting up 44 qPCR reactions, as listed below.

Reactions set up in triplicate (8 reactions x 3 = 24 reactions total):

Reactions set up in triplicate		
	Template	**Primers**
1	WT UT cDNA	*FUS1*
2	WT αF cDNA	*FUS1*
3	Mut UT cDNA	*FUS1*
4	Mut αF cDNA	*FUS1*
5	WT UT cDNA	*ACT1*
6	WT αF cDNA	*ACT1*
7	Mut UT cDNA	*ACT1*
8	Mut αF cDNA	*ACT1*

Reactions set up in duplicate (10 reactions x 2 = 20 reactions total):

Reactions set up in duplicate		
	Template	**Primers**
1	WT UT –RT	*FUS1*
2	WT αF -RT	*FUS1*
3	Mut UT -RT	*FUS1*
4	Mut αF -RT	*FUS1*
5	No DNA (H_2O)	*FUS1*
6	WT UT -RT	*ACT1*
7	WT αF -RT	*ACT1*
8	Mut UT -RT	*ACT1*
9	Mut αF -RT	*ACT1*
10	No DNA (H_2O)	*ACT1*

DAY 17 (Thu, Apr 14th), continued

(Which qPCR samples are your control samples? What is the purpose of each?)

5) Check with your TA to see which qPCR plate number and which 44 well numbers on the plate have been assigned to your lab group. Note that each plate fits up to 96 samples, so multiple lab groups will be sharing each plate. Decide which qPCR template and primer combination you will use for each of your assigned 44 well numbers (record this information in your notebook!). You may find it useful to make a table diagram (e.g. using the 96-well template sheet provided on the next page) to help keep track of your plate layout while setting up your samples.

6) Add 8 μL of the appropriate diluted primers (from the diluted primers tubes you made in Step 2) into each of your assigned 44 wells, following the layout you decided on in Step 5. Double-check before you pipet each time to make sure you add the correct primer pair into the correct well!

7) Add 2 μL of the appropriate DNA template (or sterile water) into each of your assigned 44 wells, following the layout you decided on in Step 5. Double-check before you pipet each time to make sure you add the correct template into the correct well! **Note that you are using the original "cDNA" or "–RT" samples from Day 15 for setting up this qPCR plate (and <u>not</u> the PCR samples from Day 16).**

8) When you are finished adding primers and template to your assigned wells, put a plastic adhesive seal over your wells to protect your samples and give the qPCR plate back to your TA for the teaching staff to collect. The 7.003 teaching staff later will add 10 μL of 2X KAPA SYBR Fast qPCR Master Mix to each of your wells (*what is the final concentration of the primers in the final qPCR reaction?*) and send the plate to the MIT BioMicro Center to run the following qPCR program:

Step 1) 95°C for 5 minutes
Step 2) 95°C for 10 seconds
Step 3) 60°C for 20 seconds
Step 4) 72°C for 10 seconds and measure a single acquisition
Step 5) Repeat Steps 2 – 4 a total of 45 cycles
Step 6) 95°C for 5 seconds
Step 7) 65°C for 1 minute
Step 8) Gradually increase temperature to 97°C, while measuring 5 acquisitions per each degree C (melting curve step)
Step 9) 40°C for 30 seconds (cooling step)

	A	B	C	D	E	F	G	H
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								

DAY 17 (Thu, Apr 14th), continued

Day 17 Pre-Lab Notebook Tips & Reminders:

- Remember to include Aims for all parts (Parts 4.7 and 4.8).

Day 17 Post-Lab Notebook Tips & Reminders:

- Be sure to label the gel photo such that it's clear what is loaded in each lane and what the sizes of all ladder bands are.

- You should discuss each lane of your gel (lanes/samples with similar overall results can be discussed together as a group if you want).

    - What was the expected overall result for each lane? Why? (Included expected PCR product sizes, if applicable.)

    - What can you conclude based on whether or not you saw the expected result for each lane? Explain your reasoning. If you did not get the expected results, what might be a possible reason to explain the difference?

- Remember to list which plate number you used for setting up your.qPCR samples and which well numbers were used for each of your samples.

DAY 18 (Tue, Apr 19th)

4. Analyzing RNA expression of αF-resistant mutants

Part 4.9: Analysis of the *FUS1* qPCR results

Background: Today, you will analyze your results from the *FUS1* qPCR samples you set up from Day 17. A qPCR Analysis Worksheet has been posted on Canvas to help you interpret your qPCR data. Follow all the instructions on the worksheet and answer any questions when writing up your Day 18 Post-Lab.

Day 18 Pre-Lab Notebook Tips & Reminders:

- What is your Aim for Part 4.9?

Day 18 Post-Lab Notebook Tips & Reminders:

- Follow all the instructions and answer all the questions on the qPCR Analysis Worksheet posted on Canvas when completing your Day 18 Post-Lab.

DAY 19 (Thu, Apr 21st)

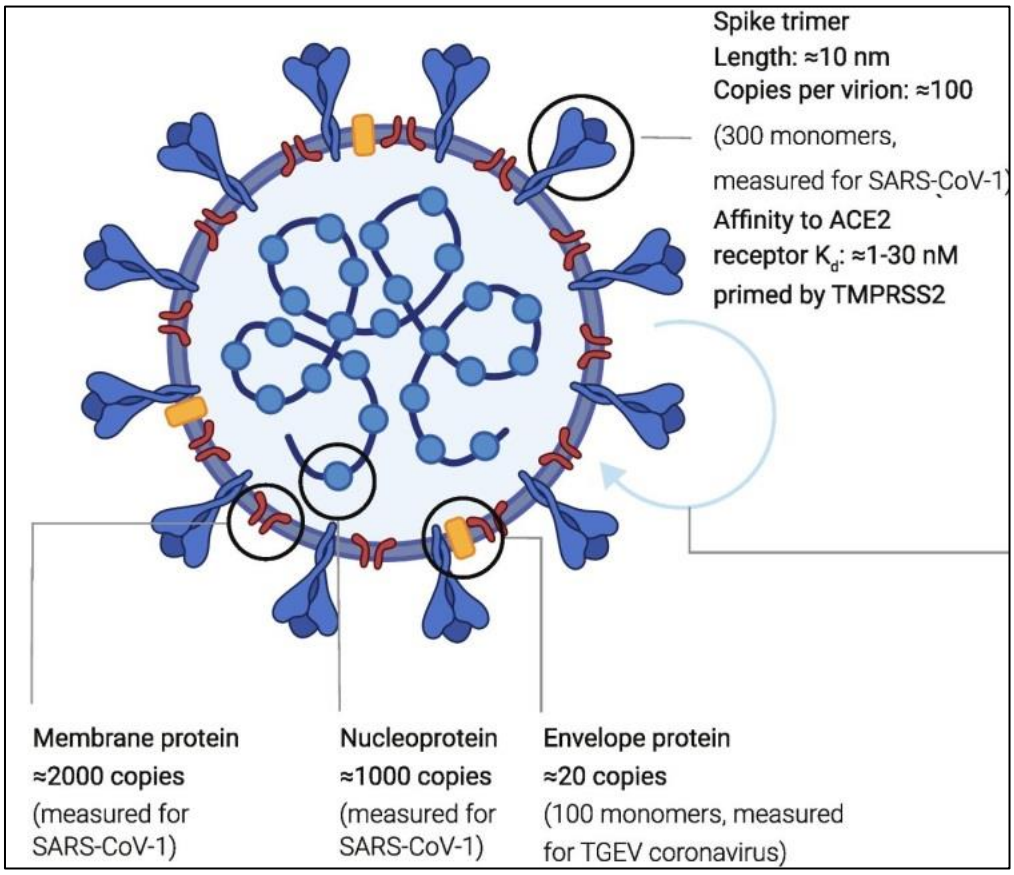
5. ChemE: Yeast surface display & protein engineering

Part 5.1: Design of SARS-CoV-2 target protein

For the remaining lab days (Days 19 – 23), the overall goal is to use yeast surface display to engineer an antibody that can bind tightly and specifically to a SARS-CoV-2 viral protein target. Such an antibody could potentially be used in a COVID-19 diagnostic test (as described in lecture). To accomplish this, you first need to obtain pure samples of SARS-CoV-2 protein to use as the target for your surface display experiments when screening for potential binders. Day 19 will cover picking an appropriate SARS-CoV-2 protein target, cloning the gene encoding that viral protein into an expression plasmid, and expressing & purifying the recombinant viral protein.

Day 19 Part 5.1 Overall Experimental Workflow

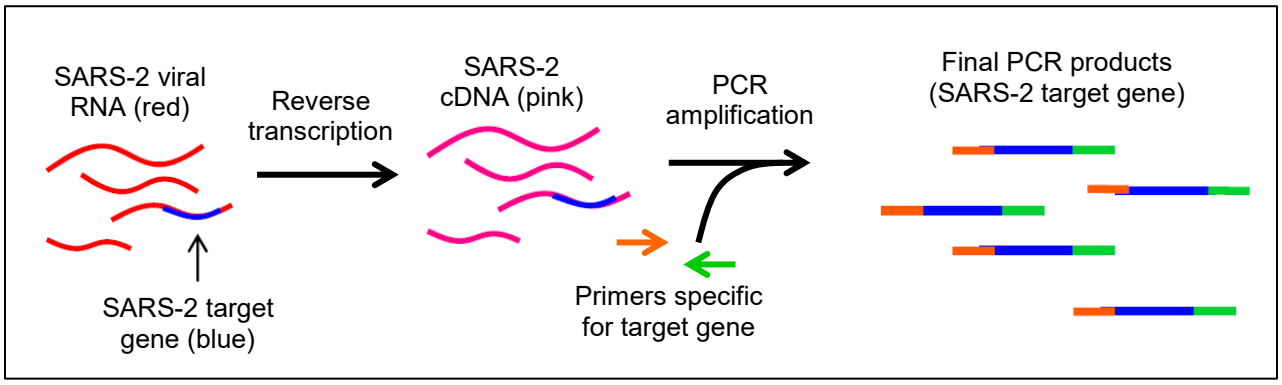
Step 1: Choose SARS-CoV-2 target protein (or protein domain).



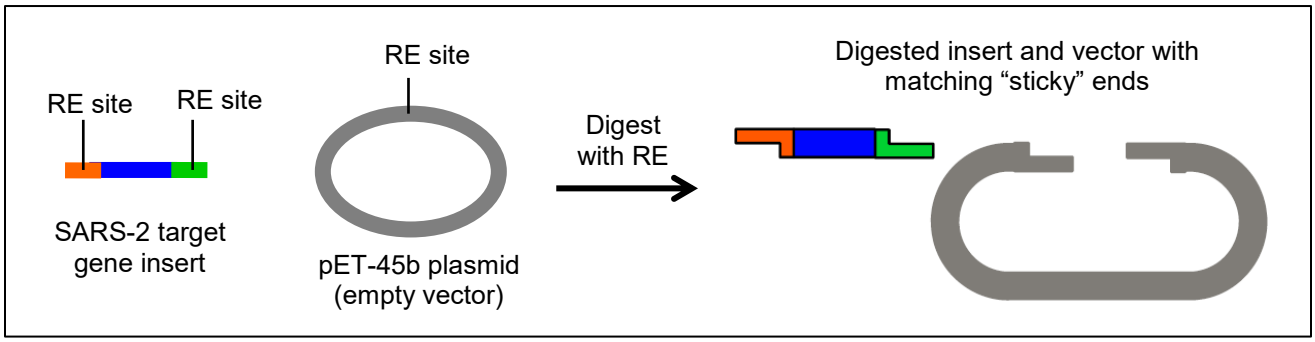
Yinon M Bar-On, Avi Flamholz, Rob Phillips, Ron Milo (2020) **Science Forum: SARS-CoV-2 (COVID-19) by the numbers** *eLife* 9:e57309. https://doi.org/10.7554/eLife.57309 ; License CC-BY.

DAY 19 (Thu, Apr 21st), continued

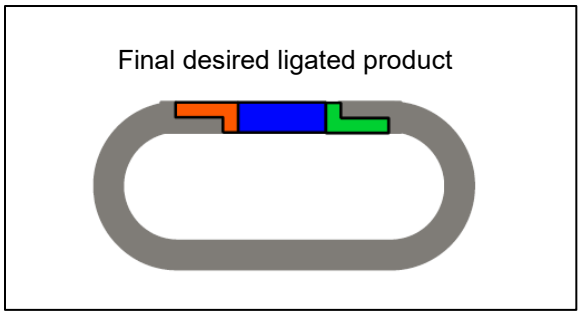
Step 2: PCR-amplify gene encoding target protein from SARS-CoV-2 cDNA



Step 3: Digest SARS-CoV-2 target PCR product and pET-45b expression vector with restriction enzymes (RE).

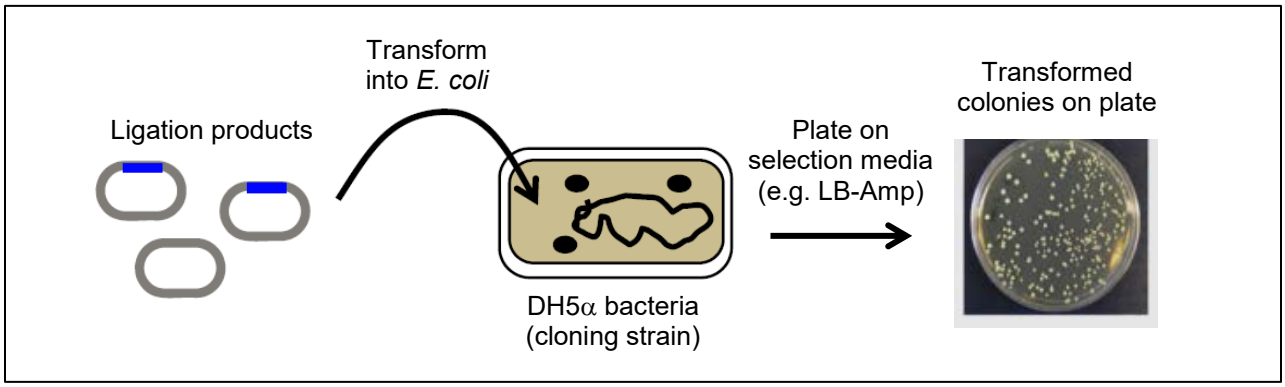


Step 4: Ligate SARS-CoV-2 target gene insert into pET-45b expression vector backbone.

