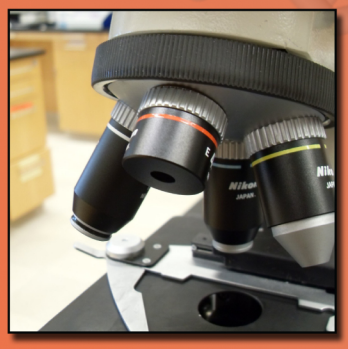
Investigations in Molecular Cell Biology

Author: Clare O'Connor

2012

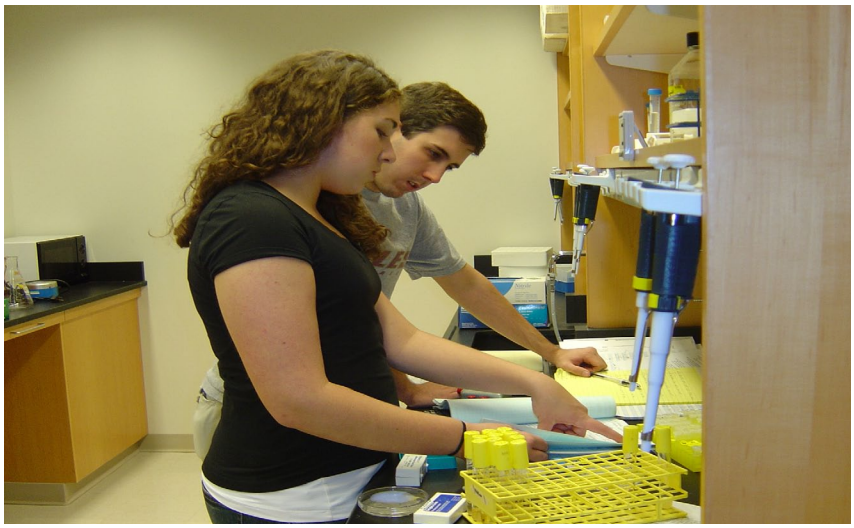




Investigations in Molecular Cell Biology

Boston College | Clare O'Connor
2012-2013

Contents

Chapter 1
Introduction



We live in the "post-genomic" era, in which the availability of complete genome sequences from a host of organisms offers exciting opportunities for undergraduate research. In BI204, we will use some of the strain and clone resources generated by the yeast genome project to investigate the evolution of genes involved in the synthesis of methionine, an essential amino acid for all living organisms.

BI204 - Investigations in Molecular Cell Biology

- Course goals
- Our research project
- Course overview
- Acknowledgments

Chapter 1

Welcome to BI204 - Investigations in Molecular Cell Biology. BI204 is a new kind of introductory lab course, which has been designed to incorporate an authentic research project. It has been said that we live in a "post-genomic" era. Large-scale genome projects have generated tremendous amounts of sequence data, and complete genome sequences are available for many different organisms. Many of the "genes" annotated by the genome projects were identified by their similarity to known genes in other organisms, but their functions have not been tested directly. These loose ends provide exciting opportunities for undergraduate students to participate in authentic functional genomics research. This course is designed as a research project in which students study the evolution of the genes involved in methionine biosynthesis. We hope that you enjoy the research experience and we look forward to your experimental results!

Course goals

Biology education at the undergraduate level is undergoing a transformation. For decades, many have viewed biology as an encyclopedic subject, because of the vast amount of content matter included in the undergraduate curriculum. A recent reevaluation of undergraduate biology education, however, is guiding biology curricula in a new direction, stressing the importance of involving students in the process of scientific investigation throughout their coursework. The reevaluation process has also challenged educators to sort through the vast amount of content in introductory biology to identify the core concepts that students should learn and the key competencies that students should acquire during their undergraduate education. This course has been designed in line with the findings of that report, "Vision and Change in Undergraduate Biology Education" (Bauerle et al., 2011).

The BI204 research project focuses on the evolutionary conservation of the genes involved in the synthesis of methionine, an essential amino acid in all organisms. The experiments in the project explore the core concepts of biology:

- Evolution - the genes coding for methionine synthesis show varied patterns of conservation during evolution.
- Structure and function - cells employ a variety of strategies to synthesize methionine.
- Information transfer - methionine synthesis requires enzymes encoded by multiple genes.
- Pathways and energy transformation - the enzymes involved in methionine synthesis constitute an energy-consuming metabolic pathway.
- Systems biology - the reactions involved in methionine synthesis intersect with many other metabolic pathways in cells.

The course is also designed to teach students research skills and to give students many opportunities to practice them throughout the semester. Students will learn experimental design, collect and analyze data, and test hypotheses. In the course of the project, students will learn the specific skills of molecular cell biology and how to use basic laboratory equipment. Students will also learn to access information in biological databases, to read the primary scientific literature, and to communicate scientific results in both oral and written presentations.

Pathways over time – our research project

During the semester, we will be investigating the evolution of the genes involved in the synthesis of methionine, an essential sulfur-containing amino acid in proteins. Most of our experiments will involve the budding yeast, *Saccharomyces cerevisiae*. We will take advantage of a large collection of *S. cerevisiae* strains with defined defects in methionine biosynthesis, which were generated as part of the yeast genome project. We will use these mutant strains as hosts for methionine genes cloned from other organisms. If the foreign gene restores the ability to synthesize methionine to its *S. cerevisiae* host, in a process known as functional complementation, we will know that gene function has been conserved over the evolutionary time frame that separates the two species.

During the 2012-2013 academic year, we will explore the conservation of methionine biosynthetic enzymes between *S. cerevisiae* and the fission yeast, *Schizosaccharomyces pombe*. Both *S. pombe* and *S. cerevisiae* are ascus-forming yeast from the phylum Ascomycota. The two species are thought to be separated by close to a billion years of evolution. Since the two species diverged from their common ancestor, the *S. cerevisiae* lineage has undergone whole genome duplications and reductions. By contrast, *S. pombe* is considered to be much closer to ancestral members of the phylum. Our results during this coming year will establish which genes involved in methionine synthesis have been functionally conserved between the two yeast species. Results from this year will also guide the future direction of the project into other branches of life. Over time, we hope that student results will allow us to construct evolutionary trees for many of the genes involved in methionine synthesis.

Course overview

The course can be viewed as a series of related modules.

- In the first experiments, student teams will receive three *S. cerevisiae* mutants, each of which is deficient in one of the genes involved in methionine synthesis. Students will use selective growth media and PCR to identify the three strains. Teams will bring forward one of the three strains for the remaining experiments of the semester.
- Teams will receive three plasmids to identify. One plasmid contains the *S. cerevisiae MET* gene that is deficient in their yeast strain. The second plasmid carries a different gene involved in methionine synthesis. The third plasmid carries the *S. pombe* homolog for the *S. cerevisiae MET* gene that is missing in their yeast strain. Students will learn how to isolate plasmids and characterize them by restriction mapping.
- Teams will transform their yeast strains with the three plasmids. Selective plates will be used to determine where complementation has occurred.
- Teams will study expression of the plasmid-genes on western blots.
- In the last segment of the semester, teams will design and conduct their own experiments, based on questions that have arisen during the previous experiments.

Chapter 1

Acknowledgments

It has been a pleasure to work with many Boston College colleagues and students on this manual. In particular, Dr. Michael Piatelli and Meghan Rice helped to design the experiments in this manual and offered many comments on its contents. Thanks are also due to the many students and teaching assistants who participated in the pilot semesters of BI204. The teaching assistants provided able leadership for their sections and gave valuable feedback about the effectiveness of the manual and suggestions for its improvement. Thanks (in no particular order) to Kim Regna, Kaila Deiorio-Haggar, David Layman, Kate Halm, Sudeshna Saha, Shannon Heyse, Marisha Collins, Evan Senter, Josh Walker, Michelle Archibald, Rashmi Dubey, Brian Nowlin, Patrick Grady, Lauren Meyer, Zeynep Onder and Rebecca Dunn.

Several undergraduate students deserve special mention. David Chou and Kathy Samley spent many hours designing and laying out the manual. Michael Lim and Jim Han read draft versions of the manual and provided student perspectives on the material.

Finally, I would like to acknowledge the support from the National Science Foundation for the Pathways over Time project.

References

Bauerle, C. et al. 2011. Vision and Change in Undergraduate Biology Education: A Call to Action. (National Science Foundation/American Association for the Advancement of Science, Washington, D.C.)

Chapter 2

Mastering the micropipette

Welcome to the microworld! In this class, you will be working with microorganisms, including yeast and bacteria, which are much too small to be detected with the naked eye. Indeed, millions would fit into a period on this page. You will also be working with costly reagents, such as plasmids and enzymes. Therefore, in every experiment, you will be required to accurately measure volumes as small as a few microliters (μL). Micropipettes will allow you to do this accurately and precisely.

Objectives

- Learn to select and adjust micropipettes
- Learn to accurately transfer microliter volumes
- Use the spectrophotometer to measure absorbance
- Understand experimental errors in measurements

Chapter 2

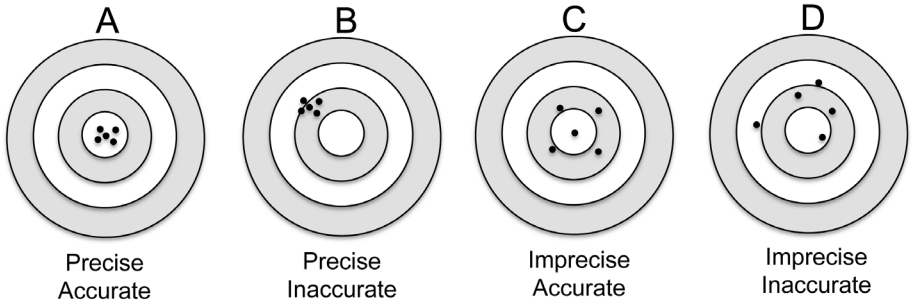
Introduction

Arguably, the most important scientific equipment that you will use this semester are adjustable micropipettes. Micropipettes are precision instruments that are designed to accurately transfer volumes in the microliter range. You may use microliters or milliliters as the units of volume in your lab notebooks and lab reports, but be careful to always state the volume unit that you're using. Recall the relationships between volume units:

1 microliter (abbreviated μL) = 10^{-3} milliliter (mL) or 10^{-6} liter (L)
(A useful tip for Mac users: The keyboard shortcut for the Greek letter μ is Alt-m)

Accuracy and precision

Ideally, micropipettes will deliver liquids with accuracy and precision. Accuracy depends on the micropipette delivering the correct volume. Precise results are reproducible. Let's use a target analogy to demonstrate the difference between accurate and precise results. Imagine that four students try to hit the bulls-eye five times. Students A and B are precise, while students A and C are accurate.



The best way to determine the accuracy and precision of micropipettes is to use them to weigh set volumes of distilled water on an analytical balance. The density of water is 1.0 gram per mL at 25˚C. The process is repeated several times during the calibration process, and the data is used to calculate the accuracy and precision of a micropipette.

Accuracy refers to the performance of the micropipette relative to a standard (the intended) value. It is computed from the difference between the actual volume dispensed by the micropipette and the selected (intended) volume. Note that this can be a negative or positive value. When micropipettes are calibrated, the accuracy is normally expressed as a percent of the selected value. In general, micropipettes are designed to operate with accuracies within a few percent (generally <3%) of the intended value. The accuracy of a micropipette decreases somewhat, however, when micropipettes are set to deliver volumes close to the lowest values in their range.

Precision provides information about reproducibility, without any reference to a standard. Precision reflects random errors that can never be entirely eliminated from a procedure. Precision is expressed as the standard deviation of the measurements. Like accuracy, precision is often expressed as a percent of the selected volume in calibration documents.

Choosing the micropipette

There are three different sizes of micropipettes in the laboratory, which we will refer to as the P20, P200 and P1000. Our micropipettes have been purchased from several different manufacturers, but the principles of operation are the same. The numbers after the "P" refer to the maximum number of microliters that the micropipette is designed to transfer.

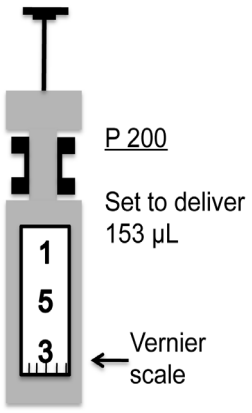
Use the chart below to select the correct micropipette for an operation. Note that there is some overlap in the ranges of the different micropipettes. For example, both the p200 and p20 can be used to transfer 15 µl, but the p20 is more accurate within that range. As a rule of thumb, always select the smallest volume pipette that will transfer the volume, since the measurement will be more precise.

Micropipette	Recommended range (µL)	Smallest increment (µL)
P20	1 - 20	0.02
P200	20 - 200	0.2
P1000	100 - 1000	2.0

Micropipettes use disposable plastic tips. The P1000 tips are larger than those used with P200s and P20s. P1000 tips may be either white or blue, depending on the vendor, while P20 and P200 tips may be either yellow or natural in color.

Specifying the transfer volume

There are three numbers on the volume indicator. With each of the micropipettes, you will specify a volume to three digits by turning the volume adjustment knob. You will also be able to extrapolate between the lowest numbers with the vernier marks on the lower dial. Most of the measurements you will make with the micropipettes will be accurate to four significant figures!

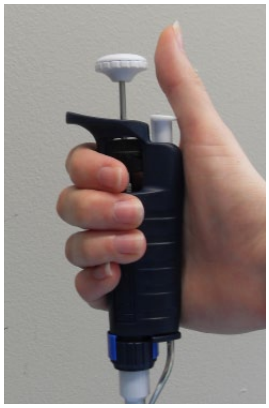


NEVER turn the indicator dial beyond the upper or lower volume limits of the micropipette! This could damage the piston.

Chapter 2

Transferring volumes accurately

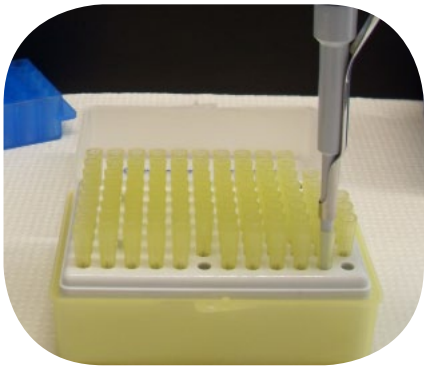
Micropipettes work by air displacement. The operator depresses a plunger that moves an internal piston to one of two different positions. The first stop is used to fill the micropipette tip, and the second stop is used to dispense the contents of the tip. As the operator depresses the plunger to the first stop, an internal piston displaces a volume of air equal to the volume shown on the volume indicator dial. The second stop is used only to dispense the contents of the tip.


Start


First stop


Second stop

Filling the micropipette



- Remove the lid from the box containing the correct micropipette tips.
- Attach the tip by inserting the shaft of the micropipette into the tip and pressing down firmly (figure on right). This should produce an airtight seal between the tip and the shaft of the micropipette.
- Replace the lid of the tip box to keep the remaining tips sterile. ***Avoid touching the tip (especially the business end), because the tips are sterile.***
- Depress the plunger of the micropipette to the FIRST stop.
- Immerse the tip a few millimeters below the surface of the solution being drawn up into the pipette. Pipetting is most accurate when the pipette is held vertically. Keep the angle less than 20˚ from vertical for best results.
- Release the plunger ***S L O W L Y***, allowing the tip to fill smoothly. Pause briefly to ensure that the full volume of sample has entered the tip. Do NOT let the plunger snap up. This is particularly important when transferring larger volumes, because a splash could contaminate the shaft of the micropipette. If you inadvertently contaminate the shaft, clean it immediately with a damp Kimwipe.

NEVER rest a micropipette with fluid in its tip on the bench!

Dispensing the contents of the micropipette

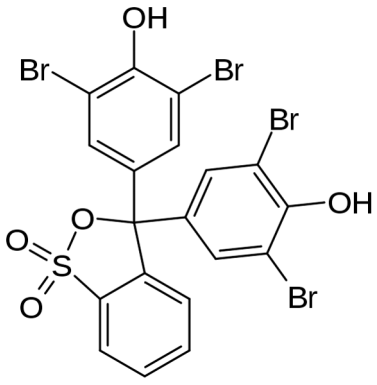
- Place the micropipette tip against the side of the receiving test tube. Surface tension will help to dispense the contents of the micropipette. Do NOT attempt to eject the contents of the micropipette into "thin air."
- Smoothly depress the plunger to the first stop. Pause, then depress the plunger to the second stop. The contents of the pipette should have been largely released at the first stop. The second stop ensures that you've released the "last drop."
- Use the tip ejector to discard the tip.

WARNING: The most common - and serious - operator error is depressing the plunger to the second stop before filling the micropipette tip. DON'T DO THIS!!!

Lab Exercises

Since you will be using micropipettes for all of your experiments, the quality of your results will depend on proper operation of the micropipette. Today's laboratory will lead you through some exercises that will show you how to use micropipettes correctly and point out some common pitfalls associated with their use. Your results will also provide information about whether the pipettes are functioning properly.

In these exercises, we'll be using the spectrophotometer to determine if your pipetting is accurate and precise. You will be using micropipettes to combine various volumes of water and solutions of a blue dye, bromophenol blue (BPB). You will measure the absorbance of the resulting solutions at 590 nm (A_{590}), which is close to the absorbance maximum of bromophenol blue at neutral pH. Measuring errors will be reflected in the spectrophotometer readings.



Consult the appendix on laboratory equipement for instructions on using the GeneSys 20 spectrophotometer.

Chapter 2

Good laboratory notebooks are essential to success in the laboratory.

Carefully record your results in your lab notebook as you perform these exercises. Since this is the first experiment of the semester, you will be given a template that you can use to record your results. In the future, you'll be expected to prepare your lab notebook in advance of the experiment. Some guidelines:

- Include a brief outline of the procedures that you will be doing in the lab - do NOT copy this manual!
- Modify your notebook as you work in the lab. You may want to check off steps as you complete them. Cross out entries that no longer apply. Add dates and times as needed.
- Drawings and flow diagrams are recommended, since they provide clarity.
- Lab notebooks are not always neat, but they need to be accurate!

As a rule of thumb, another scientist should be able to reproduce your experiment from your notes!

Exercise 1 - Getting the feel of micropipettes

Concept: Micropipettes work by an air displacement mechanism

1. Set the P200 to deliver 200 µL. Be careful not to overshoot, which could damage the pipette piston.
2. Grip the pipette by wrapping your fingers around the barrel.
3. Use your thumb to depress the plunger to its first stop.
4. Next press the plunger to the second stop.
   Compare the distance that the plunger moved during the first and second strokes.
5. Set the P200 to deliver 20 µL and depress the plunger to its first stop.
   Compare the distance that the plunger moved when the P200 was set to 200 or 20 µL.
6. Depress the plunger to the second stop.
   How does the distance between the first and second stops compare for 200 and 20 µL?
7. Set the P20 to deliver 20 µL. Depress the plunger to the first stop.
   Compare the distance to the first stop when a P20 and P200 are set to deliver 20 µL.

Concept: The filling and dispensing strokes are different.

1. Place a tip on the shaft of the P200.
2. Set the P200 to deliver 50 µL.
3. Draw up 50 µL of 0.05% BPB solution into the pipet.
4. Dispense the BPB into a microcentrifuge tube down to the first stop, holding the tip against the wall of the tube. Note whether all of the dye has been expelled. Push the plunger down to the second stop to release any remaining BPB.

Exercise 2 - How NOT to pipette!

In this exercise, you'll determine the error that results from deliberate incorrect pipetting. (Hopefully, this is the ONLY time that you ever do this!)

1. Use the P1000 to add 990 µL of water to two microcentrifuge tubes. Label the tubes A and B. Dispose of used tips in the containers provided.
2. Use a P20 to correctly transfer 10 µL of 0.05% BPB to tube A. Make a mental note of what fraction of the pipet tip is filled with the dye. Use the vortex mixer to disperse the BPB in the water.
3. Use a P20 to ***INCORRECTLY*** transfer 10 µL of 0.05% BPB to tube B. Do this by depressing the plunger to the second stop before you take up the BPB solution. Make a mental note of how well the dye fills the tip this time.
4. Set the wavelength of the spectrophotometer to 590 (A_{590}). Pipette 1 mL of water into a plastic cuvette and blank the spectrophotometer at this wavelength.
5. Read the A_{590} of the solutions in tubes A and B, in the spectrophotometer.
   How do the two readings compare?
   What kind of error results from drawing solution into the pipette incorrectly?

Exercise 3 - How precise are your transfers?

The standard method used to determine the accuracy of a micropipette is to weigh a specified volume of distilled water on an analytical balance. Unfortunately, we don't have enough analytical balances available for this, so we will transfer specified volumes of BPB to deioinized water, and we'll then measure the A590 of the solution. (We will assume that there is no error in the measurement of the water!)

Work in groups of three. One person in the group should work with the P-20, another with the P-200 and the third with the P-1000. Each person should prepare three identical samples and then determine the A_{590} of the three samples. From the data, you will be able to determine if the micropipette is measuring volumes correctly.

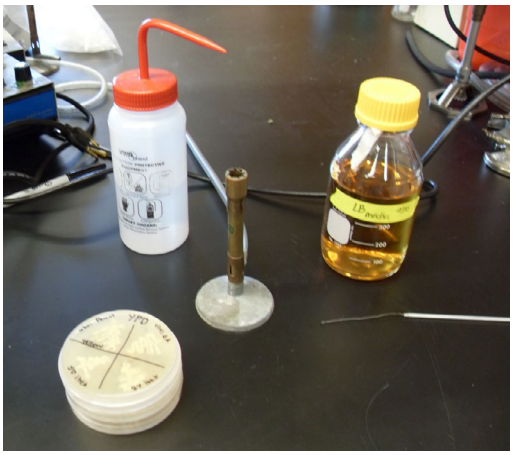
1. Set up three microcentrifuge tubes and label them appropriately. The final volume (water + BPB) in each tube will be 1.0 mL. Calculate the volume of water that will need to be combined with each of the following to give 1.0 mL, and record your calculations in your lab notebook:
   P-20: 10 μL of 0.05% BPB
   P-200: 100 μL of 0.005% BPB
   P-1000: 300 μL of 0.005% BPB
2. To minimize our plastic waste, strategize how to minimize the number of tips that you use without contaminating the stock solutions. A tip can be used multiple times, but a tip that has been used for BPB cannot be used to subsequently transfer deionized water. Combine the BPB solution and water to give a final volume of 1.0 mL.
3. Measure the A_{590} of the three solutions and record the data in your notebook.
4. Compute the mean and standard deviations for your three measurements, using either a calculator or Excel.
5. Enter these values on the chart that your TA has prepared on the whiteboard. Compare the individual values that you obtained for the three pipettes with the combined class measurements for each tube.

What conclusions can you make about the behavior of your micropipettes?

Notify your TA if any of the micropipettes are not performing properly. Your TA will follow up on your observations and test the micropipettes with the gravimetric test described at the beginning of this exercise.

Chapter 3

Working with Yeast



This lab will introduce you to standard techniques used in microbiology. Very similar techniques are used to culture yeast and bacteria, although the culture conditions are optimized for each organism. In this lab, you will learn sterile techniques required for maintaining the integrity of strains in the lab, as well as methods for culturing cells and estimating cell numbers.

Objectives

- Understand the phases of microbial growth
- Learn and practice sterile techniques used to culture yeast and other microorganisms
- Learn how to use spectrophotometry and spot plating to estimate the number of cells in yeast cultures

Chapter 3

Throughout the semester, you will be working with cultures of yeast. Methods for culturing yeast in the laboratory are well-developed. The techniques used for yeast and bacteria are similar, except that the media composition and culture temperature are optimized for individual organisms. In general, culture media contain a carbon source, nitrogen source, salts, vitamins and essential minerals. During the course of the semester, you will use a number of different strains of yeast and different kinds of media. Your success in the lab will depend on your ability to use sterile technique, which is needed to maintain the integrity of the different strains. An equally important element in laboratory success is careful bookkeeping!

In this lab, you will:

- Prepare stock cultures of *S. cerevisiae met* mutant strains on streak plates
- Compare the number of viable cells in log phase and stationary phase cultures of *S. cerevisiae* and S. *pombe* using spot plates
- Use light scattering to quickly estimate cell densities of these same yeast cultures

Sterile technique

Sterile technique is ESSENTIAL when working with microorganisms! This semester, we will be working with several different strains of yeast and bacteria. The strains have defined genotypes that have been generated by careful planning and experimentation. It's important to protect strains from contamination with other laboratory strains and from the many undefined microbes in the environment. Large numbers of diverse microorganisms are all around us - in the air, on laboratory surfaces, on your skin and on your clothing. True to their name, microorganisms are too small to be detected by the eye, but they grow rapidly in laboratory culture media. Correct transfer techniques and the use of sterile reagents are usually enough to prevent contamination of valuable laboratory strains.

Some simple precautions will reduce the possibility of contamination:

- Before working with strains, wipe down a small working area on the lab bench with 70% ethanol.
- Use sterile reagents, micropipette tips, and test tubes. Tips and test tubes should be kept in covered containers when not in use.
- Minimize contamination from clothing and body surfaces. Pull back and secure long hair. Avoid touching or breathing on sterile surfaces that will contact microorganisms.
- Avoid talking when you are transferring strains.
- Minimize the time that the caps are removed from vessels containing microorganisms or sterile media. Caps should always be kept right-side up to prevent contamination from airborne microbes falling into the caps.

The culture media and reagents that we will use have been sterilized by either autoclaving or filtration. An autoclave is a chamber that uses pressurized steam to kill cells on surfaces and in solutions, using temperatures near 121˚C and pressures from 30-40 psi. (For comparison, atmospheric pressure is ~15 psi.) Filtration is used in the place of autoclaving when solutions contain temperature-sensitive compounds. The pores in the filters used to remove microorganisms are typically 0.2 or 0.45 μm, which are sufficiently small to prevent the passage of bacteria. It's not difficult to keep stocks of media and reagents sterile as long as you work quickly and follow the directions above.

Yeast growth media

For routine culture, scientists usually use rich media that supply all the nutrients that cells need to grow. The individual components of rich media are often undefined. For example, yeast are commonly grown in a medium known as **YPD**, which is simple and inexpensive to prepare. The "**Y**" in YPD refers to a yeast extract, which contains the water-soluble compounds generated when yeast are forced to self-digest. (Those of you who have visited Australia may have encountered yeast extract in the popular spread, Marmite.) The "**P**" refers to peptone, a mixture of peptides and amino acids prepared by digesting animal protein with proteases. The "**D**" refers to dextrose, or glucose, which is the favored carbon source of yeast.

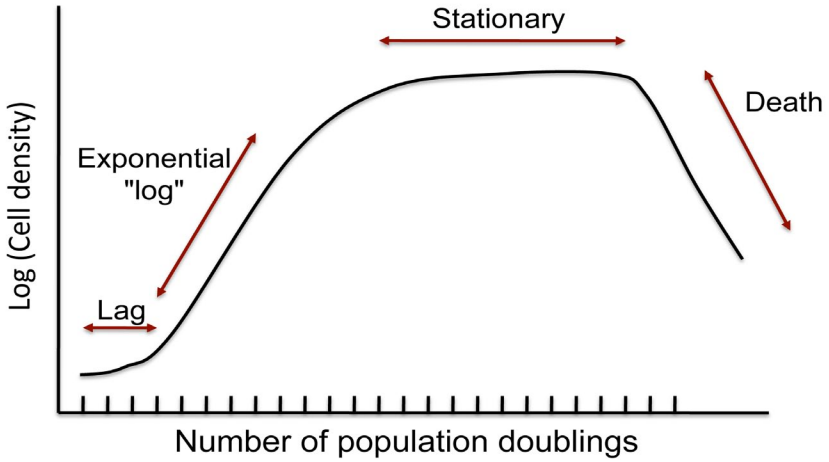
Because YPD is composed largely of crude extracts, its composition may show significant batch-to-batch variation. This variation is rarely a problem, however, because YPD contains more than enough essential nutrients to satisfy the metabolic requirements of cells. Many experiments, however, require media with a defined composition. To meet this need, the yeast community has developed several varieties of defined synthetic media that support the growth of most strains. Individual components of the synthetic media may then be manipulated to suit the needs of an experiment. (Later this semester, we will use defined media to select for particular genotypes.)

Yeast can be grown in liquid media or on the surface of plates containing solid media. Agar is usually added to liquid media, causing it to solidify. When cells are grown in liquid media, it is impossible to distinguish cells that have different genotypes from one another. By contrast, cells grow in colonies on solid media. Each cell in a colony is derived from a common ancestor and the cells are therefore genetically very similar, if not identical, to each other. For most of our experiments this semester, we will use solid media, because we need to distinguish cells with different genotypes from one another.

Chapter 3

Yeast growth phases

When yeast are grown in liquid YPD medium, the culture follows a well-established pattern for microbial growth. Cultures are usually started by inoculating media with a small number of cells. A lag phase follows the inoculation, during which cells become acclimated to the new environment and cells begin to condition the media with their own metabolites. ***Lag phase*** is followed by an ***exponential, or log, phase***, when the number of cells increases exponentially.



The exponential growth of yeast can be described by the equation:

$$N = N_0 e^{kt}$$

where N represents the number of cells at any time (t), N_0 represents the number of cells at time zero. Scientists often find it convenient to think of the growth constant k in terms of the doubling time of the culture. In this rendering, k = ln2/T (T = the doubling time of the culture). The growth rate of yeast varies with temperature. Yeast grow well at room temperature, but they grow more rapidly at 30˚C, which we'll use for our experiments. At 30˚C, wild-type yeast have a doubling time of ~90 minutes in YPD. We will also make sure that the cultures are well-aerated by growing them on either rotary shakers or a rotating wheel.

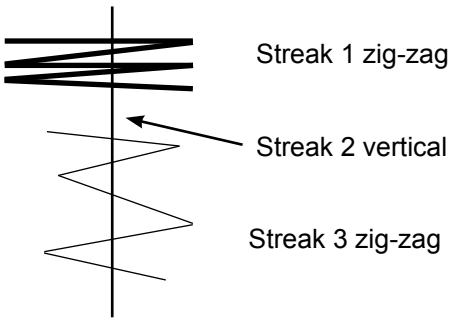
After a few doubling times, cells begin to deplete the nutrients in the culture, their growth rate slows, and the cells enter ***stationary phase.*** Cells in stationary phase have a different transcriptional program that allows them to survive in the less favorable environment, sometimes for considerable lengths of time. In stationary phase, the rate of cell division is similar to the rate of cell death, so the number of cells does not change appreciably. Eventually, cells enter ***death phase*** if conditions do not improve.

Exercise 1 – Streak plates

Microbiologists like to begin their experiments with a single colony, because the cells in a colony are the progeny of a single cell. A concern in all genetic experiments is unknown mutations that arise spontaneously and may affect the phenotype being studied. Spontaneous mutations arise constantly in all cells, with a rate of approximately 10^{-8}/base/generation. For *S. cerevisiae*, with a genome of 12 Mbp, most cells will have accumulated at least one mutation by the time that they've undergone 9-10 divisions. A colony, which has hundreds of millions of cells, is therefore a population of genetically similar, but not necessarily identical, organisms.

Researchers commonly use streak plates to isolate single colonies. A streak plate is actually a serial dilution of an existing culture on solid media. Researchers begin a streak by picking up a small sample of yeast or another microorganism with a sterile loop, wooden applicator stick or toothpick. They then spread the culture by making a series of zig-zag strokes across the surface of the plate. The number of cells on the loop or toothpick decreases as the streak progresses. Consequently, streaks appear thickest at their starting points, and the streak thickness decreases until it is possible to detect well-isolated single colonies near the end of the streak. Because it may be difficult to resolve colonies from a single streak, many labs use a series of streaks on the same plate to separate colonies. Each new streak is done with a freshly sterilized loop or toothpick that picks up cells by crossing over the tracks of the previous streak, before beginning a new series of zig-zags. In our experiments, we'll use a multi-streak protocol, which allows us to culture multiple strains on a single plate of culture medium. (See the figure below.) The streak plates that you prepare will be used as stocks for future experiments. As you streak your cultures, pay careful attention to detail to avoid cross-contamination or confusion about the identities of individual strains.

Streak plate with three sectors.
Plate has been divided into three clearly labeled sectors. Three streaks were used to spread the cells in each sector. The third streak in each sector contains well-separated colonies that can be used for genetics experiments.



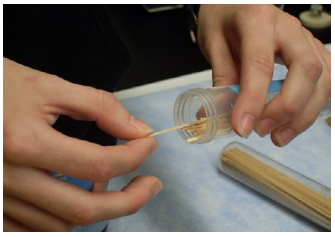
Chapter 3

Preparing a streak plate

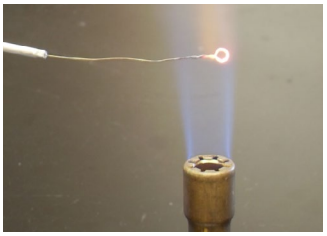
1. Your team will be assigned three different *S. cerevisiae met* strains to culture. Gather the parent strains to be propagated, an inoculation loop or sterile toothpicks, and agar plate(s) with the appropriate fresh media.