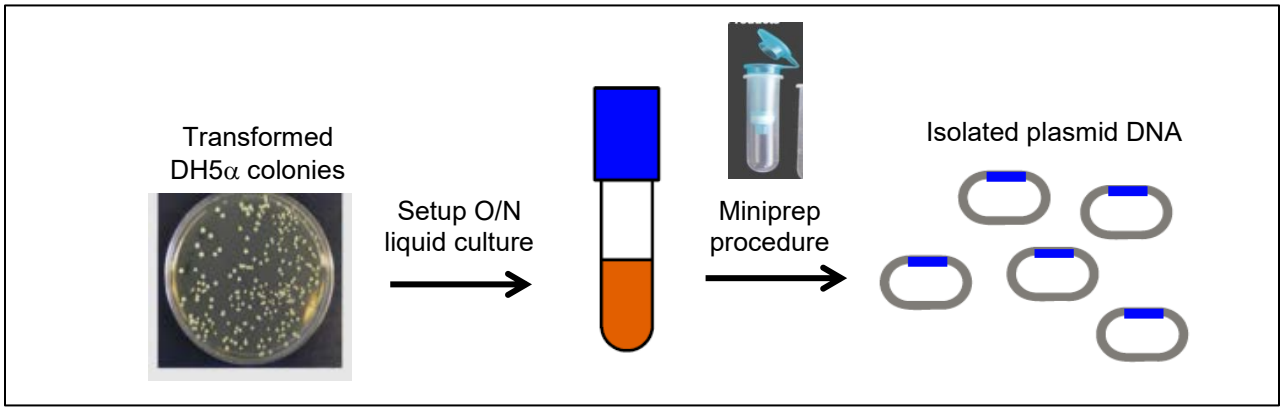


DAY 19 (Thu, Apr 21st), continued

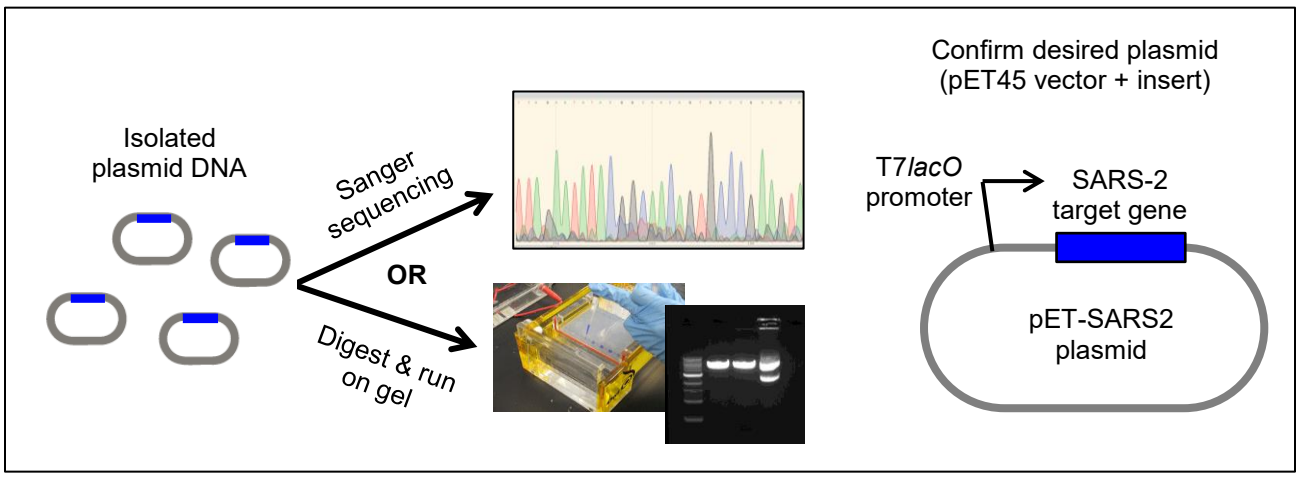
Step 5: Transform ligation products into DH5α *E.coli* bacteria cloning strain.



Step 6: Isolate miniprep plasmid DNA from transformed DH5α colonies.

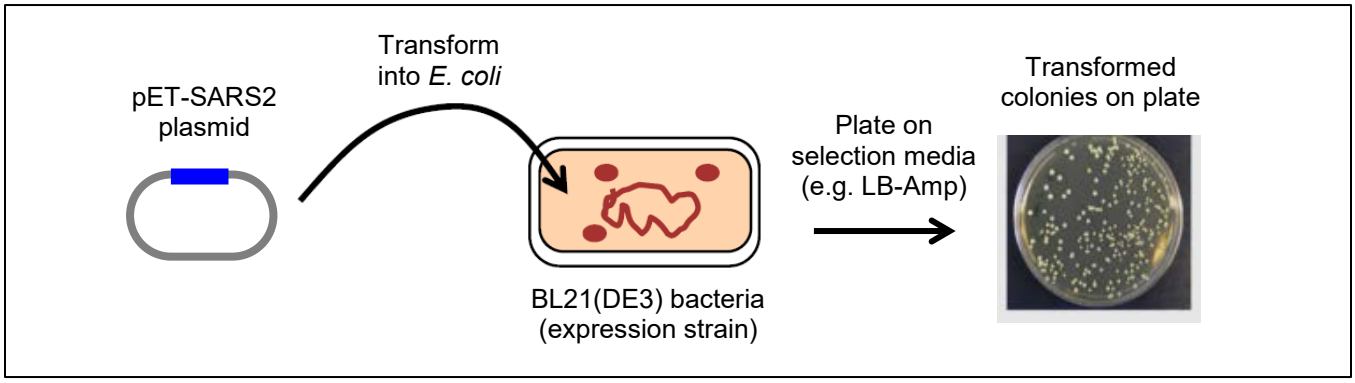


Step 7: Confirm successful insertion of SARS-CoV-2 target gene into pET-45b vector backbone with Sanger sequencing or analytical digest of miniprep plasmid.

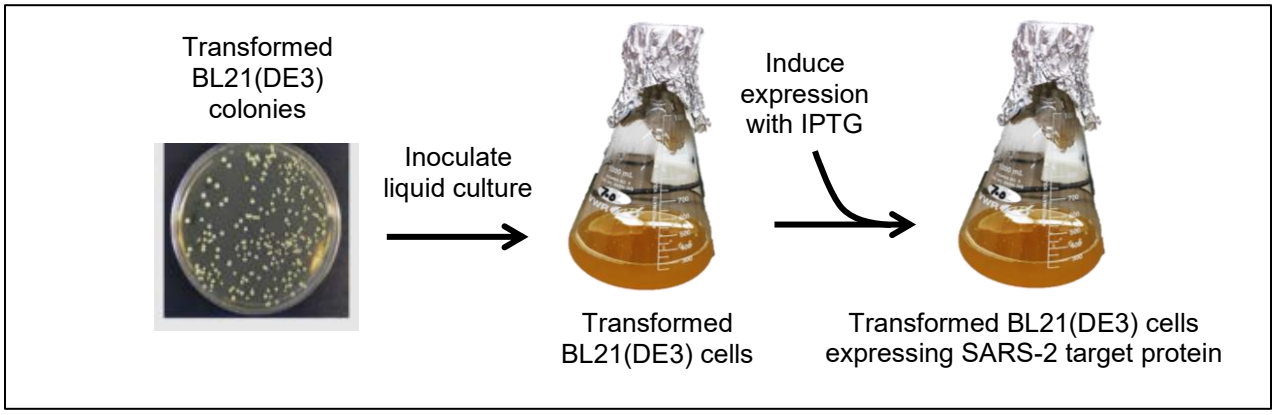


DAY 19 (Thu, Apr 21st), continued

Step 8: Transform correct plasmid into BL21(DE3) *E. coli* bacteria expression strain.

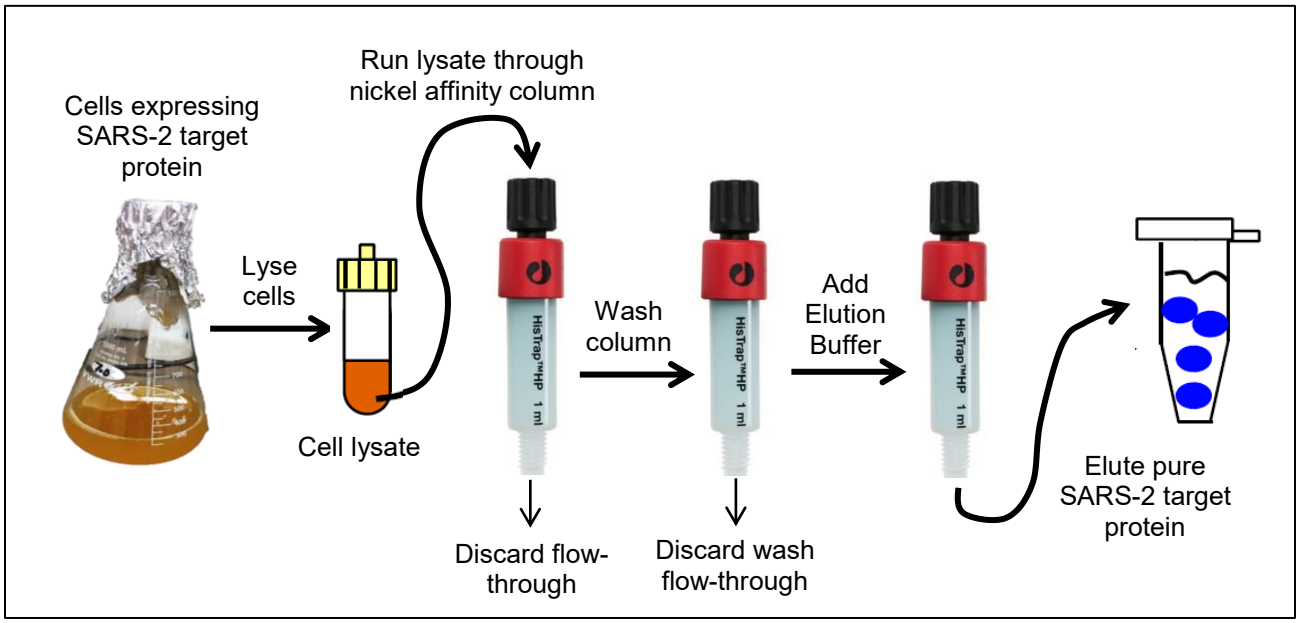


Step 9: Induce expression of recombinant SARS-CoV-2 target protein from T7*lacO* promoter on pET-45b plasmid in transformed BL21(DE3) cells (e.g. by using IPTG induction).



DAY 19 (Thu, Apr 21st), continued

Step 10: Purify SARS-CoV-2 target protein from induced cells (e.g. by using nickel-affinity chromatography column if the protein was His-tagged).



HisTrap column image adapted from
https://www.gelifesciences.com/en/us/shop/chromatography/resins/affinity-tagged-protein/histrap-hp-histidine-tagged-protein-purification-columns-p-00250

Note: Steps 5 – 10 above are similar to what was done in 7.002 to create the mutagenized pET-ClpX plasmid and then express and purify the His-tagged mutant ClpX protein from bacteria. We will focus mainly on Steps 1 – 4 for 7.003 Day 19 (*see next page)*.

DAY 19 (Thu, Apr 21st), continued

The following six Snapgene .dna files are posted on the course site under Lab Day 19:

- SARS-CoV-2 complete genome sequence
- 229E human coronavirus complete genome sequence
- NL63 human coronavirus complete genome sequence
- OC43 human coronavirus complete genome sequence
- HKU1 human coronavirus complete genome sequence
- pET-45b expression plasmid vector sequence

Note: 229E, NL63, OC43, and HKU1 are four common human coronaviruses (i.e. it's likely for a human to have been previously infected by at least one of these four viruses).

Follow the instructions and answer all nine questions below to complete your Day 19 Post-Lab on LabArchives.

1) Which SARS-CoV-2 protein do you choose as your target viral protein (pick from either the E, M, N, or S proteins)?

2) Will you be using the full-length protein as a target or will you only be using a portion of the protein as a target? If you will only be using a particular region of the protein, list which basepairs of the gene sequence or which amino acid residues of the protein sequence you will be using.

3) Perform a multiple protein alignment that aligns your SARS-CoV-2 target protein with the corresponding homologous protein from all of the four common human coronaviruses (229E, NL63, OC43, and HKU1). Take a screenshot of the protein alignment (focusing on the region of interest within your target protein, if applicable). Insert a JPG of that protein alignment screenshot in your Day 19 Post-Lab on LabArchives.

Detailed instructions for performing a multiple protein alignment (using either SnapGene or NCBI COBALT) are provided on the site under Lab Day 19.

4) Briefly explain your answers to Questions 1 and 2: Why did you choose that particular protein (and protein region) to use as your target viral protein for engineering antibodies that can detect SARS-CoV-2 virus?

DAY 19 (Thu, Apr 21st), continued

5) Design PCR primers to amplify your SARS-CoV-2 target gene (region) for cloning into the pET-45b expression plasmid such that your SARS-CoV-2 protein can be expressed from the plasmid's T7*lacO* promoter with a 6xHis-tag (for purification of the protein later).

a. Write your forward and reverse primer sequences, labeling the 5' and 3' ends of each primer.

b. Underline any parts of your primer sequences that bind to any SARS-CoV-2 gene sequence.

c. List any restriction enzyme sites present in your primers and use a different color font or color highlight to indicate where the restriction sites are in your primer sequences.

d. List the T_m (melting temperature) for each primer. (SnapGene will calculate approximate T_m's for primers, or you can use the NEB online T_m calculator at https://tmcalculator.neb.com)

6) List the length of your expected PCR product (in basepairs).

7) Write a PCR program to amplify your SARS-CoV-2 target gene (region) using your primers from Question 5. List the temperatures and times of all steps in your PCR thermocycler program.

8) List what restriction enzymes you will use to digest your PCR product (target gene insert) and what restriction enzymes you will use to digest the pET-45b plasmid for cloning.

9) Create a SnapGene .dna file consisting of your SARS-CoV-2 target gene region inserted into the pET-45b vector, following your cloning strategy from Question 8.

a. Annotate or label the SARS-CoV-2 gene sequence in the plasmid as a new feature (highlight the desired sequence, then from the top menubar, go to Features → Add Feature…).

b. Add both your forward and reverse PCR primers from Question 5 (from the top menubar, go to Primers → Add Primer…).

c. Save the .dna file as "pET-SARS2" with your initials and protein name (e.g. "VC pET-SARS2 M protein.dna").

d. Upload this pET-SARS2 .dna file as an attachment in your Day 19 Post-Lab on LabArchives.