2. Divide the plates with fresh media into sectors by marking the bottom of each plate with a magic marker. CLEARLY label each sector with a code for the strain that will be streaked in it. Keep the labels at the rim of the plate and use small letters. ***Note your initials and the date.***

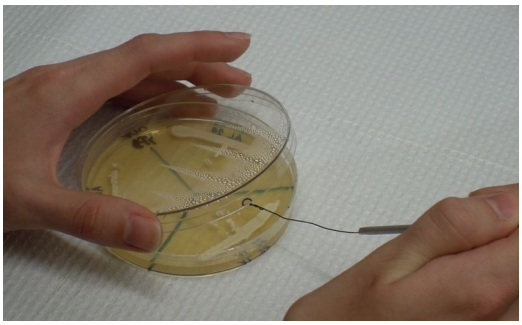
3. Carefully shake the container of sterile toothpicks until you can grab the tip of one toothpick without touching any of the others in the container with your fingers. Carefully draw it out of the container. (Note: toothpicks and applicator sticks will have been sterilized in the autoclave, so they should not be placed in the flame.)



If you are using an inoculation loop, sterilize the loop by holding it in the flame of a Bunsen burner until it glows red. Cool the loop by briefly touching the surface of the agar plate before proceeding.



4. Partially remove the lid of the petri dish containing the parent strains with one hand and hold the lid at an angle as you work. With the other hand, lightly touch a colony with the tip of a sterile toothpick or inoculation loop. Cultures should be *barely* visible on the tip. Avoid removing too many cells. Parent cultures are very concentrated, and a barely perceptible drop contains millions of cells. If the starting volume is too high, it may be difficult to separate single colonies on the new plate.



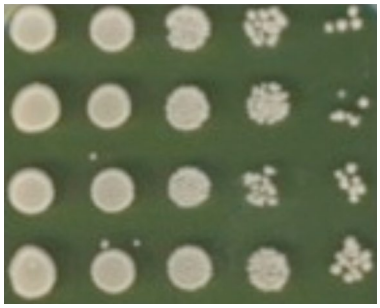
5. Transfer cells to the plate with fresh media. Make several zigzags across the outside edge of one sector with the toothpick or loop. *LIGHTLY* touch the agar surface as you move the toothpick. Think of pushing a hockey puck across an ice rink, rather than digging a ditch. Replace the lid and discard the toothpick. If you are using an inoculation loop, sterilize it in the flame, and then touch it to the agar plate in an area without cells to cool it down.

6. Make a second vertical streak from the rim of the plate toward the center, staying within the sector. The streak should cross the zigzags in the first streak.

7. Make a new series of zigzags that cross back and forth over the straight second streak, beginning at the outer edge of the plate and moving toward the center. Be careful to stay within the sector. Invert the plate and incubate it at 30˚C until individual colonies are visible.

Exercise 2 - Spot plates

In this lab, you will use spot plates to estimate the cell densities of log phase and stationary phase cultures of *S. cerevisiae* and *S. pombe*. Scientists use spot plates both to calculate the number of cells in cultures and to obtain information about the growth properties of strains on different media. The figure below shows an example of a typical spot plate. Each row represents a dilution series from a different yeast culture. The same volume of diluted culture is used for each spot. The dilution series is planned so that the most dilute spots contains a small number of individual colonies that can be distinguished from one another, typically less than ten.

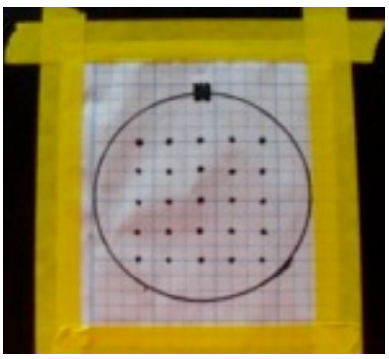


Spot plate.
Each row on the plate represents a series of 1:10 dilutions of a liquid culture of *S. cerevisiae*. Five µL of each dilution was spotted on the plate. The plate was incubated for two days at 30˚C. Individual colonies are apparent at the highest dilution of each extract.

Most commonly, investigators make a series of 1:10 dilutions in sterile (**NOT deionized**) water and then spot a few microliters of each dilution in a row. In this experiment , 5 µL aliquots were spotted from the serial dilutions. Note that it's possible to count individual colonies in the most dilute samples. This in turn enables you to calculate the number of viable cells in the original culture. In the top row, you can distinguish 4 colonies in the sample that has been 100,000-fold. The original culture would have contained 400,000 cells in 5 µL, which corresponds to 80 million cells per mL (8 x 10^7 cells/mL).

Preparing a spot plate

1. Alignment grids are useful for preparing good-looking spot plates! Prepare an alignment grid on a piece of graph paper (right). Trace the outline of a petri dish. Mark the target positions for culture dilutions. Place an orientation mark at one point along the circumference. Tape the grid to the lab bench.



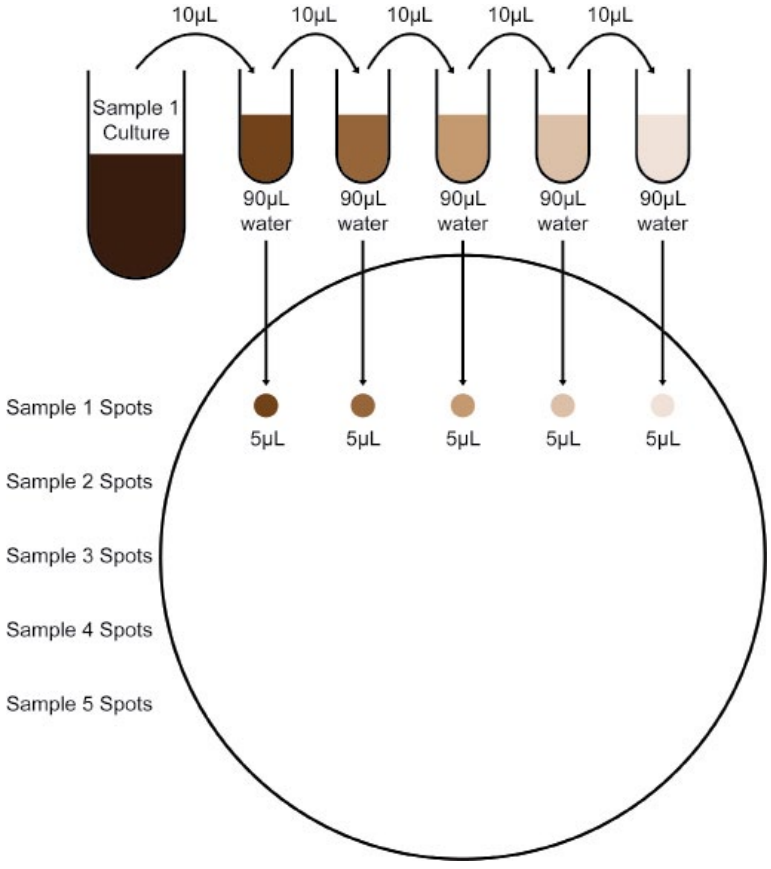
2. Label each petri dish with your initials and date with small letters around the BOTTOM rim of the dish. Put a hash mark on the bottom edge of the plate to serve as an alignment marker.

3. Prepare a series of five 1:10 dilutions from each culture using sterile distilled water. (Diagrams in your lab notebook are often helpful in designing dilution series.) To prepare a serial dilution, first pipette 90 µL sterile water into five microcentrifuge tubes. Next, use a P20 to transfer 10 µL from the original culture into the first tube. Vortex the cell suspension, and then transfer 10 µL from this tube to the second tube in the series, and so on. Use the same P20 pipette tip for the entire dilution series. Eject the tip into the appropriate waste container.

4. Beginning with the last dilution in the series, spot 5 µL spots in a row. Again, you will be able to use a single pipette tip for a dilution series, since you started with the most dilute sample.

5. Repeat step 3 for each culture that you are analyzing. Be careful to note in your lab notebook which culture has been spotted into each row on the plate!

6. Leave the plate right side up for ~30 minutes, to allow time for the yeast to settle and adsorb to the medium.



7. Invert the plates and incubate them at 30°C. Plates are inverted to prevent water droplets that form on the inner surface of the lid from falling on the colonies. Plates can also be kept at room temperature, but cells will grow more slowly. Do NOT incubate the cells above 30°C, which stresses the yeast.

8. When colonies are large enough to count (typically 2-3 days), remove the plates from the incubator. If you're not ready to analyze the data, seal the plates with parafilm and place them in the refrigerator or cold room.

9. Record your data with the scanner. To do this, remove the top from the plate and invert both the plate and the lid. Place the bottom half of the dish on the scanner and leave the inverted lid on the bench. (The lid is inverted to avoid contamination from spores and microorganisms that may be present in the air.) Place a black piece of cardboard or a folder over the plates before lowering the lid of the scanner.
10. Use spots where you can count individual colonies to calculate the density of cells in the original cell culture, correcting for the dilutions that you used and the volume of the spot.

Exercise 3 - Estimating cell densities with a spectrophotometer

The spectrophotometer provides a "quick and dirty" way to estimate the density of cells in a culture. In contrast to spot plates, which must be incubated for several days before colonies appear, spectrophotometer readings can be instantly converted into cell densities. On the other hand, the method does not discriminate between living and dead cells. The spectrophotometric method to determine cell number is based on light scattering. Most visible light cannot penetrate a cell. When the light beam in a spectrophotometer hits a cell, the light is deflected from the light path, so some of the light never reaches the detector. The greater the number of cells in a sample, the more light scattering that occurs. The light scattering ability of a cell depends on its size and geometry, so a calibration curve is necessary to extrapolate optical density measurements to cell number. For example, the same number of yeast cells would scatter light more than the same number of bacterial cells, because the bacterial cells are much smaller.

Read the background material on the spectrophotometer in the Laboratory Equipement chapter before proceeding.

Light scattering is measured with the spectrophotometer set to report absorbance. Because the principles used to measure light scattering and absorbance are different, the amount of light scattered by a solution is referred to as its "optical density" rather than its "absorbance." The optical density of a sample analyzed at 600 nm is abbreviated OD_{600}, with the subscript indicating the wavelength used for the measurement.

Chapter 3

Estimating cell densities with the spectrophotometer
Follow the directions in the Laboratory Equipment chapter for operating the GeneSys 20.

1. Turn on the GeneSys 20 spectrophotometer. Adjust the wavelength of the monochromator to 600 nm.

2. Fill a cuvette with 1.0 mL deionized water and orient the cuvette in the holder so that the flat side of the cuvette faces the front of the instrument. (Note: it is not necessary to use culture medium as the blank.)

3. Close the lid and press the "0 Abs/100%T" button to establish a baseline value for further measurements.

4. Remove the cuvette from the instrument and replace the water with 1 mL of undiluted culture. Close the lid and read the OD_{600}. Record this value in your lab notebook. If the optical density of the sample is greater than 1.0, dilute the sample 1:10 with deionized water and read the optical density again. (The linear relationship between the OD_{600} and cell density is lost when OD_{600} values exceed 1.0) Record the new value in your lab notebook, noting how you diluted your sample. Dispose of all cell material in the white liquid waste container.

5. Repeat step 4 with each of your cultures.

6. Calculate an approximate cell density for each sample, assuming that an OD_{600} of 1.0 corresponds to approximately 1.3×10^7 cells/mL. Use only data where the OD_{600} is less than 1.0 for these calculations.

Chapter 4

Introduction to Databases

The computer belongs on the benchtop in the modern biology lab, along with other essential equipment. A network of online databases provides researchers with quick access to information on genes, proteins, phenotypes, and publications. In this lab, you will collect information on a *MET* gene from a variety of databases.

Objectives

- Learn how information is processed in databases
- Become familiar with literature, molecular and organism databases that you will use in this semester's research project
- Understand how the *Saccharomyces* Genome Project provided the reference sequences for *S. cerevisiae* genes
- Use primary and derivative databases to locate information on a *MET* gene

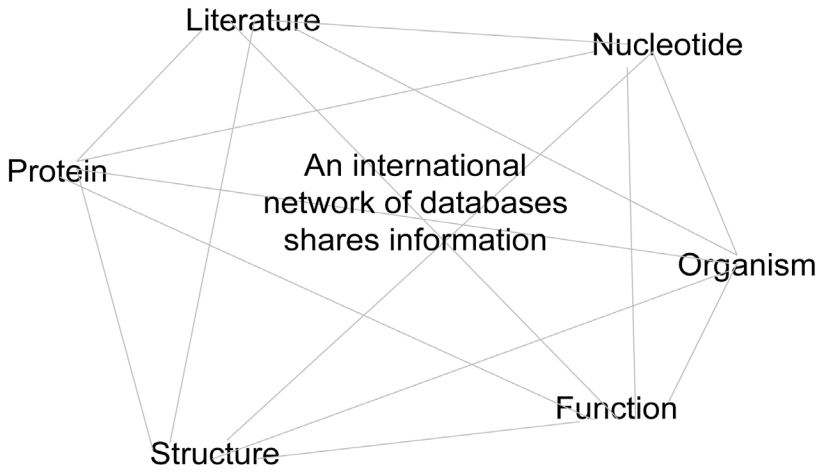
Chapter 4

Your team has been assigned a *MET* gene that you will be studying for the semester. By the end of the semester, you will hopefully have determined if the function of the *MET* gene has been conserved between *S. cerevisiae* and *S. pombe*. As you begin your experiments, you will want to find more information about the protein encoded by your gene and its role in metabolism. You will also be interested in tapping into the wealth of sequence information from genomic projects to determine if functional homologs of your gene product might be present in other species, including S. pombe. A wealth of online databases and database tools will help you to find this information.

The best way to learn about databases is to use them! In this chapter, you will be given a quick overview of how databases are organized and tailored to fit the research needs of different communities. In this lab, you will search for information on the *S. cerevisiae MET* gene that your group is studying in several databases. As you progress through this lab, you may feel like you're going in circles at points, because you are! The records in databases are extensively hyperlinked to one another, so you will often find the same record via multiple paths. As you work through this chapter, we recommend that you record your search results directly into the lab manual.

An international network of databases

Information on genes and proteins is organized into many databases, with missions that vary widely in their size and focus. This chapter will introduce you to some of the large, international databases that are freely accessible to the biological research community. These databases receive support from governments, and they work cooperatively with one another. The figure below shows the various kinds of databases that are widely used in molecular biology.



By far, the largest collection of databases is housed at the National Center for Biotechnology Information (NCBI) in the United States. NCBI includes literature, nucleotide, protein and structure databases, as well as powerful tools for analyzing data. On the other side of the Atlantic, the European Union supports the EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics Institute) collection of databases and bioinformatics tools. Both NCBI and EMBL-EBI exchange nucleotide sequence information on a daily base with each other and the with smaller DNA Database of Japan (DDNJ). Much of the information is the same in NCBI and EMBL-EBI databases, but each database has its own touch and feel. Consequently, most researchers have their favorite entry points in the network of databases.

It's important to keep in mind that information in databases is not static. Scientists make mistakes and technology continues to improve. It is not uncommon to find changes in a database record. Scientists with an interest in a particular gene are well-advised to check frequently for updates!

Investigators submit information to primary databases

The ultimate source of information in databases is the research community, which submits their experimental data to primary databases, Primary databases ask investigators for basic information about their submission. A record that meets the database's standards is accepted and assigned a unique accession number that will remain permanently associated with the record. Each database has its own system of accession numbers, making it possible to identify the source of a record from its accession number. Once a record is accepted into a database, professional curators take over. Curators are professional scientists who add value to a record by providing links between records in different databases. Curators also organize the information in novel ways to generate derivative databases. Derivative databases, such as organism databases, are often designed to fit the needs of particular research communities. In this course, we will be using both primary and derivative databases. Let's look at a few databases.

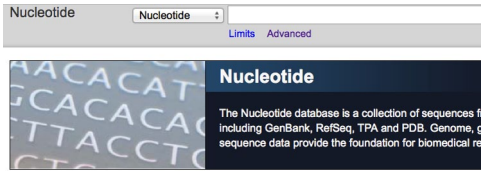
Pubmed

It's hard to overemphasize the importance of PubMed. PubMed is the primary literature database maintained by NCBI. PubMed has no counterpart in any other database. Because PubMed serves as the international database for biomedical literature and aims to be comprehensive, it accepts articles that are not written in English. (The vast majority of entries are written in English, however.) PubMed, which is housed in the National Library of Medicine, is the web portal to MEDLINE, an index of over 5000 biomedical journals. PubMed entries are submitted by publishers and assigned a PMID accession number. PubMed currently contains over 21 million citations!

In addition to basic citation information, most PubMed entries have an abstract of the article being referenced, and hyperlinks are supplied to related articles in PubMed. The usability of PubMed continues to grow. More recent PubMed entries from open access journals provide a brief preview of the entire article, including expandable thumbnails of the figures and tables in a paper. Users are able to use a clipboard, save their searches, and arrange for RSS feeds when new search results enter PubMed. There is also a mobile version of the interface.

The articles referenced in PubMed provide the experimental evidence for the pathways that we are studying this semester. You can access PubMed at pubmed.gov or through the BC Library's database portal. An advantage of using the library's portal is that you will be able to use the powerful "Find It" button to access the actual articles.) Expect to use PubMed extensively in upper-division biology courses, graduate school, and medical school.

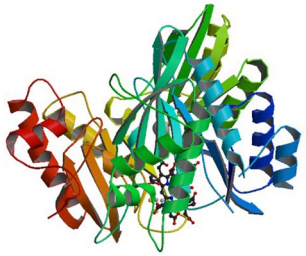
GenBank



GenBank is the portal for nucleotide sequence submissions at NCBI. GenBank is a massive databank that was established in 1982, when DNA sequencing methods had just been developed and individual investigators manually sequenced one gene at a time. Over the years, investigators have contributed many separate records for individual genes, transcripts, plasmids, and segments of chromosomes. The rate of GenBank submissions has increased in pace with advanced in DNA sequencing technologies. Today, there are over 135 million records in GenBank, which is now part of NCBI's Nucleotide database. Individual investigators continue to make single submissions to GenBank at the same time that genome projects use automated processes to submit whole genome sequences.

Not surprisingly, GenBank contains multiple records that refer to the same gene. For example, GenBank contains several hundred entries for *S. cerevisiae MET* genes. GenBank has now been incorporated into NCBI's Nucleotide database together with some other derivative databases. With good search techniques, it's not difficult to find the record that you're looking for, but many researchers find it simpler to use derivative databases where curators have done the work for them. In this lab, you will use both the primary and derivative databases to search for information on your gene.

Protein data bank - (pdb.org)



Researchers submit the atomic coordinates and structural models for biological macromolecules to the protein data bank (PDB). The PDB is part of an international consortium that accepts data for protein and nucleic acids. The vast majority of PDB records have been obtained by X-ray diffraction, although the database also accepts models obtained with nuclear magnetic resonance (NMR), electron microscopy, and a some other techniques. The number of entries in the PDB databases is orders of magnitude smaller than the number of predicted proteins in GenBank, reflecting the difficulties inherent in determining structures

of macromolecules. In this project, we are interested in the structure of Met proteins, since their structures are intimately linked to their functions. Later in the course, we will work with PDB file and spend some time comparing Met protein structures across species.

Derivative databases organize primary data

Hundreds of derivative databases are available, organizing the information in primary databases into new formats that are more useful to research communities. We will use a variety of derivative databases in this course, taking advantage of the work that the curators have already done. Two derivative databases will be particularly useful for this semester's project: the RefSeq sequence database and the *Saccharomyces* Genome Database organism database.

RefSeq Database

We will use NCBI's RefSeq database to obtain sequence information for this project. The RefSeq database is designed to provide a set of non-redundant gene, transcript and protein sequences for many different organisms. For *S. cerevisiae*, these reference sequences were generated by the Saccharomyces genome sequencing project (SGP), which was completed in 1996. The *Saccharomyces* genome project (SGP) involved an international consortium that employed a high standard of accuracy to obtain the complete sequence of strain 288C (Goffeau et al., 1996). A single strain was chosen for the project, since *S. cerevisiae* laboratory strains naturally accumulate mutations over time and diverge from one another (evolution in action!). The RefSeq sequences are perfect for our project, because we are using strains and plasmids produced by the SGP.

RefSeq records have been incorporated into NCBI's Nucleotide database. RefSeq records for *S. cerevisiae* genes have accession numbers with the format NC_###### (chromosome sequence), NM_###### (mRNA sequence) or NP_ ###### (protein sequence). The "N" in the accession number indicates that experimental evidence is available for the transcript and/or protein sequence. (The "N" can also serve as a mnemonic for "non-redundant.") To understand the relationship between the three types of RefSeq records, it's helpful to consider how the genome project worked. *S. cerevisiae* had been a popular model organism for several decades before the RefSeq databases were established in 2000. By the time that the SGP began, the yeast community had contributed a large number of GenBank records for individual gene sequences. Many of the GenBank records had been submitted by geneticists, who established linkage relationships between various traits. These linkage relationships provided a rough map of the yeast chromosomes that would provide landmarks for aligning data generated by the SGP.

NC_ records: DNA sequences were aligned into groups representing chromosomes. The raw data from the SGP consisted of thousands of DNA sequences that had been randomly generated ("shotgun" sequencing) from yeast genomic DNA. The project was designed so that each chromosomal region would be represented in multiple sequence records. Individual

sequencing reactions generated 500-1000 bp of sequence information, so thousands of individual sequences were required to generate the complete sequence of a chromosome. To determine the chromosome sequence, investigators first identified regions where the DNA sequences overlapped with each other. Groups of overlapping sequences were assembled into longer scaffold sequences that represented larger segments of chromosomes. The positioning of scaffolds along the length of the yeast chromosomes was facilitated by the work that yeast investigators had already done with individual genes. By the time that the SGP started, yeast geneticists had constructed recombination maps of the chromosomes, and many individual genes had been cloned and sequenced. As you will see, the *MET* genes that we are studying this semester had all been mapped to chromosomes, and the sequences of many *MET* genes had also been determined. When the SGP was finished, the lengths of the 16 yeast chromosomes were found to vary in size from 230 to 1530 kbp.

NM_ records: potential genes were identified as open reading frames. The NM_ records contain the open reading frames (ORFs) predicted from the chromosome sequences. ORFs are the probable coding sequences (CDS) for proteins. ORFs (almost always) begin with an ATG initiation codon and terminate with a stop codon in the same reading frame. Initial ORF identification was generated computationally, and human annotators then verified the ORF assignments and looked for similar sequences in GenBank that could provide a clue to the function of the ORFs. There are approximately 6000 ORFs in the *S. cerevisiae* genome. At the time the SGP was completed, only about half of the ORFs could be verified as protein coding genes, but that number has increased to about 75%.

NP_ records: protein sequences are predicted from NM_ sequences. The amino acid sequences in NP_ records were generated by automatic translation of the NM_ sequence records. The NP_ records are stored in the NCBI Protein database. The Protein database contains some amino acids that have been experimentally determined, but the number of these records is very small. We will use NP_ records when we study the conservation of Met proteins between species.

RefSeq records for many other organisms, particularly microbes, are not as well-verified as those for S. cerevisiae and other model organisms. In many cases, the RefSeq have been generated by computational predictions of whole genome sequences, and no experimental evidence is available for that particular organism. In these cases, records have prefixes beginning with X or Y (XC_, XM_, XP_, etc.).

***Saccharomyces* Genome Database (SGD)**

You will probably find the SGD to be particularly useful for finding information about your gene. SGD has a clear mission - facilitating research on *S. cerevisiae*. SGD curators work closely with the yeast research community to maintain the SGD. The SGD provides links to information in a wide variety of databases, including sequences in NCBI databases, primary literature articles in PubMed, protein information, structural data and much more. Investigators are encouraged to submit data from their research about the functions of *S. cerevisiae* genes and the phenotypes that result when a gene is mutated. SGD is an essential tool for yeast researchers.

(Should you want to access SGD on the run, you can download an app for the iPhone and iPad.) Similar derivative databases exist for other model organisms. These databases serve as central points of information for their community and greatly facilitate interactions between researchers.

Exercise 1: Finding gene records in NCBI databases

Homepage: Point your browser to the NCBI homepage: ***ncbi.nlm.nih.gov***

Hint: Bookmark this page. You will use it often.

NCBI is a large collection of databases. Clicking on the dropdown box brings up a list of individual databases for more targeted searching. For a comprehensive search, use the "All databases" setting. Write the name of your *MET* gene in the search box and click "Search."

Entrez summary page: The Entrez page summarizes the number of hits in each of the many NCBI databases. The number is probably quite large! Take a look at the results. In your notebook, record the number of records in the PubMed, Nucleotide, Protein, and Structure categories.

- Modify the search term by adding "AND Saccharomyces cerevisiae" to the search box. Record the number of records in each of the categories used above. The numbers have probably dropped significantly! Why do you think that this happened? This simple comparison may give you some idea of the sheer volume of records in the NCBI databases. You may not receive any hits in the Structure category, since the vast majority of proteins have not been crystallized or studied with NMR.

NCBI Nucleotide:

- Click on the nucleotide link, which brings you to NCBI's Nucleotide database. The Nucleotide database aggregates records from multiple databases, including GenBank and RefSeq. Do all the records in your search results refer to *S. cerevisiae* sequences? Probably not! Any mention of "Saccharomyces cerevisiae" in a record is enough to bring it up in an unrestricted search, even if the sequence comes from a different organism.
- Narrow down the search to records that actually contain S. cerevisiae sequences by clicking the Saccharomyces cerevisiae link in the Top Organism list on the right. Note that clicking on the tree adds an additional search term. You will now see RefSeq records in the new list.
- Use the hyperlinks at the right to filter you results to the RefSeq database. You should see a single NC_ and a single NM_ record in the list. (Remember the results are non-redundant!
- Let's look at the NC___ record first.

Record the accession number _____

Which chromosome is represented in the record? _____

How many nucleotides are in the chromosome (bp)? _____

- Click to open the NC_ record. Near the top, you will see a link(s) to articles in the primary literature. Scroll down a bit in this very long record and look at a few genes. As you scroll down, you are moving from one end of the chromosome to the other, and you will see annotation information for the ORFs identified by the SGP. Each ORF has a description of its gene, mRNA, and CDS. You may see some genes where introns are predicted to occur. Intron-containing genes can be identified by the word "join" in the first line of the mRNA and CDS descriptions.

- Now let's take a look at the NM_ record for your gene. Use the back button on your browser to return to your search results. Open the NM_ record.

  Record the accession number _____

  How many nucleotides are in the coding sequence (bp)? _____

  Is there an intron in your gene? _____
  (Hint: Check the CDS to see if there are interruptions in nucleotide numbers)

- Find the predicted amino acid sequence in the record.
  What is the accession (NP) number for the protein sequence? _____

Think: is the NM_ record the actual sequence of the mRNA for your gene? (Do mRNAs all begin with AUG and end with a stop codon?)

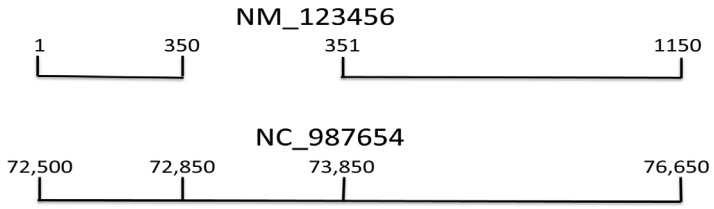
Use the back button on your browser and click "All" to find all the *S. cerevisiae* records. Do any of the records look like they may be an mRNA sequence? Pay special attention to early GenBank submissions, which are near the bottom of the list. The earliest records were often submitted by investigators studying individual *MET* genes. If you do find an mRNA record, write its accession number in your notebook for future reference. How does its length (bp) compare to that of the NM_ sequence?

Exercise 2: Locating your gene on its chromosome

Using NCBI's BLAST alignment tool

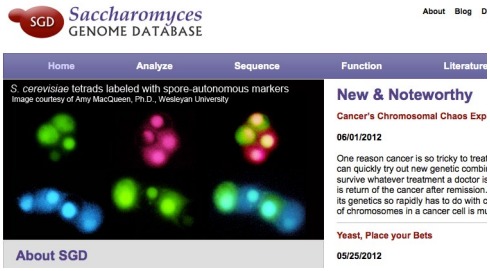
The ~6000 ORFs in the *S. cerevisiae* chromosome are divided between 16 different chromosomes, that vary in size from ~230 to ~1500 kbp. Consequently, it can be difficult to find your gene in a NC_ record! In this exercise, you will use NCBI's BLAST (Basic Local Alignment Search Tool) tool to find the position of your gene on its chromosome. The BLAST tool is a powerful tool for aligning either nucleic acid or protein sequences. In the next few weeks, we will review the theory underlying the BLAST tool, but today we won't concern ourselves with the theory.

- Choose BLAST from the list of resources on the right.
- From the list of BLAST tools, choose nucleotide blast. This brings you to the query page, where you can either paste in sequences or use some (but not all!) kinds of accession numbers. Fortunately, RefSeq accession numbers can be used directly in the query box. Enter the NM_ accession number for your gene as the query sequence.
- Click the box "Align two or more sequences. " This brings up the subject sequence box. Enter the NC_ number for your gene here. Click the BLAST button.
- The results page shows the alignment data, as well as numerical scores about the strength of the alignment. Cursor down to the sequence alignment at the bottom of the page. You won't be surprised to see that the sequences are identical.
- Which nucleotide in the NC_ record corresponds to nucleotide 1 of the NM_ record? ...to the last nucleotide in the NM_ record? Were there any gaps in the alignment consistent with an intron? Make a sketch of the alignment, including the nucleotide numbers for the NM_ and NC_ records. (How long is the intron in this figure?)



Exercise 3: Using the Saccharomyces Genome Database

Direct your browser to yeastgenome.org



In addition to summarizing information about yeast genes, SGD serves as a meeting place for the yeast community. Note the meeting announcements and the "New and Noteworthy" articles. The Community tab at the top of the page will bring you to a variety of resources including a wiki for researchers to exchange information.

- Type the name of your *MET* gene in the search box. This brings up the summary page for your gene.

  Record the standard name for your gene _____
  Are there multiple genes for your name? *What are they?*

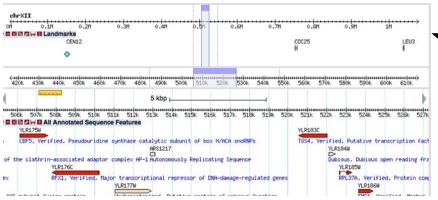
  Record the systematic name for your gene _____

Locating genes on *S. cerevisiae* chromosomes

Information in the SGD is organized by gene locus, each of which has a systematic name assigned by the SGP that denotes its precise position and orientation on one of the yeast chromosomes. You already know which chromosome your gene is located on from its NC_ record. All locus names begin with a "Y" (for yeast). What do the other characters in the locus name mean?

- Under Chromosomal Location, ***click on GBrowse***, which brings you to the genome browser.

  GBrowse is an interactive genome browser that provides users with an overview of a chromosomal segment. Users are able to move the viewing window and to set the window size (kbp) of the genome viewer. The figure shows a (grainy) screenshot from the GBrowse.



Click along the chromosome to reposition the viewer

Individual ORFs in the window are shown below the chromosome map

- Locate your gene. You should see both its standard name (MET_) and its systematic name. Now look to the left and right on the chromosome. Each of the arrows denotes an ORF identified by the YGP.

- Use the GBrowse tools to move along the chromosome. As you do, look for genes with introns - introns are shown by thin lines connecting the exons, which are shown as thicker bars. Note how densely ORFs are packed into the S. cerevisiae chromosomes. Contrast this packing to the human genome, where only 2% of the DNA is found in ORFs.

- What do you notice about the second letter in the locus names? The second letter in the locus name identifies the chromosome where the gene is situated. Chromosome numbers had been assigned before the genome project began. The genome project gave each chromosome a letter (A=chr. 1; B=chr. 2; C=chr.3, etc.). Does the letter in your gene's systematic name agree with the information in the NC_ record?

- What do you notice about the third letter in the locus names in neighboring ORFs? What do you notice about the locus numbers, as you move from left to right?

  Gene positions are designated as either L (left) or R (right) relative to the centromere. By convention, chromosomes are read from left to right. Do the ORF numbers increase or decrease as you read from left to right?

  Find the centromere, which will appear as a blue circle in the GBrowse window. Where is the centromere relative to your gene?

- What is different in the locus names of the ORFs with leftward and rightward-facing arrows?

  The "W" (Watson) or "C" (Crick) designation indicates which of the two strands of DNA has the coding sequence. RNA polymerase will move in the direction shown by the arrow during transcription.

  Does RNA polymerase move away or toward the centromere when your gene is transcribed?

Chapter 4

What role does the product of your *MET* gene play in metabolism?

Now that we've looked at the structure of your gene, let's consider its function. The *MET* genes that we are studying all encode enzymes. Return to the SGD summary page for your gene and travel down to "Pathways" in the sidebar. You will see several links to the right, which bring you to the MetaCyc database's information for *S. cerevisiae* enzymes. MetaCyc, the online Encyclopedia of Metabolic Pathways. MetaCyc is a nonredundant database that catalogs pathways supported by experimental evidence. MetaCyc has extensive collections about reactions in humans, other model organisms and some more obscure organisms.

- List the MetaCyc pathways that your enzyme is involved in. Prepare a concept map showing how the pathways are related to one another.

- Click on one of the pathway links. What is name of the enzyme encoded by your *MET* gene?

Under the name of your enzyme, you will see a number with 3 decimal points. This is the official classification given to the enzyme by the Enzyme Commission, which categorizes enzymes in very fine detail. The first number indicates the broad class of enzyme, e.g. hydrolase, transferase, oxidoreductase. The subsequent numbers drill down to the kinds of bonds altered in the reaction and finally to specific substrates. Enzymes from different organisms with the same EC number are *expected* to catalyze the same reaction. Record the E.C. number.

- Click on the E.C. number to see the reaction catalyzed by the enzyme. What are the substrates and products for your enzyme? Draw the structure of the substrate and product that are intermediates in methionine synthesis.

Mutant phenotypes - what happens your *MET* gene product is missing?

- Return to the summary page for your gene. Look at the mutant phenotypes for your gene that have been discovered by classical genetics.

- What phenotypes are detected in mutants lacking your gene product's function? (***Note:*** *you can use the tab at the top of the page to obtain more detailed information about the phenotypes, as well as literature references.*)

- Mutations in some *MET* genes have phenotypes associated with tellurium accumulation and resistance. What's tellurium? Find tellurium on the periodic chart. How do you think tellurium is working? (Tellurium is fairly toxic, so we won't be working with it in this course.)

Gene expression

The expression of a gene in response to environmental stimuli often provides clues to its physiological importance. The absolute level of gene expression may change in an experiment, and it's also important to see which genes show similar changes in expression. Microarray technology has allows investigators to study the simultaneous expression of hundreds of genes. Since the completion of the SGP, expression of *S. cerevisiae* genes has been studied in hundreds of high-throughput experiments.

- Click on the Expression summary. This brings you to a graph showing the number of experiments in log2 units (0 indicates no change, 1 a 2-fold change, 2 a 4-fold change, etc.) where the level of gene expression changed. Were both decreases and increases observed? How large were most of the changes observed in experiments? The *MET* genes encode enzymes that catalyze discrete steps in the synthesis of methionine. Consider:

  Would large changes in the concentration of a Met protein be required to alter the concentration of methionine in a cell? Would a change in the concentration of a single catalyst in the methionine biosynthetic pathway be expected to change methionine concentrations? Would changes in the concentrations of multiple enzymes be required to change methionine concentrations?

- Click on the SPELL link. This will bring you to a summary of many high throughput gene expression studies under a variety of environmental conditions. SPELL gives a list as well as graphical output of the genes that are most similar to your gene in their expression patterns. Green labels indicate increased expression, and red labels indicate reduced expression. Scroll right to see the results of experiments that don't fit on the screen. Note that SPELL gives you the literature citations for the experiments that produced the experimental data.

- Record the list of the six genes that were most frequently co-expressed with your gene. Look up the reactions catalyzed by their gene products. What do you notice about genes that are coordinately expressed with your *MET* gene?

Protein information

- Click on the protein tab at the top of the gene summary page. This page gives you a lot of useful information about your protein, which we'll use later in the course.

- Investigators used a microscopic method to estimate how many molecules of each protein were present in a log phase yeast cell (Ghaemmaghami et al., 2003). Numbers varied from less than 50 to a~1 million molecules/cell.
  How many molecules of your enzyme are present in a log phase cell?
  Is your gene highly expressed? moderately expressed? Are you surprised?

- Note the physical characteristics of your enzyme.
  How many amino acids are encoded in its sequence?
  Is your enzyme acidic or basic? (Check the pI).

- Click on the PDB Homologs under Homologs to access structural information on your enzyme. Has the *S. cerevisiae* enzyme been crystallized? ...a homolog from another species? If so, note the species and the 4-character PDB accession number(s). (Hint: check the PDB number to make sure that the E.C. number is the same as that of your Met protein.)

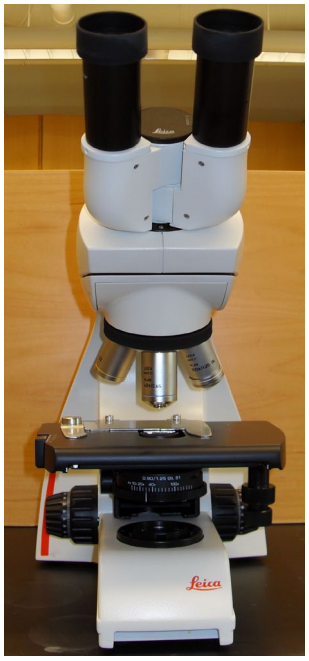
References

Ghaemmaghami, S., Huh, W.K., Bower, K., Howson, R.W., Belle, A., Dephoure, N., O'Shea, E.K., and Weissman, J.S. (2003). Global analysis of protein expression in yeast. *Nature* **425**, 737-741.

Goffeau, A., Barrell, B.G., Bussey, H., Davis, R.W., Dujon, B.., Feldmann, H., Gailbert, F., Hoheisel, J.D., Jacq, C., Johnston, M., Louis, E.J., Mewes, H.W., Murakami, Y., Philippsen, P., Tettelin, H., and Oliver, S.G. (1996). Life with 6000 genes. *Science* **274**, 546, 563-567.

Chapter 5

Looking at yeast



The budding yeast, *Saccharomyces cerevesiae*, is an important model organism that is easily cultured in the laboratory. *S. cerevisiae* is separated by ~1 billion years of evolution from the fission yeast, *Schizosaccharomyces pombe*. In this lab, you will use the light microscope to compare cultures of *S. cerevisiae* and *S. pombe*.

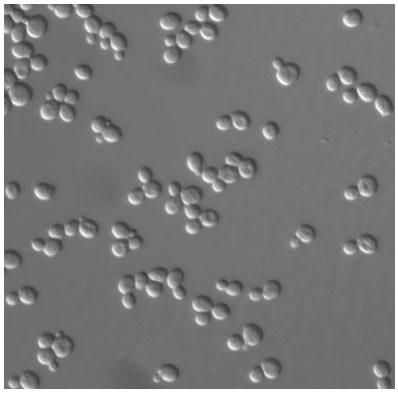
Objectives

- Be able to identify the components of a compound light microscope
- Be able to adjust a light microscope for a particular specimen
- Compare the morphological characteristics of log phase and stationary phase yeast cultures
- Compare the morphological characteristics of *S. cerevisiae* and *S. pombe* cultures

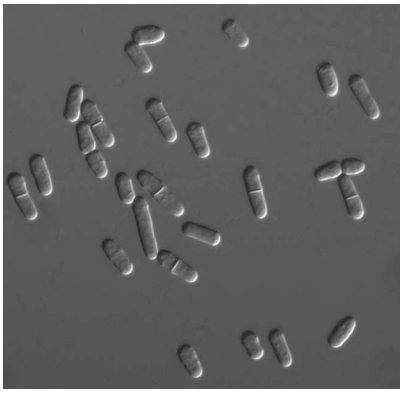
Chapter 5

Introduction

The budding yeast, *Saccharomyces cerevisiae*, and the fission yeast, *Schizosaccharomyces pombe*, derive their common names from their mechanisms of cell division. These differences in growth properties are clearly apparent under a light microscope. The images below were obtained using differential interference contrast (DIC) microscopy, a kind of light microscopy that provides detail about the surface properties of living cells.



Saccharomyces cerevisiae



Schizosaccharomyces pombe

This lab will show you how to observe yeast with a compound light microscope. Our laboratory microscopes do not have the sophisticated optics used to obtain the images above, but you will be able to distinguish cells at various points in the cell cycle and to distinguish the two species of yeast. At the end of this lab, you should be able to distinguish S. cerevisiae and S. pombe by their growth properties and appearance under the light microscope.

You will receive two cultures of both *S. cerevisiae* and *S. pombe* before beginning these experiments. One culture will contain cells in log phase growth and the other, denser culture will contain cells in stationary phase. All of the cultures have been grown in YPD medium. Can you distinguish the two phases by looking at the culture tubes?

Students should work in groups of two for these experiments.

Light microscopes

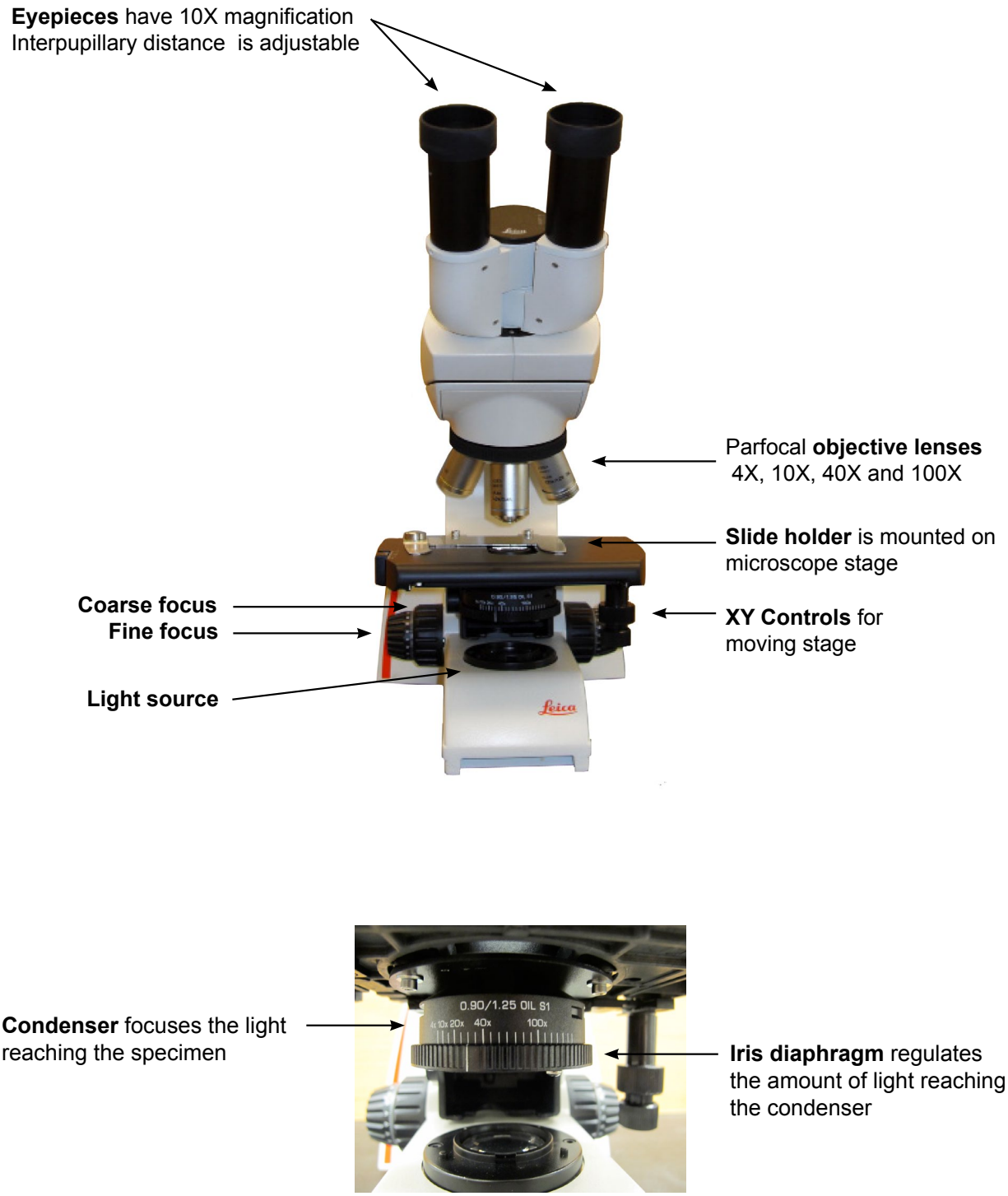
Microscopes are essential for viewing microorganisms. Yeast cells typically have diameters of ~10 μm, while bacteria have diameters of ~1 μm, far too small to be seen without considerable magnification. Light microscopes use a system of lenses to gather and focus light passing through a specimen and to project the image on the viewer's retina. (The lens in the viewer's eye forms part of this path as well.) Our labs are equipped with Leica DM500 brightfield light microscopes that effectively allow one to view microorganisms. The microscope has four, interchangeable objective lenses, with magnifications of 4X, 10X, 40X and 100X. Ocular lenses in the eyepieces magnify specimens an additional 10-fold, producing final magnifications of 40X, 100X, 400X and 1000X. When working with the microscope, ***always begin with the lowest power objective***, which is easiest to focus, and work your way to the higher power objectives. The lenses on the DM500 are parfocal, meaning that specimens remain reasonably well-focused when the lenses are changed. (We will not be using the 100X lens, which requires immersion oil.)

In the DM500 microscopes, light from an LED source at the base of the microscope enters a condenser that focuses the light reaching the specimen on the microscope stage. Users are able to control the amount of light reaching the specimen by adjusting the intensity of light emitted from the source and/or by opening and closing the aperture diaphragm, essentially an iris that controls the diameter of the light beam that enters the condenser. Slides containing specimens are mounted on the stage in a slide holder. The position of the slide with respect to the light path can be finely adjusted by moving the stage with X- and Y-control knobs.

Using the microscope

1. Adjust the positions of the eyepieces to fit the distance between your eyes.
2. Rotate the 4X objective lens into position, and adjust the aperture diaphragm to the recommended opening for the 4X lens. Turn on the light source using the switch on the lower right of the microscope stand. Place a slide in the slide holder and use the XY stage controls to center the coverslip in to the microscope light path.
3. Use the coarse focus knob to bring the specimen into focus. Use the fine focus knob to sharpen the image. Adjust the light intensity to a comfortable level by using the illumination control on the left side of the stand or by turning the aperture diaphragm.
4. Rotate the 10X objective into position and focus on the specimen. Because the lenses are parfocal, you may be able to sharpen the image with only the fine focus knob. Adjust the light, as in step 3. Yeast cells should be visible, but small.
5. Rotate the 40X objective into position and readjust the lighting and focus. You may find that closing the aperture slightly improves the appearance of unstained yeast preparations.

Leica DM500 Light microscope



Precautions to take with lenses!!!

- Be careful while focusing. The objective should not touch the slide at any time! Lenses are fragile and expensive!
- Clean lenses with lens paper only. Kimwipes™ and other paper may scratch a lens.
- The 100 X objective contains an oil immersion lens. A drop of immersion oil must be placed on the coverslip before using the lens. If you use the 100X lens, be sure to wipe the oil off the lens with ***lens paper*** when you are finished. Do NOT use oil with any of the other lenses, which will be damaged by the oil.

Exercise 1 – using the compound light microscope

1. Identify the parts of the microscope. Note the positions of the objectives, the coarse and fine focus adjustments, the adjustable light switch and the condenser diaphragm.
2. Locate the four objective lenses on the microscopes. The magnification of each lens (4x, 10x, 40x, and 100x) is stamped on its casing. Rotate the 4x objective into position. Adjust the position of the aperture diaphragm to the corresponding 4x position.
3. Turn on the microscope lamp and adjust the dimmer switch until the light is not too intense when you look through the eyepieces. You may need to adjust the distance between the eyepieces to fit your eyes.
4. Place the transparent ruler on the microscope stage. Use the coarse focus knob to bring the ruler into focus. You may also need to adjust the light. Make additional adjustments with the fine focus knob. The ruler is graduated into 1 mm divisions. (Remember that 1 mm is equal to 1000 μm, the unit of distance usually used by microscopists.)

   What is the diameter of the field of view with the 4x objective?
5. Use the stage manipulators to move the ruler to either the right or the left.

   What direction does the image move?
6. Dial the 10x objective into position and adjust the condenser diaphragm.

   How does the distance between the specimen and the objective change?

   Adjust the focus with the coarse and fine focus adjustment knobs.

   What is the diameter of the field of view with the 10x objective?

   Can you find a mathematical relationship between the magnification and the diameter of the field of view?
7. Swing the 40x objective into position and adjust the condenser diaphragm. Adjust the focus using ONLY the fine focus knob.

   What happens to the working distance and the field of view?

Chapter 5

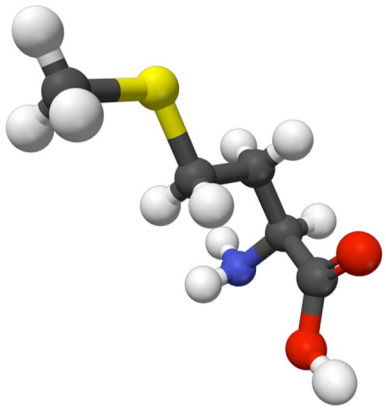
Exercise 2 - observing yeast cultures with the microscope

As yeast progress through the cell cycle, they undergo characteristic morphological changes. A log phase culture will have cells in the G1, S, G2 and M phases of the cell cycle. In this experiment, you will observe both log phase and stationary phase cultures of *S. cerevisiae* and *S. pombe*. The two yeasts use very different modes of division. In *S. cerevisiae*, buds begin to form when cells enter S phase. The size of the bud, which will become the daughter cell, continues to grow until the cells divide in M phase. At the time that the cell divides, the daughter cell is still smaller than the mother cell. The daughter cell will need to grow a bit before it enters another round of cell division. By contrast, *S. pombe* divides by medial fission. Cells grow in length until they are 12-15 μm, at which point a septum forms and the cell divides. In this experiment, you will use the compound light microscope to observe samples of the four cultures.

1. Concentrate the cells in your log phase yeast cultures by spinning them for a count of 10 in a microcentrifuge set at top speed. Use a transfer pipet to remove most of the culture medium, until the medium just covers the cell pellet. Resuspend the cells with the vortex mixer. (It is not necessary to centrifuge the stationary phase cultures, which are more concentrated than the log phase cultures.
2. In this experiment, you will prepare a series of slides, each of which contains two different samples for easy comparison. The slides are large enough to accommodate two samples (and two coverslips). In your notebook, be sure to record which sample is closer to the frosted area on the slide. (If your slide does not have a frosted area, mark one end of the slide with a magic marker.) First, let's compare the two yeast species. Prepare a slide with log phase cultures of *S. pombe* and *S. cerevisiae*. Spot 5 μL of each cell suspension and cover each sample with a coverslip.
3. Use the same sequence of microscope adjustments that you used in the ruler exercise to visualize the cultures. Start at low magnification and gradually increase the magnification, making changes in the condenser diaphragm as needed. (Play with the position of the aperture diaphragm a bit to maximize the quality of the image. The cultures have not been stained, so they may show up better with less light than you expect.) In your notebook, draw some examples of the forms that you see in the cultures and the relative proportions of each form. Comment on both the sizes and shapes of the cells.
4. Rinse the slide and coverslips with deionized water and dry them with a paper towel. Spot 5 μL of a stationary phase culture of *S. cerevisiae* on the slide and apply a coverslip. For the second spot, combine 2.5 μL of stationary phase cells with 2.5 μL of log phase cells. *Can you distinguish stationary phase cells from exponentially growing cells?* Comment on any differences in size and morphology.
5. Rinse and dry the slide and coverslips. Prepare a third slide with *S. pombe* cultures similar to the one that you prepared in step 4.

Chapter 6

Genetic Analysis



The growth properties of mutant organisms can often provide information about the gene products involved in biochemical pathways within cells. In this experiment, you will use selective media to identify which of the *MET* genes have been inactivated in yeast strains that are unable to grow in the absence of methionine.

Objectives

- Become familiar with the biochemical pathways involved in methionine synthesis
- Understand how selective media are used in genetics
- Design and carry out a strategy to distinguish *met* mutants by their nutritional requirements
- Spot plate yeast *met* strains on selective media containing different sources of organic sulfur

Chapter 6

Introduction

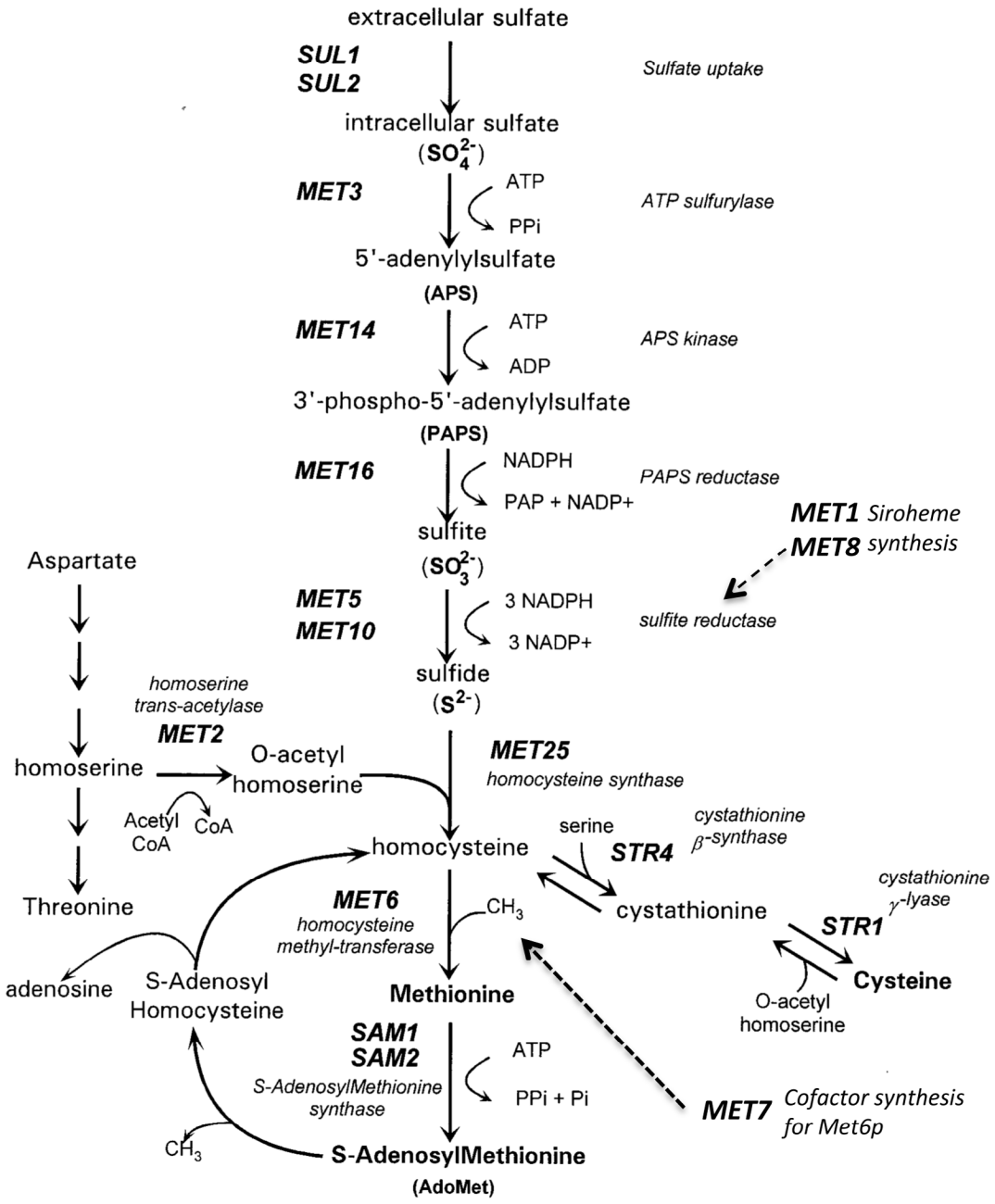
Mutant organisms provide powerful tools to study biochemical pathways in living cells. This semester, we are working with yeast strains that are unable to synthesize methionine because one of the genes involved in the biosynthetic pathway has been inactivated. Methionine is an essential amino acid for all organisms. Consequently, these *met* mutants must receive methionine or another compound that they can convert to methionine from their diet.

The biochemical pathway for methionine synthesis in yeast has been elucidated from the combined experiments of many different biochemists and geneticists. In each of our *met* mutant strains, a *MET* gene has been replaced with a bacterial kanamycin resistance gene by homologous recombination (Gelperin et al., 2005). Depending on the position of the missing gene in the methionine biosynthetic pathway, which is shown on the opposite page, these *met* mutants may be able to grow in the absence of methionine if another source of organic sulfur is present. In this lab you will use selective media to identify the affected *MET* gene in three different mutant strains.

Genetic nomenclature

When working with strains, it's important to use the correct genetic nomenclature. Pay close attention to italics and capital letters. Gene names are placed in italics, while proteins and phenotypes are referred to with normal font. Gene names that begin with capital letters refer to dominant alleles, while gene names beginning with lower case letters refer to recessive alleles. (One oddity about budding yeast: *S. cerevisiae* gene names are unique in that dominant alleles are described with three capital letters. In almost all other species, dominant alleles would be referred to as *Met6* with only the first letter capitalized.) Gene names consist of three letters, followed by a number. There may be many different gene names that begin with the same three letters, e.g. there are over 20 different *MET* genes in *S. cerevisiae*, but the number at the end of the gene name is specific for a particular gene. If some molecular detail is available for a particular mutant allele, the number may be followed by a hyphen and additional information (see below).

As an example, let's look at the nomenclature that would be used for the *MET6* gene from *S. cerevisiae*. *MET6* is one of over twenty different *MET* genes. The *MET* prefix is used because most of the *MET* genes were isolated in genetic screens based on the inability of mutant strains to live in the absence of methionine. The *MET6* gene acquired its name after genetic complementation studies, but before its gene product had been identified as homocysteine methyltransferase, the last step in methionine synthesis. The list below describes the naming conventions for genes, proteins, and strains related to *MET6*. These same rules apply for other genes in *S. cerevisiae* as well.



Methionine biosynthesis in yeast.
The proteins catalyzing individual steps in methionine biosynthesis are listed next to each step in the pathway. The names of the genes encoding the activities are shown in capital letters, following S. cerevisiae conventions. The *MET1* and *MET8* genes encode proteins that are involved in synthesizing siroheme, an essential cofactor for sulfite reductase. The *MET7* gene product is involved in synthesizing a different cofactor for Met6p, homocysteine methyltransferase.

MET6	Dominant allele of the *MET6* gene or the chromosomal locus
met6	Recessive allele of the *MET6* gene (allele found in a *met6* mutant)
met6-12	Recessive allele - number after the parentheses refers to specific mutation
met6-Δ1	Recessive allele - *met6* allele has a specific deletion (*Δ* indicates a deletion)
met6::LEU2	Recessive allele -insertion of a dominant *LEU2* gene into the *MET6* locus on the chromosome has inactivated the host *MET6* gene
Met6p	Protein encoded by the *MET6* gene, i.e. homocysteine methyltransferase

To write the genotype of a particular strain, begin with the mating type and follow with the mutant alleles in the strain. For example, we are using met strains constructed by inserting a bacterial kanamycin resistance gene into yeast strain BY4742, which is auxotrophic for histidine, leucine, lysine and uracil. Thus, the genotype of our met6 mutant would include the BY4742 mutations and be written: *MAT***a** *his3-Δ1 leu2Δ0 lys2Δ0 ura3Δ0 met6::KAN*^{R}
(Note: *MAT***a** has a unique designation in that the "a" is not italicized and is shown in bold font.)

Auxotrophs and selective media

The *met* mutants are methionine auxotrophs, meaning that they are unable to grow in media that does not contain methionine. Auxotrophic strains are very useful in the genetics laboratory. Many laboratory strains carry multiple mutations that interfere with the synthesis of essential nutrients. The BY4742 strain, for example, carries mutations that affect the synthesis of histidine, leucine, lysine and uracil. These compounds must therefore be provided by the culture medium. Researchers often use auxotrophic strains as hosts for plasmid transformation (Chapter 12). The plasmids used for transformation carry functional alleles of the gene that is defective in the host strain, making it possible to identify transformants by their ability to grow on media lacking an essential nutrient.

Synthetic media are an essential tool for culturing and studying auxotrophs, because all of the components are defined. Yeast researchers have developed a variety of different formulations for synthetic media. All synthetic media contain a carbon source (usually D-glucose), a nitrogen source, and essential vitamins and minerals. The vitamins and minerals are usually purchased in a formulation known as yeast nitrogen base (YNB). The supplements added to synthetic media vary widely and are tailored to support or select against the growth of particular genotypes. In this course, we will use a minimal medium that supplies only those supplements required for survival. The growth rate of wild type strains in minimal media is considerably slower than that in rich media like YPD, but the strains survive. The table on the following page shows the composition of a defined medium with a rich supply of amino acids and nucleotide bases. This medium, known as YC, supports the growth of most laboratory strains (http://labs.fhcrc.org/gottschling/Yeast%20Protocols/yc.html).

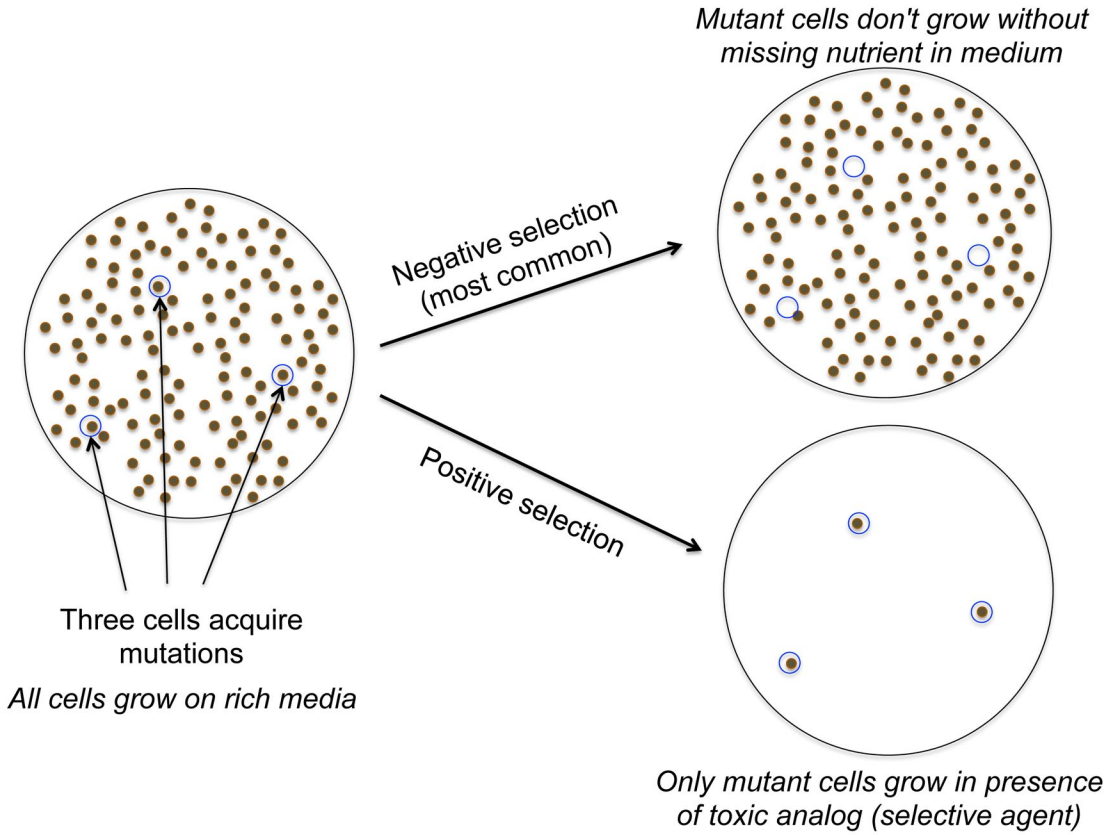
Composition of Yeast Complete (YC) Medium					
Component	**grams/liter**	**Component**	**mg/liter**	**Component**	**mg/liter**
YNB*	1.7	arginine	100	tyrosine	50
$(NH_4)_2SO_4$	5	aspartic acid	50	lysine	100
D-glucose	20	isoleucine	50	methionine	50
		phenylalanine	50	tryptophan	100
		proline	50	leucine	100
		serine	50	histidine	50
		threonine	100	uracil	10
		valine	50	adenine	10

YNB is a complex mixture of vitamins, minerals and salts. Final concentrations in YC:
Vitamins (µg/liter): biotin (2), calcium pantothenate (400), folic acid (2), inositol (2000), niacin (400), p-aminobenzoic acid (200), pyridoxine hydrochloride (400), riboflavin (200), thiamine hydrochloride (400).
Minerals (µg/liter): boric acid (500), copper sulfate (40), potassium iodide (100), ferric chloride (200), manganese sulfate (400), sodium molybdate (200), zinc sulfate (400).
Salts (mg/liter): potassium phosphate monobasic (1000), magnesium sulfate (500), sodium chloride (100), calcium chloride (100).