DAY 20 (Tue, Apr 26th)

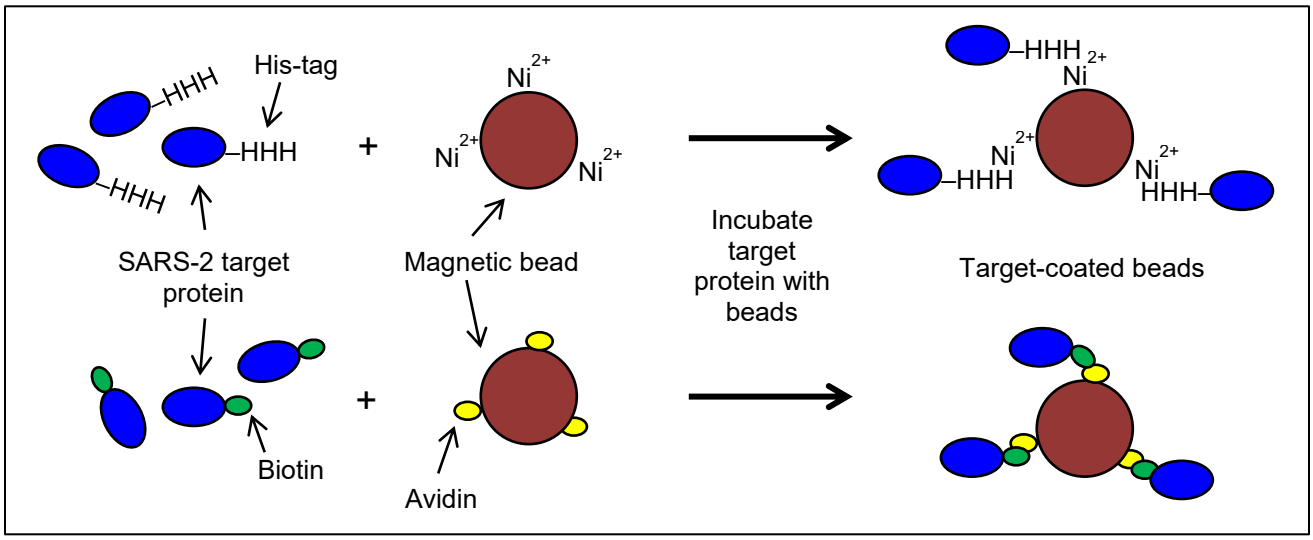
5. ChemE: Yeast surface display & protein engineering

Part 5.2: Yeast display magnetic bead sorting

Having expressed and purified a SARS-CoV-2 protein (from Day 19), you can now use it as a target to engineer antibodies that can recognize and bind to that target protein. You will use yeast surface display, where a library of yeast cells each express a different antibody variant on their cell surface. For Day 20, you will perform a bead sorting, using magnetic beads coated with the SARS-CoV-2 target protein to screen the library for antibody binders to the viral target protein.

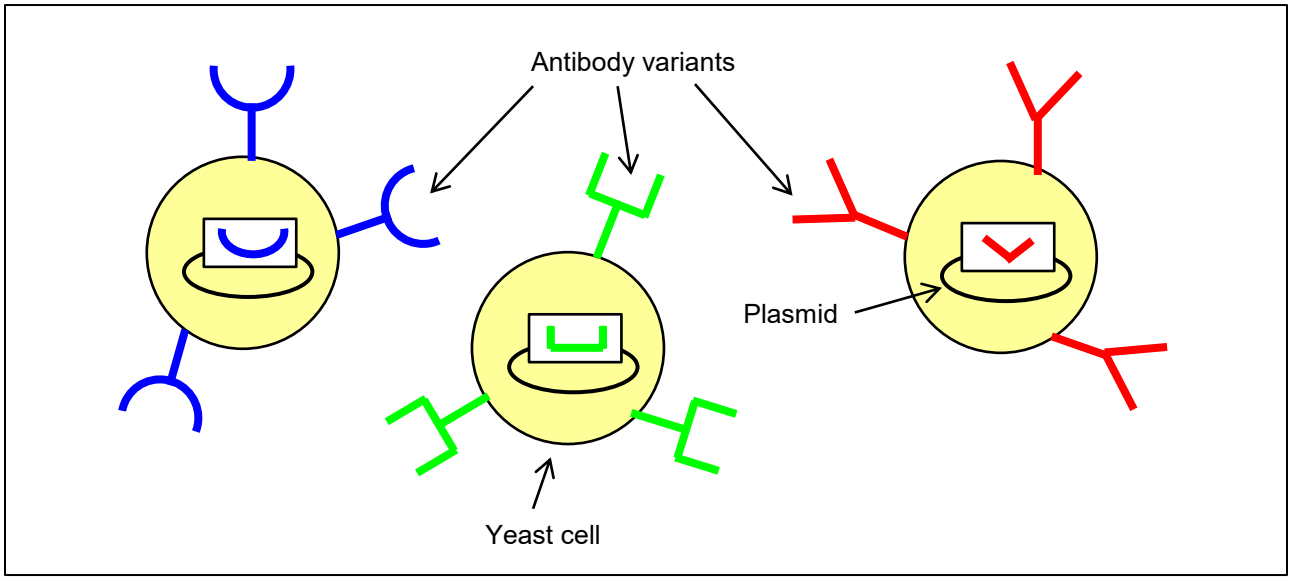
Day 20 Part 5.2 Overall Experimental Workflow

Step 1: Coat magnetic beads with SARS-CoV-2 target protein. Options include having 6xHis-tagged target protein bind to Ni^{2+}-NTA-beads or having biotinylated target protein bind to streptavidin-conjugated beads.

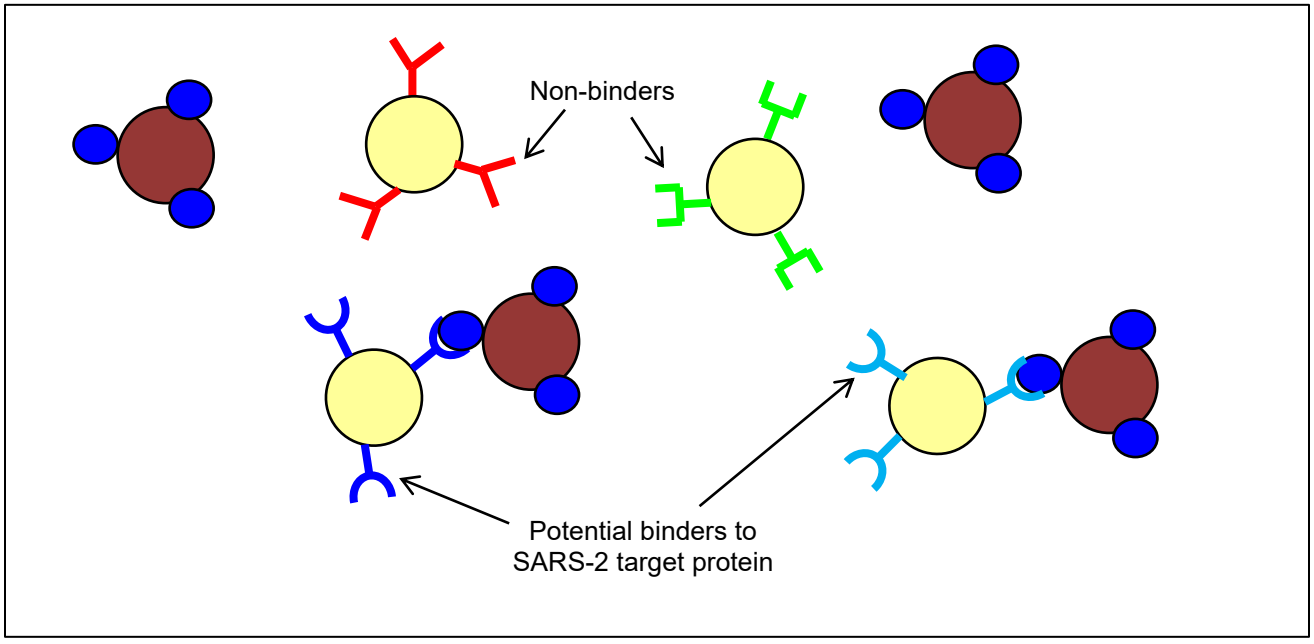


DAY 20 (Tue, Apr 26th), continued

Step 2: Prepare a yeast library of potential binders (antibody variants). Each yeast cell in the library contains a plasmid encoding a different antibody variant that is expressed on the cell surface.

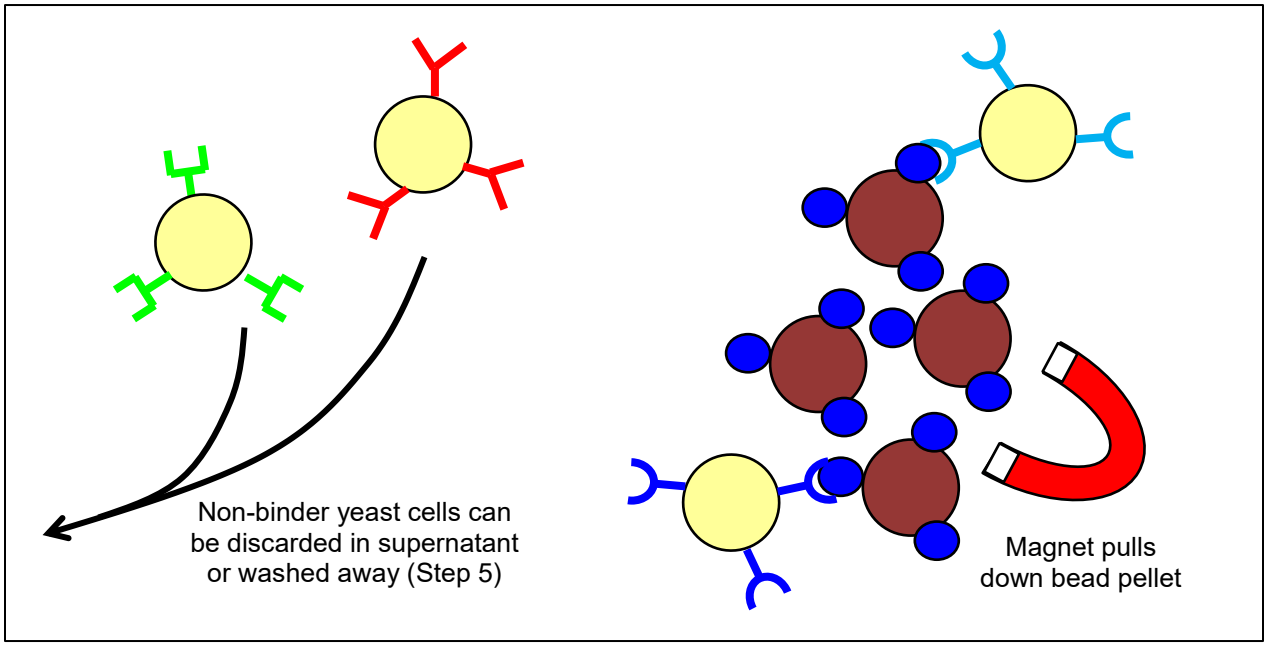


Step 3: Incubate antibody library with target-coated beads to allow any yeast cells expressing potential target-binders to bind the beads (note: Steps 3 – 6 are all taking place in an Eppendorf tube).



DAY 20 (Tue, Apr 26th), continued

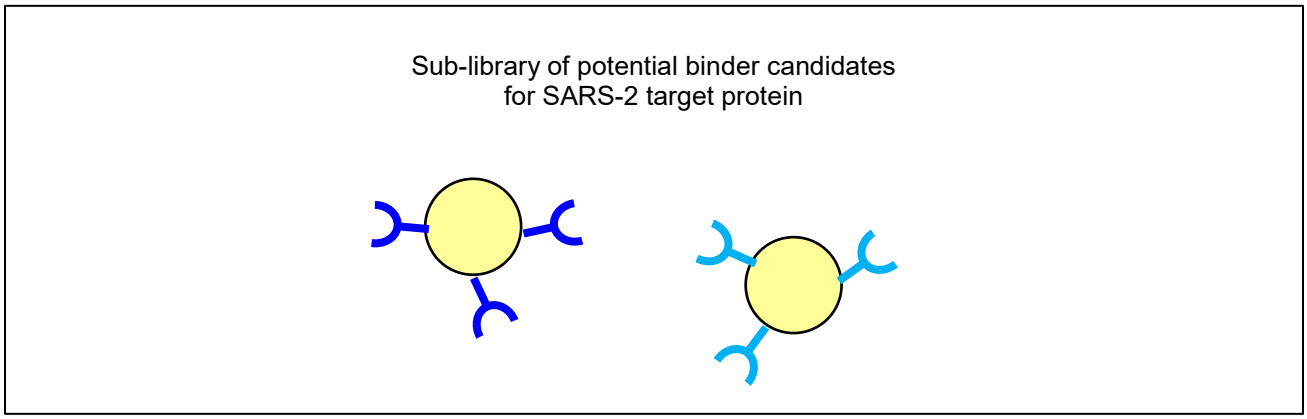
Step 4: Use a magnet to pull down and pellet the magnetic beads, along with any yeast cells also bound to the beads via a binder-target interaction.



Step 5: Wash beads to remove any non-binder yeast cells (or yeast cells that are only binding non-specifically or very weakly to the target viral protein on the beads).

DAY 20 (Tue, Apr 26th), continued

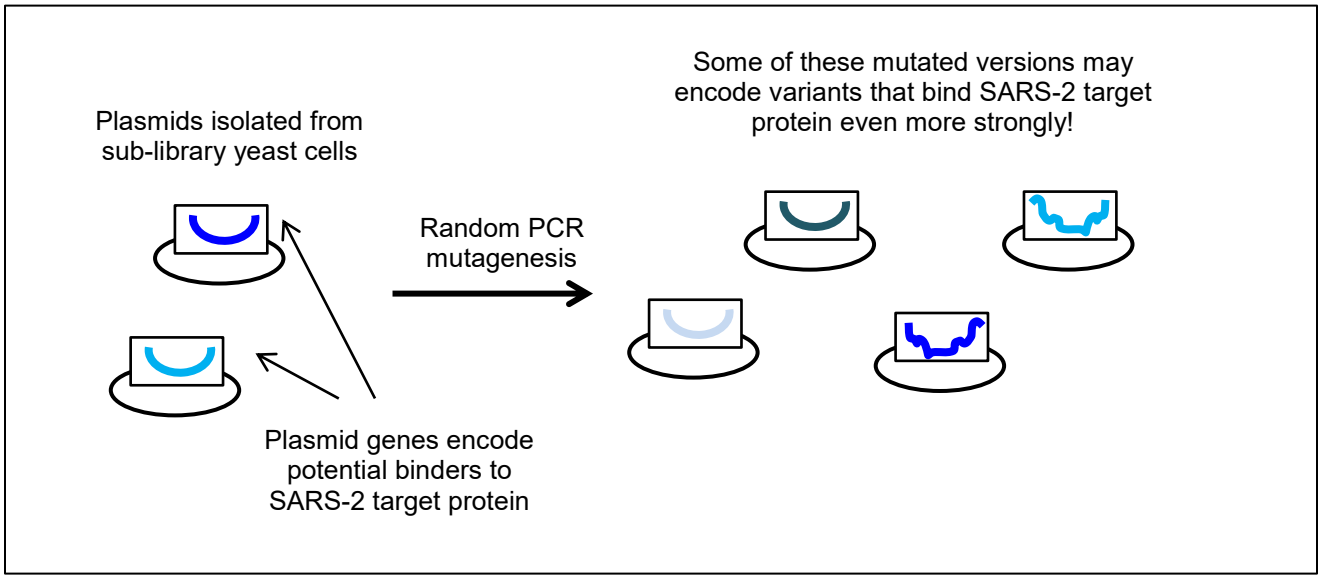
Step 6: Collect and save the pelleted bead fraction to isolate the remaining binder yeast population. This sub-library should now predominantly consist of only those cells from the original library that express an antibody variant that can bind the SARS-CoV-2 target protein.



Future steps: The sub-library can be used for additional rounds of sorting, using either magnetic beads again (Steps 3 – 6) or FACS (as will be done on Lab Day 21).

Screening conditions with beads or FACS can be varied during these repeated sorting rounds to optimize isolation of stronger binders or binders with certain properties.

The sub-library plasmids can also be randomly mutagenized to introduce new genetic variation into the binder population, with the goal of possibly producing a binder with even stronger affinity to the target protein (i.e. “directed evolution” of a stronger binder).



DAY 20 (Tue, Apr 26th), continued

Follow all instructions and answer all four questions below for your Day 20 Post-Lab.

1) State a modification you could do to your overall magnetic bead sorting protocol that would likely help increase the sensitivity of selecting binders for your SARS-CoV-2 target protein.

2) Having decided on the conditions for your magnetic bead sorting protocol, you wish to perform a test run to assess the effectiveness of your sorting protocol. For your test run beads, you use magnetic beads coated with the c-Myc epitope peptide. You use a test starting population containing exactly:

2×10^4 yeast cells expressing a Myc antibody on their surface
1×10^6 yeast cells that do not express anything on their surface

You perform your test run bead sorting protocol using the above reagents. Your final isolated "sub-library" after this bead sorting test run contains exactly:

1.2×10^4 yeast cells expressing a Myc antibody on their surface
2.5×10^5 yeast cells that do not express anything on their surface

Calculate the percent sensitivity and percent specificity for your overall magnetic bead sorting protocol (please show your work).

3) You have successfully performed your magnetic bead sorting screen and isolated a sub-library of yeast cells expressing antibody variants that potentially bind your SARS-CoV-2 target protein. Your instructor is concerned though that some of the binders in your sub-library may not be specific for your actual desired target (the SARS-CoV-2 protein).

Design two different magnetic bead sorting experiments you could perform on your sub-library to remove any potential non-specific binders from the population. For each experiment, state what type of beads you would use and explain what fraction you would save when isolating the desired population.

4) You have isolated the plasmids from the yeast cells in your sub-library and now wish to randomly mutagenize the antibody genes on the plasmids, with the hopes of introducing mutations that might produce a variant with increased binding affinity for the SARS-CoV-2 target protein. Design an experiment to randomly mutagenize the antibody genes on your sub-library plasmids.

DAY 21 (Thu, Apr 28th)

5. ChemE: Yeast surface display & protein engineering

Part 5.3: Optimization of magnetic bead sorting protocol

Background information: Today, you will perform a magnetic bead sorting experiment to separate yeast cells that can or cannot bind to a target of interest. You and your lab partner will decide what particular protocol conditions to adjust to try to optimize both the sensitivity and the specificity of your magnetic bead selection protocol.

For this optimization experiment, you will use an artificial library containing just two types of yeast cells: binder yeast cells (L7.5.1 strain) and non-binder yeast cells (EBY100 strain) (see strain genotypes below). The L7.5.1 binder yeast cells have the same genotype as EBY100 cells, except that the L7.5.1 cells also express a protein variant on their surface that has been engineered to bind strongly to the enzyme lysozyme (*what is lysozyme?)*. This lysozyme-binder is expressed in the L7.5.1 cells from the pCTCON2 plasmid, which has a *TRP1*$^+$ auxotrophic selection marker (see Day 20 ILQs Question 1). The presence or absence of this plasmid will allow you to easily differentiate between L7.5.1 binder cells and EBY100 non-binder cells by plating the cells on YPD or SC-Trp plates (*which type of cells would you expect to be able to grow on YPD plates? How about on SC-Trp plates?)*.

EBY100 strain genotype (non-binder): *MATα ura3-52::[pIU212 = GAL1pr-AGA1; URA3], his3Δ200, leu2Δ1, trp1Δ, pep4Δ::HIS3, prb1Δ1.6R, can1*

L7.5.1 strain genotype (binder): Same as EBY100, with the addition of *leu2D1::[pRS305-GAL1pr-YFP; LEU2]* and *[pCTCON2 extrachromosomal plasmid = GAL1pr-AGA2-L7.5.1; TRP1]*

You will be provided with a mixed population of EBY100 and L7.5.1 yeast cells in a pre-determined ratio and streptavidin magnetic beads coated with biotinylated lysozyme. You will choose how to modify the specific parameters of your bead selection protocol (based off of a general standard protocol template) to try to achieve the maximum sensitivity and specificity for your selection.

Materials:
- Magnetic Dynabeads (Invitrogen) coated with biotinylated lysozyme (2 x 10^7 beads/mL in PBSA)
- Mixed yeast culture of EBY100 non-binders (1 x 10^6 cells/mL) and L7.5.1 binder cells (2 x 10^4 cells/mL) in PBSA
- PBSA (Phosphate-Buffered Saline with 1 mg/mL Bovine Serum Albumin)
- 3 YPD plates (single blue stripe)
- 3 SC-Trp plates (single red stripe)
- Magnetic rack
- Sterile inoculating loops

DAY 21 (Thu, Apr 28th), continued

Procedure:

The general standard bead selection protocol is summarized below:

A) In a 1.5-mL Eppendorf tube, mix the following in a final total volume of 1 mL PBSA:

   1×10^6 EBY100 yeast cells *(note: this parameter cannot be modified!)*
   2×10^4 L7.5.1 yeast cells *(note: this parameter cannot be modified!)*
   5×10^5 magnetic beads coated with 0.1 μg of lysozyme

B) Incubate the tube on a rotator in the 4°C cold room for 15 minutes.

C) Place the tube on the magnet for 3 minutes to let the beads settle to the bottom of the tube. Remove and discard the supernatant, leaving the beads in the tube.

D) Remove the tube from the magnet. Wash the beads once by adding 1 mL of PBSA to the tube and gently resuspending the beads. Place the tube back on the magnet for 3 minutes and remove and discard the supernatant as before.

E) Remove the tube from the magnet and resuspend the beads in 200 μL of PBSA *(note: this parameter cannot be modified!)*. This is your final selected yeast population that is ready for preparing serial dilutions and plating (see the following pages).

1) You and your lab partner will perform a total of three different magnetic bead selections today. Determine what specific modifications (if any) you will make to the above standard bead selection protocol for each of your three bead selections. You might choose to modify the same parameter(s) to varying degrees for all three selections or you might choose to design completely different conditions for each of the three selections – the choice is up to you and your lab partner.

2) Work through the necessary calculations to determine what volume of lysozyme-coated magnetic beads and what volume of mixed EBY100 + L7.5.1 yeast culture you would need to add for each selection. *(Check your work with your TA!)*

3) Label three sterile 1.5-mL Eppendorf tubes with “A,” “B,” and “C,” and your bench number. You will be setting up your three bead selections in these tubes. Record which bead selection will correspond to “A,” “B,” and “C.”

4) Add the appropriate amount of magnetic beads and mixed yeast culture to each of your three bead selection tubes (Part A from the standard protocol).

DAY 21 (Thu, Apr 28th), continued

5) Incubate the three bead selection tubes on a rotator for the appropriate amount of time at the appropriate temperature (Part B from the standard protocol). During this incubation, you can label tubes/plates for serial dilutions and/or work on ILQs.

6) Place the three tube on the magnet for 3 minutes to let the beads settle to the bottom of each tube. When the 3-minute magnet incubation is done, you should see a small faint brownish patch on the side of each tube by the magnet – these are the beads. Carefully open the caps of the three tubes (while still leaving the tubes on the magnet). Using a P1000, slowly remove the supernatant from each tube without disturbing the beads on the side of the tube. Discard the supernatant in a plastic waste beaker. (This is Part C from the standard protocol.)

7) Remove the three tubes from the magnet. Perform the appropriate number of washes for each bead selection (Part D from the standard protocol).

8) Gently resuspend the beads in each of the three tubes in a final volume of 200 μL PBSA (Part E from the standard protocol). Your final enriched bead selections are ready for making serial dilutions and plating.

9) Label each of your three bead selection tubes (A – C) with "#1." These three tubes should now be labeled "A #1," "B #1," and "C #1."

10) To prepare the serial dilutions, you will need three more sterile 1.5-mL Eppendorf tubes for each of the three different bead selections "A" – "C" (nine additional tubes total). For each of the three different bead selections, label three 1.5-mL Eppendorf tubes "#2," "#3," and "#4." Also label each tube with its appropriate bead selection ("A," "B," or "C").

11) Add 180 μL of PBSA to each of the "#2," "#3," and "#4" Eppendorf tubes you labeled from Step 10.

12) To prepare the serial dilutions for bead selection "A," pipet 20 μL of the undiluted bead selection from the "A #1" tube into the "A #2" tube. Make sure the bead selection is well-mixed before you pipet it.

13) Pipet 20 μL from the "A #2" tube into the "A #3" tube. Make sure everything is well-mixed before you pipet it.

14) Finally, pipet 20 μL from the "A #3" tube into the "A #4" tube. Again, make sure everything is well-mixed before you pipet it.

15) Repeat Steps 12 – 14 using bead selections "B" and "C" to prepare serial dilutions of those bead selections.

DAY 21 (Thu, Apr 28th), continued

***Note: It's best to prepare the serial dilutions one bead selection at a time (i.e. do all the "A" serial dilutions together before you start the "B" serial dilutions, etc). This minimizes tube mix-ups and cross-contamination between bead selections.

16) You will need three YPD plates and three SC-Trp plates (one YPD and one SC-Trp plate for each of the three different bead selections). Write your bench number on all six plates. Label the three YPD plates and the three SC-Trp plates "A," "B," and "C." Divide each plate into four equal quadrants. For each plate, label the four quadrants "1" – "4."

17) To plate bead selection "A," carefully pipet 20 μL from each of the four "A" serial dilution tubes onto its corresponding quadrant of the YPD "A" plate (i.e. 20 μL from "A #1" tube goes onto Quadrant 1, 20 μL from "A #2" tube goes onto Quadrant 2, etc). **Make sure the tubes are well-mixed before you pipet from them and make sure you pipet each serial dilution onto the correct quadrant.**

18) Using a **NEW sterile inoculating loop** for each droplet, gently spread each droplet throughout the entire quadrant until it is mostly absorbed into the agar surface.

- Be careful not to go over the quadrant boundaries or get too close to the edge of the plate while spreading (if cells are pushed too close to the edge of the plate, this can make it difficult to count them accurately). The plate does not have to be completely spread to dryness, but you do not want to leave too much liquid sitting on top of the agar, or else the cell solution may leak and spread into the wrong quadrant.

- Be careful not to push too hard on the agar or else you will scratch or puncture the agar surface – this may cause some of the yeast cells to enter the agar medium, making it difficult to accurately count colonies later.

19) Repeat Steps 17 – 18 with the SC-Trp "A" plate to plate the "A" serial dilutions on the SC-Trp plate.

20) Repeat Steps 17 – 19 using the serial dilutions from bead selections "B" and "C" to plate these bead selections on both YPD and SC-Trp plates.

21) Once you are done plating all the serial dilutions for all three bead selections onto both YPD and SC-Trp plates, check your plates for moisture. If your plates are all dry, tape them together and incubate them upside-down in the 30°C warm room to let them grow for three days. The 7.003 teaching staff will then store them in the cold room until the next lab session.