Genetic screens

Synthetic media provide an important tool for genetic screens designed to identify mutants in biochemical pathways. Classical genetic screens begin with a mutagenesis, in which the parent strain is treated with a chemical or radiation that induces mutations in DNA. Yeast, like all organisms, have a low spontaneous mutation rate of ~10^{-8}/base/generation. The yeast genome is ~12 Mbp, so most cells undergo cell division without having accumulated a mutation. The spontaneous mutation rate would be much too low for a practical genetic screen, so investigators adjust the mutagen dose to one that kills up to ~50% of the cells. The mutagenized cells are then used for the genetic screen. In the screen, large populations of cells (many plates with many colonies per plate) are plated on selective media that will identify mutants. The easiest kinds of screens employ positive selection, which allows only mutant cells to grow. More often, investigators are analyzing pathways important for cells to grow - in these cases, mutant cells will fail to grow on selective media. In these cases, which employ negative selection, cells are first plated on permissive media that allows all cells to grow. Replicas of these master plates are made, and copies of the colonies are then plated on both permissive and restrictive (selective) media. Since only wild-type cells grow on the restrictive media, researchers look for colonies on the permissive plate that are missing on the restrictive plate. These negative screens are more difficult to do than screens that use positive selection, but they are critically important for sorting out essential

biochemical pathways. The figure below provides a graphical comparison of genetic screens using positive and negative selection strategies.



Selection strategies used to isolate yeast mutants.
After the initial mutagenesis, yeast are grown on a plate containing rich (or complete synthetic) media. In this figure, the mutagenesis has generated three different mutants in the gene of interest. The mutant colonies are surrounded by an empty circle. Replicas of the master plate are copied to selective media. In a negative selection scheme, the selective plate lacks a component that is normally present in complete synthetic media. In a positive selection scheme, the media contains a selective agent, which is toxic to normal cells, but tolerated by mutant cells. The selective agent is often a toxic analog of a normal cellular metabolite.

The genes required for methionine synthesis were primarily discovered with negative selection schemes. Wild type yeast are able to synthesize methionine using only sulfate as a sulfur source, but *met* mutants require an external source of methionine (or an alternative source of organic sulfur) to grow. Large numbers of met mutants were isolated in a variety of screens carried out in different laboratories. Investigators exchanged strains, did complementation experiments, and performed biochemical analyses that elucidated the metabolic pathways involving methionine.

Exercise 1 - Predicting growth properties of met strains

The *met* mutants that we are using for our experiments were derived from the parent strain BY4742, which has the genotype *MATa his3-Δ1 leu2Δ0 lys2Δ0 ura3Δ0*. Each strain contains one additional mutation in a *MET* gene. **The minimal media (MM) that we will be using this semester all contain leucine, lysine and histidine.** Depending on the experiment, we will also use plates supplemented with methionine and/or uracil.

Normally, yeast are able to synthesize methionine *de novo*, using sulfate as the sulfur source. The sulfur source in MM is ammonium sulfate. A mutation in a *MET* gene, however, will prevent a strain from using sulfate as a sulfur source. Depending on the position of the disrupted *MET* gene in the biosynthetic pathway, *met* mutants may be able to grow on different sources of sulfur (Thomas and Surdin-Kerjan, 1997).

In this experiment, you will use synthetic media to distinguish between various *met* strains. Construct a table in your notebook similar to the one below that predicts the ability of *met* strains (*met1,2,3,5,6, 7,8,9,10,14,16,25*) to grow in the media shown at the top of each column. Place a plus (+) when you predict that the strain will grow on the plate and a minus (-) when you do not expect the strain to grow.

	YPD	**MM**	**MM+Met**	**MM+Ura**	**MM+Ura +Met**	**MM+Ura +Cys**	**MM+Ura +SO_3**
met1	+						
met2	+						
etc...							

Which strains are able to grow using cysteine as the sole sulfur source?

Which strains should be able to grow with sulfite as the sole sulfur source?

Chapter 6

Exercise 2 - Identifying strains by nutritional requirements

Your team will be given three strains, each of which carries a different met mutation. You will be given the names of the mutations, but you will not know which mutation corresponds to each strain. You will also be given a series of selective plates containing some of the media shown in the table in Exercise 1. Prepare spot plates to distinguish the three strains that your team has been given, following the procedure in Chapter 3.

Each member of the team should prepare serial dilutions of a single strain.

1. Spot your dilution series on each of the plates that your team received. Spot the complete dilution series on one plate before proceding to the second plate.

2. Use the same pattern of strains/rows on each of the different selective plates. ***Make sure that the plates are properly labeled so that you can identify which strain corresponds to which row!***

3. Incubate the plates at 30 °C for at least 3 days, when colonies should become apparent. Note the size of the colonies. Rapidly growing strains produce larger colonies than slowly growing strains. Record any observations in your notebook. Note that it may take longer than 3 days for some of the strains to produce single colonies.

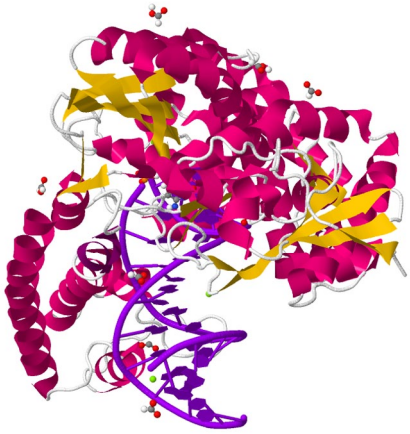
References

Gelperin, D.M. White, M.A., Wilkinson, M.L., Kon, Y., Kung, L.A., Wise, K.J., Lopez-Hoyo, N., Jiang, L., Piccirillo, S., Yu, H., Gerstein, M., Dumont, M.E., Phizicky, E.M., Snyder, M., and Grayhack, E.J. (2005). Biochemical and genetic analysis of the yeast proteome with a movable ORF collection. *Genes Develop.* **19**: 2816-2826.

Thomas, D., and Surdin-Kerjan, Y. (1997). Metabolism of sulfur amino acids in Saccharomyces cerevisiae. *Microbiol. Mol. Biol. Rev.* **61**, 503-532.

Chapter 7

Yeast Colony PCR



The *S. cerevisiae met* strains that we are using this semester were constructed as part of the *Saccharomyces* Gene Deletion Project. In the project, yeast investigators systematically replaced each ORF in the yeast genome with an ORF-specific cassette containing a bacterial kanamycin resistance (KAN^R) gene. In this lab, you'll design and carry out PCR reactions to identify which *MET* genes have been replaced by KAN^R genes in your yeast strains.

Objectives

- Understand the reactions occuring at the different temperatures used in PCR
- Understand the principles of primer design and hybrization
- Understand how to optimize PCR conditions for specific products
- Use PCR to distinguish between yeast colonies with different genotypes

Chapter 7

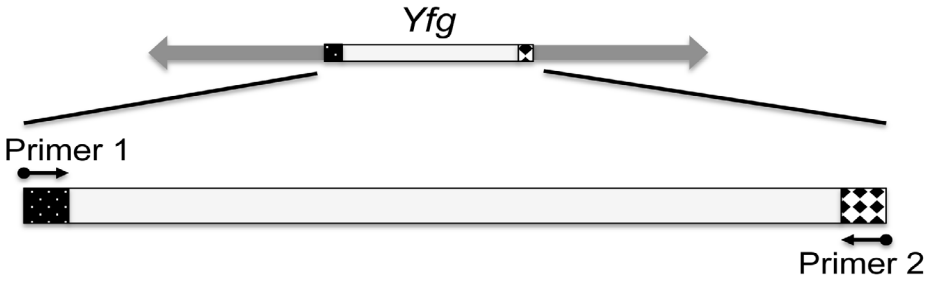
In this lab, you will use the polymerase chain reaction (PCR) to identify YMP met strains. This chapter begins an overview of the PCR and the *Saccharomyces* Gene Deletion Project. You will use this knowledge to design a PCR strategy for identifying the disrupted *MET* gene in a yeast colony.

Polymerase chain reaction overview

The polymerase chain reaction (PCR) revolutionized molecular biology. With PCR, researchers had a tool for amplifying DNA sequences of interest from vanishingly small amounts of material. Indeed, billions of copies can be synthesized from a single DNA molecule in a typical PCR reaction. The development of PCR grew out of research on DNA polymerases and the discovery of thermostable DNA polymerases able to withstand extended heat treatments that denature most proteins (Sakai et al., 1988). Today, PCR is a standard technique that is widely used to analyze DNA molecules and to construct novel recombinant molecules.

Thermostable DNA polymerases are central to PCR. The first description of PCR used a DNA polymerase from *E. coli*, which denatured and had to be replaced after each round of DNA synthesis (Sakai et al., 1985). The procedure was much-improved by replacing the *E. coli* polymerase with a DNA polymerase from *Thermus aquaticus*, a bacterium that thrives in thermal springs at Yellowstone National Park. The *T. aquaticus* DNA polymerase, or *Taq* polymerase, functions best at temperatures of 70-75˚C and can withstand prolonged (but not indefinite) incubation at temperatures above 90˚C without denaturation. Within a few years, the *Taq* polymerase had been cloned and overexpressed in *E. coli*, greatly expanding its availability. Today, the selection of polymerases available for PCR has increased dramatically, as new DNA polymerases have been identified in other thermophilic organisms and genetic modifications have been introduced into *Taq* polymerase to improve its properties.

PCR involves multiple rounds of DNA synthesis from both ends of the DNA segment that is being amplified. Recall what happens during DNA synthesis: a single-stranded primer binds to a complementary sequence in DNA. This double-stranded region provides an anchor for DNA polymerase, which extends the primer, ALWAYS traveling in the 5' to 3' direction. Investigators control the start sites for DNA replication by supplying oligonucleotides to serve as primers for the reaction (shown below for *Your favorite gene Yfg*). To design the primers, investigators need

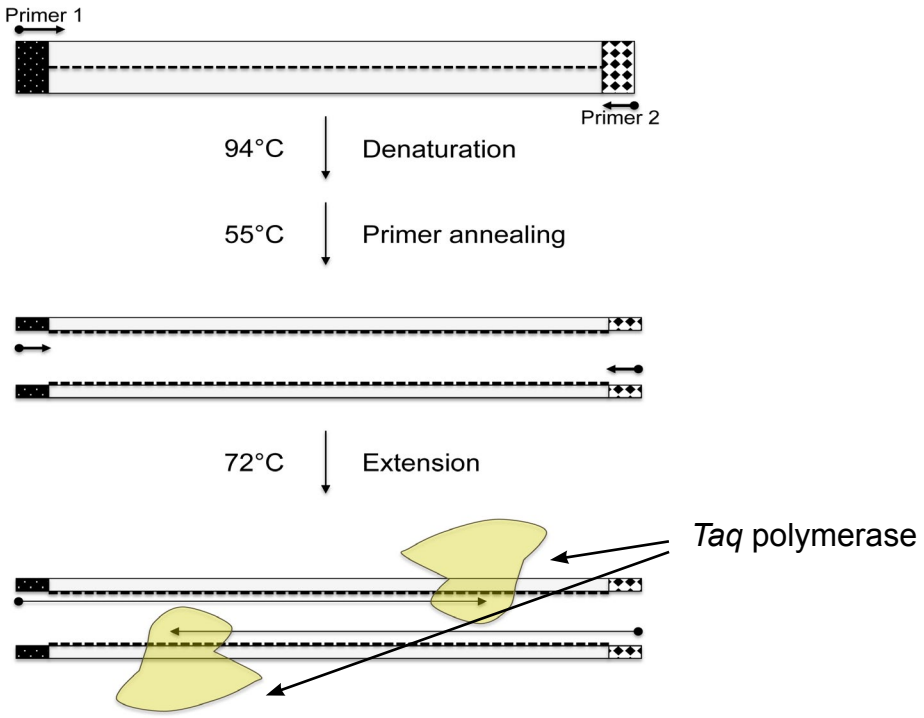


accurate sequence information for the DNA segment of the target DNA. One primer binds each strand of the DNA helix. Because the strands in a DNA helix are anti-parallel, DNA polymerases travel in opposite directions when they copy the Watson and Crick strands of DNA.

PCR reactions typically begin with an initial period in which the reaction mixture is heated for several minutes at a temperature high enough to break the hydrogen bonds that hold the two strands of the DNA helix together. Effective denaturation of DNA is critical, because DNA polymerase requires single-stranded DNA to serve as a template. The first cycle of denaturation is longer than subsequent denaturation steps, because PCR templates are often long, complex molecules held together by many hydrogen bonds. In subsequent cycles, shorter templates will predominate (see below).

Following the initial denaturation, PCR involves a series of 30-35 cycles with three segments, as outlined and discussed in greater detail below.

- A denaturation step - commonly 94˚C
- A primer annealing step - commonly 55˚C
- An extension step - commonly 72˚C



PCR reactions include multiple cycles at three different temperatures

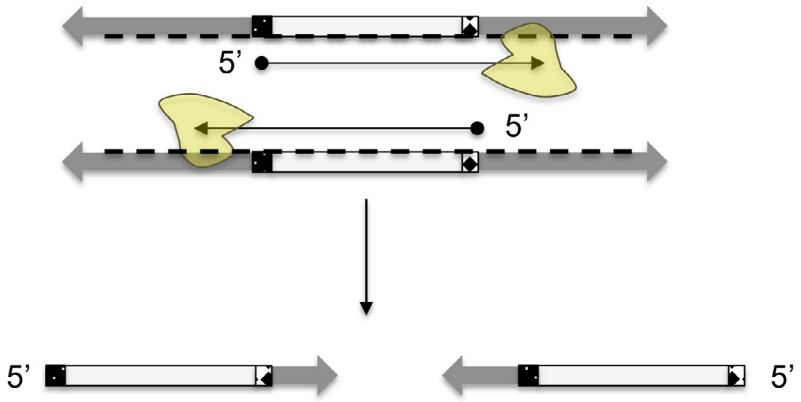
Each cycle of a PCR reaction includes three different temperatures. During the first denaturation step, the hydrogen bonds holding DNA helix together are broken. In the following annealing step, oligonucleotide primers bind to single-stranded template molecules, providing starting points for processive DNA polymerases that will extend the primer sequence. DNA polymerases become more active at the extension temperature, which is close to its temperature optimum. Investigators frequently adapt the temperatures and times of the steps above for different primers, templates and DNA polymerases.

In the early days of PCR, investigators (or their students!) manually transferred tubes between three water baths programmed for one of the three temperatures used in the procedure. Today, PCR is performed in automatic thermocyclers that rapidly adjust temperatures in a block containing the PCR reactions.

PCR products accumulate exponentially

PCR is indeed a chain reaction, since the DNA sequence of interest roughly doubles with each cycle. In ten cycles, a sequence will be amplified ~1000 fold (2^{10}=1024). In twenty cycles, a sequence will be amplified ~million fold. In thirty cycles, a sequence can be theoretically amplified ~billion fold. With this tremendous amount of amplification, it's not hard to see how a single molecule can be detected in a biological (crime scene?) sample by PCR. PCR reactions in the lab typically involve 30-35 cycles of denaturation, annealing and extension. To understand PCR, it's important to focus on the first few cycles. The first two cycles set the stage. Exponential amplification of the desired end-product only begins in the third cycle.

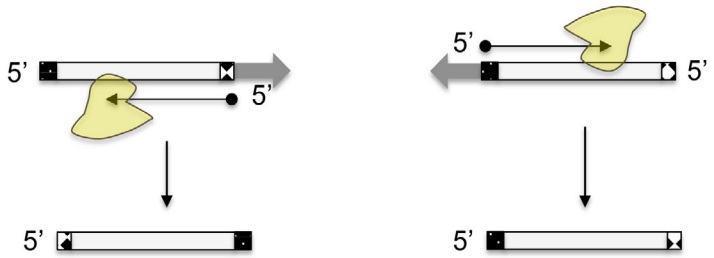
During the first cycle, the thermostable DNA polymerases synthesize DNA, extending the 3' ends of the primers. DNA polymerases are processive enzymes that will continue to synthesize DNA until they literally fall off the template. Consequently, the complementary DNA molecules synthesized in the first cycle have a wide variety of lengths. Each of the products, however, has a defined starting position, since it is "anchored" in the primer sequence. These "anchored" sequences will become templates for DNA synthesis in the next cycle, when fragments of the intended length first appear.



First cycle of PCR
During the first PCR cycle, DNA polymerases synthesize complementary strands of the template DNA, beginning at the primer site. The lengths of the products are quite variable and depend on the processivity of the DNA polymerase.

The starting template for PCR will continue to be copied in each subsequent cycle of PCR, yielding two "anchored" products with each cycle. Because the lengths of the anchored products are quite variable, they will not be detectable in the final products of the PCR reaction.

DNA strands of the desired length first appear during the second cycle. Replication from these "anchored" fragments begins at one primer site and terminates at the second primer site. The number of these defined length fragments will double in each new cycle and quickly become the predominant product in the reaction.



PCR fragments of the desired length first appear in the second cycle.
The "anchored" fragments generated during the first cycle of replication begin with either the primer 1 or primer 2 sequence. During the second cycle, replication begins at the other primer site, generating a PCR fragment capped at both ends with primer sequences.

Most PCR protocols involve 30-35 cycles of amplification. In the last few cycles, the desired PCR products are no longer accumulating exponentially. As in any enzymatic reaction, substrates have been depleted and the repeated rounds of incubation at 94˚C have begun to take a toll on Taq polymerase, which begins to denature.

Primer annealing is critical to specificity in PCR

Good primer design is critical to the success of a PCR reaction. PCR works best when the primers are highly specific for the target sequence in the template DNA. Mispriming occurs when primers bind to sequences that are partially complementary, causing DNA polymerase to copy the wrong DNA sequences. Fortunately, investigators are able to adjust experimental parameters to maximize the probability that primers will hybridize with the correct targets. Researchers usually begin by focusing on the melting temperature of the primer-target DNA hybrid and the ratio of target to primer molecules.

PCR primers used are typically synthetic oligonucleotides between 18 and 25 bases long. When designing primers, researchers consider its T_m, the temperature at which half of the hybrids formed by the primer will melt. In general, the thermal stability of a hybrid increases with the length of the primer and its GC content. The following formula provides a rough estimate of the T_m of oligonucleotide hybrids. In this formula, *n* refers to the number of nucleotides, and the concentration of monovalent cations is expressed in molar (M) units.

$$T_m = 81.5^{\circ}C + 16.6\,(\log10[K+ + Na+]) + 0.41\,(\%[G + C]) - (675/n)$$

When possible, researchers design primers that are similar in length and have a 40-60% GC composition. The sequences are designed so that the T_ms of the primer-DNA hybrids are a few degrees higher than the annealing temperature. Adjusting the T_ms of the primers to be close to the annealing temperature favors specific hybrids over less specific hybrids that may contain a

few mismatched bases. A hybrid formed between a primer and a non-target sequence with even one mismatched base has a T_m that is lower than that of the fully hydrogen-bonded hybrid. When mis-priming appears to be a problem in a PCR reaction, investigators have several options. They can increase the length and/or GC content of the primers, alter the salt concentrations (results may be hard to predict) or increase the annealing temperature.

When designing a PCR reaction, investigators also consider the nature of the template DNA. A variety of DNA templates can be used for PCR. Depending on the purpose of the experiment, investigators could choose to use genomic DNA, a plasmid or a cDNA (complementary DNA generated by a reverse transcriptase from mRNA). PCR can also be done with much cruder preparations of DNA, such as a bacterial or yeast colony. The more complex the template (its length in bp), the greater the probability that it will contain another sequence that is very similar to a primer sequence. For example, the haploid yeast genome is 12 Mbp long and contains only one copy of each *MET* gene. The probability of a non-target sequence in the yeast genome that is similar to a 20-nucleotide primer for a *MET* gene is reasonably good. A 12 kbp plasmid carrying the same *MET* gene, however, will have one copy of the gene per plasmid molecule. Thus, the same quantity of yeast genomic DNA will contain 1/1000 the number of desired target molecules and many more non-target competitors than plasmid DNA. With complex templates such as genomic DNA, investigators can sometimes reduce the impact of mismatched hybrids by decreasing the amount of template DNA in the reaction. (More is not always better.....)

The components of a PCR reaction are simple, consisting of the DNA template, primers, dNTPs, a buffer containing $MgCl_2$ (polymerases use dNTPs complexed with Mg^{2+}), and the thermostable polymerase. For our experiments, we will be using a master mix that contains all of the components except the template DNA and the primers. The use of a master mix ensures that all reactions have identical reagents and it also reduces the number of transfers requiring micropipettes. The smaller number of transfers is particularly advantageous, because it reduces the opportunities for cross-contamination of reagents. PCR is an exquisitively sensitive procedure. Many researchers use special barrier tips for their micropipettes, which contain filters that prevent samples from reaching the barrel of the micropipettes.

Exercise 1 - Design PCR primers for a MET gene

In one of the next lab sessions, you will begin using plasmids that contain the sequence of your *MET* gene (Gelperin et al., 2005). During the construction of the plasmids, the *MET* sequences were amplified with PCR and then cloned into the pBG1805 overexpression plasmid (more on pBG1805 later). The pBG1805 plasmids drive overexpression of Metp proteins with 167 additional amino acids at their C-termini. To amplify the coding sequences for Metp proteins from genomic DNA, Gelperin et al. used a forward primer containing the initiator codon (ATG) and a reverse primer containing the complementary sequence for the last several codons from the *MET* gene, but not the stop codon. This exercise will bring you through the thought process that the investigators used.

1. Find the summary page for your gene in the SGD.
   Under the Sequence Information section, retrieve the CDS for your gene.

2. Design a forward primer. Copy the first 25 nucleotides in your notebook.
   (*Hint: experienced molecular biologists usually write the letters in groups of 3*)
   Mark the 5' and 3' end of the sequence.

   Aim for a primer that is 40-60% GC and has a T_m between 55 and 60˚C. If possible, try to have your primer end with a "G" or a "C." Such a "GC-clamp" at the end of a primer facilitates DNA polymerase binding and extension of the primer.

   Use an online calculator to estimate the Tm of your primer. Some popular calculators can be found at:
   http://www.basic.northwestern.edu/biotools/oligocalc.html
   http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/

   Record the sequence of the primer that you would recommend using.

3. Design a reverse primer.
   Copy the last 30 nucleotides in your notebook. Mark the 5' and 3' end of the sequence.

   Put a box around the stop codon at the end of the CDS. The stop codon will not be included in the primer, because the plasmid codes for a fusion protein with additional amino acids at the C-terminus of the Met protein.

   Write the complementary sequence, marking the 5' and 3' ends.

   Suppliers of oligonucleotides follow normal conventions, in that nucleotide sequences are written from 5' to 3' (left to right). If the sequence above is not written with the 5' end at the left, rewrite it.

   Use the oligo calculator to estimate the Tm of your primer.

   How well-matched are your forward and reverse primers? Why or why not?

   What would you do to change either the forward or reverse primer?

Chapter 7

Exercise 2 - Assessing potential problems in PCR

In this exercise, you will use one of the primers that you designed in Exercise 1 to look for sequences in the yeast genome that are partially complementary to your primer sequence and may therefore cause false priming in the PCR reaction. The most like sequences to cause mispriming are sequences with identical nucleotides in the 3' half of the sequence. Researchers often find BLAST to be a useful tool in assessing potential primer binding to non-target sequences.

- Direct your browser to the NCBI site and access the BLAST tool. Select Nucleotide BLAST. Paste one of your primer sequences into the query sequence box.
- Choose the search set. You want to limit the search to RefSeq NC_ records for yeast. Select the Reference genomic sequences database. Under organism, enter Saccharomyces cerevisiae S288c. Click BLAST.

The BLAST results bring up a table with a line for each of the *S. cerevisiae* chromosomes, listed in descending order according to the goodness of the alignments. The top match should be to the chromosome locus where your gene is located! Each chromosome should have multiple matches to your primer sequence, but the alignments will be relatively short. Do a quick survey of the results for each chromosome. List only those sequences where 10 or more nucleotides to the 3' end of the primer match the query sequence. (*Hint: If you are using a 25-nucleotide primer sequence as the query, the last number of the query in the graphic alignments should be 25.*) A 10-nucleotide sequence is expected to occur by chance alone (4^{10}) about once for every million base pairs. Complete the table below, noting the number of sequences with ten or more perfectly matched bases at the 3' end of the primer.

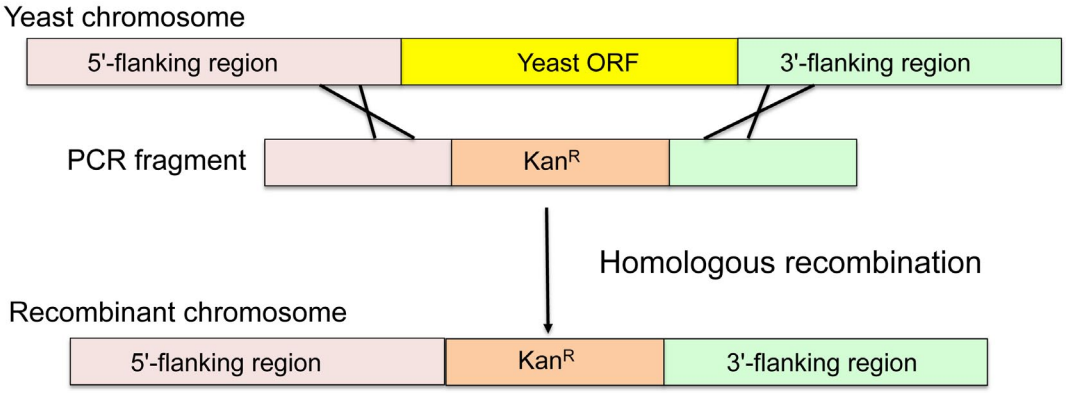
Chromosome	**Length (kbp)**	**Matches with 10+ bp**	**Perfect match**
I (A)			
II (B)			
III (C)			
IV (D)			
V (E)			
VI (F)			
VII (G)			
VIII (H)			
IX (I)			
X (J)			
XI (K)			
XII (L)			
XIII (M)			
XIV (N)			
XV (O)			
XVI (P)			

This exercise has hopefully pointed out the difficulties of designing PCR primers that can be used with complex templates, such as genomic DNA. The *Saccharomyces* Genome Deletion Project grappled with these issues as they set out to make systematic deletions to the *S. cerevisiae* ORFs.

Saccharomyces Genome Deletion Project

The publication of the yeast genome sequence opened new opportunities for yeast geneticists. Knowing the DNA sequence of the yeast genome, geneticists could take advantage of the high frequency with which yeast exchange genes using homologous recombination to generate mutants of their own design. Homologous recombination normally occurs during meiosis and during certain kinds of DNA repair. During homologous recombination, two closely related DNA sequences align with one another, the DNA strands break and strand exchange occurs when the breaks are healed. Investigators who want to use homologous recombination for gene replacement use molecular cloning to construct a replacement cassette in which a marker gene is flanked on either side by sequences that flank the gene to be replaced in the target chromosome.

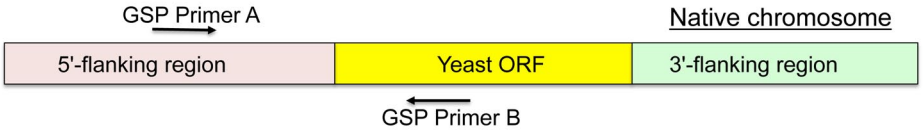
The strains that we are using were generated by this approach during the *Saccharomyces* Genome Deletion Project (Winzeler et al., 1999). After the publication of the DNA sequence, yeast researchers began to systematically replace all of the predicted ORFs in the *S. cerevisiae* genome with a kanamycin resistance (*KAN*^{R}) gene. The figure below outlines the process used to generate the deletion strains. For each ORF, researchers used a series of PCR reactions to construct cassettes in which the *KAN*^{R} gene was flanked at either site by short DNA sequences upstream and downstream of the targeted ORF on the *S. cerevisiae* chromosome. The PCR fragments were used to transform the BY4742 strain of *S. cerevisiae*, and strains that had incorporated the *KAN*^{R} gene were selected on plates containing kanamycin.



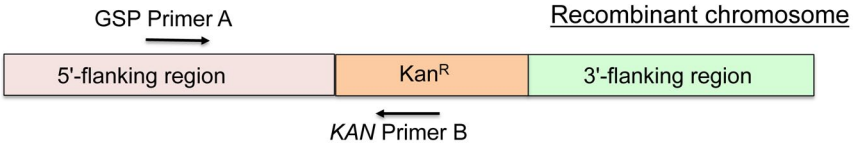
All of the deletion strains were analyzed by PCR to confirm that the targeted ORF was replaced by the *KAN*^{R} cassette. Thus, we can be reasonably certain about the genotypes of our *met* mutants. The Deletion Project also verified that strains were able to grow and mate, showed the appropriate auxotrophies and had functional mitochondria. (See the Deletion Project website for additional details: http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html.) With the large number of strains generated in the project, it was not possible to do a thorough phenotypic analysis of individual strains. Those analyses are left for you to do!

Exercise 3 - Design yeast colony PCR reactions

In this laboratory, we will use the primers designed by the *Saccharomyces* Deletion Project to analyze the *MET* gene disruptions in your yeast strains. As shown in the figure below, we have obtained two gene-specific primers (GSP) for each of the *MET* genes that we are working with. One of the GSPs, GSP Primer A, is located 200-400 bp upstream of the initiation codon. The second GSP, GSP Primer B, is an antisense primer that binds the ORF. We also have an antisense primer that binds 250 bp within the *KAN*^{R} gene (*KAN* Primer B).



Gene-specific (GSP) primers A and B give a product from the native chromosome, but not the recombinant chromosome



Gene-specific (GSP) primer A and KAN primer B give a product from the recombinant chromosome, but not the native chromosome

Use your results from the selective plating experiment to devise a strategy that will allow you to positively identify your met yeast strains. Each team will be able to perform six PCR reactions. List the primer pairs that you will use for the reactions.

Exercise 4 - Yeast colony PCR

Each team will be able to do six PCR reactions. You'll first mix the primer pairs with a VERY SMALL number of yeast cells that you transfer from a colony to the tube with the tip of a P-20 or P-200 micropipette. The colony and primers will then be heated at 98˚C for 15 minutes to disrupt the yeast cells. At that point, you will add an equal volume of a PCR master mix, containing nucleotides and the *Taq* polymerase, to each tube. The tubes will then be returned to the thermocycler for a typical PCR reaction.

1. ***Label the PCR tubes.*** The tubes are very small, so develop a code that you can use to identify the tubes. (Don't forget to include the code in your notebook. The code should indicate which primers and strains are mixed in each tube.)

2. ***Prepare the primer mixture.*** The final volume of the PCR reactions will be 20 µL. The primer mixture accounts for half the final volume, or 10 µL. The primers stock concentrations are 2.0 µM each. Pipette 5.0 µL of the two primers that you would like to use into each PCR tube. *What will the final concentration of each primer be in the actual PCR reaction?*

NOTE: Because of the extraordinary sensitivity of PCR reactions, it is very important not to cross-contaminate tubes with primers that don't belong in a reaction. To save pipette tips, pipette the water into the tubes first. Change tips between every primer transfer that you do.

3. ***Transfer a small quantity of yeast cells to each PCR tube.*** Lightly touch the tip of a P20 or P200 micropipette to a yeast colony. Twirl the micropipette tip in the primer tip to release the cells. The most common error is transferring too many yeast cells, which will interfere with the PCR reaction. The appropriate amount of yeast would fit on the tip of a pin.

4. ***Lyse the yeast cells.*** Place the tubes in the thermocycler for 15 min at 98˚C.

5. ***Set up the PCR reactions.*** Remove the tubes from the thermocycler and add 10 µL of PCR master mix to each tube.

6. ***Amplify the target gene sequences.*** Return the tubes to the thermocycler and start the PCR program.

95°C for 2 minutes	
95°C for 30 sec.	
55°C for 30 sec.	Repeat 35 times
72°C for 1 minute	
72°C for 10 minutes	

Store the reactions at 4°C for subsequent analysis.

Chapter 7

References

Gelperin, D.M. White, M.A., Wilkinson, M.L., Kon, Y., Kung, L.A., Wise, K.J., Lopez-Hoyo, N., Jiang, L., Piccirillo, S., Yu, H., Gerstein, M., Dumont, M.E., Phizicky, E.M., Snyder, M., and Grayhack, E.J. (2005). Biochemical and genetic analysis of the yeast proteome with a movable ORF collection. *Genes Develop.* **19**, 2816-2826.