*****Note: Day 21 continues on the next page!!!*

DAY 21 (Thu, Apr 28th), continued

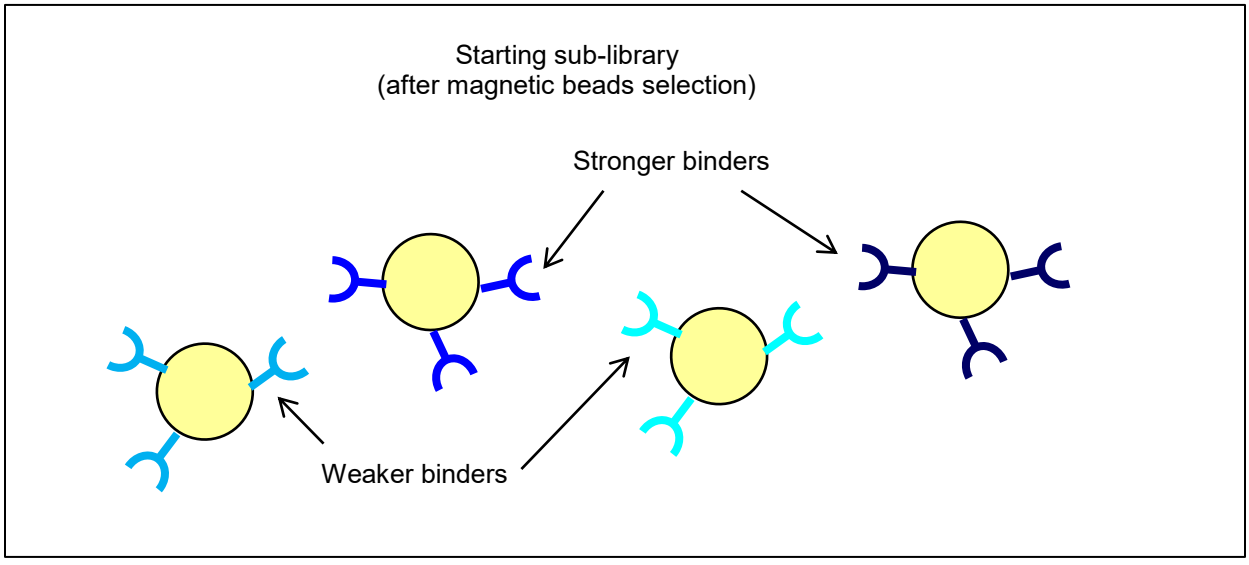
Part 5.4: Yeast display FACS sorting

After performing magnetic bead sorting as in Parts 5.2 and 5.3, you have a sub-library of surface display yeast cells expressing potential binders to the SARS-CoV-2 target protein. This sub-library of binders likely consists of a mixture of both strong and weak binders to your target protein. The next step is to use flow cytometry with FACS (Fluorescence-Activated Cell Sorting) to further screen your sub-library and select only those binders with the strongest affinity for the target protein – these strongest binders would be most effective for any downstream applications like diagnostic tests or therapeutic drugs.

Note: If you would like an animated overview of flow cytometer, watch this video: Abingdon Health. "See How a Lateral Flow Immunoassay Works." April 8, 2019. YouTube.

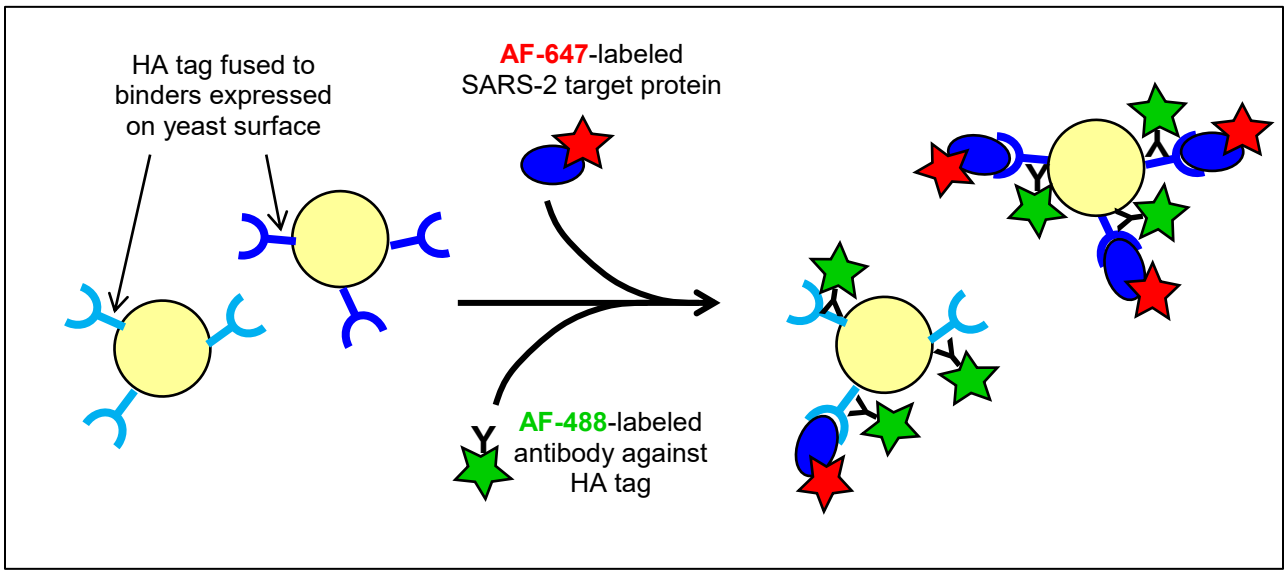
Day 21 Part 5.4 Overall Experimental Workflow

Step 1: Start with your sub-library of potential binders to the SARS-CoV-2 target protein (obtained from magnetic bead sorting from Day 20). Dark blue binders represent strong binders to the target, and light blue binders represent weak binders.



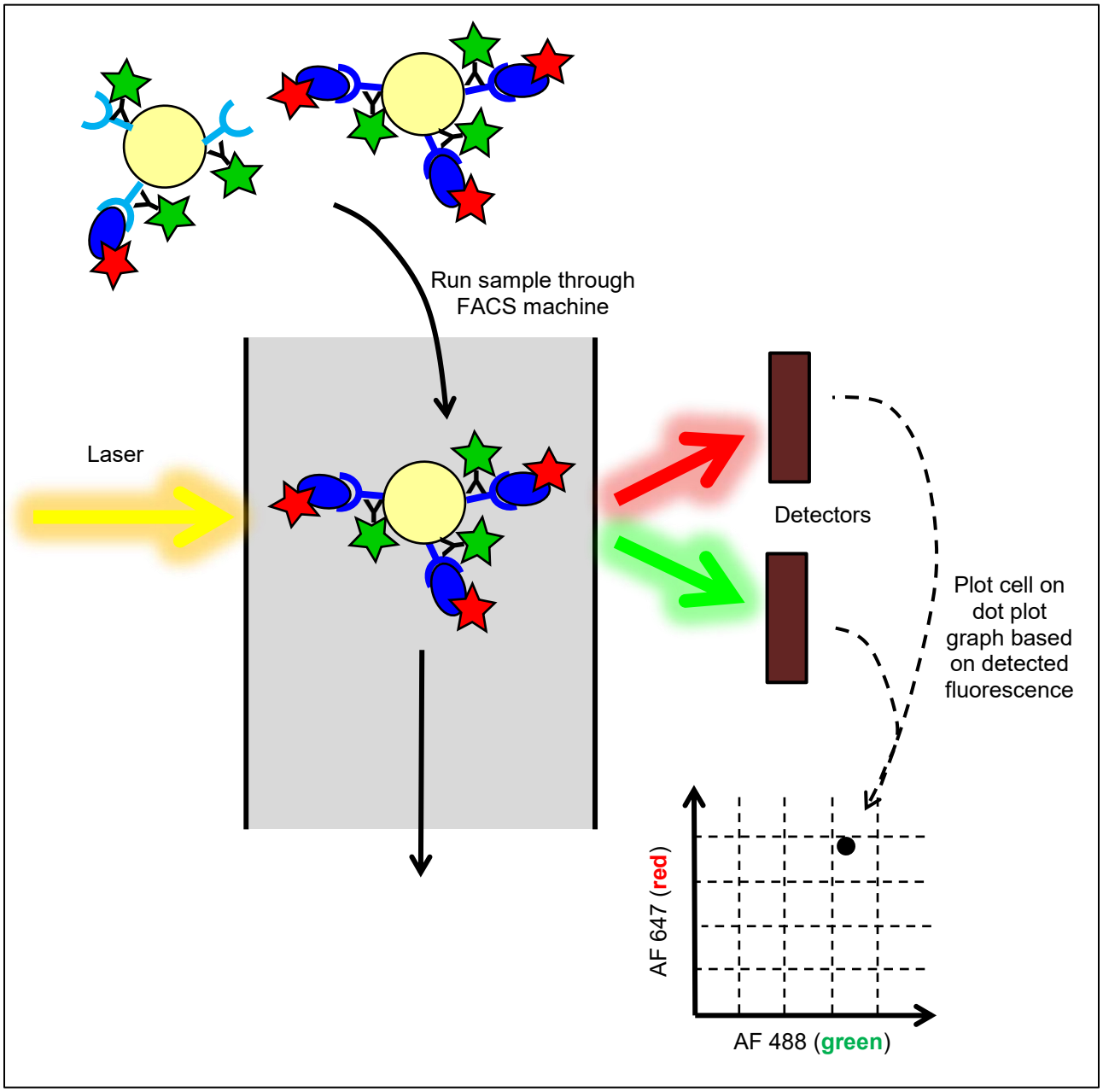
DAY 21 (Thu, Apr 28th), continued

Step 2: Incubate the sub-library with the SARS-CoV-2 target protein labeled with a fluorophore (e.g. AF 647 (red)). Also include an antibody that recognizes one of the tags (e.g. HA tag) expressed with the binder on the surface of the yeast cell. This antibody should be labeled with a different color fluorophore (e.g. AF 488 (green)). Allow both the SARS-CoV-2 target protein and the anti-tag antibody to bind their respective partners on the yeast cells of the sub-library. Note that yeast cells expressing stronger binders will have a greater percentage of the binders on their cell surface bound to the virus target compared to yeast cells expressing weaker binders.



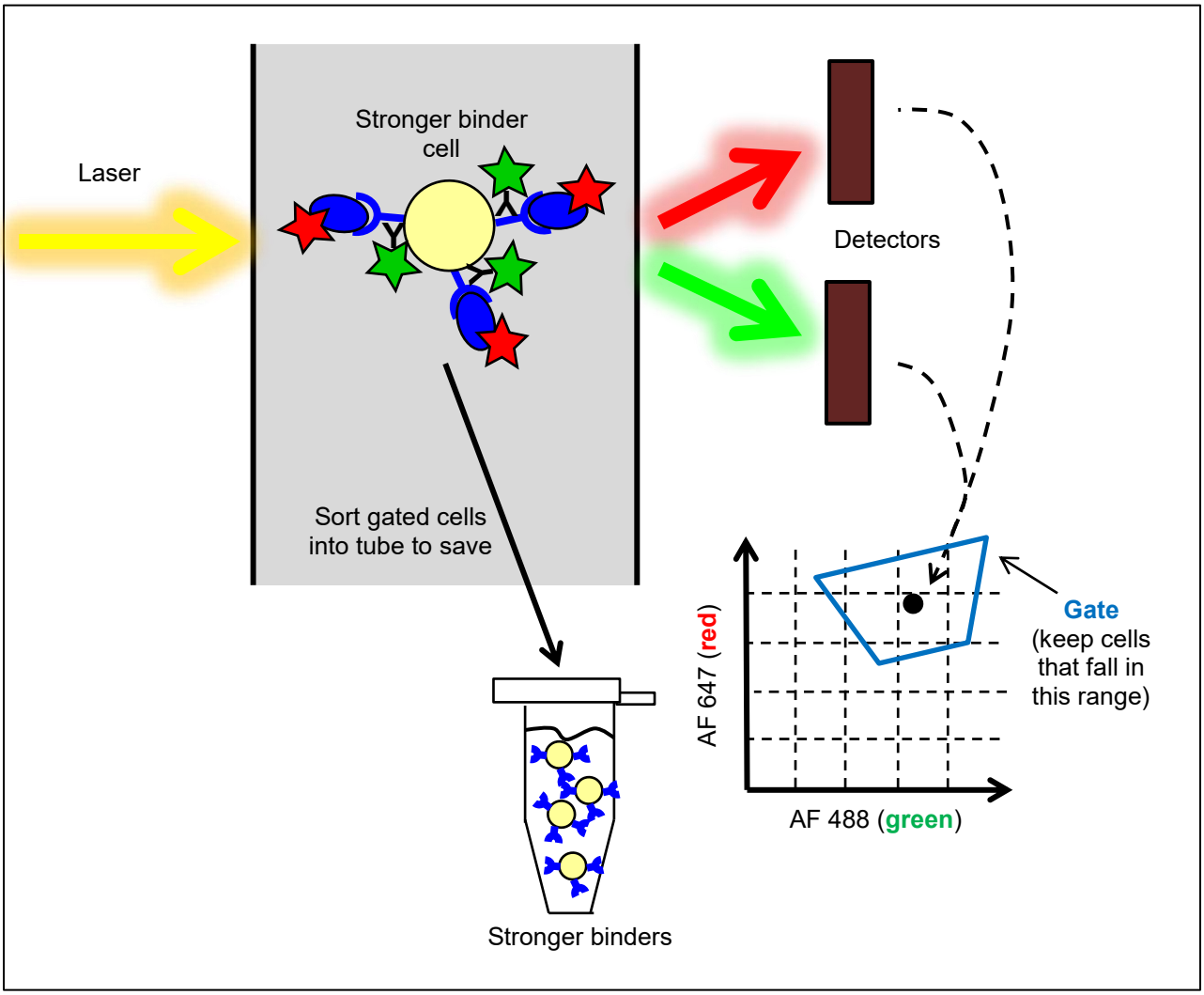
DAY 21 (Thu, Apr 28th), continued

Step 3: Run the mixture through a FACS machine. Cells pass individually through a laser beam that excites any attached fluorophores, and the amount of emitted fluorescence for each cell is detected. Most FACS machines have multiple channels so they can simultaneously measure fluorescence at different wavelengths or colors for each individual cell (e.g. green and red).