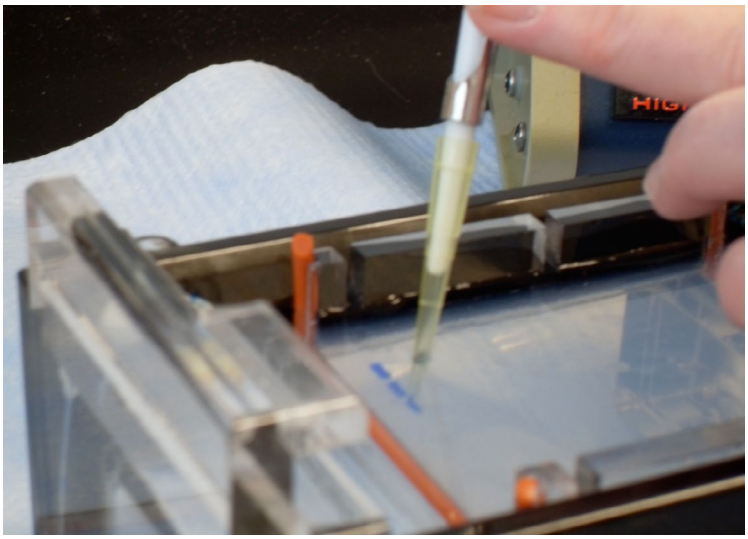
Sakai, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., and Amheim, N. (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**: 1350-1354.

Sakai, R.K., Gelfand, D.H., Stofel, S., Scharf, S.J., Higucki, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**: 487-491.

Winzeler, E.A., Shoemaker, D.D., Astromoff, A., Liang, H., Anderson, K., Andre, B., Bangham, R. *et al.* 1999. Functional characterization of the *Saccharomyces cerevisiae* genome by gene deletion and parallel analysis. *Science* **285**: 901-906.

Chapter 8

Agarose gel electrophoresis



Agarose gels are used to analyze DNA molecules. These gels are simple to construct, because they rely only on the gelling properties of agarose. Molecules are separated by size and visualized with fluorescent intercalating dyes. In this lab, you'll analyze the products of the PCR reactions from the previous lab.

Objectives

- Prepare agarose gels for separating DNA molecules
- Separate DNA molecules by electrophoresis
- Visualize DNA molecules on gels using intercalating dyes
- Calculate the size of DNA molecules using molecular weight standards

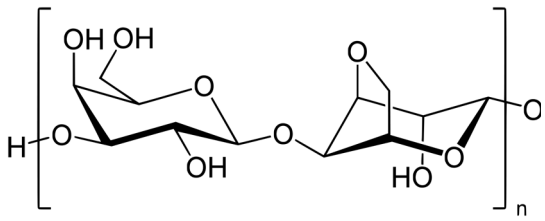
Agarose gel electrophoresis

Background

Agarose gels provide a simple method for analyzing preparations of DNA. Although the base compositions of individual DNA molecules vary, the basic chemical structure of DNA is the same for all DNA molecules. DNA molecules share the same charge/mass ratio, which imparts similar electrophoretic properties to DNAs of widely varying lengths.

Agarose gels are porous matrices

Agarose is a polysaccharide purified from red algae, or seaweed. Agarose is more highly purified (and significantly more expensive!) than agar, which is obtained from the same seaweed. Agarose molecules are long, linear polymers of the repeating disaccharide (1 3)-β-D-galactopyranose-(1 4)-3,6-anhydro-α-L-galactopyranose (right). A typical agarose molecule contains over one hundred monomers. The agarose used for electrophoresis has been highly purified. The purification process removes contaminants that would interfere with the enzymes used in molecular cloning, such as restriction endonucleases. The process also generates an agarose preparation with desirable electrophoretic properties and minimal background fluorescence, which is important for visualizing DNA molecules.



Agarose molecules are able to form gels with relatively defined pore sizes because of the chemical properties of agarose molecules. Agarose demonstrates hysteresis - its melting temperature is higher than its gelling temperature. Agarose molecules dissolve at about 90°C, forming random coils in solution. Gels form when the temperature falls to approximately 40°C. As the gel forms, the agarose molecules first assemble into helical fibers, which then further aggregate to form networks of supercoiled helices stabilized by hydrogen bonds. The sizes of the pores, which typically range from 50 to 200 nm, depend on the concentration of agarose. As the agarose concentration increases, the average diameter of the pore decreases.

Several factors affect the migration of DNA through agarose gels

Because of the negative charge of the phosphate residues in the DNA backbone, DNA molecules move toward the positive pole (anode) of the electrophoresis apparatus. The uniformity of DNA structure gives molecules the same charge/mass ratio, which makes it straightforward to calculate the sizes of DNA fragments. (Molecular weights are not always very meaningful with DNA fragments, since a single molecule can contain the sequences of multiple genes.) In general, the migration of DNA molecules is inversely proportional to the logarithm of their lengths, or number of base pairs.

Although the relationship between migration rate and DNA length holds true in most cases, the actual migration rate of DNA molecules in a particular experiment is affected by multiple factors. Some of these factors are intrinsic to the DNA molecules, while other factors relate to the electrophoretic conditions. Because of this variability, DNA standards with known

sizes should always be run on the same gels with samples being analyzed. Importantly, the standards need to have a similar structure (e.g. linear or supercoiled) and to be subjected to the same chemical modifications as the DNA samples being analyzed.

Size and conformation of DNA

The migration rate of a DNA molecule depends on both its size and its conformation. Within a certain size range dictated by the gel conditions, the migration rate of linear DNA molecules is inversely proportional to the $\log_{10}$ (number of base pairs). The migration of more structured DNA molecules, such as circular plasmids, is much less predictable. The migration rates of these more structured forms are influenced by the density of coils, the presence of nicks, and other structural features.

Intercalating agents

The migration rates of DNA molecules are reduced when they are complexed with intercalating agents such as ethidium bromide or SYBR™ Green. DNA molecules bind large quantities of these intercalating agents, which increases both the length and stiffness of the DNA molecule.

Buffer systems

The migration rates of DNA molecules are also affected by the composition of the gel and running buffer. The migration rate of a DNA molecule decreases as the concentration of agarose in the gel increases. The migration rate of a DNA molecule is somewhat higher in a gel made with TAE (Tris: acetate: EDTA) buffer than with TBE (Tris: borate: EDTA) buffer.

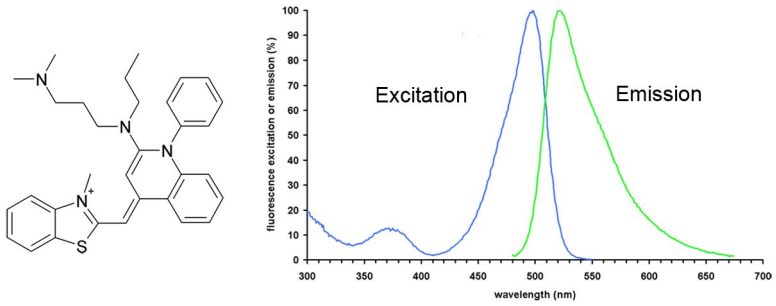
Fluorescent intercalating agents are used to visualize DNA fragments in gels

Nucleic acids are visualized by fluorescent dyes that bind strongly to DNA. The dyes are intercalating agents that insert into the DNA helix and into structured regions of single-stranded nucleic acids. The fluorescence of these dyes increases by an order of magnitude when they bind nucleic acids, so the background fluoresence remains low. The most commonly used dyes are ethidium bromide and variants of SYBR Green.

SAFETY NOTE: All DNA intercalating agents are potential mutagens. Be sure to wear gloves when handling solutions with intercalating dyes.

DNA intercalating agents have an absorbance maximum in the long ultraviolet range, so they are viewed with transilluminators that emit light with wavelengths close to the absorbance maximum of the dyes. Bands on the gel have an orange-like color. The figure below shows the structure and spectral data for SYBR Green I.

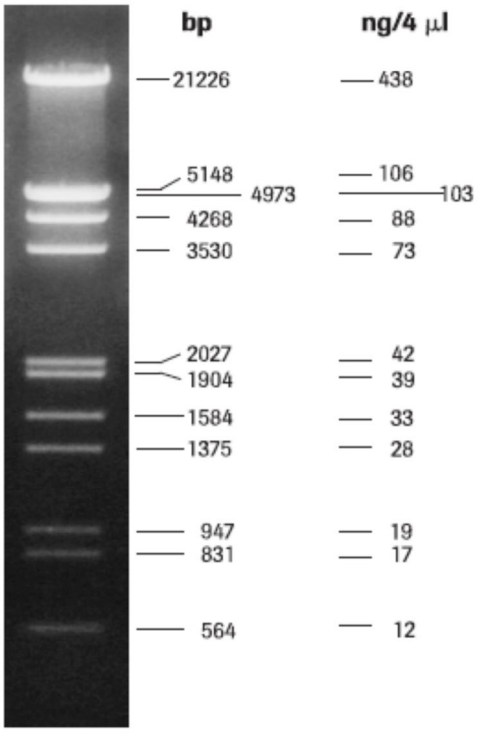
SAFETY NOTE: Be careful to wear protective eyewear if you look at an unshielded transilluminator. UV light is damaging to the eye!



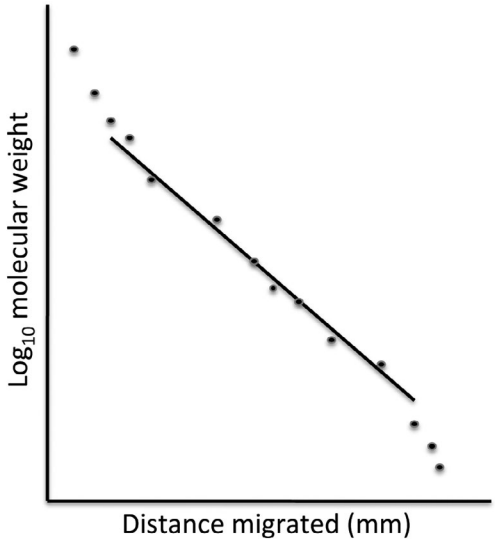
SyBr Green I fluorescent dye
Left: Chemical structure
Right: Absorbance and emission spectra of SyBr Green I. SyBr I has an absorbance maximum of 488 nm and an emission maximum of 522 nm.

The sizes and quantities of DNA fragments can be calculated using standards

Agarose gels provide information about the sizes of DNA fragments, if they are calibrated with appropriate size standards. Agarose gels also provide information about the quantity of DNA in a particular fragment, because the amount of intercalating agent that a DNA molecule binds is proportional to its mass. Consequently, the intensity of a band reflects the amount of DNA in the band.



The standard in the gel on the right is a preparation of bacteriophage λ DNA that has been digested with two restriction endonucleases, EcoRI and HindIII. The intact λ phage is 48,502 bp in length. The enzymes generate a series of fragments, varying in length from 564 to 21.2 kbp, as shown on the right. Because all of the fragments are present in equimolar amounts, it is also possible to use this standard to visually estimate the quantity of DNA in an unknown sample. In the figure, the standard is diluted so that 4 µL contains 1000 ng DNA. (We'll use half this amount, or 500 ng, in our gels.)



To calculate the sizes of bands in other lanes of the gel, you would first construct a standard curve like the one at the left from the migration of the λ restriction fragments.

Plot the $\log_{10}$(number of base pairs) for each fragment on the y-axis against the distance that each fragment migrated on the x-axis. You will be able to estimate the sizes of fragments in your sample by interpolating on the standard curve.

Note: Always visually confirm that your estimated sizes are correct!!

Prepare the agarose gel

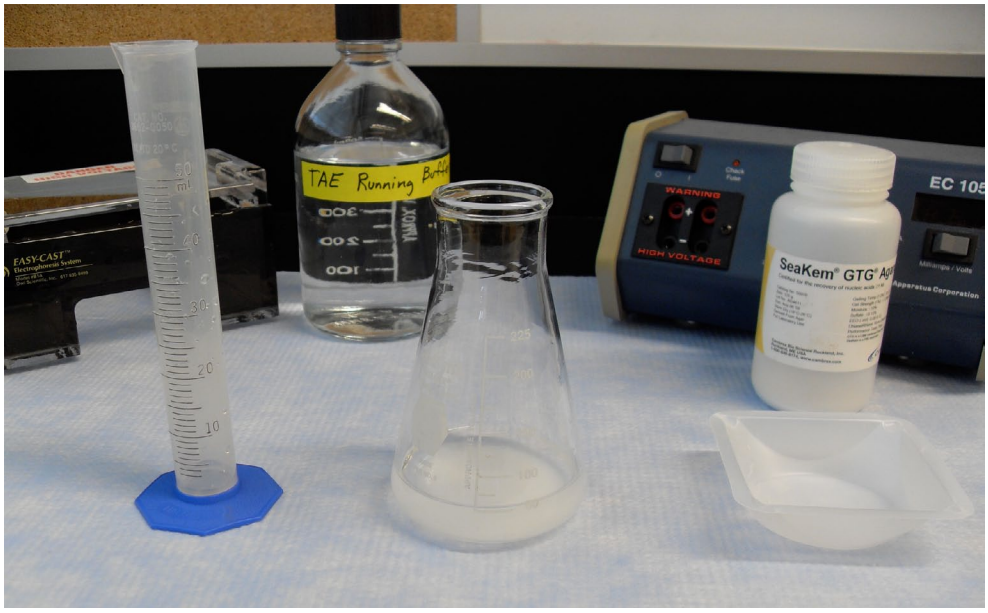
In this course, we will use agarose gels to separate DNA molecules produced in PCR reactions and restriction digests. These molecules are well-resolved on 1% agarose gels prepared in TAE buffer, which provides good separation of molecules ranging in size from 500 bp - 10 kbp. Place the casting tray into the gel apparatus. If you are using the BioRad apparatuses, position the casting gate at each end of the casting tray. (If you are using a different apparatus, you will need to tape the end of the casting tray, following your TA's instructions.

1. Determine the amount of agarose that you will need for a 1% (1 g/100 mL) agarose gel that fits your casting platform. Most of the gel apparatuses in the lab are the BioRad Mini-Sub GT systems, which have a 7 cm x 7 cm casting tray. These apparatuses accommodate 30-40 mL gels. If you are using a different apparatus, you will need to adjust the volume for the area of the casting tray. Check your calculations with your teammates before you proceed.

2. Fill a graduated cylinder with the appropriate volume of TAE buffer. Pour the solution into a small flask.

3. Weigh out the appropriate amount of agarose. Sprinkle the agarose onto the surface of the TAE in the flask. Note: the agarose will not dissolve until it is heated.

4. Dissolve the agarose by heating the solution for intervals of 15-20 seconds in a microwave oven. After each interval, remove the flask and gently swirl it around a bit to disperse the contents. Note if the agarose particles are still apparent or if the agarose has dissolved. The best gels are made from agarose that has NOT been overcooked.



SAFETY NOTE: The agarose solution will be very HOT when you remove it from the microwave! Please use caution when handling the flask. Be particularly careful not to contact the steam that will be coming through the opening of the flask. Fold several paper towels and wrap them around the neck of the flask when you handle it. If you do happen to spill some hot agarose on your skin, wash it immediately with cold water and alert your TA.

5. Allow the agarose solution to cool until you can comfortably touch the flask with your hands. Agarose solutions over 60˚C will warp the casting tray! Pour the gel. Place the sample comb in place. Do not move the casting platform until the gel sets. You will know that the gel is set when it becomes opaque. Allow the gel to cure for about 20 minutes after it sets.

Sample preparation

Prepare your samples for electrophoresis while the gel is curing. Add 1 volume (vol) of 6X loading dye to 5 vol of each sample. (You would add 1 µL of loading dye to 5 µL of sample.) The loading dye contains two dyes, bromophenol blue and xylene cyanol, which migrate with "apparent" molecular weights of ~5 kb and ~0.5 kb, respectively. Loading dye also contains glycerol, which makes the sample dense enough to sink to the bottom of the sample well.) Briefly, centrifuge each sample to mix the dye and samples, if necessary.

Load and run the agarose gel

1. When the gel has set, ***carefully*** remove the comb.

2. Orient the gel in the electrophoresis tank such that the wells (holes made by the comb) are oriented toward the black (negative) electrode. The DNA fragments will move from the well toward the red (positive) electrode. Cover the gel with ~5-10 mm of TAE buffer.

3. Load one sample to each well. The wells should hold up to 20 µL of sample. For PCR reactions, load 5-10 µL of the original PCR reaction (6-12 µL after sample buffer has been added). Try to avoid air bubbles as you load the samples.

4. Load 5 µL molecular weight standard to one lane of the gel. Make sure that you have accurately recorded the location of each sample in the gel.

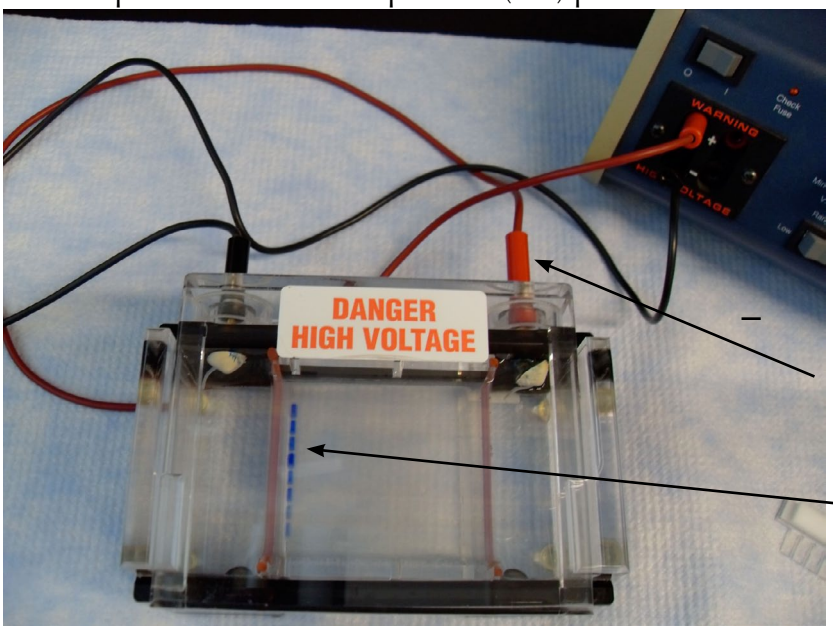
5. Place the lid on the electrophoresis tank and connect the electrodes to the power supply (black-to-black and red-to-red). ***Make sure that the polarity is correct before continuing!***

6. Turn on the power and apply a constant voltage of 75 V.

7. Pay careful attention to the gel as it runs. Turn off the power when the bromophenol blue is ~ 1 cm from the end of the gel. ***Do not allow the dye to run off the gel, since small DNA molecules will be lost.***

Proper set-up of an agarose gel

The gel is submerged in running buffer. Samples will elecctrophorese toward the postivie (red) pole.



Red electrical leads are attached to the positive pole of the power pack.

Samples are loaded into wells.

Agarose gel electrophoresis

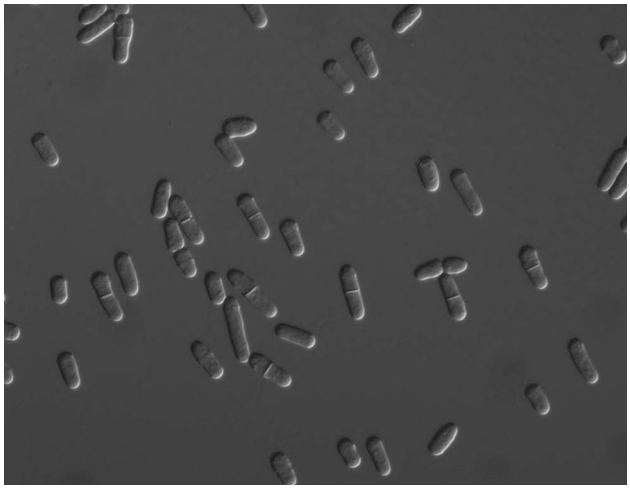
Stain and analyze the agarose gel

SAFETY NOTE: Wear safety goggles, lab aprons and disposable gloves when staining gels. Gloves are important when working with intercalating dyes, which are potential mutagens.

1. Remove the gel from the apparatus and transfer the gel to a small tray. Cover the gel with 100 mL deionized water. Add 5 µL of ethidium bromide (EtBr) solution (10 mg/mL), following your TAs instructions. What is the concentration of EtBr in the staining solution? Note: EtBr stocks are light sensitive, so they are stored in the dark.
2. Place the tray on a rocking platform and rock gently for 30 minutes.
3. Drain the EtBr solution in to the appropriate waste container.
4. Cover the gel with deionized water and rock gently for 2-5 minutes.
5. With a spatula, carefully place the gel on the transilluminator and close the cover to the Gel-Logic apparatus. (Drain the wash solution into the waste container.)
6. Turn on the transilluminator light and photograph the gel. Turn off the transilluminator immediately after you photograph the gel. Save the picture and email a copy to yourself.
7. Open the door of the GelLogic apparatus. Use the spatula to transfer the gel to a waste container set up for EtBr-stained gels.
8. Construct a standard curve using data from the photograph. Measure the distance that each fragment migrated from the sample well. Make a table in your notebook with the size of each DNA standard and the distance that each fragment migrated on the gel. Plot the $\log_{10}$(fragment length) of each standard on the y-axis and the distance that each standard migrated on the x-axis.
9. Determine the approximate length of the DNA fragments in your samples by interpolating on the standard curve. Are the sizes consistent with your expectations?

Chapter 9

S. pombe homologs



The budding yeast, *Saccharomyces cerevisiae*, and the fission yeast, *Schizosaccharomyces pombe*, are separated by close to a billion years of evolution. Our research goal for the semester is to determine if the proteins involved in methionine biosynthesis have been conserved between these evolutionary divergent species. In this lab, you will use bionformatics tools to search databases for potential *S. pombe* Met proteins.

Objectives

- Learn the one letter code for amino acids
- Understand how amino acid side chain chemistry is reflected in the BLOSUM62 matrix
- Understand how BLAST algorithms for sequence alignments work
- Use BLASTP and BLASTN to find *S. pombe* orthologs

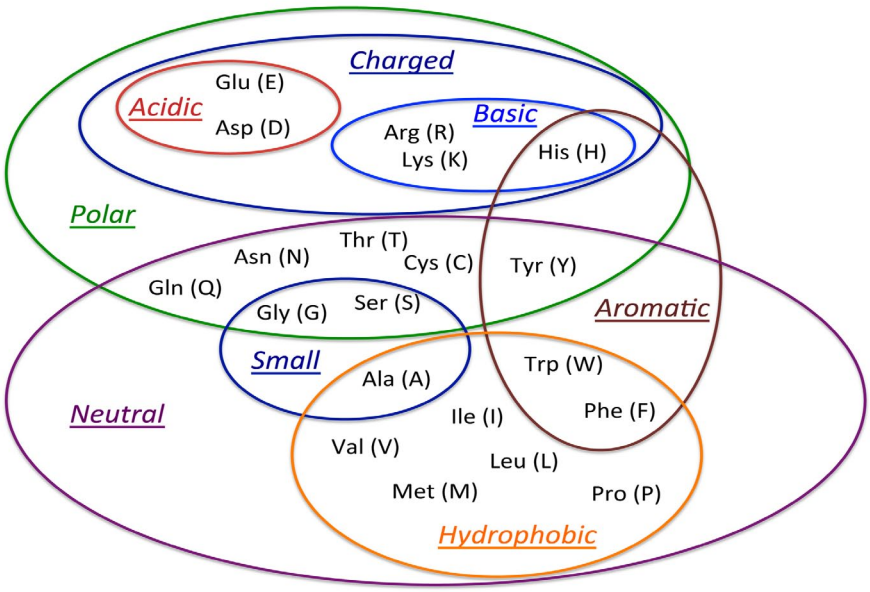
Chapter 9

As species evolve, their proteins change. The rate at which an individual protein sequence changes varies widely, reflecting the evolutionary pressures that organisms experience and the physiological role of the protein. Our goal this semester is to determine if genes for methionine biosynthesis are functionally conserved between *S. pombe* and *S. cerevisiae*, species that are separated by close to a billion years of evolution. In this lab, you will search databases for homologs of *S. cerevisiae* sequences in the *S. pombe* genome. Homologs show high sequence similarity to each other. When the sequences are found in different species, they are also referred to as orthologs. (Similar sequences within the same genome, the result of genome duplication events, are considered paralogs.)

Protein function is intimately related to its structure. You'll recall that the final folded form of a protein is determined by its primary sequence, the sequence of amino acids. Strongly conserved proteins generally contain regions with closely related amino acid sequences. To use the protein databases effectively, it is essential to know the one letter code for amino acids.

Amino acid R groups have distinct chemistries

Each of the 20 amino acids commonly found in proteins has an R group with its own distinctive chemistry. R groups differ in their size, polarity, charge and bonding potentials. When thinking about evolutionary changes in proteins, it is helpful to group the amino acids by their chemistry in a Venn diagram, such as shown below. In general, replacing one amino acid with a second amino acid from the same sector can be considered a conservative change. Size is also important. R groups vary considerably in the bulkiness of their chains. Substitution of a large R group for a small one can significantly alter the function of a protein.



Exercise 1 - The 1-letter code

You may find NCBI's Amino Acid Explorer helpful for this exercise. Access it at:
http://www.ncbi.nlm.nih.gov/Class/Structure/aa/aa_explorer.cgi

Met-Glu-Asn-Asp-Glu-Leu-Pro-Ile-Cys-Lys-Glu-Asp-Pro-Glu-Cys-Lys-Glu-Asp

What is the net charge of this peptide? (Assign -1 for each acidic amino acid and +1 for each basic amino acid. Add up the total charges.)

How many hydrophobic amino acids are found in this peptide?

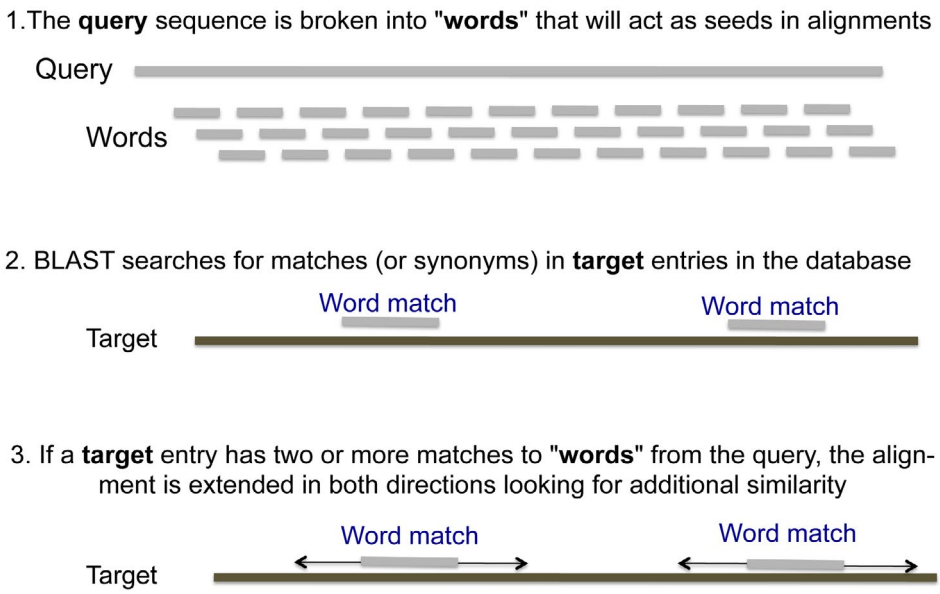
Write the name of a music group that you enjoy. Then transpose the name into an amino acid sequence. Pass the amino acid sequence to a friend and have him/her decode it. (Note: the 1-letter code uses all of the alphabet, except B, J, O, U, X and Z).

BLAST algorithms are used to search databases

There are many different algorithms for searching databases, but BLAST algorithms are some of the most popular. BLAST is an acronym for Basic Local Alignment Search Tool, which aptly describes its function. The original BLAST algorithms were developed and published by a group of investigators in 1990 (Altschul et al., 1990). Since that time, investigators have refined and diversified the original BLAST programs so that a whole family of BLAST programs is currently available. BLAST programs are widely used because they are able to quickly search through large amounts of sequence data. The key to BLAST's speed is its use of local alignments that serve as seeds for more extensive alignments. Other algorithms perform global sequence alignments that match the entire sequence of a query sequence against database information. Global sequence alignments are more sensitive than BLAST in finding related sequences, but they require considerably more processing time. BLAST searches are quite adequate for our purposes, and we will use the online BLAST tools available at the NCBI site throughout this course.

Either nucleotide or protein sequences can be used as the query sequence for BLAST searches. The BLASTN and BLASTP algorithms are designed for nucleotide and protein

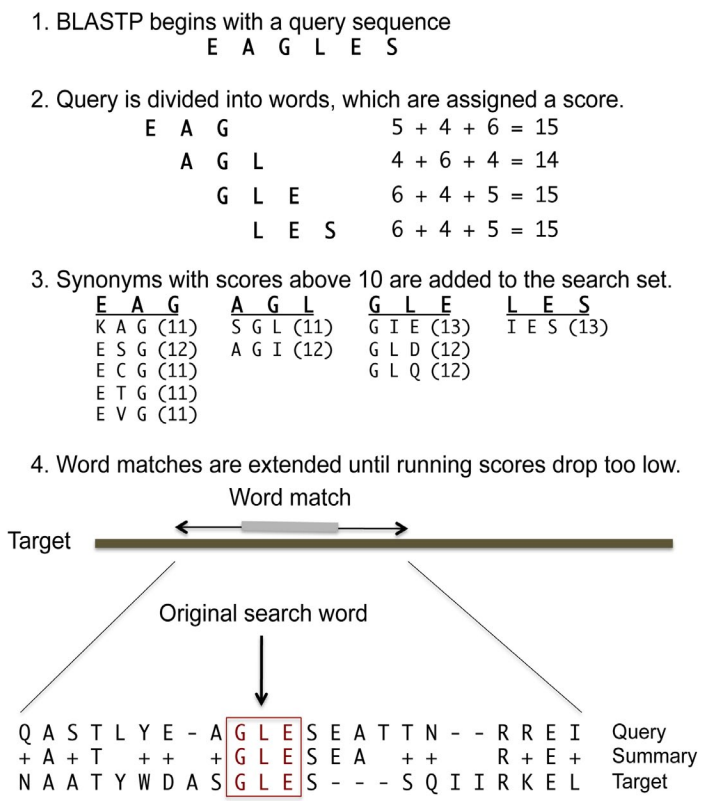
queries, respectively, and use different scoring matrices and search parameters. You have already used BLASTN to align nucleotide sequences, but BLASTP is much more useful for comparing protein sequences. Both BLAST algorithms begin by breaking down query sequence into short "words" and assigning numerical values to the words. A scoring matrix is used to assign numerical values to the words. Words and synonyms above a threshold value are then used to search databases. The default word size for BLASTN is 28 nucleotides, while the default word size for BLASTP is 3 amino acids.



Overview of the strategy used in BLAST algorithms
BLASTN and BLASTP use a rolling window to break down a query sequence into words and word synonyms that form a search set. At least two words or synonyms in the search set must match a target sequence in the database, for that sequence to be reported in the results.

We will primarily be using BLASTP in this course, so let's take a closer look at how BLASTP works. In BLASTP, a sequence in broken into all possible 3-letter words using a moving window. A numerical score for each word is assigned by adding the up values for amino acids from a scoring matrix. Using the popular BLOSUM62 scoring matrix (see below), the minimum score for a word would be 12. BLASTP next scans through potential synonyms that differ from the word at one position, and those synonyms that exceed a defined threshold value are included in the search terms as well. The threshold can be set by the user. NCBI BLASTP uses a default threshold of 10 for synonyms. Using this search set, BLAST rapidly scans a database and identifies sequences in which two words/synonyms in the search set are found. These sequences are set aside for the next part of the BLASTP process, when these short matches serve as seeds for more extended alignments in both directions from the original match. BLAST keeps a running raw score as it extends the matches. Each new amino acid either increases or decreases the raw score.

Penalties are assigned for mismatches and for gaps between the two alignments. Again, users can adjust the gap penalties, which are usually very severe. In the NCBI default settings, the existence of a gap brings a penalty of 11, which increases by 1 for each missing amino acid. Once the score falls below a set level, the alignment ceases. Raw scores are then converted into bit scores by correcting for the scoring matrix used in the search and the size of the database search space.



Overview of the BLASTP process.
The query sequence EAGLES into broken into three-letter words or synonyms that are used as a search set against records in a protein or translated nucleotide database. See the text for additional details.

The output data from BLASTP includes a table with the bit scores for each alignment as well as its E-value, or "expect score". The E-value indicates the number of alignments with that particular bit score that would be expected to occur solely by chance in the search space. Alignments with the highest bit scores (and lowest E-values) are listed at the top of the table. For perfect or nearly perfect matches, the E-value is reported as zero - there is essentially no possibility that the match occurs randomly. The E-value takes into account both the length of the match and the size of the database that was surveyed. The longer the alignment, and/or the larger the database search space, the less likely that a particular alignment occurs strictly by chance. An E-value of 1 does not necessarily imply, however, that the match has no biological relevance. More nuanced analysis may simply be needed to evaluate the match.

Chapter 9

BLOSUM62 scoring matrix

The results obtained in a BLASTP search depend on the scoring matrix used to assign numerical values to different words. A variety of matrices are available, whose utility depends on whether the user is comparing more highly divergent or less divergent sequences. The BLOSUM62 matrix is used as the default scoring matrix for BLASTP. The BLOSUM62 matrix was developed by determining the frequency of all 2^{10} possible amino acid substitutions in conserved blocks within protein families. The sequences used for the BLOSUM62 matrix were more than 62% identical. Thus, the matrix is based on observed amino acid changes in homologous proteins. The BLOSUM62 score for a particular substitution is a log-odds score that provides a measure of the biological probability of a substitution relative to the chance probability of the substitution. For a substitution of amino acid *i* for amino acid *j*, the score is expressed:

$$S_{ij} = (1/\lambda)\ log\left(\frac{p_{ij}}{q_i q_j}\right)$$

where p_{ij} is the frequency of the substitution in homologous proteins, and q_i and q_j are the frequencies of amino acids i and j in the database. The term (1/λ) is a scaling factor used to generate integral values in the matrix.

The BLOSUM62 matrix is shown below. The matrix is consistent with strong evolutionary pressure to conserve protein function. As expected, the most common substitution for any amino acid is itself. Overall, positive scores are less common than negative scores, suggesting that most substitutions have a negative effect on protein function. The most highly conserved amino acids are cysteine, tryptophan and histidine, which have the highest scores. Interestingly, these latter amino acids have unique chemistries and often play important structural or catalytic roles in proteins.

BLOSUM 62 scoring matrix

Ala	**A**	4																		
Arg	**R**	-1	5																	
Asn	**N**	-2	0	6																
Asp	**D**	-2	-2	1	6															
Cys	**C**	0	-3	-3	-3	9														
Gln	**Q**	-1	1	0	0	-3	5													
Glu	**E**	-1	0	0	2	-4	2	5												
Gly	**G**	0	-2	0	-1	-3	-2	-2	6											
His	**H**	-2	0	1	-1	-3	0	0	-2	8										
Ile	**I**	-1	-3	-3	-3	-1	-3	-3	-4	-3	4									
Leu	**L**	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4								
Lys	**K**	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5							
Met	**M**	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5						
Phe	**F**	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6					
Pro	**P**	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7				
Ser	**S**	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4			
Thr	**T**	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5		
Trp	**W**	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11	
Tyr	**Y**	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7	
Val	**V**	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4
		A	**R**	**N**	**D**	**C**	**Q**	**E**	**G**	**H**	**I**	**L**	**K**	**M**	**F**	**P**	**S**	**T**	**W**	**Y**	**V**

Henikoff, S. and Henikoff, J.G. (1992) Amino acid substitution matrices from protein blocks. *Proc. Natl. Acad. Sci. USA* **89**:10915-10919.

Exercise 2 - The BLOSUM62 matrix

1. The highest values in the matrix are observed when no substitution has occurred. Which amino acids have the 3 highest values? What does this suggest about the amino acids?

2. Compare the number of positive values (shaded) to negative values in the matrix. What does this suggest?

3. Hypothesize why relatively high values (2 and above) occur for the substitutions:

   Asp to Asn

   Asp to Glu

   His to Tyr

   Ile to Val

4. Find the amino acid composition for your Met protein.
   - Locate the amino acid sequence for your protein from its NP_ or NM_ record at NCBI or from the Protein page for your gene in the SGD.
   - Paste the amino acid sequence into the Protein Parameters tool at the European Bioinformatics Institute: http://web.expasy.org/protparam/
     (Note: you may see some unfamiliar amino acids, selenocysteine (Sec) and pyrrolysine (Py1). Neither of these amino acids are found in S. cerevisiae proteins. In the organisms where these amino acids are found, one of the stop codons has been recruited to serve as a codon.)

   What are the five most common amino acids in your protein? What are the BLOSUM scores for these amino acids? What do you notice about their chemistry?

   What are the three least common amino acids? What are the BLOSUM scores for these amino acids?

   Are any amino acids missing in your protein? Which ones?

   Which amino acids would you expect to be conserved in the *S. pombe* ortholog?

Chapter 9

Exercise 3 - BLASTN

Locate the NM_ and NP_ accession numbers for your protein from your notebook or a database. You will use these as search terms.

Direct your browser to the BLAST tools at NCBI:
http://blast.ncbi.nlm.nih.gov

Select nucleotide blast, which brings you to the query page.

- Enter the NM_ number for your gene in the query box. (Don't forget the NM and underscore character in the record number.)
- For the database, select Reference RNA sequences from the drop down box
- Type pombe in the organism box, which brings up the full species name to select.
- Under program selection, choose "Somewhat similar sequences (blastn)"
- Click BLAST.

On the results page, note the graphic summary at the top which gives you an instant idea about the extent and strength of the match with *S. pombe* sequences.

Does the match extend the full length of the transcript?

How many orthologs are present in *S. pombe*?

Use the information in the data table to record the:

name of the *S. pombe* gene (It may be different from the *S. cerevisiae* name.)

total score

how much of the query sequence was aligned (coverage)

E-value for the alignment

Beneath the data table, locate the actual alignment.

Record the first and last nucleotides in the aligned sequences.

Take a screen shot of the alignment and save it for future reference.

Exercise 4 - BLASTP

Return to the BLAST tools page and choose protein blast (BLASTP)

- Enter the NP_ number for the S. *cerevisiae* Met protein in the query box
- For the database, select reference proteins
- Select *S. pombe* for the organism
- Click BLAST

How many *S. pombe* orthologs did BLASTP identify? Record the NP_ number(s).

As in the previous exercise, record the:

total score

how much of the query sequence was aligned (coverage)

E-value for the alignment

Locate the alignment and take a screen shot.

Note the numbers of the first and last amino acids in the alignment.

Take note of the center row in the alignment which summarizes the homology between the protein sequences. If an amino acid is conserved between the two species, its 1-letter code name is shown in that row.

What do the pluses in the center row indicate?

Did BLASTP add gaps to one or both of the sequences in the alignment?

How do the starting and ending points for the alignment compare to those from BLASTN?

Find the positions of the three most infrequent amino acids (Exercise 2) in the alignment. Are they conserved between the two yeast species?

Compare the statistics for the BLASTN and BLASTP searches. What differences do you note? Why do you think this is?

Click on the link to the NP_ record for the S. pombe ortholog. Record the EC number for the protein. Is it identical to that for the *S. cerevisiae* enzyme?

Chapter 9

Exercise 5 - Investigate your Pombase record

Like *S. cerevisiae*, *S. pombe* is a model organism with its own large community of researchers. A relatively new database, Pombase, brings together information on *S. pombe*, functioning much like the SGD.

- Direct your browser to Pombase: pombase.org

- Enter the name of your *S. pombe*homolog in the search box. In general, *S. pombe* genes have not been as well-studied as their counterparts in *S. cerevisiae*. You may need to use the systematic name, which begins with SPAC, SPBC or SPCC, corresponding to chromosomes I, II, and III, respectively. These systematic names were given to all the ORFs identified in the *S. pombe* genome project.

- Record the systematic name for your gene.

- Look at the genome browser. Note that the numbering system is different from that used for *S. cerevisiae*.

- Spend a little time seeing what kind of information is available for your *S. pombe* ortholog. You may find Pombase to be a helpful resource for your lab reports.

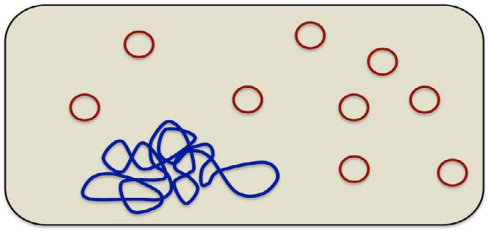
References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool *J. Mol. Biol.* **214**: 403-410. PMID 2231712

Henikoff, S., and Henikoff, J.G. (1992) Amino acid substitution matrices from protein blocks. *Proc. Natl. Acad. Sci. USA* 8**9**: 10915-10919.

Chapter 10

Plasmids



Plasmids were the first cloning vectors to be widely used in molecular biology and they continue to play important roles in the laboratory. Plasmids are small, circular pieces of DNA that replicate independently of the host chromosome. The first plasmids used in the lab were derivatives of naturally-occurring plasmids. Since then, investigators have added many features to plasmids to suit varioius applications.

Objectives

- Understand the structure and replication of plasmids
- Understand some of the features that have been engineered into plasmids for molecular cloning
- Learn how the physical properties of plasmids are used in their purification
- Isolate a plasmid from *E. coli*
- Use ultraviolet spectroscopy to estimate the concentration of plasmid DNA

Chapter 10

Plasmids are the workhorses of molecular biology. Plasmids are small, circular DNA molecules that replicate independently of the chromosomes in the microorganisms that harbor them. Plasmids are often referred to as vectors, because they can be used to transfer foreign DNA into a cell. Plasmids can be engineered to carry large amounts of foreign DNA and they can be easily isolated from microorganisms for manipulation in the lab. In this laboratory, you will study the features of the plasmids that we are using this semester and you will isolate plasmid DNA from a bacterial culture. In the next few weeks, you will use these plasmids to transform yeast.

Introduction to plasmid biology

Plasmid replication depends on host cell polymerases

Plasmids are found naturally in many microorganisms. Plasmids can be transferred between species by transformation or conjugation, but they generally have a restricted host range. When you think of plasmids, you probably also think of bacteria, but plasmids are not restricted to bacteria. In fact, most *S. cerevisiae* strains carry a large plasmid known as the 2 micron or 2 μm plasmid. Multiple copies of the 2 μm plasmid are usually present in the nucleus of a yeast cell, and the plasmid number is stable through many rounds of cell division.

Although plasmids replicate independently of the chromosomal DNA, they rely on host enzymes to catalyze their replication. Host DNA polymerases bind to origins of replication within the plasmid replicon. Plasmids that replicate in bacteria have origins of replication that bind bacterial DNA polymerase, while plasmids that replicate in yeast have distinct origins of replication that bind yeast DNA polymerase. Our plasmids are considered shuttle vectors because they can "shuttle" between two different species. The shuttle vectors that we will use contain both bacterial and yeast origins of replication. Like most laboratory plasmids, they are multi-copy plasmids, which are present anywhere from tens to hundreds of copies per cell. In this class, we will propagate the shuttle vectors in bacteria, because bacteria grow more rapidly than yeast and the yield of plasmid is usually higher from bacteria than from yeast. We will harvest the plasmids from bacteria and then use them to transform yeast cells.

Laboratory plasmids carry selectable markers

The plasmids used in molecular biology are derived from a small number of naturally-occurring plasmids and their usefulness was initially quite limited. Since that time, many bells and whistles have been engineered into plasmids, vastly expanding their utility. For plasmids to be useful in molecular biology, investigators must be able to distinguish transformed cells that carry the plasmid from cells without plasmids. To this end, molecular biologists have engineered a variety of selectable markers into laboratory plasmids. Without the selective advantage conferred by the marker, plasmids would most likely be lost from transformed cells, because of the extra toll they place on host metabolism. The markers used to select transformed bacteria often carry genes that confer antibiotic resistance, while the markers used to select transformed yeast strains often carry genes that complement nutritional deficiencies in auxotrophic strains.

Overexpression plasmids drive expression of a foreign protein in the host cell

The plasmids that we will use this semester are overexpression plasmids that can drive expression from ORFs cloned into the plasmid sequence. Our plasmids all contain the promoter sequence for the yeast *GAL1* gene. Transcription from the *GAL1* promoter is normally regulated by regulatory proteins that sense glucose and galactose levels in yeast. In the plasmids, the *GAL1* promoter has been placed at the 5'-ends of protein coding sequences for *S. cerevisiae* Met proteins or their *S. pombe* homologs. The presence of the *GAL1* promoter will allow you to manipulate expression of the Met proteins in transformed yeast cells. The proteins that will be expressed from our plasmids are expected to be longer, however, than the natural Met proteins because *MET* gene stop codons were dropped during cloning and the cloning vectors added coding sequences for some convenient tags. The fusion proteins expressed from the vectors are therefore fusion proteins with additional tags at the C-termini that can be used for western blots, interaction analyses or protein purification.

The plasmids containing *S. cerevisiae* ORFs are based on a different cloning vector than the plasmids carrying the *S. pombe* ORFs. ORFs for the *S. cerevisiae* Met proteins were cloned into the pBG1805 cloning vector in a genome-wide experiment (Gelperin et al., 2005). The "p" in pBG1805 denotes that it is a plasmid, while the remainder of the plasmid name is a code used by the laboratory that constructed it. Often, the letters in a plasmid's name contain the initials of the researcher who performed the last step in its construction. In this case, "BG" refers to Beth Greyhack, one of the paper's authors. The structure of pBG1805 is outlined on the next page.

The plasmids containing the *S. pombe* ORFs were constructed by the BI204 staff and are based on a commercial cloning vector, pYES2.1. The plasmids carrying the *S. pombe* ORFs all begin with pSPM, for *S. pombe* methionine, followed by the number of the *S. cerevisiae MET* gene. Thus, pSPM3 carries the CDS of the *S. cerevisiae MET3* gene. ***A warning: S. pombe Met genes do not always have the same number as their orthologs. For example, the ortholog of S. cerevisiae MET2 in S. pombe is Met6.***

Exercise 1 - Map the yeast overexpression vectors

In this exercise, you will use the web-based PlasMapper program (Dong et al., 2004) to identify and compare elements in the pBG1805 and pYES2.1 cloning vectors. When the sequences of circular plasmids are entered into PlasMapper, the program matches the sequence against a library of commonly used elements, including promoters, affinity tags, origins of replication, selectable markers and restriction sites. The program also has an ORF finder that identifies ORFs that are longer than an adjustable cutoff length. The program generates both graphical and text maps of the plasmid that can be easily downloaded and used in reports, etc.

Chapter 10

Direct your browser to: **http://wishart.biology.ualberta.ca/PlasMapper**

1. Enter one of the plasmid sequences: FASTA files are available for both pBG1805 and pYES2.1 without added yeast ORFs in the cloning sites. FASTA files are text files that begin with a title line with the format ">(some text) - hard return." The title line is followed by the DNA sequence. You can upload the file directly into PlasMapper. Alternatively, you can open the FASTA file and then paste its contents into the search box.

2. Adjust the controls for the map. Leave the feature options checked, but uncheck the restriction site(s) box. (We'll work with restriction enzymes soon.)

3. Click the Graphic Map box, which brings up a nicely annotated map of your plasmid.

4. Save the map to your computer.

5. Repeat Steps 1-3 for the other plasmid.

6. Compare the two plasmid maps. Draw rough sketches of the plasmids in the space below.

Which elements do the two plasmids have in common?

Note the sizes of the two plasmids. What accounts for the difference in size between the two? Which plasmid appears to be more highly engineered?

Plasmid maps like these provide a natural history of the plasmid. Almost all common plasmids are derived from a few progenitors constructed in the 1970s. Since that time, researchers have tailored the sequences of cloning vectors to meet their experimental needs. In many cases, vectors maintain features that are no longer needed for current research needs.

Plasmids are easily isolated from bacterial cells

Plasmid isolation takes advantage of the unique structural properties of plasmids. Plasmids are small, supercoiled circular pieces of DNA. Unlike the much larger bacterial chromosome (which is also circular), plasmids are quite resistant to permanent denaturation. Today, most laboratories use commercial kits for plasmid isolations, because the kits are convenient and relatively inexpensive. The kits give good yields of high-quality DNA, while avoiding the need for organic denaturatants. A variety of less expensive, but somewhat more time-consuming, procedures have been described for investigators who want to make their own reagents. These procedures generally give good yields of DNA that is slightly less pure than DNA purified with the kits. Whatever the isolation procedure, the general principles of plasmid isolation are the same. The paragraphs below summarize the steps and general principles used for plasmid isolation.

1. ***Denaturation*** - The first step in plasmid isolation is lysis of the bacterial cells carrying the plasmids. Strong denaturating conditions are required for this step, because of the tough bacterial cell wall. The most common procedures use a combination of strong base and a detergent to lyse the cells. The detergents help to solubilize lipids in the cell wall, allowing the denaturants to enter the cell. Proteins, because of their fragile structures, are irreversibly denatured by this treatment. The treatment also breaks the hydrogen bonds that hold the two strands of the chromosomal and plasmid DNA together.
2. ***Neutralization*** - The extracts are then neutralized, allowing DNA strands to reanneal. Plasmids are able to renature during this step because they have supercoiled structures that remain associated even when the plasmids were denatured. Chromosomal DNA is not able to renature, however, because its longer strands are mixed with precipitated protein and complementary sequences require longer times to find their former bonding partners. Samples must be mixed gently at this step to prevent fragmentation of the long, chromosomal DNA into pieces that might be able to reanneal and co-purify with the plasmids.
3. ***Physical separation of plasmid DNA from precipitated macromolecules*** - During the neutralization step, the denatured proteins and chromosomal DNA form large aggregates. Precipitated proteins and chromosomal DNA are removed from the extracts by centrifugation or by phenol extraction.
4. ***Purification of plasmid DNA*** - Solutions containing plasmids are further purified by organic extraction or by adsorption to a resin. Most kits for plasmid purification contain silica resins that bind the plasmid DNA under high salt conditions. Contaminants are washed from the column before the plasmid DNA is eluted under low salt conditions. Alternatively, plasmids can be extracted several times with phenol and/or chloroform, followed by precipitation of the plasmid DNA with either ethanol or isopropanol. The purity and yield of the extraction procedure can be monitored by ultraviolet spectroscopy.

Chapter 10

Exercise 2 - Plasmid isolation with the Zyppy™ kit

Concentrate the plasmid-bearing bacterial cells

1. Your group will receive 3 bacterial cultures that were grown overnight in Luria Bertani (LB) media containing 100 µg/mL ampicillin. The density of cells in the culture is expected to be 3-4 X 10^9 cells/mL. *What is the purpose of the ampicillin? How does it work?*
2. Transfer 1.5 mL of each 3 mL culture to a microcentrifuge tube. Concentrate the cells by centrifuging them at maximum speed (~14,000 rpm) for 1 min. The cells will form a white pellet at the bottom of the tube.
3. Remove the culture medium above the cell pellet by gently decanting the supernatant. Add the remaining 1.5 mL of bacterial culture to the tube containing the cell pellet.
4. Concentrate the cells once more by centrifugation at maximum speed for 1 min.
5. Use a P200 to aspirate off as much of the culture medium as possible.

Alkaline lysis of bacterial cells harboring the plasmids

6. Re-suspend the pellet in 600 µl of TE buffer (Tris-HCl, EDTA pH=8.0) using the vortex mixer.
7. Add 100 µL of 7X Blue Zyppy Lysis buffer to the tube. Mix the buffer and cells by gently inverting the tube 4-6 times. Be gentle! Too much mechanical stress will fragment the bacterial chromosomal DNA and contaminate your plasmid preparation. The solution should turn from a cloudy blue to a clear blue. **NOTE: *This step is time-sensitive!! Proceeed to step 3 within 2 minutes.***

Separate plasmid DNA from denatured proteins and chromosomal DNA

8. Add 350 µL of cold Yellow Zyppy Neutralization buffer (w/RNAase A) to the tube, and mix the contents thoroughly by inverting several times. The solution will turn yellow when neutralization is complete, and a yellowish precipitate will form. Invert the sample an additional 3-4 times to ensure complete neutralization.
9. Centrifuge the mixture at maximum speed for 3 minutes to remove denatured proteins and chromosomal DNA. Notice that the tube contains a yellow precipitate that has collected on one side of the tube. The pale yellow supernatant contains the plasmid DNA.

Purify plasmid DNA by adsorption to a silica resin.

10. Using a P200, carefully transfer the pale yellow supernatant (~900 µL) onto a Zyppy spin column. Be careful not to transfer any of the yellow precipitate!
11. Place the column with the collection tube attached into a centrifuge and spin at maximum speed for about 15 seconds. It is best to use the "pulse" button on the centrifuge and count to 15 or 20 seconds for this centrifugation step.
12. Remove the column and discard the flow through in the collection tube.
13. Place the column back into the collection tube and add 200 µL of Zyppy Endo-Wash solution. (Endo-Wash contains guanidine hydrochloride and isopropanol, which will remove denatured proteins from the resin.)
14. Centrifuge for 15-20 seconds, and discard the flow through.
15. Place the column back into the collection tube then add 400 µL of Zyppy Column Wash buffer. (This steps removes contaminating salts.) Centrifuge for 30-40 seconds.

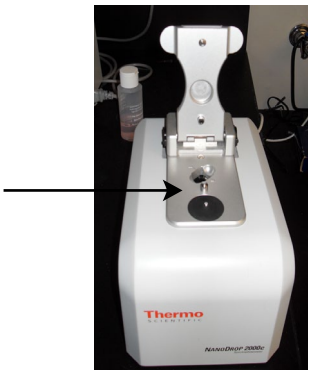
Elute the plasmid DNA

16. Transfer the Zyppy column to a clean (and appropriately labeled) 1.5 mL centrifuge tube, leaving the lid of the tube open.
17. Carefully, add 100 µL of TE buffer directly on top of the white column bed. Place the pipette tip as close as you can to the white column bed without poking it. Slowly dispense the TE on top of the resin bed.
18. Allow the buffer to percolate into the column by letting the column stand upright in the microcentrifuge fuge tube for 10 minutes.
19. Centrifuge the column at maximum speed for 30 seconds. Again, it's fine to leave the cap open during this spin.
20. Remove the column, cap the tube and place it on ice. This tube should now contain plasmid DNA.

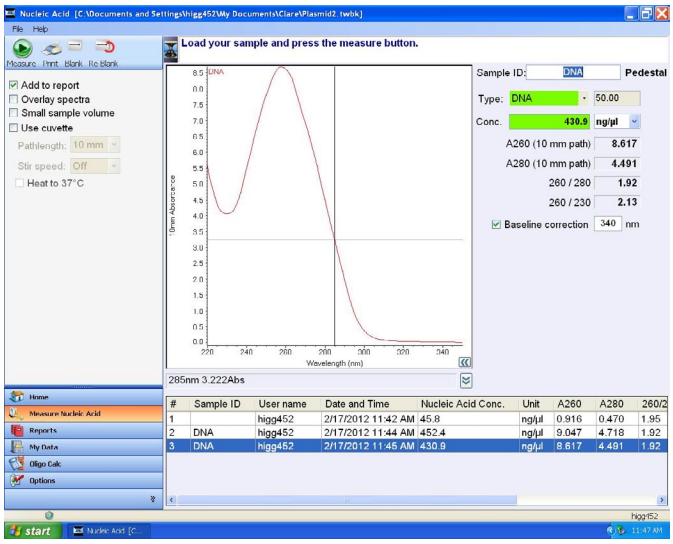
Exercise 3 - Measure the plasmid DNA concentration

The concentration and purity of DNA samples can be estimated from their absorbance of ultraviolet (UV) light. Nucleic acids absorb light strongly in the UV range, with an absorbance maximum of ~260 nm. An A_{260} of 1 corresponds to ~50 µg/mL DNA. Proteins also absorb UV light due to their aromatic side chains, but much less strongly than nucleic acids. Proteins have a different absorbance maximum of ~280 nm. An A_{280} of 1 corresponds to 1 mg/mL protein. Because of the differences in protein and nucleic acid absorbances, the A_{260}/A_{280} ratio of a sample is useful in determining the purity of a DNA sample. Pure DNA has an A_{260}/A_{280} of ~1.8. Protein or chemical contamination will lower this ratio.

In this laboratory, you will use an instrument called the NanodropTM to obtain an ultraviolet absorbance spectrum for your plasmid DNA samples. The Nanodrop software will also calculate the concentration of DNA and the A_{260}/A_{280} ratio. The NanoDrop uses fiber optic technology and the surface tension of a sample to provide accurate absorbance measurements over a large dynamic range of DNA concentrations. From a practical point of view, this means that you need to sacrifice only small amounts of your precious DNA sample. The NanoDrop is amazingly simple to use, and your TA will demonstrate the appropriate use of the instrument.



1. Pipette 1 µL of your plasmid sample on the pedestal on top of the pin (shown at the end of the arrow at left).
2. Close the arm and record the absorbance.
3. When the measurement is complete, open the arm and wipe the pedestal surface with a lint-free wipe in preparation for the next sample.
4. Record the absorbance readings in your notebook.



The Nanodrop results screen shows the UV spectrum of the sample and the estimated DNA concentration

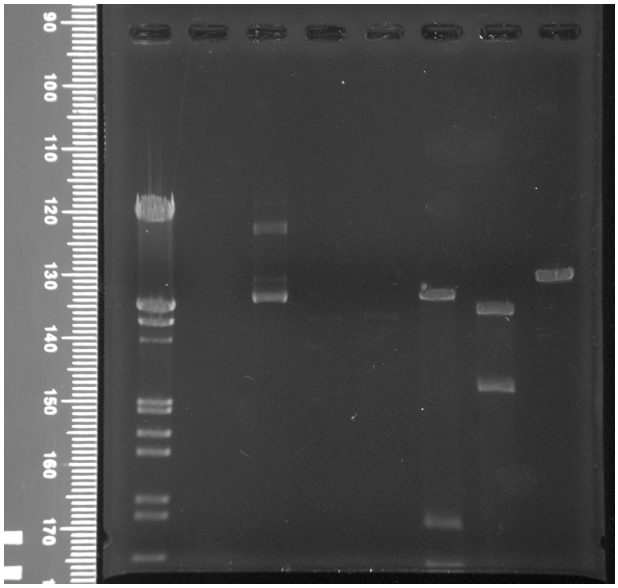
References

Dong, X., Stothard, P., Forsythe, I.J., and Wishart, D.S. (2004). PlasMapper: a web server for drawing and auto-annotating plasmid maps. *Nucleic Acids Res.* **32**, W660-W664.

Gelperin, D.M. White, M.A., Wilkinson, M.L., Kon, Y., Kung, L.A., Wise, K.J., Lopez-Hoyo, N., Jiang, L., Piccirillo, S., Yu, H., Gerstein, M., Dumont, M.E., Phizicky, E.M., Snyder, M., and Grayhack, E.J. (2005). Biochemical and genetic analysis of the yeast proteome with a movable ORF collection. *Genes Develop.* **19**, 2816-2826.

Chapter 11

Restriction mapping



Restriction endonucleases (REs) are part of bacterial defense systems. REs recognize and cleave specific sites in DNA molecules. REs are an indispensable tool in molecular biology for both constructing recombinant molecules and analyzing DNA molecules. In this lab, you will use REs to distinguish plasmids carrying *S. cerevisiae* and *S. pombe* ORFs.

Objectives

- Understand the biological origins and functions of REs
- Understand the structures of restriction sites in DNA
- Devise a strategy to distinguish between three yeast overexpression plasmids using RE digests
- Analyze restriction digests on agarose gels

Chapter 11

In the last experiment, you isolated plasmids carrying *S. cerevisiae MET* or *S. pombe Met* genes from transformed bacteria. Each group was given three different plasmids. One of the three plasmids, cloned into the pBG1805 plasmid, carries the *MET* gene that has been inactivated in your yeast strain. A second plasmid carries a different *S. cerevisiae MET* gene. The third plasmid, constructed in the pYES2.1 plasmid, carries the *S. pombe* homolog for your *MET* gene. In this lab, you and your team will design and carry out a strategy to distinguish between the plasmids using restriction endonucleases. In the next lab, you will separate the products of the restriction digests, or restriction fragments, by agarose gel electrophoresis, generating a restriction map.

Restriction endonucleases

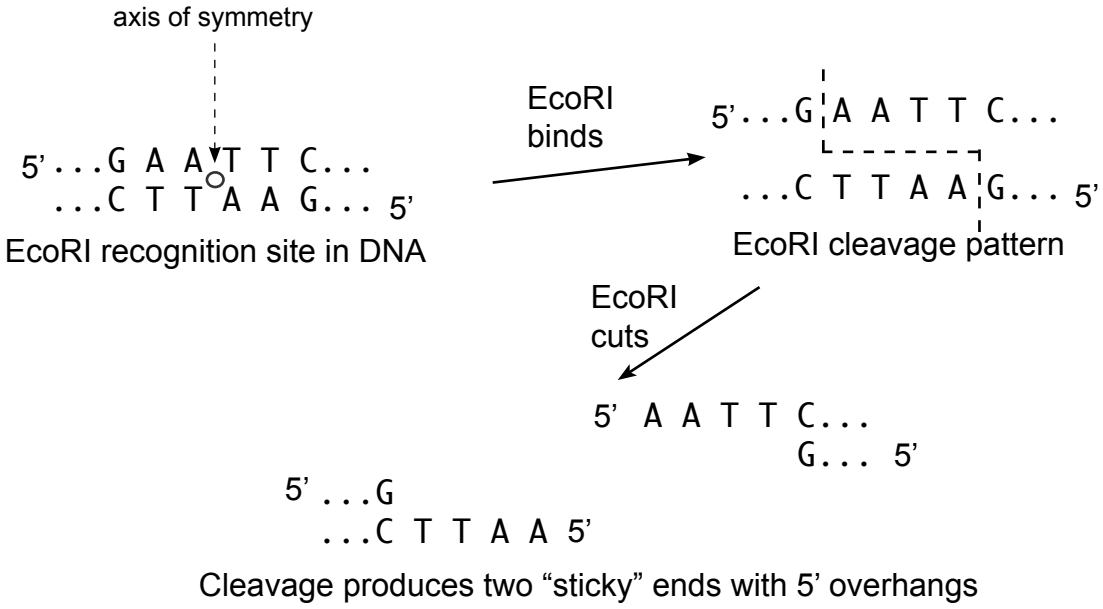
Bacterial restriction/modification systems protect against invaders

The discovery of restriction enzymes, or restriction endonucleases (REs), was pivotal to the development of molecular cloning. REs occur naturally in bacteria, where they specifically recognize short stretches of nucleotides in DNA and catalyze double-strand breaks at or near the recognition site (also known as a restriction site). To date, thousands of REs with distinct specificities have been described. You might wonder why bacteria harbor these potentially destructive enzymes. REs are part of a bacterial defense system against foreign DNA, such as an infectious bacteriophage. The bacteria protect their own DNA from cleavage with a methyltransferase that modifies the same restriction sites in the bacterial DNA. The combined activities of the endonuclease and methyltransferase are referred to as a restriction/modification system. In Type I enzymes, the endonuclease and methyltransferase activities are part of the same complex. The REs used in laboratory are Type II enzymes. The Type II endonucleases and methyltransferases do not form a complex and function independently.

To understand how REs work, it may be helpful to look at a single enzyme in greater detail. One of the best-studied restriction enzymes is EcoR1. Although the names of individual REs may sound a bit like baby talk, the nomenclature is actually very systematic and is based on its biological source. EcoRI is found naturally in the RY13 strain of *Escherichia coli*, or *E. coli*. Its name begins with the genus and species (Eco for *E. coli*), followed next by a strain identifier (R for RY13), and ends with a Roman numeral that distinguishes the different enzymes in the strain. Strain RY13 of *E. coli* contains multiple endonucleases, but only EcoRI and EcoRV, are widely used in molecular biology. Purification of REs from their natural source is a laborious procedure that requires large quantities of bacteria, since the REs are not abundant proteins in bacteria. To facilitate the production of these useful enzymes for routine laboratory use, molecular biologists cloned the coding sequences of the endonucleases into bacterial expression plasmids. Today, most commercially available enzymes are recombinant proteins overexpressed in bacteria. (Along the way, scientists also introduced some mutations that generated enzymes with enhanced stability or higher activity.)

Restriction enzymes cleave specific sites in DNA

Restriction enzymes like EcoRI are frequently called 6-cutters, because they bind to a 6-nucleotide sequence. Assuming a random distribution of A, C, G and Ts in DNA, probability predicts that a recognition site for a 6-cutter should occur about once for every 4000 bp (4^6) in DNA. Of course, the distribution of nucleotides in DNA is not random, so the actual sizes of DNA fragments produced by EcoRI range from hundreds to many thousands, but the mean size is close to 4000 bp. A DNA fragment of that length is very useful in the lab, since it is long enough to contain the coding sequence for some proteins and it is easily resolved on common agarose gels. The sequence that EcoRI recognizes in double stranded DNA is G A A T T C. The sequence is a palindrome with a two-fold axis of symmetry, because reading from 5' to 3' on each strand gives the same sequence. The palindromic nature of the restriction site is more obvious in the figure below. The circle in the center of the restriction site denotes the axis of symmetry. EcoRI binds DNA at the restriction site and catalyzes the hydrolysis of the phosphodiester bond between G and A on either strand.