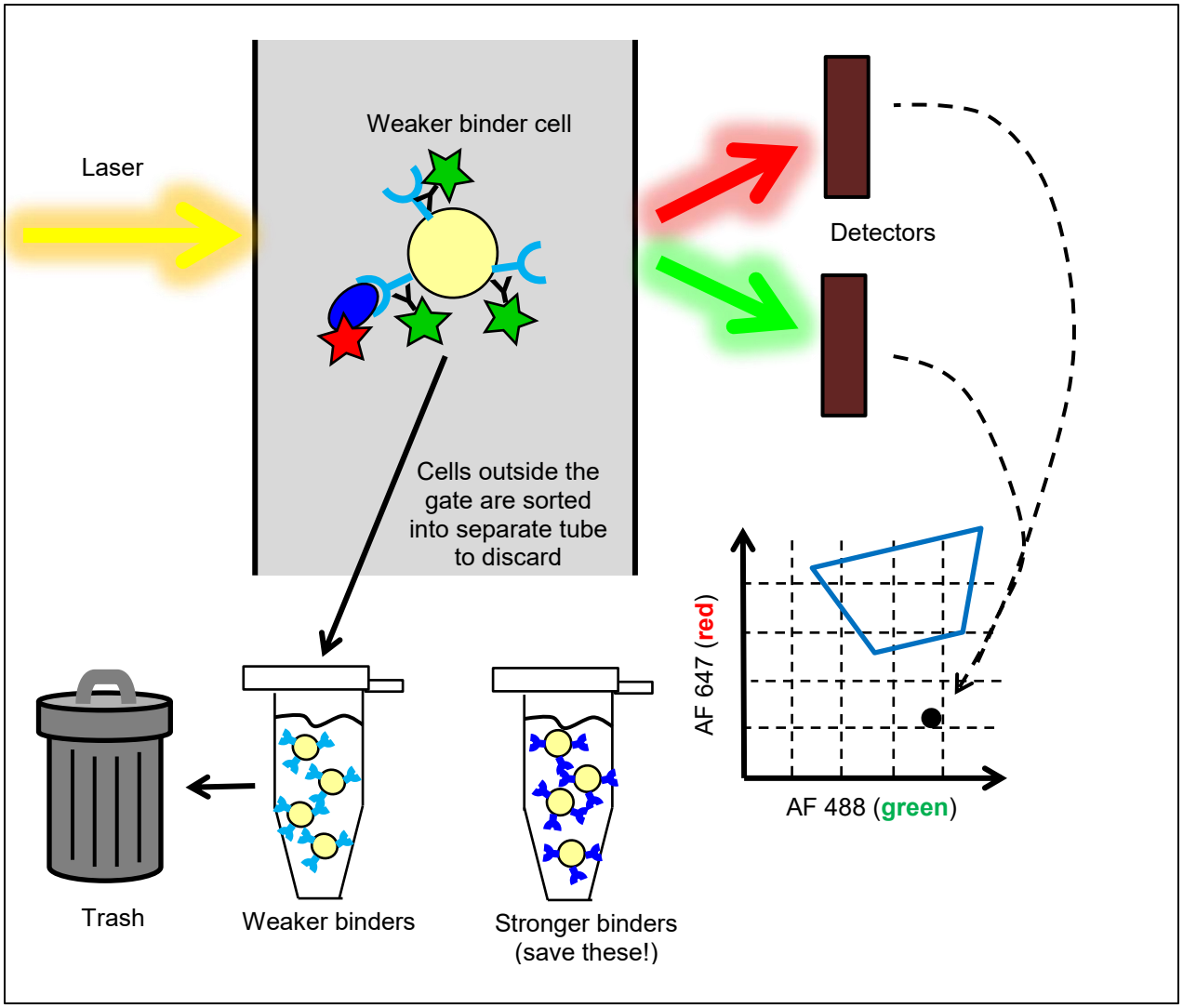
DAY 21 (Thu, Apr 28th), continued

Step 4a: Sort the cells as they pass through the FACS machine based on the level of fluorescence per cell for each of the measured color channels. You can arbitrarily set a pre-defined range or "gate," where all cells that have fluorescence levels that fall within that gate are sorted and saved into a tube.



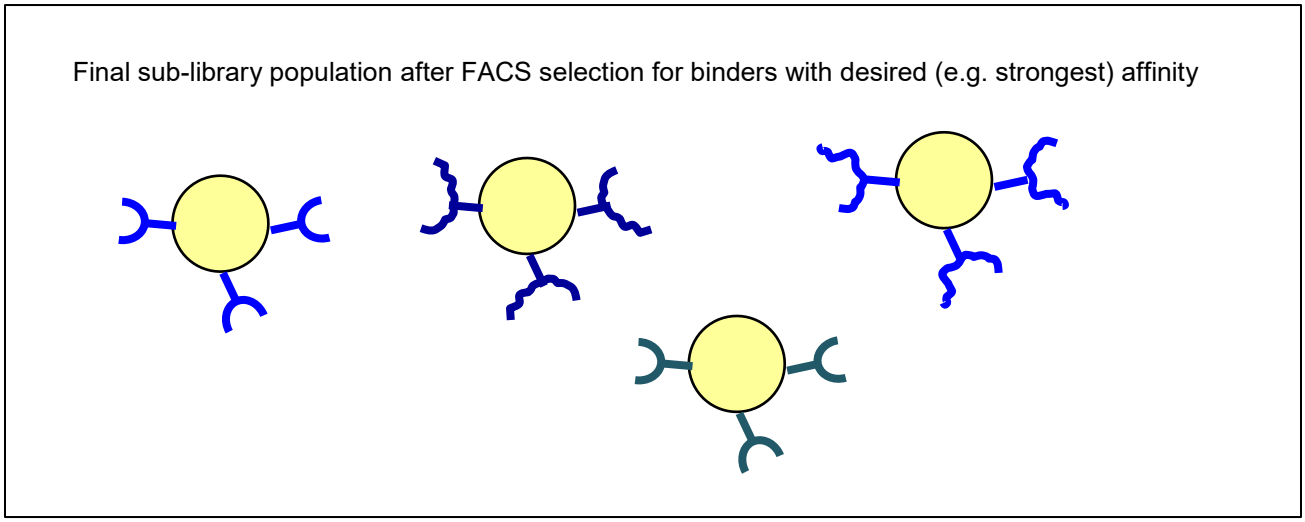
DAY 21 (Thu, Apr 28th), continued

Step 4b: Cells that fall outside of the gate range you set will be sorted into a separate tube (e.g. to be discarded later).



DAY 21 (Thu, Apr 28th), continued

Step 5: If you set your gate conditions correctly, your final sub-library population after FACS selection should consist of only those yeast cells expressing binders with the desired level of affinity for the SARS-CoV-2 target (e.g. binders with stronger affinity).



Future steps: This newly selected sub-library population can be used for additional rounds of sorting with FACS with even more stringent conditions to screen for only the very strongest binders. This can be coupled with random mutagenesis of the library plasmids to introduce genetic variation for directed evolution of binders with even tighter affinity to the target, as previously described on Day 20.

DAY 21 (Thu, Apr 28th), continued

Follow all instructions and answer all five questions below for your Day 21 Post-Lab.

1) List what were the protocol conditions you changed from the standard protocol for each of your three magnetic bead selections A – C from Part 5.3.

2) You plan to do three rounds of FACS selection when screening your surface display sub-library for binders to the SARS-CoV-2 target protein. State one way you can modify your overall FACS screening protocol set-up to increase your selection stringency each round (i.e. a protocol modification that will screen for stronger binders with each successive round).

3) The FACS dot plots of your results after each of the three rounds of screening are posted on Canvas. For each of the three dot plots:

a. Draw lines to indicate the quadrants on each FACS plot.

b. Draw lines to indicate the area where you will gate and collect the cells on each FACS plot.

c. Insert the image of all three FACS plot (labeled with the quadrants and gate) into your Day 21 Post-Lab on LabArchives.

4) Explain in general why you chose that region on the FACS plot for drawing your gate.

5) Design a possible control FACS experiment you could perform to confirm that you are selecting for yeast cells expressing binders specific for the SARS-CoV-2 target protein (and not for any other unwanted non-specific target). Indicate the expected result or outcome of your control experiment if your binders are in fact specific for the desired viral target protein.

DAY 22 (Tue, May 3rd)

5. ChemE: Yeast surface display & protein engineering

Part 5.5: Analysis of magnetic bead sorting optimization results

On Day 21, you performed three different magnetic bead selection protocols with various modifications to try to optimize the sensitivity and specificity of the bead sorting protocol. You plated serial dilutions of your final bead selections on YPD and SC-Trp plates. By counting the colonies that grew on these plates, you will be able to determine the total number of cells present in each sample after the bead sorting process. Because you plated your bead selections on both YPD and SC-Trp plates, you will also be able to distinguish between EBY100 non-binder and L7.5.1 binder colonies and thus determine how many of the cells present in the final bead selection are L7.5.1 binder cells (*how will you differentiate between EBY100 and L7.5.1 colonies?*). Altogether, this information will allow you to calculate sensitivity, specificity, and accuracy for each bead selection and evaluate how well you were able to optimize the bead selection protocol.

Obtain your six bead selection plates (three YPD and three SC-Trp plates). Count and record the total number of colonies on each quadrant of all six plates. Some quadrants may contain too many colonies and cannot be accurately counted (e.g. there is a lawn of cells) – if this is the case, you do not have to count the colonies for that quadrant and can simply record that there were too many colonies to count for that quadrant.

Follow all instructions and answer all six questions below for your Day 22 Post-Lab. (Note that there will be no regrades for the Day 22 Post-Lab.)

1) Record your colony counts for each quadrant on all six plates. Remember that zero colonies is still a result!

2) Calculate how many EBY100 yeast cells and how many L7.5.1 yeast cells were in each of your final bead selections (A, B, and C). For each plate, use the quadrant counts that will likely give you the most accurate data (*how will you determine this?*). Remember to take into account any serial dilutions.

3) For each of your bead selections A, B, and C, calculate the sensitivity, specificity, and accuracy. Accuracy is a quantitative measure of a selective procedure that takes into account both the sensitivity and specificity of that procedure.

$$\text{Accuracy} = \frac{\text{Final \# of True Positives + Final \# of True Negatives}}{\text{Total number of starting binders and non-binders}}$$

DAY 22 (Tue, May 3rd)

4) How did each of your selections perform relative to what you were expecting based on the protocol modifications you made? Did your overall observed results match what you were expecting, or did some bead selections perform better or worse than predicted? What are some possible explanations why?

5) What might you conclude about the particular protocol parameter(s) you modified in terms of how they affect the overall success of the magnetic bead selection procedure?

6) Which of your three bead selections (A, B, or C) do you think performed the best overall? Explain your reasoning.

DAY 23 (Thu, May 5th)

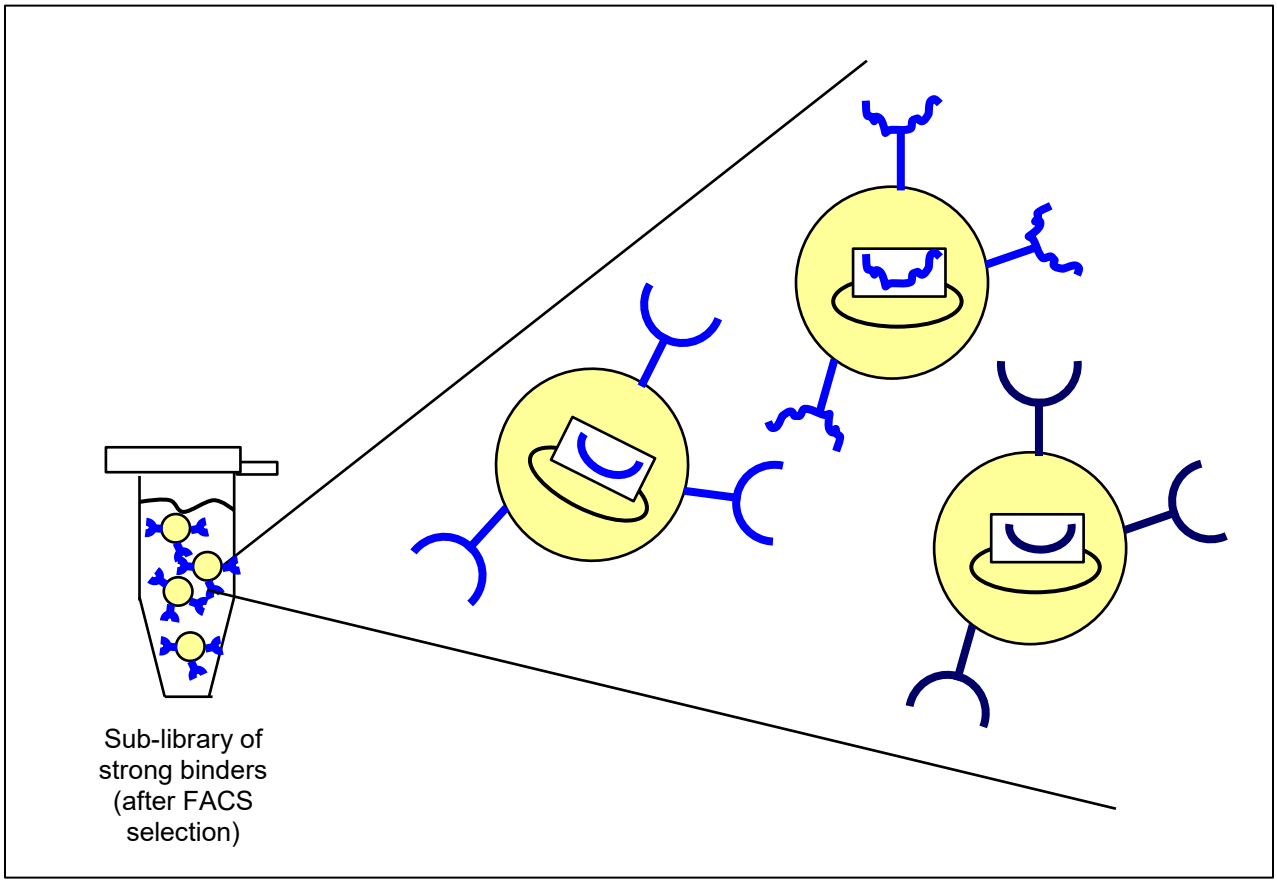
5. ChemE: Yeast surface display & protein engineering

Part 5.6: Clonal analysis of potential target binders

After the FACS selection on Day 21, you have your final sub-library population of cells, with each cell presumably expressing a binder variant on their surface that has a strong affinity to the SARS-CoV-2 target protein. For Day 23, you now need to isolate the plasmid DNA from each yeast cell so that you can sequence the gene on the plasmid encoding the binder variant (and thus learn the amino acid sequence of the binder variant as well).