EcoRI catalyzes the cleavage of a palindromic recognition site.
The recognition site for EcoRI has a two-fold axis of symmetry. Cleavage generates two fragments with 5′-sticky ends.

The structure of EcoRI is consistent with an enzyme that catalyzes identical reactions at two different sites. Crystallization data shows that EcoRI is a homodimer, an enzyme with two identical subunits. The figure on the next page (PDB datafile 1QPS) shows two different views of EcoRI bound to DNA. In the left view, we're looking down the axis of the DNA helix. The right view is a side view. Take a close look at both structures, using the DNA helix as the center. In these models, alpha helices and beta sheets are given different colors or shades. The symmetry in the enzyme is quite apparent.

Crystallization structures show that EcoRI is a symmetric homodimer that binds DNA.

Selecting restriction enzymes for experiments

The first step in an experiment involving restriction enzymes is to analyze the nucleotide sequence of the target DNA molecules with a software program that detects restriction sites, such as the Plasmapper program that you used in the previous lab. These programs usually generate a graphical output as well as tabular data with details about the lengths and positions of the restriction site in the DNA sequence. The list of enzymes that cut a particular sequence is always impressive, but only a few enzymes usually turn out to be practical for the purpose of the experiment. When choosing enzymes, investigators consider the number of lengths of the fragments that will be generated in a digest, the position of the restriction sites relative to a gene of interest, as well as the kind of ends in the restriction fragment.

The kind of end generated by an RE is particularly important when investigators are constructing a recombinant DNA molecule. In the figure on the previous page, you saw how EcoRI produced DNA fragments with 5' overhangs. Other enzymes produce fragments with 3' overhangs, while still others produce blunt ends with no overhangs. Ends with overhangs are often referred to as "sticky" ends, since they will form hydrogen bonds with a complementary sequence on a second DNA fragment. By contrast, fragments with blunt ends can anneal with any other blunt end, regardless of the adjoining sequence. DNA ligases supply the "paste" for stitching restriction fragments together, by catalyzing the formation of phosphodiester bonds. In this course, we will be using plasmids that were constructed by a series of steps, most of which involved restriction digests and ligase reactions. We will not be constructing any recombinant molecules in this course, but it is helpful to understand the process by which they were generated.

Handling restriction endonucleases in the laboratory

The REs that we are using in the lab are highly purified (and expensive!) proteins that have been purified from recombinant bacteria. Like all enzymes, each restriction endonuclease functions optimally under a defined set of reaction conditions, including variables such as temperature, pH, metal ions and salts. The manufacturer's supply restriction enzymes with buffers that generate reaction conditions close to those that are optimal for the enzyme. Some of the buffers

contain bovine serum albumin (BSA), an abundant protein from cow serum. BSA is included largely for its stabilizing properties. In general, enzymes are more stable at higher protein concentrations. BSA does not affect the enzymatic activity of the REs, but protects particularly sensitive REs from denaturation. BSA may also saturate non-specific binding sites for REs on the sides of the test tubes used in the digests.

Like all enzymes, REs are subject to spontaneous denaturation over time, so they need to be handled with care. (By comparison, DNA is an exceptionally stable molecule.) The rate of protein denaturation increases as the temperature increases, so enzymes are kept on ice while reaction mixtures are being prepared. Denaturation is also accelerated at air/water interfaces, so it's important to minimize air bubbles and freeze/thaw cycles for solutions containing restriction enzymes. Manufacturers attempt to minimize denaturation by supplying the solutions in a solution of 50% glycerol, which does not freeze when the enzymes are stored at -20˚C.

Follow some simple rules when you use REs.

- Be sure to use the recommended buffer for a particular RE.
- Keep the reactions on ice until the incubation begins
- Be careful not to introduce bubbles. Mix the reactions by flicking the tubes with your fingers, rather than using the vortex mixer.

Chapter 11

Exercise 1 - Plan the restriction digest

Assign each person in your group a different plasmid to analyze. You will use the sequence information for the Met protein coding sequence that has been cloned into the cloning vector. Recall that the S. cerevisiae MET genes have been cloned into the pBG1805 vector and that the S. pombe Met genes have been cloned into the pYES2.1 vector. Yo will use this information to predict which REs will cut the plasmid and/or your gene coding sequence. You will also be able to predict the sizes of restriction fragments generated with different REs.

First, you need to construct the sequence of the plasmid containing your gene:

1. Find the coding sequence of gene in its NM_ record.
   Record the number of nucleotides in your gene's coding sequence.

2. Open the sequence file for the BG1805 or pYES2.1 vector sequence posted on Blackboard.
   Paste the coding sequence from the NM_ record to the end of the plasmid sequence. (*Note that these are text files, not Word files.*)

   After you paste the coding sequence at the end of the vector sequence, delete the last three nucleotides, which comprise the gene's stop codon. (Stop codons are not included in the vector, which encode fusion proteins with additional amino acids at the C-terminus of your protein.)

   Remember that the first nucleotide of your MET is one nucleotide higher than the last nucleotide in the vector. For example, the BG1805 sequence contains 6573 nucleotides. The first nucleotide of a MET gene inserted into pBG1805 will be nucleotide 6574.

Next, you need to find useful restriction sites in the plasmid.

3. Paste the sequence from step 2 into the search box in the NEBCutter tool

tools.neb.com/NEBcutter2/

   Check the box to indicate that the plasmid is circular. You might also want to give your plasmid a name. The NEB site will store your queries for 24 hours, which can be very convenient. Click submit.

   The search tool will return results for a bewildering number of REs. The vast majority of the RE sites are not very useful, because the fragments are too large or too small, the enzyme is not available in the lab, or the endonuclease is sensitive to DNA methylation (which can be unpredictable).

4. Perform custom digests with enzymes that look promising.

   Click the custom digest link. This brings up a chart of RE that cut the plasmid, their recognition sites, the number of recognition sites, and the amount of enzyme activity in each of the four buffers used for digests. ***Your TA will tell you which REs are available in the lab.*** We will be analyzing the restriction fragments on agarose gels, which do well resolving fragments ranging in size from ~200 bp to ~5000bp. See which of the REs available in the lab will cut your plasmid.

5. With your team, prepare a table summarizing the RE predictions for your three plasmids.

   Make a table indicating the sizes of the restriction fragments generated with each RE. It may be helpful to include the length of the plasmid in the table. One possible organization for the table is shown below. The table uses a fictitious RE named PacMan and notes the lengths of the predicted restriction fragments.

	Plasmid 1	***Plasmid 2***	***Plasmid 3***
size	8330 bp		
Restriction Enzyme			
RE #1 - PacMan	2533, 5573*		
RE #2 - name			
etc.			
as many REs as you like			

*PacMan cuts the vector at 2 positions, generating 2 fragments. Note that the total size of the two fragments is equal to the length of the plasmid.

With your team, decide which enzymes would be useful for DNA fingerprinting your plasmids. Identify enzymes that would enable you to distinguish the three plasmids.

Exercise 2 - Set up the restriction digest

The concentrations of RE and plasmid DNA need to be matched in a restriction digest. Manufacturers assay the activity of each batch of RE and express the activity in units of activity/ µL. A unit of activity (U) is assessed in a standardized assay. Restriction digests are usually set up to contain 2-5 U per µg plasmid DNA. We will be using 5 µL of plasmid miniprep DNA in each reaction. The REs that you will be using have been diluted to a concentration of 0.5 U/µL.

In your lab notebook, note which RE(s) you have decided to use. Check which 10X salt solution that you should use from the manufacturer's chart of : http://www.neb.com/nebecomm/tech_reference/restriction_enzymes/buffer_activity_restriction_enzymes.asp?

- Set up the reactions to have a final volume of 10 µL (10.1 µL if you're adding BSA). For each reaction, combine in this order:

  ____ µL deionized water (to bring total volume to 10 µL)*
  1.0 µL 10X buffer
  0.1 µL BSA (from a 10 mg/mL 100x stock) - IF recommended
  5.0 µL plasmid - to give a final concentration of 0.2 - 0.4 µg plasmid DNA
  2.0 µL (1.0 U) restriction enzyme

- Add the restriction endonuclease(s) last and mix by gently tapping the tube.
- Incubate the samples at 37 °C for 2 hr. Store the samples in the freezer.

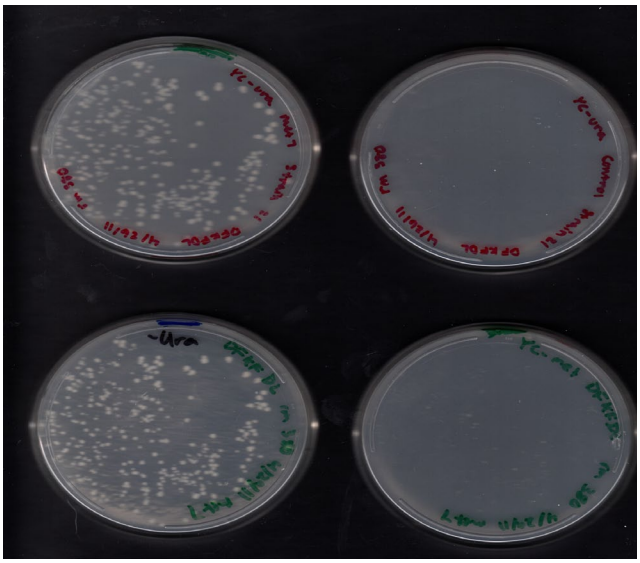
Exercise 3 - Analyze the restriction digest on an agarose gel

1. 1. Plan your gel. Each group of students will prepare one agarose gel. Each student will run one lane with undigested plasmid and a second lane with plasmid that has been digested with restriction endonuclease(s). An additional lane should be reserved for molecular weight standards. Record in your notebook how you plan to load your gels.

2. 2. Prepare, load and run your gels as described in Chapter 8. Be sure to record the details of every step in your lab notebook.

Chapter 12

Yeast transformation



Techniques for transforming microbial organisms with foreign DNA are essential in modern molecular biology. In this lab, you will transform a *S. cerevisiae met* strain with three different plasmids and use complementation to detect transformed cells. You will use replica plating to determine if *S. pombe met* genes are functionally equivalent to *S. cerevisiae MET* genes.

Objectives

- Understand the principles of microbial transformation
- Understand how plasmid-encoded genes can complement *met* mutations
- Understand selection schemes used to isolate transformed cells
- Transform *met* strains with plasmids carrying *S. cerevisiae* and *S. pombe* genes involved in methionine synthesis
- Test transformants for their ability to grow in the absence of methionine

Chapter 12

In this lab, you may receive a preliminary answer to the semester's research question. In this lab, you will transform an *S. cerevisiae met* strain with expression plasmids carrying homologs of the missing *MET* gene. You will use complementation analysis in two different experiments. In the first experiment, you will transform your yeast *met* strain with your three different plasmids and isolate transformants on media lacking uracil. Uracil selection depends on complementation. The expression plasmids we are using all carry a normal copy of the yeast *URA3* gene, as well as the *URA3* promoter, so the gene is regulated much like a normal chromosomal gene. Our yeast strains are derived from BY4742, which has the *ura3Δ0* allele. The Ura3p protein produced from the plasmid-encoded *URA3* gene compensates for the *ura3* deletion in the yeast chromosome, allowing transformed cells to grow in the absence of uracil. In the second experiment, we will use replica plating to determine if the transformed cells are able to grow in the absence of methionine. Growth should only occur if the plasmid-encoded *MET* or *Met* gene has complemented the *met* deletion in your yeast strain.

Transformation is an enabling technology in the molecular biology lab, so it's useful to review the process before beginning this lab.

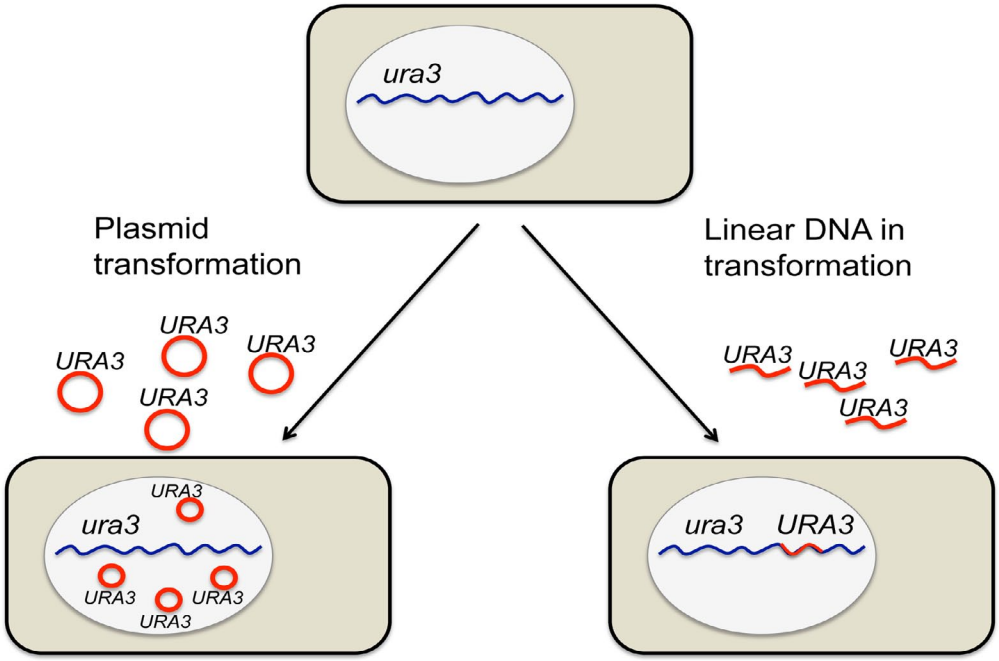
Transformation

Transformation refers to the uptake of DNA by a cell, causing a change in its phenotype. Naturally-occurring transformation was first described in 1928 by Frederick Griffith (Griffith, 1928), who described a heat-stable "transforming principle" from virulent *Streptococcus pneumoniae* that could transform non-virulent *S. pneumoniae* to an encapsulated, virulent form. The "transforming principle" was subsequently identified as DNA by Avery and colleagues in 1944 (Avery *et al.*, 1944). Since then, transformation has become an indispensable tool in the molecular biology laboratory. The physical basis for transformation is still poorly understood, but researchers have empirically developed conditions that give fairly consistent transformation in the lab. Reliable transformation techniques have been developed for bacteria and many eukaryotes, ranging from yeast to mammalian cells.

Transformation conditions have been developed empirically

The challenge in laboratory transformation is to devise conditions under which DNA will pass across the cell wall and plasma membrane of living cells, which are normally impermeable to DNA. Very few cells are naturally competent, or able to take up DNA. Consequently, researchers use a variety of chemical treatments to render cells competent. In general, these chemical treatments have some kind of destabilizing effect on the plasma membrane. The introduction of DNA into these competent cells can be further encouraged by a physical stress, such as a pulse of electric current or a rapid temperature change. Transformation is not a very efficient process, but because large numbers of microorganisms can be cultured in the laboratory, useful numbers of transformants can be obtained with most strains.

Techniques for yeast transformation are now standard in the laboratory. Depending on the details of the experimental procedure, reactions can yield as many as 10^6 transformants per µg DNA. The structure of the DNA used for transformation greatly affects the transformation efficiency. Transformation efficiencies are considerably higher with supercoiled plasmid DNA than with linear pieces of DNA. The reasons for this difference in efficiency are not entirely clear and probably arise from several factors. Linear DNA and plasmids may be taken up by cells at different rates. Linear DNA is more susceptible to destruction by nucleases than supercoiled plasmid DNA, which would lower its stability in the yeast cytoplasm. In contrast to plasmids, which have their own origins of replication, linear pieces of DNA also must be integrated into the chromosomal DNA before they can bring about the heritable change in phenotype associated with transformation. The differences between transformation with plasmids and linear pieces of DNA are outlined on the figure below.



Transformation of yeast cells with plasmid DNA and linear pieces of DNA
Competent *ura3* yeast cells are transformed with either plasmid DNA with the *URA3* gene (left) or a linear DNA cassette with the *URA3* gene (right). Both transformants are able to grow on media lacking uracil. Strains transformed with plasmids must be continuously grown on media lacking uracil to preserve the plasmid. Strains transformed with linear DNA fragments stably incorporate the *URA3* gene into their chromosomes, so selective pressure is no longer needed.

The most commonly used yeast transformation methods use a combination of lithium acetate, single-stranded DNA and polyethylene glycol (PEG). Although no one knows exactly how these components promote transformation, a number of hypotheses have been advanced. Lithium ions neutralize the negative charges on DNA molecules and the phospholipid bilayer, and they may also generate small holes in the plasma membrane that allow the passage of nucleic acids. Single-stranded DNA acts as a carrier for the DNA to be transferred into the cell and it

may help to protect the latter from endonucleases. The source of the carrier DNA is unimportant. Since the carrier DNA concentration is considerably higher than that of the DNA to be introduced into the cell, the carrier DNA is usually isolated from an inexpensive source, such as salmon sperm. It is particularly important that the carrier DNA for transformations be single-stranded. Either boiling (followed by rapid chilling to prevent reannealing of the helix) or base treatment can be used to denature the DNA helix before it is used for transformations. PEG may help bring the DNA into closer apposition with the membrane. PEG is often used to promote membrane fusion and is thought to alter water structure around the plasma membrane.

Transformants are isolated on selective media

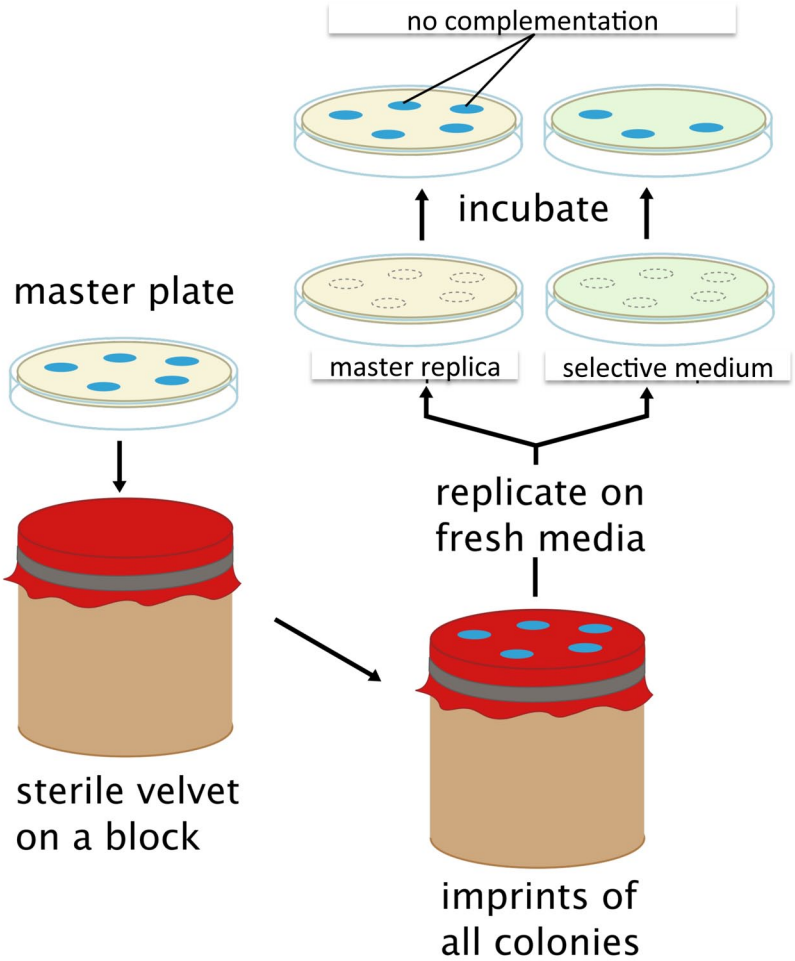
The DNA being used for transformation must carry a selectable marker whose presence can be detected by screening. Following the transformation, cells are plated on selective media that will allow transformed, but not untransformed, cells to grow. Consequently, it is critical to know the genotypes of the strains that one uses for transformations. For example, DNA carrying *URA3* genes must be transformed into *ura3* mutants in order to (1) detect transformants and (2) to maintain the plasmid in transformed cells (see below). In this case, the Ura3p encoded by the plasmid compensates for the deficiency in the *ura3* gene, an example of plasmid complementation.

Another common approach in transformation experiments is to introduce foreign genes that provide the recipient cells with protection against an environmental stress. The *S. cerevisiae* deletion strains that we are using this semester provide excellent examples of this kind of experiment. The deletion strains were generated as part of a systematic gene deletion project in which every *S. cerevisiae* ORF was replaced with a kanamycin resistance (KAN^R) gene. In this project, researchers used PCR reactions to prepare transformation cassettes with a kanamycin resistance (KAN^R) gene for each of the *S. cerevisiae* ORFs. Transformants were selected on plates containing kanamycin, and strains were established from single colonies.

The structure of the transformed DNA also affects the selection regimen that must be used to maintain the transformed strains. Transformed strains carrying plasmids are not stable under non-selective conditions. These strains must be propagated continually on selective media to maintain the plasmid. In the absence of selection, plasmid-bearing cells generally have a competitive disadvantage compared to cells without plasmids and will ultimately be lost from cultures. By contrast, strains derived by transformation and incorporation of linear pieces of DNA are stable. Selection is only required to establish the strains initially. Once the DNA has become incorporated into the chromosome, the marker is maintained by normal mitotic mechanisms.

Replica plates accelerate the screening process

As noted above, transformation is an inefficient process, so researchers want to make the most of every cell that has been transformed. In our experiments, we will be isolating transformed cells for their ability to grow in the absence of uracil, but we are really interested in their ability to grow in the absence of methionine. Replica plating offers a means to quickly screen a plate of cells for their ability to grow in a wide range of media, while retaining information about individual colonies. As shown below, the original plate of transformants becomes the "master plate." An imprint of the master plate is made by *GENTLY* tapping the inverted plate on a piece of sterile velveteen immobilized on a block. This imprint can then be transferred to plates with different kinds of selective media, establishing the genotype of the transformants. In our experiments, we will make transfer replicas of the transformation reactions (isolated on -URA plates) to plates lacking methionine and containing either glucose or galactose as a carbon source.



Replica plating provides a rapid screening method for analyzing phenotypes.
Colonies on a master plate are transferred to a sterile piece of velveteen. Copies of the mater plate are transferred to additional selective or indicator media to monitor phenotypes under additional conditions.
Adapted from http://en.wikipedia.org/wiki/Replica_plating#mediaviewer/File:Replica-dia-w.svg

Chapter 12

Exercise 1 - Yeast transformation

The following protocol is a slight modification of the "Quick and Dirty" transformation protocol described by Amberg *et al.* (2005). With careful attention to detail and cooperative strains, this procedure can yield thousands of transformants per µg plasmid DNA. Modifications to this method can increase its efficiency by several orders of magnitude, which would be required for linear pieces of DNA (Gietz and Schiestl, 2007).

Prepare a transformation master mix

1. Prepare a transformation master mix. The following ingredients provide enough reagents for five transformation reactions. Combine in a microcentrifuge tube:

    100 µL sterile 2 M lithium acetate (freshly prepared)
    400 µL sterile 50% PEG-3350
    4 µL 2-mercaptoethanol (STINKY!! add this in the fume hood!)

Set up individual transformation reactions - for each transformation:

2. Add 15 µL of the denatured salmon sperm DNA (2 mg/mL) to ***labeled*** microcentrifuge tubes.

Note: It is important for the salmon sperm DNA to be single-stranded for this procedure to work well. Boil the DNA for 5 minutes to denature the DNA. Quick chill the DNA by placing it immediately on ice. Keep the DNA on ice until you are ready to use it.

3. Add 5 µL of miniprep plasmid DNA to the appropriately labeled microcentrifuge tube.
4. Add 100 µL of transformation mix from step 1 to the microcentrifuge tube. Vortex for 10-15 seconds to mix the contents.
5. Using a sterile toothpick, scrape a large yeast colony (or the equivalent of a "match head" of yeast) from a YPD plate. Transfer the yeast to the microcentrifuge tube containing the transformation/DNA solution (step 4) by twirling the toothpick several times. Be sure that the cells are uniformly suspended before proceeding.
6. Transfer the transformation mixture from step 5 to a sterile round bottom culture tube. (Replace the cap on the microcentrifuge tube and save it for step 8.)

Repeat steps 2-6 for each of your transformation reactions. ***Be sure to include a control with contains no plasmid DNA.***

7. Incubate the transformation mixtures at 37˚C with shaking for 30 min.

Plate the transformed cells on selective media lacking uracil

8. Remove 10 µL of the resuspended cells to 90 µL of sterile water in a microcentrifuge tube. This sample will be serially diluted for a spot plate (step 10) that you will use to calculate the transformation efficiency.
9. Plate the remainder of the mixture on a selective media lacking uracil. Transfer the transformation reaction to the plate, and then shake out ~4 sterile glass beads that will spread the cells. Cover the plates and spend 0.5-1 minutes agitating the plates so that the beads spread the transformation mixture evenly over the surface of the plate. Discard the glass beads into the appropriate waste containers, so they can be used again. Incubate the plates at 30˚C until colonies can be detected. The earliest that colonies will be visible is usually 2 days. If the colonies are small, allow them to grow an additional day(s) at 30˚C. If you need to incubate the plates beyond 3 days, seal the plates with parafilm to prevent them from drying out. Count the number of cells on the plate.

Determine the number of viable cells in the transformation mixture.

10. Prepare a series of 4 additional dilutions of the cells set aside in step 8. Use these dilutions for a spot plate on YPD media. Each row on the plate should contain cells from a different transformation reaction. Incubate the cells at 30˚C or room temperature until individual colonies can be detected. ***Do not allow the plate to overgrow, because you need to distinguish individual colonies.***

Calculate the transformation efficiency

11. Calculate the fraction of cells that were transformed. For each transformation reaction, divide the number of transformed cells (step 9) by the total number of cells in the transformation mixture. Use the spot plate data from step 10, correcting for dilution, to calculate the total number of cells in the transformation mixture.

14. Transformation efficiencies are usually expressed by the number of cells transformed per µg DNA. Use your Nanodrop data to calculate the concentration of DNA used for the transformation and your data from step 9 to obtain the number of transformed cells.

Chapter 12

Exercise 2 - Complementation

Your initial selection of transformants was done on plates that lacked uracil, but contained methionine. You next will test the ability of your transformed strains to grow on media lacking methionine using replica plating. We will use methionine-free media containing either glucose or galactose for replicas, and you will also prepare a fresh master plate. Predict which transformants will grow on each of the plates.

It is important to have a light touch during replica plating!! The goal is to transfer a small portion of cells from each colony on the master plate (the plates carrying your transformants) to a number of plates containing different media.

1. Place an orientation mark with a magic marker on the perimeter of your master plate as well as the plates that will be used for replicas.
2. Place a piece of sterile velveteen with the nap face up on the replica plating block.
3. Remove the lid from your master plate and invert the plate on the block, aligning the orientation marker on the plate with the marker on the block. *GENTLY* and *EVENLY* tap on the bottom of the plate to transfer cells to the velveteen. Remove the master plate and replace the lid.
4. Repeat step 3 with plates containing the following media:
    - Medium without methionine, containing glucose
    - Medium without methionine, containing galactose
    - Mediuam without uracil, containing glucose and methionine

References

Amberg, D.C., Burke, D.J., and Strathern, J.N. (2005). Methods in Yeast Genetics. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) Pp. 113-114.

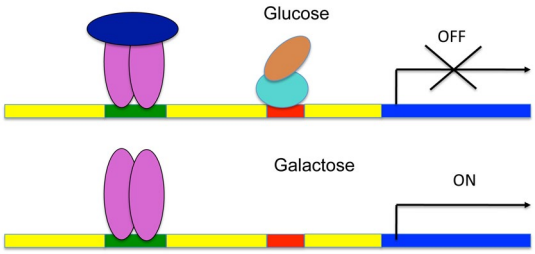
Avery, O. T., MacLeod, C. M., and McCarty, M. (1944). Studies on the chemical nature of the substance inducing transformation of pneumococcal types: Induction of transformation desoxyribonucleic acid fraction isolated from pneumococcus type III. *Journal of Experimental Medicine* **79**, 137–157.

Gelperin, D.M. White, M.A., Wilkinson, M.L., Kon, Y., Kung, L.A., Wise, K.J., Lopez-Hoyo, N., Jiang, L., Piccirillo, S., Yu, H., Gerstein, M., Dumont, M.E., Phizicky, E.M., Snyder, M., and Grayhack, E.J. (2005). Biochemical and genetic analysis of the yeast proteome with a movable ORF collection. *Genes Develop.* **19**, 2816-2826.

Gietz, R.D., and Shiestl, R.H. (2007). High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nature Protocols* **2**, 31-34.

Griffith, F. (1928). The significance of pneumococcal types. *Journal of Hygiene* **27**, 113–159.

Chapter 13
Protein overexpression



In this lab, you will use various carbon sources to manipulate the expression of Met fusion proteins in cells that have been transformed by overexpression plasmids. Expression of the Met proteins is controlled by the inducible *GAL1* promoter in the plasmid. You will prepare extracts from cells grown under both repressed and induced conditions for subsequent analysis by SDS-PAGE.

Objectives

- Understand regulation of the yeast *GAL1* promoter by carbon sources.
- Understand the experimental challenges to preparing cell extracts from yeast.
- Culture yeast with different carbon sources to either induce or repress expression from the *GAL1* promoter
- Prepare extracts from transformed yeast grown under repressed and induced conditions

Chapter 13

Over the next few weeks, you'll be analyzing the expression of *S. pombe* and *S. cerevisiae* Met fusion proteins in your transformed strains as well as untransformed parental *met* strains. You have already tested the ability of the overexpression plasmids to complement the *met* mutations in your yeast strains. Complementation depends on the presence of functional Met proteins. If you observed a failure to complement the *met* deficiencies, this could indicate that proteins were not expressed from the plasmids. Alternatively, the overexpressed proteins may not function normally. Remember that the proteins expressed from the BG1805 and pYES2.1 plasmids are fusion proteins with additional sequences at their C-termini (Gelperin et al., 2005). The biochemical activities of these fusion proteins have not been evaluated.

In this experiment, you will prepare extracts from yeast for later experiments (Chapters 14 and 15) in which you will determine if the fusion proteins are actually expressed in the transformed yeast strains and how the expression of Met proteins varies with carbon sources. (Unfortunately, these experiments will not tell you whether the proteins are enzymatically active. You might want to think about how to figure out if the overexpressed proteins are active....)

Regulation of the GAL1 promoter

Protein expression from both the pBG1805 and pYES2.1 plasmids is controlled by the powerful *S. cerevisiae GAL1* promoter. The *GAL1* promoter is positively regulated by galactose and negatively regulated by glucose. In yeast, glycolysis plays a major role in energy production, and glucose is far and away the preferred carbon source. Genes involved in the metabolism of other carbon sources are usually repressed when glucose is available. Glucose repression is a complex phenomenon that includes the binding of repressor proteins to control regions of genes involved in the metabolism of alternative carbon sources, such as *GAL1*. When glucose is not available, however, yeast activate genes that metabolize other available energy sources, such as galactose. Galactose increases the transcription of several genes in a pathway that culminates in the conversion of galactose into glucose-6-phosphate (G6P), an intermediate in glycolysis. The first gene in the pathway induced by galactose, *GAL1*, encodes galactokinase, which phosphorylates galactose to galactose- 1-phosphate. (Check out the *GAL1* pathways link in SGD.) The *GAL1* promoter is exquisitely sensitive to galactose. In the presence of galactose, expression of the *GAL1* gene increases ~1000-fold above the level observed in the presence of glucose.

In this lab, you will prepare extracts from transformed *met* strains grown in either glucose or galactose. For this experiment, we will "wean" the yeast from glucose by growing them for several days in raffinose before inducing expression with galactose. Raffinose is a trisaccharide composed of galactose, fructose and glucose. Raffinose relieves glucose repression, but does not induce expression from the *GAL1* promoter. Cultures will then be given glucose or galactose for a few hours to either repress or induce expression from the *GAL1* promoter before the extracts are prepared.