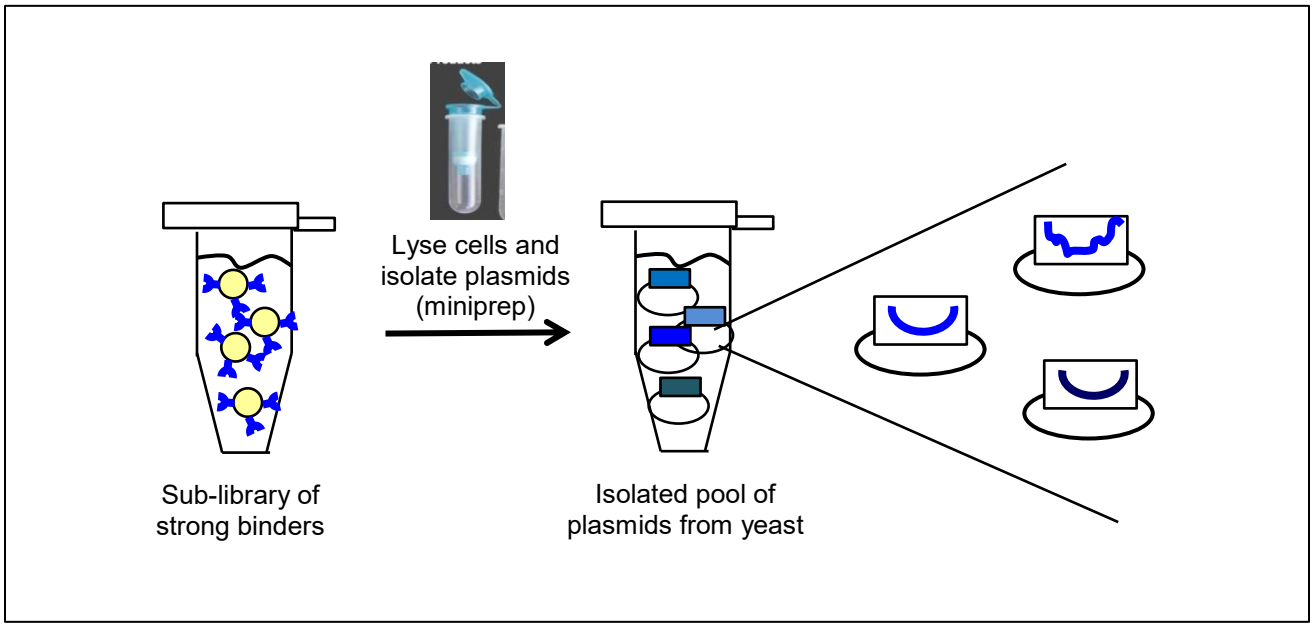
Day 23 Part 5.6 Overall Experimental Workflow

Step 1: Start with your sub-library of potential strong binders to the SARS-CoV-2 target protein (obtained from FACS selection from Day 21). Each yeast cell contains a plasmid with the gene encoding the binder variant on the cell surface.

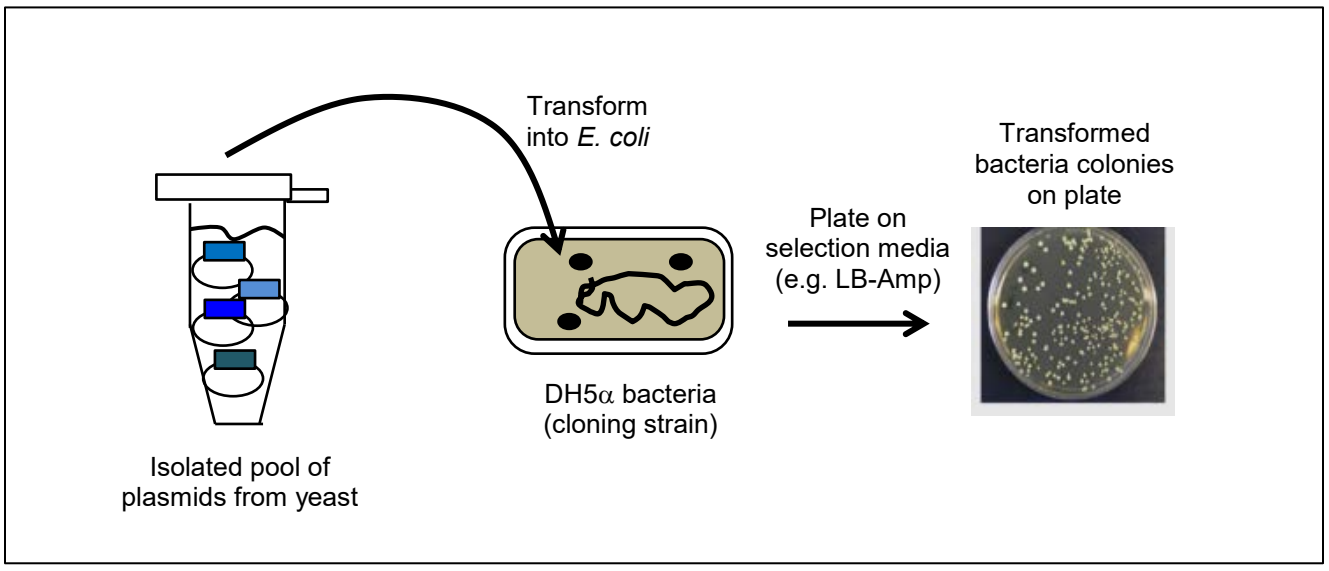


DAY 23 (Thu, May 5th), continued

Step 2: Isolate the plasmids from the pool of yeast cells. This process is similar to the bacteria miniprep procedure for isolating plasmids from bacteria (although the miniprep plasmid isolation is less efficient in yeast and results in a much lower overall plasmid yield as compared to a bacteria miniprep).

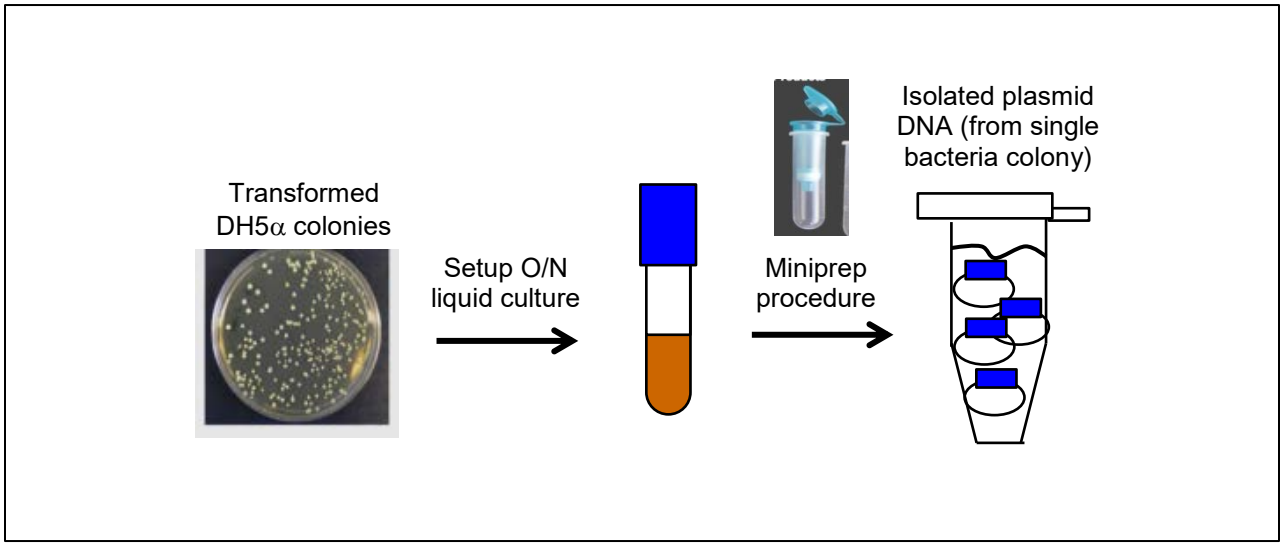


Step 3: To amplify and separate out individual plasmids, the yeast plasmid miniprep sample is transformed into *E. coli* bacteria and plated on selective media (e.g. LB-Amp plates) to select for successfully transformed colonies.

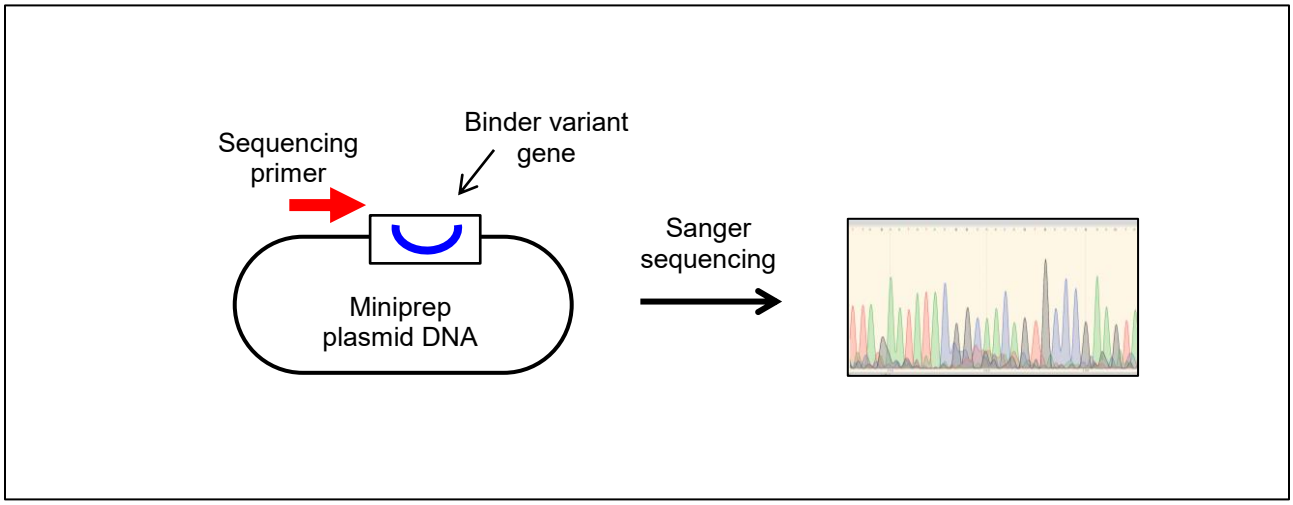


DAY 23 (Thu, May 5th), continued

Step 4: Perform a bacteria miniprep to isolate the plasmid from individual transformed colonies.

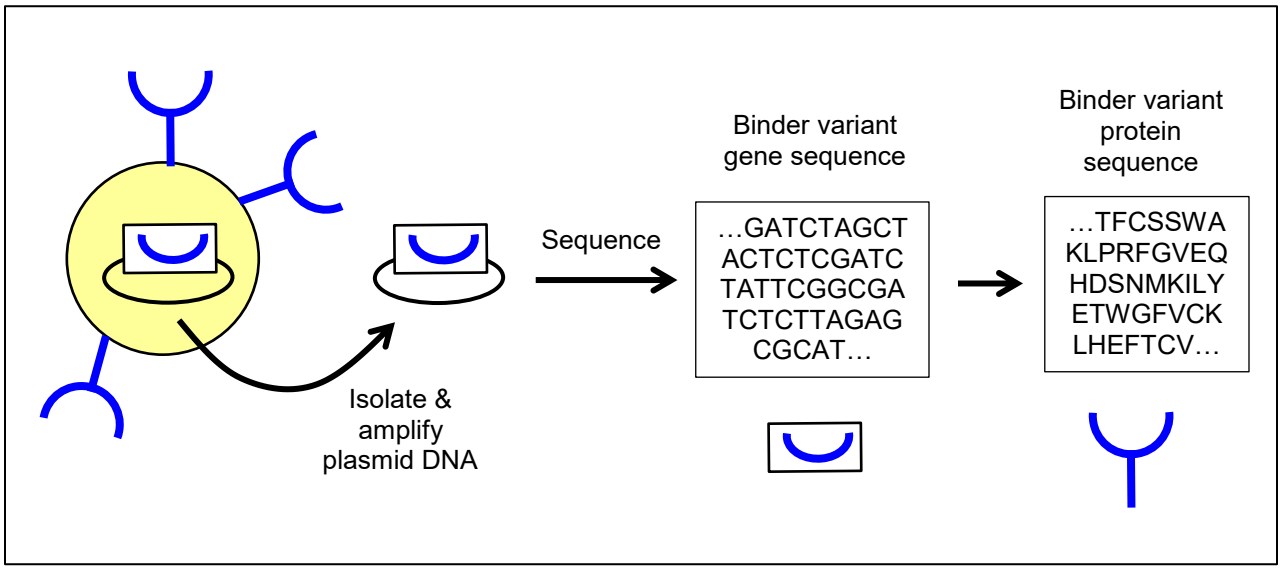


Step 5: Send miniprep plasmid samples for Sanger sequencing to determine the sequence of the binder variant gene. Your sequencing primer would anneal to a region of the plasmid backbone near binder variant gene.



DAY 23 (Thu, May 5th), continued

Step 6: Analyze the sequence results to determine the amino acid sequence of the binder variant proteins.



Future steps: Once you have the gene sequence (and thus the corresponding amino acid sequence), you can analyze the variety of amino acid residues represented to learn more about the biochemical properties of the binding interface. The binder proteins themselves and their binding activity can be further characterized, e.g. to measure their K_d dissociation constant and binding affinity for the SARS-CoV-2 target. The binder gene sequence can also be cloned into expression plasmids so that the binder protein can be recombinantly expressed in large quantities for use in diagnostic tests or therapeutics.

Follow all instructions and answer all six questions below for your Day 23 Post-Lab. Note that the Day 23 Post-Lab is **due by 11:59 PM on Thursday, May 5th** (i.e. the same evening as Lab Day 23) and there are no regrades for the Day 23 Post-Lab. Students are encouraged to finish the Day 23 Post-Lab before leaving class today so you may check your answers with a TA or instructor.

You have sequenced the binder variant gene from seven different miniprep plasmids (i.e. independent clones each isolated from a separate transformed bacteria colony). The DNA sequence result for these seven binder genes (Binders 1 – 7) are in the "Day 24 In-Lab Questions (PDF)" file.

The DNA sequence of the “wild-type” (WT) binder gene is also in this file. This WT sequence encodes the original scaffold protein (which presumably would not have

DAY 23 (Thu, May 5th), continued

any natural binding affinity to the SARS-CoV-2 target on its own) that the surface display library is based on. To create the original library (e.g. for using in the initial magnetic bead sorting), random mutations were introduced into this scaffold protein to generate genetic diversity. The seven binder gene clones you isolated after your FACS screening are all derived from the randomly mutated variants of this original WT scaffold gene.

1) Translate the DNA sequence for each of the seven binder genes (Binders 1 – 7) and the original WT scaffold gene into their corresponding amino acid sequence. Perform a protein alignment for all eight proteins (Binders 1 – 7 and the WT scaffold) using either SnapGene or NCBI COBALT (refer back to the Day 19 Post-Lab Worksheet if you need instructions on how to perform the alignment). Take a screenshot of this protein alignment and insert the JPG of that alignment into your Day 23 Post-Lab on LabArchives.

2) How many different unique binder proteins are represented in the seven clones that you sequenced?

3) How many variable amino acid residues are there among the seven binders with respect to the WT scaffold?

4) Which region(s) of the scaffold protein most likely make up the binding interface between the binder protein and the SARS-CoV-2 target? Explain your reasoning.

5) Of the variable amino acid residues you counted from Question 3, how many of those are identical in all seven of the binders you sequenced? What might be the significance of these residues? Pick any one of these identical residues and suggest a hypothesis about why that particular residue was changed from the WT scaffold sequence in all seven binders isolated.

6) Of the variable amino acid residues you counted from Question 3, how many of those show variances within the seven binders you sequenced? What might be the significance of these residues? Pick any one of these residues and suggest a hypothesis about how that particular residue may be affecting the interaction of the binder protein to the SARS-CoV-2 target.

Appendix: Related Literature and References:

Bardwell, L. (2004). A walk-through of the yeast mating pheromone response pathway. *Peptides*. **25:** 1465 – 1476.
General review article of the yeast mating pathway.

Burns, N., Grimwade, B., Ross-Macdonald, P.B., Choi, E., Finberg, K., Roeder, G.S., and Snyder, M. (1994). Large-scale analysis of gene expression, protein localization, and gene disruption in *Saccharomyces cerevisiae*. *Genes Dev*. **8:** 1087 – 1105.
This reference describes the mTn3 transposon library and the overall transposon mutagenesis procedure used in 7.003.

Gietz, R.D. and Woods, R.A. (2002). Transformation of yeast by the Liac/SS carrier DNA/PEG method. In: Methods in Enzymology, vol. 350 (Christine Guthrie and Gerald R. Fink, ed.), Academic Press, San Diego, pp. 87 – 96.
This reference describes the yeast lithium acetate transformation procedure used in 7.003.

Philippsen, P., Stotz, A., and Scherf, C. 1991. DNA of Saccharomyces cerevisiae. In: Methods in Enzymology, vol. 194: 169 – 182.
This reference describes the yeast genomic DNA isolation procedure used in 7.003.

Ross-Macdonald, P.B., Sheehan, A., Friddle, C., Roeder, G.S., and Snyder, M. (1998). Transposon Tagging I: A novel system for monitoring protein production, function, and localization. In: Methods in Microbiology, vol. 26: Yeast Gene Analysis (Alistair Brown and Mick Tuite, ed.), Academic Press, San Diego, pp. 161 – 180.
This reference describes the pRSQ2 plasmid used in the plasmid recovery procedure.

Quintara Biosciences website: https://www.quintarabio.com

New England Biolabs (NEB) 2018 – 2019 Catalog & Technical References

E.Z.N.A. Cycle Pure Kit manual (Omega)

Qiagen RNeasy Mini Kit manual

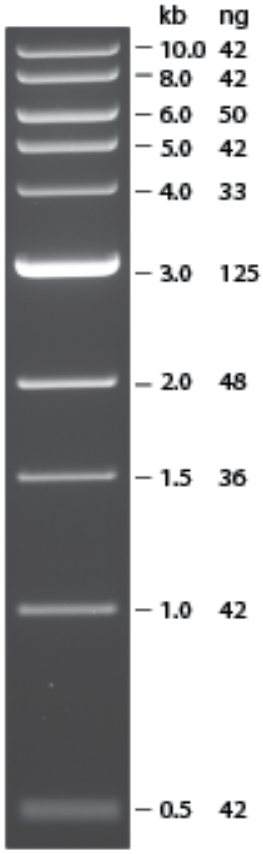
Replica-plating block instructions (Scienceware, Bel-Art Products)

Note: Special thanks to Peter Pryciak and Duane Jenness at UMass Medical School Worcester for the generous use of their yeast strains, plasmids, and advice for this module.

We also thank New England Biolabs (NEB) for their generous donation of restriction enzymes, DNA ligase, DNA Ladder, and Phusion PCR reagents for use in this module.

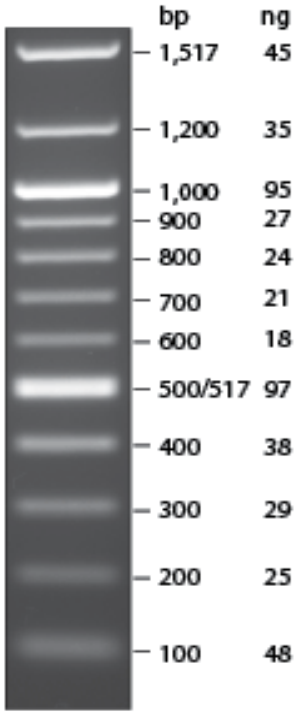
Appendix: DNA Ladders and Yeast Strain Genotypes

1-kb DNA Ladder (NEB)



1 kb DNA Ladder
0.8% TAE agarose gel.
Mass values are for
0.5 µg/lane.

100-bp DNA Ladder (NEB)



100 bp DNA Ladder
1.3% TAE agarose gel.
Mass values are for
0.5 µg/lane.

Yeast Strain Genotypes:

PPY295: *MAT***a** *cry1 his4 leu2 lys2 trp1 tyr1 ura3 cyh2 SUP4-3 bar1-1*

PPY144: *MAT*α *ade1 arg4 aro2 his7 lys5 met4 ura2*

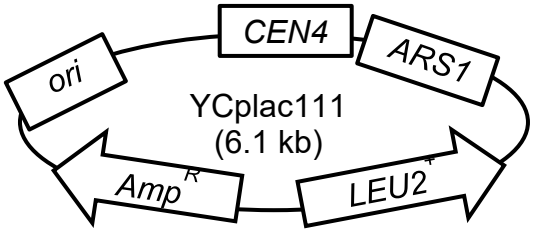
PPY917: *MAT***a**/*MAT*α *cry1/cry1 ade2/ade2 ade3/ade3 his4/his4 leu2/leu2 lys2/lys2 trp1/trp1 ura3/ura3 SUP4-3/SUP4-3*

ste2Δ: *MAT***a** *cry1 ade2 his4 leu2 lys2 trp1 tyr1 ura3 SUP4-3 ste2*Δ*::LEU2*

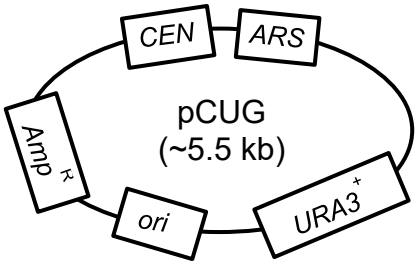
ste11Δ: *MAT***a** *cry1 ade2 ade3 his4 leu2 lys2 trp1 ura3 SUP4-3 ste11*Δ*::hisG*

Appendix: DNA Plasmid maps

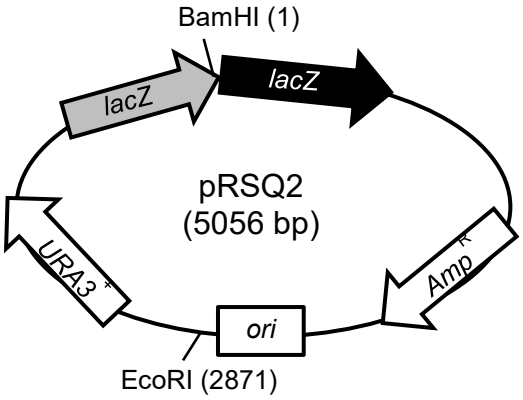
YCplac111 plasmid map



pCUG plasmid map



pRSQ2 plasmid map



(Note: What is the purpose of each DNA element present on each of the plasmids above?)

Appendix: Eppendorf BioSpectrometer Instructions

Measuring DNA concentration:

***** You will be using special (read: expensive!) microcuvettes to measure your DNA. Please use them under the supervision of your TA until you are completely familiar with their use. When you are done, wipe them with a Kimwipe and return them to your TA. *****

1) Turn the power on (the switch is in the back left corner of the spec). Wait for the "SELF-TEST" to complete. Press "OK" to go to the Main Menu.

2) On the Main Menu, press the right arrow once to highlight "Favorites." Press the right and down arrows to highlight "dsDNA 1 mm" in the Methods column. ("ds" stands for double stranded DNA, and 1 mm is the path length for the special microcuvette we will be using to measure DNA).

3) Press the white oval "enter" button – this will take you to the "dsDNA 1 mm" parameters page. Press "Next" to proceed (note: do NOT change any parameters!).

4) Now you are ready to measure your blank solution. Take a microcuvette out of its metal box and lift one glass arm to expose the two black circular wells to hold your sample. Pipet 2 μl blank solution into one of the wells and close the arm by snapping gently. Look from the side to make sure that you have indeed generated a column between the two pieces of glass. (If the sample does not form a continuous column between the two wells, lift the arm and snap it closed again carefully.)

5) Lift the blue sample compartment door on the spec and insert the cuvette with your blank into the sample holder. Make sure the cuvette is in the correct orientation in terms of light path! Also, make sure to use the microcuvette each time in the same orientation as you did with your blank (e.g. the side which says Eppendorf facing you or not).

6) Close the sample compartment door. Press the white round "blank" button to zero (or blank) the spec. The display panel should give a concentration reading of 0.

7) Open the sample compartment door and remove the cuvette with your blank. Lift the cuvette arm and use a Kimwipe to carefully wipe off the blank solution from the cuvette.

8) Load 2 μl of your DNA sample into the microcuvette as previously described. Insert the cuvette with your sample and close the compartment door. Press the blue round "sample" button and the spec will display the DNA concentration and the A_{260}/A_{280} ratio for your sample. Repeat for any additional samples you have. Always make sure to wipe off the microcuvette with a clean Kimwipe before/after measuring each sample.

9) At any point, you can press the blue oval "exit" button to return to the Main Menu.

Appendix: Eppendorf BioSpectrometer Instructions, cont.

Measuring a single wavelength:

1) Turn the power on (the switch is in the back left corner of the spec).

2) Wait for the "SELF-TEST" to complete. Press "OK" to go to the Main Menu.

3) On the Main Menu, press the down arrow once to highlight "Absorbance." Press the right arrow twice to highlight "Single λ" in the Methods column.

4) Press the white oval "enter" button – this will take you to the "Single λ" parameters page.

5) If needed, press "Edit" to change the wavelength. Leave the "Cuvette" setting at 10mm (or 1 cm, the standard pathlength of the cuvettes used in most labs). Use the number keypad to change the "Wavelength" setting to your desired wavelength (e.g. 280 nm or 600 nm, etc). Press "Save" to save any changes you made.

6) Once the wavelength setting is correct, press "Next>" to proceed.

7) Lift the blue sample compartment door and insert the cuvette with your blank into the sample holder. Make sure the cuvette is in the correct orientation such that the light path passes directly through the sample!

8) Close the sample compartment door. Press the white round "blank" button to zero (or blank) the spec. The display panel should give an absorbance reading of 0.

9) Open the sample compartment door and remove the cuvette with your blank.

10) Insert the cuvette with your sample and close the compartment door. Press the blue round "sample" button and the spec will display the measured absorbance for your sample. Repeat for any additional samples you have.

11) At any point, you can press the blue oval "exit" button to return to the Main Menu.

Appendix: Guidelines for writing lab notebook entries

7.003 Pre-Lab Notebook Entry Guidelines

(The Pre-Lab is due by 1:00 PM the day of that lab)

Aims:

- What you will be doing
- How you will be doing it
- Why you are doing it

Note: If your entire daily Aims is longer than ~5 sentences, you're probably including more detail than you need!

7.003 Post-Lab Notebook Entry Guidelines

(The Post-Lab is due by 1:00 PM the following lab day)

Data & Observations:

- Indicate if any major deviations from the protocol were made.

- Indicate any unique strains/reagents/etc used by your group that are not specified in the lab manual.

- Include any results/raw data/observations/counts/calculations/etc (including those asked for in the lab manual).
    - Gels: Include the gel photo and label all lanes and all ladder band sizes.

    - Bacteria/yeast plates: Include all colony counts and/or observations for all plates (note: "Zero colonies" is still a result and should be recorded!). If applicable, include any plate photos, properly labeled.

- All images and annotations should be legible.
    - For best results, import your original image into PowerPoint or similar program to label your images. Save the slide as a .png file. You can then drag and drop the .png file into your LabArchives notebook entry.

Summary & Conclusions:

- Expected vs observed results:
    - Indicate the expected (or "ideal") specific results for each sample, including both experimental samples and control samples (note: you only need to include this on the day when you obtain the actual results for your samples, not on the day when you just set up the samples).

Appendix: Guidelines for writing lab notebook entries, cont.

- Briefly discuss if your observed group results were consistent with or different from expected results for each sample. What is the significance if your observed results either matched or didn't match the expected results for each sample?

- If there were any differences between your observed results and the expected results, suggest a potential reason for the difference and briefly comment on any implications.

- Additional tips for discussing gel results and bacteria/yeast plate results:

    - Comment on <u>each</u> lane of the gel and on <u>each</u> plate!

    - For gels, band size is just as important as whether or not you see band on a gel. What are the approximate sizes of all the bands you observe on your gel? What does each different-sized band represent? Do they match the sizes you would expect to see for each lane?

- To save space/time, if multiple different samples have an overall similar purpose/result, you may discuss the samples together as a group (e.g. if Sample A and B had similar purposes and results, you can talk about Samples A and B together, instead of discussing first Sample A separately, and then Sample B separately).

- If applicable, briefly discuss the overall significance of any results/data and any conclusions that can be made based on your experiments. (Hint: Think back to the "Why are you doing it" part of your Aims section – what was the purpose of doing the experiment in the first place? Was your experiment successful in fulfilling that purpose? What did you learn from your experimental results in connection to your original experimental Aims?) Be sure to include answers to any questions explicitly asked for in the lab manual. (Note: Even if your overall experiment didn't "work" properly, you should still try to make what conclusions you can, even if you may not be 100% sure – just be sure to include any qualifications.)

Note: While it is perfectly acceptable to use phrases like "expected results" or "my experiment worked/was successful, etc" in your lab notebook entries (which are a more informal scientific record of your experiments), please keep in mind that such subjective terminology should not be used when writing a formal scientific manuscript (e.g. the SciComm paper you will be writing).

7.003 Applied Molecular Biology Lab

Spring 2022