Preparing protein extracts from yeast cells

Proteins comprise about half of the dry weight of most cells and include the myriad of structural proteins, catalysts, receptors and signaling proteins responsible for cell function. To understand cell function, scientists often want to analyze the protein composition of cells. Protein analysis begins with the preparation of a cell extract, ideally under conditions that minimize protein degradation. Preparing good cell extracts is something of an art, and many factors need to be considered during the design of an extraction protocol. In this course, we will be analyzing protein function in yeast. An average haploid yeast cell contains ~6 pg protein (Sherman, 2002). Although yeast cells have many advantages for genetic studies, they are notoriously difficult to use for biochemical studies. Nonetheless, scientists have been able to develop procedures for extracting yeast proteins that circumvent many of these experimental barriers.

The first consideration in designing an extraction procedure is the compartmentalization of cells. All cells are surrounded by a plasma membrane and eukaryotic cells contain additional membranes that surround organelles. Fungal cells also have cellulose-based cell walls that protect the cells against mechanical and osmotic forces. Cell extraction procedures begin with the disruption of the plasma membrane and cell wall by mechanical and/or chemical treatments. Mechanical disruption of yeast cells must be fairly vigorous because their cell walls are very tough. Mechanical methods commonly used to disrupt yeast include sonication, high pressure, and "beating" with glass beads. These vigorous treatments run the risk of denaturing proteins because of the heat and foaming generated during the processes.

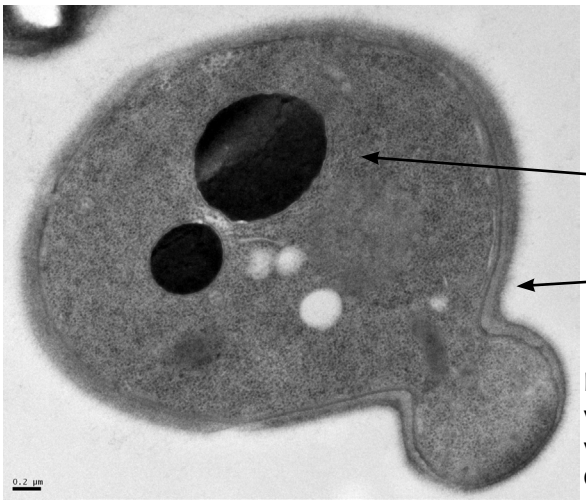


Figure 1.
Electron micrograph of an *S. cerevisiae* cell.

Vacuole contains many proteases.

Polysaccharide-based wall surrounds the cell.

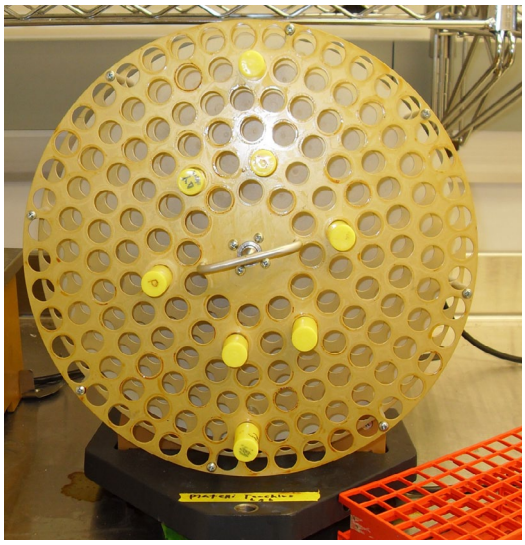
Image by Christopher Bruser, reproduced with permission from www.celllibrary.org CIL10452

Chemical treatments offer a gentler alternative to mechanical disruption for preparing extracts. Chemical extraction procedures frequently include detergents that solubilize membrane lipids, thereby allowing proteins to diffuse out of the cell. Most detergents do not discriminate between intracellular and plasma membranes, so a detergent extract usually contains proteins from multiple organelles as well as cytoplasmic proteins. Detergents can be either nondenaturing or denaturing. Denaturing detergents destroy protein structures by breaking the thousands of

weak bonds that normally hold proteins together. A good example of a denaturing detergent is SDS, or sodium dodecyl sulfate, which we will be using to prepare our cell extracts. By contrast, non-denaturing detergents bind to the surfaces of proteins and consequently preserve enzyme activity.

When preparing extracts, care must be taken to protect proteins from degradation by cellular proteases. Cells contain proteases with many different specificities that are responsible for normal turnover of proteins in cells. Cell disruption often releases proteases from compartments such as lysosomes, providing them access to cytoplasmic proteins. Yeast are notoriously rich in proteases. In an intact yeast cell, many of these proteases are located in the yeast vacuole, which is analogous to the mammalian lysosome. The protocol that we'll use in this course (Amberg et al., 2005) relies on chemical disruption and rapid protein denaturation to denature the yeast proteases. Extracts prepared by this method are suitable for SDS-PAGE and western blot analysis.

Exercise 1 - Prepare cellcultures for extraction



First lab session of the week

1. Inoculate a 2 mL of YC-URA containing raffinose with a single yeast colony.

2. Repeat step 1 for each of your transformed strains.

3. Place the cultures on the wheel at 30˚C, ***taking care that tubes are properly balanced.*** Turn the wheel on.

Don't forget to turn the wheel back on after removing or adding cultures!!

~Four hours before the next lab session

1. Remove your cultures from the wheel.

2. Remove 1 mL of the culture and read its OD_{600}. Determine the volume of cells that is needed to prepare a fresh 2 mL culture with an OD_{600} of 0.4 mL. Transfer this quantity to two empty culture tubes. Add enough induction medium (medium with galactose) to one tube and repression medium (medium with glucose) to the other tube to bring the volumes up to 2 mL. Be sure that the tubes are labeled with your initials and the sugar source.

3. Place the cultures back on the wheel until class time.

Exercise 2 - Preparing cell extracts

Prepare cells for the extraction

1. Grow the cells under various conditions as instructed.
2. Determine the cell concentration. Transfer 100 µL of each cell culture to 900 µL deionized water and measure the OD_{600} of cultures in the spectrophotometer. Calculate the volume of the culture that you will need to obtain the number of cells equivalent to 2.5 OD_{600} units.
3. Collect the cells by centrifugation using either the table top centrifuge or the microcentrifuge. (This latter may require multiple spins in the microcentrifuge.) Your goal is to have 2.5 OD_{600} from each culture in a separate, clearly labeled 1.5ml microcentrifuge tubes. Collect the cells by centrifugation for 1 minute at top speed- in a microcentrifuge. Decant and discard the supernatant.
4. Rinse the cells. Add 1 mL deionized water to each tube. Resuspend the cell pellet by gently drawing the cells in and out of a micropipette tip, taking care to prevent premature lysis of the cells. This rinse step removes proteins from the culture medium that may be present in the cell pellet.
5. Collect the cells by centrifuging the suspension for 1 minute at top speed. Decant and discard the supernatant.
6. Resuspend the cells in 100 µL deionized water.

Prepare the protein extract

1. Transfer the cells to an appropriately LABELED 1.5 ml screw-cap microcentrifuge tube.
2. Add 100 µL of 0.2N NaOH to each tube, and incubate the cells for 5 minutes at room temperature. (The addition of NaOH does not lyse the cells, but it makes them more permeable and more fragile.)
3. Pellet the cells again in the microcentrifuge and remove the supernatant.
4. Resuspend the cells in 50 µl 2 X SDS-PAGE sample buffer*. IMMEDIATELY place the tubes in a boiling water bath. Leave the cells in the water bath for 3 minutes. This treatment effectively denatures the proteins. Yeast cells contain many proteases that could degrade other cellular proteins, so it's important that the proteases are denatured before they have the chance to attack other cellular proteins.

Note: The 2 X SDS-PAGE sample buffer contains 2-mercaptoethanol (also known as beta-mercaptoethanol, or BME). Use appropriate caution and work quickly when handling this reagent. BME is a volatile reagent with a strong odor reminiscent of rotten fish.

5. Remove insoluble cellular debris from the protein extract by centrifuging the extract for 2-3 minutes in the microcentrifuge. The insoluble residue in the pellet contains the cell wall, DNA and some insoluble proteins. Carefully remove the supernatant, which contains the proteins to a new, properly labeled microcentrifuge tube.

6. Store the samples in the freezer for future use.

*2 X SDS-PAGE sample buffer consists of:

120 mM Tris/HCl, pH 6.8
10% glycerol (glycerol is significantly more dense than water)
4% SDS
8% 2-mercaptoethanol
0.004% bromophenol blue (a tracking dye for electrophoresis)

References

Amberg, D.C., Burke, D.J., and Strathern, J.N. (2005). Methods in Yeast Genetics (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). P. 121

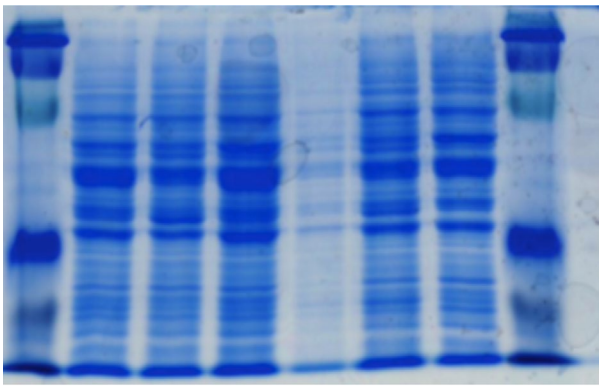
Gelperin, D.M. White, M.A., Wilkinson, M.L., Kon, Y., Kung, L.A., Wise, K.J., Lopez-Hoyo, N., Jiang, L., Piccirillo, S., Yu, H., Gerstein, M., Dumont, M.E., Phizicky, E.M., Snyder, M., and Grayhack, E.J. (2005). Biochemical and genetic analysis of the yeast proteome with a movable ORF collection. *Genes Develop.* **19**, 2816-2826.

Johnston, M. (1987). A model fungal gene regulatory mechanism: the GAL genes of Saccharomyces cerevisiae. *Microbiol. Rev.* **51**, 458-476.

Sherman, F. (2002) Getting started with yeast. Methods Enzymol. 350, 3-41.

Chapter 14

SDS-PAGE



This lab will introduce you to SDS-PAGE, a simple and inexpensive method for resolving proteins in complex mixtures. SDS-PAGE gels provide the starting materials for western blots and for some proteomic techniques. In this lab, you'll use SDS-PAGE to analyze the protein extracts that you prepared from yeast strains overexpressing Met fusion proteins.

Objectives

- Understand the theory governing protein separation by SDS-PAGE
- Learn how to cast and run SDS-PAGE gels
- Understand how stains are used to visualize proteins in gels
- Learn how to estimate the molecular weight of a protein from its migration on SDS-PAGE gels

Chapter 14

This lab will introduce you to SDS-PAGE (sodium dodecyl sulfate - polyacrylamide gel electrophoresis), a simple and inexpensive method for resolving proteins in complex mixtures. You will use SDS-PAGE gels to analyze the yeast protein extracts that you prepared in the last lab. You will make two gels in this lab. You will run one gel today and stain it with Brilliant Blue G-250 to visualize the proteins in the extracts. You will store the other gel until the next lab period, where you will use it for a western blot.

Background

SDS-PAGE is widely used to analyze the proteins in complex extracts. The most commonly used methods are derived from the discontinuous SDS-PAGE system first described by Laemmli (1970). The system actually consists of two gels - a resolving (aka running) gel in which proteins are resolved on the basis of their molecular weights (MWs) and a stacking gel in which proteins are concentrated prior to entering the resolving gel. Differences in the compositions of the stacking gel, resolving gel and electrophoresis buffer produce a system that is capable of finely resolving proteins according to their MWs.

Gel electrophoresis of macromolecules

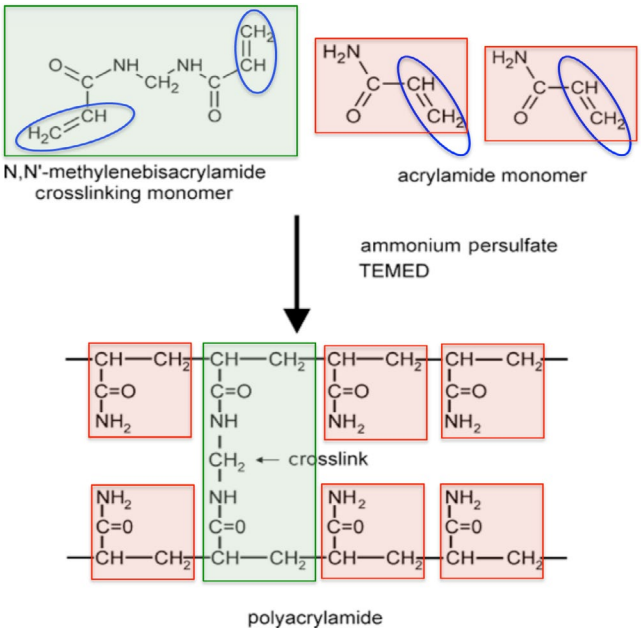
In gel electrophoresis, an electric field is used to move charged molecules through a matrix of a polymerized substance such as agarose or polyacrylamide. The rates at which individual molecules move through the gel depend on the properties of both the separation system and the molecules themselves. Gel matrices are permeated with networks of pores through which the molecules move. The amount of resistance that the matrix presents to the movement of a molecule depends on the diameter of the pore as well as the size and geometry of the molecule. Researchers can control the size of the pore by adjusting the concentration of gel monomer within a certain range. In general, smaller, more highly charged molecules migrate more rapidly through gels than larger or less charged molecules. The mobility of a molecule is also affected by the buffer system and the strength of the electrophoretic field, i.e. voltage, used for the separation.

You have already used agarose gel electrophoresis to separate DNA molecules. DNA molecules are much larger than protein molecules and they have a uniform charge to mass ratio. Consequently, electrophoretic analysis of DNA molecules is more straightforward than the separation of protein molecules. The agarose gels used to separated DNA molecules have much larger pores than the polyacrylamide gels typically used to separate protein molecules. (However, small DNA molecules, e.g. less than 1000 bp, may be separated in polyacrylamide gels.)

Chemistry of acrylamide polymerization

Proteins are usually separated on polyacrylamide gels formed by the chemical polymerization of acrylamide and a cross-linking reagent, N,N'methylenebisacrylamide (Figure 1). Investigators are able to control the size of the pores in the gel by adjusting the concentration of acrylamide, as well as the ratio of acrylamide to bisacrylamide. Raising either the concentration of acrylamide or bisacrylamide, while holding the other concentration constant,

will decrease the pore size of the gel. Polymerization occurs because of free oxygen radicals that react with the vinyl groups in acrylamide and bisacrylamide, as shown in the figure below. The oxygen radicals are generated from the catalyst, ammonium persulfate (APS), when it reacts with a second catalyst, N,N,N',N'-tetramethylethylenediamine (TEMED). Aqueous solutions of APS are unstable, but they can be stored for about a week in the refrigerator or for several months in the freezer without losing potency. .



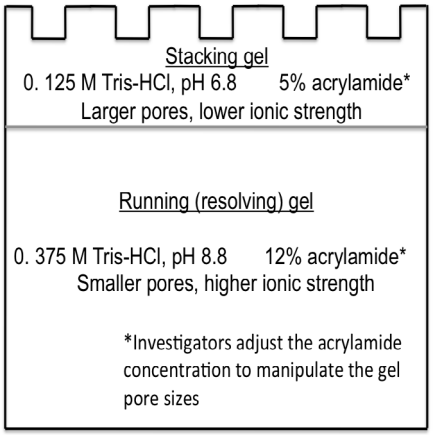
Proteins are denatured prior to electrophoresis

Compared to DNA molecules, proteins are structurally very diverse. Crystallographers have described over 1000 structurally distinct protein folds. Proteins also show tremendous variation in their amino acid compositions and in the distribution of amino acids in the folded structure, features with important implications for electrophoresis. Because the hydrophobic effect is an important contributor to protein folding, hydrophobic amino acids tend to be more frequently found in the interior of proteins than on their surfaces. The surfaces of proteins have a higher frequency of polar and charged amino acids than the interior of the protein, because these side chains are compatible with the aqueous cytoplasm. Even so, protein surfaces are mosaics with respect to the distribution of charged, polar and hydrophobic groups. Consequently, folded proteins lack the uniform charge to mass ratio that characterizes DNA molecules.

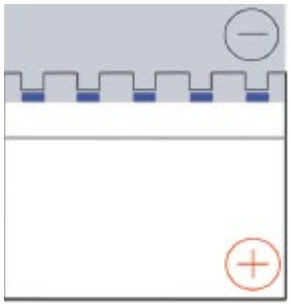
To resolve the proteins in a cell extract, investigators must to reduce the structural complexity of proteins in their samples and impart a uniform charge to mass ratio to the proteins. In SDS-PAGE, the solution is to denature the proteins by boiling them with the anionic detergent, sodium dodecyl sulfate (SDS) and 2-mercaptoethanol. The combination of heat and detergent is sufficient to break the many noncovalent bonds that stabilize protein folds, and 2-mercaptoethanol breaks any covalent bonds between cysteine residues. Like other detergents, SDS is an amphipathic molecule, consisting of a hydrophobic 12-carbon chain and a hydrophilic

sulfate group. The SDS hydrocarbon chain binds to the many hydrophobic groups in proteins, reducing the protein to a random coil, coated with negatively charges along its length. Denatured proteins bind quite a lot of SDS, amounting to ~1.4 g SDS/g protein, or ~one SDS molecule for every two amino acids.

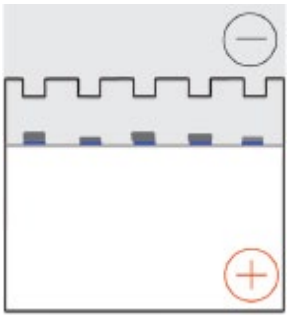
Discontinuities between the stacking and running gels underlie the resolving power of the SDS-PAGE gels



The Laemmli SDS-PAGE system can be considered a 3-component system. The stacking and running (resolving) gels have different pore sizes, ionic strength and pH. The third component is the electrophoresis buffer (25 mM Tris, 192 mM glycine,, 0.1% SDS, pH ~8.3), which contains large amounts of glycine. The ionization state of the glycine is critical to the separation. At neutral pH, glycine is a zwitterion, with a negatively charged carboxyl group and a positively charged amino group. The pK_a of the amino group is 9.6, considerably higher than the pH of the chamber buffer. Consequently, very little glycine has a negative charge in the chamber buffer or stacking gel, and significant ionization does not occur until the glycine enters the more alkaline pH 8.8 environment of the running gel. Let's follow the progress of protein samples during SDS-PAGE to see how differences in the composition of these three components generate the high resolving power of SDS-PAGE gels.

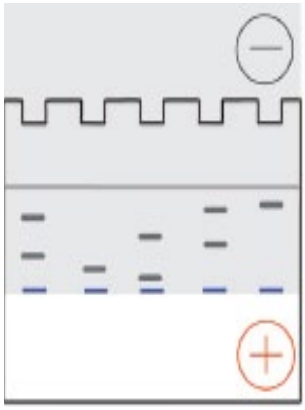


The sample buffer used for SDS-PAGE contains a tracking dye, bromophenol blue (BPB), which will migrate with the leading edge of the proteins being separated on the gel. The sample buffer also contains glycerol, which allows the protein samples to settle into the bottom of the gel wells. The gel is vertically positioned in the electrophoresis apparatus and covered with chamber buffer containing glycine (shaded).



Once a voltage is applied, the chloride ions in the sample buffer and stacking gel move rapidly toward the positive pole, forming the leading edge of a moving ion front. Glycine molecules have very little charge in the stacking gel, so they migrate at the rear of the moving ion front. This difference in chloride and glycine mobility sets up a steep voltage gradient in the stacking gel that sweeps along the negatively charged protein-SDS complexes. The large pores of the stacking gel present very little resistance to the movement of protein-SDS complexes, which then "stack up" into a very concentrated region at the interface between the running and stacking gels. Protein-SDS complexes remain concentrated at the interface until the slowly migrating glycine molecules reach the boundary between the two gels.

Dramatic changes occur as the glycine ions enter the running gel. The pH of the running gel is closer to the pK_a of the glycine amino groups, so a significant fraction of the glycine molecules assume a negative charge. Negatively charged glycine molecules begin to move at the same rate as the chloride ions, thereby eliminating the voltage difference that controlled protein mobility through the stacking gel. The pores in the running gel are much smaller than those of the stacking gel, so the pores present frictional resistance to the migration of proteins. Proteins begin to migrate at different rates, because of the sieving properties of the gel. Smaller protein-SDS complexes migrate more quickly than larger protein-SDS complexes. Within a certain range determined by the porosity of the gel, the migration rate of a protein in the running gel is inversely proportional to the logarithm of its MW.



Proteins are visualized with stains.

With few exceptions, naturally-occurring proteins are invisible on SDS-PAGE gels. Consequently, researchers often use pre-stained protein standards to monitor the approximate positions of proteins during electrophoresis. The pre-stained standards are produced by covalently attaching a large number of chromophores to a protein. The addition of the chromophores increases the MW of the protein and also produces more diffuse bands on the gel. The diffuseness of the bands reflects variation in the number of dye molecules attached to individual protein molecules. We will use prestained standard proteins in our gels, so you will be able to visualize the separation that is occurring. Yeast proteins will not be visible, however, because they have not been modified with chromophores.

To visualize the positions of proteins after electrophoresis is complete, investigators stain the gels with various dyes that bind noncovalently and with very little specificity to proteins. During the staining process, proteins are also "fixed" in the gel, meaning that proteins become insoluble and unable to diffuse out of the gel. The most commonly used dyes are the closely related Brilliant Blue R-250 and G-250 dyes, which bind proteins nonspecifically through a large number of ionic and Van der Waals interactions. Although Brilliant Blue R-250 staining is slightly more sensitive than G-250 for detecting proteins, the G-250 staining procedure is more rapid and does not require destaining with organic solvents. We will use the commercial Simply Blue™ reagent, which contains a colloidal suspension of Brilliant Blue G-250, to stain the gels. In this procedure, gels are rinsed with water to remove the buffer salts used for electrophoresis and then treated with the colloidal G-250 suspension. Protein bands appear rapidly, and when necessary, the gels can be destained with deionized water to lower the gel background. Brilliant Blue staining intensity is considered a quantitative procedure, because the intensity of a stained band is directly proportional to the amount of protein in a band.

The figure below illustrates these points. The same sets of unstained and pre-stained protein standards were separated on either 12% or 15% SDS-PAGE gels. The prestained standards in lanes 1-5 are visible without staining, but they become much more pronounced after staining. The unstained standard in lane 6 requires staining to become visible, but the bands are much more discrete and will give more reliable values wen calculating MWs of unknown proteins. The data in lanes 2-5 also demonstrate that Brilliant Blue staining is a quantitative procedure, because the intensity of bands in each lane increases in direct proportion to the amount of protein loaded in that lane.

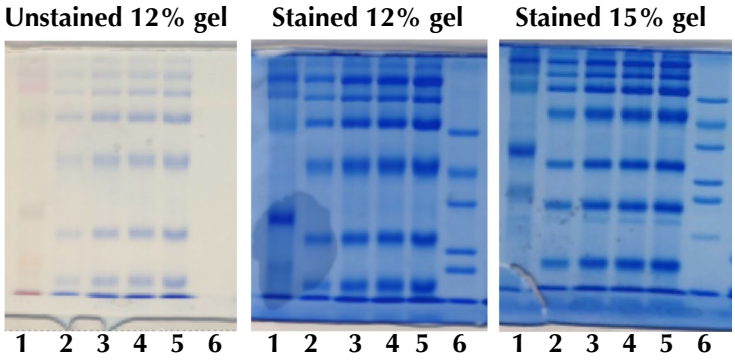


Figure 4. Molecular weight standards
1-Kaleidoscope protein standards (BioRad)
2-6.25 µg prestained protein standard
3-12.5 µg prestained protein standard
4-15 µg prestainded protein standard
5-18.75 µg prestainded protein standard
6-Unstained protein standard

Protein molecular weights can be calculated from their migration on gels

The sizes of proteins in an extract can be calculated by comparing their migration to a set of standard proteins run on the same gel. Researchers select standard proteins that are well- resolved on the particular gel that they are running. For example, an investigator using a 7.5% gel will select standards with higher molecular weights (MWs) than an investigator using a 15% gel, which is better suited to the analysis of small proteins. The principles used to estimate MWs are the same used for agarose gel electrophoresis. A plot of the $\log_{10}$MW of the standard proteins against the distance that each protein migrated on the gel will give a straight line in the region where the gel has good resolving power. (Note: MW is not the same as the mass of a protein. MW is a dimensionless term. For example, myoglobin has a mass of 16.7 kDa and a MW of 16,700.) The sizes of unknown proteins can be estimated by interpolating experimental values on a graph of standard proteins. Proteins whose molecular weights fall out side this range will not be well-resolved on the gel.

When analyzing experimental data, remember to consider any amino acids that have been added during the cloning procedure. The Met proteins that you are working with are fusion proteins with additional amino acids at the C-termini the Met proteins. The BG1805 plasmid encodes HA and His6 epitopes, as well as an immunoglobin binding domain. Together these sequences add a walloping ~19 kDa to the expected mass of S. cerevisiae Met proteins (Gelperin et al., 2005). The pYES2.1 plasmid adds only 33 amino acids, including a V5 epitope tag and a $(His)_6$ purification tag to the C-termini of overexpressed proteins. Together, these amino acids add 3710 to the MW of the protein.

Casting SDS-PAGE gels

These instructions are designed for constructing two 12% SDS-PAGE gels with the BioRad Mini Protean system. We will need only one gel in this lab, but the extra gel provides insurance against leaks! If neither gel leaks, you will use the second gel for westesrn blots in the next lab.

Assemble the gel casting apparatus

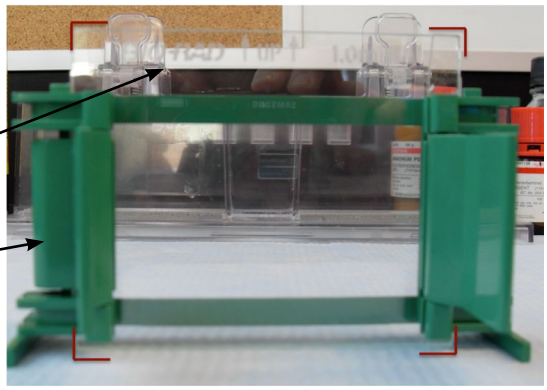
1. Assemble the components that you will need for casting the gel: a tall glass plate with attached 1 mm spacers, a small glass plate, a green casting frame and a casting stand.
2. Place the green casting frame on the bench with the green "feet"resting firmly against the bench and the clamps open (perpendicular to the frame) and facing you.
3. Place the two gel plates in the frame. Insert the taller spacer plate with the "UP" arrows up and the spacers facing toward you into the casting frame (the BioRad logo should be facing you). Insert the short glass plate in the front of the casting frame.
4. Secure the plates in the casting frame by pushing the two gates of the frame out to the sides. IMPORTANT: the bottom edges of the two plates should be flush with the lab bench before you clamp the frame closed to ensure a watertight seal.
5. Clamp the casting frame with glass plates into the casting stand, with the gates of the casting frame facing you.
6. Check to see if the assembled plates in the casting stand are sealed properly by pipetting a small amount of deionized water into the gap between the plates. If the glass plates hold water and don't leak, you are ready to make the gels. Pour the water out by holding the entire casting platform over a liquid waste container or sink. Use paper towels or tissues to absorb any residual water. If the gel leaks, disassemble the frame, dry the plates and go back to step 3.

Assembling plates in the casting frame.

Large spacer plate with spacer goes in rear, oriented so the arrows point up and the logo is readable. Small plate is placed in front. (Corners of the plates are marked with bracket marks.)

Green gates of casting frame are open.

Casting frame "feet" and bottom edges of plates are flush against the benchtop.



Prepare two resolving gels.

SAFETY NOTE: Acrylamide and bisacrylamide monomers are neurotoxins. Gloves, goggles and aprons should be used when working with acrylamide.

Assemble the chemicals that you will need to pour the gels. The table below shows the quantities of each chemical that you will need to pour two gels with the Mini-Protean system. Polymerization occurs rapidly, so be sure to follow the step-by-step instructions below.
NOTE: catalysts should NOT be included into the mixture until you are ready to pour the gels!!

Reagent	**Resolving gel**	**Stacking gel**
Deionized water	3.5 mL	1.4 mL
30% acrylamide:bis-acrylamide (29:1)	4.0 mL	0.42 mL
1.5 M Tris-HCl, 0.4% SDS, pH 8.8	2.5 mL	------
0.5 M Tris-HCl, 0.4% SDS, pH 6.8	------	0.675
10% ammonium persulfate	100 µL	20 µL
TEMED	10 µL	5 µL

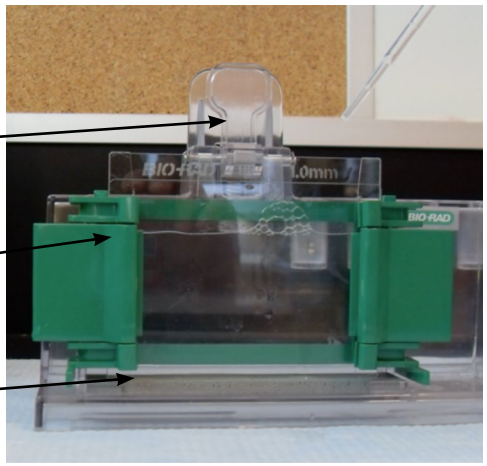
1. Label two 15 mL conical tubes "Resolving gel" and "Stacking gel".
2. Prepare the resolving gel. Mix the acrylamide solution, **pH 8.8** Tris buffer and water, as shown in the chart above. Mix the ingredients gently, trying not to introduce air. Oxygen inhibits polymerization of acrylamide gels.
3. To the resolving gel mixture, add 100 µL of a 10% ammonium persulfate (APS) solution. Gently mix the solution, trying not to introduce air. Oxygen inhibits acrylamide polymerization.
4. Add 10 µL of TEMED catalyst. Once again, gently mix in the catalyst trying not to introduce air bubbles. CAUTION: TEMED has an unpleasant odor. Cover the tube immediately after you aliquot this reagent.
5. Working quickly, use a plastic transfer pipette to fill the space between the two plates until the resolving gel solution reaches a height just above the green clamps on the gel casting frame. Draw up any remaining acrylamide into the transfer pipet. (You will know that the acrylamide has polymerized when you can no longer push the acrylamide out of the pipet.)
6. Using a transfer pipet, add deionized water so that it gently flows across the surface of the polyacrylamide mixture. The water layer ensures that the polyacrylamide gel will have a level surface once it polymerizes.
7. Allow the gel to polymerize, which takes ~10-15 minutes. You will note that the interface between the polyacrylamide and water overlay disappears temporarily while the gel polymerizes. A sharp new interface then forms between the two layers, indicating that polymerization is complete. (You can also check the remaining polyacrylamide in the transfer pipette to see if it has polymerized.)
8. When polymerization is complete, remove the water from the top of the resolving gel by tilting the gel to the side and using a paper towel or Kimwipe to wick out the water.

Casting the SDS-PAGE gel.

Casting stand and plates are clamped into the casting stand

Fill plates with running gel solution to the top of the closed green gates of the casting frame.

Bottom edges of plates are flush against the grey pad of the casting stand.



Pouring the stacking gel

1. Prepare the resolving gel. Mix the acrylamide solution, **pH 6.8** Tris buffer and water, as shown in the chart above.
2. Add 20 µL 10% APS and 5 µL TEMED to the stacking gel acrylamide mixture. Mix the contents by gently inverting the tube twice.
3. Use a transfer pipette to pipette the stacking gel on top of the resolving gel between the two glass plates. Add enough stacking solution until it just reaches the top of the small plate.
4. Carefully, but quickly, lower the comb into position, being careful not to introduce air bubbles. (The Bio Rad logo on the comb should be facing you.) Adding the comb will force some solution out of the gel, but this is fine. If air bubbles become trapped below the comb, remove the comb and reposition it.
5. Remove the comb when the gel has polymerized (10-15 minutes). You will be able to see faint schlieren lines (indicators of a change in refractive index) at the top of the spacer gel when polymerization is complete. You can also check the remaining solution in the 15 mL tube for polymerization.

Chapter 14

Running SDS-PAGE gels

Set up the electrophoresis apparatus

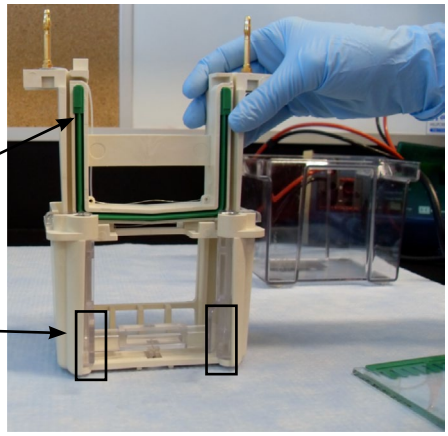
1. After polymerization of the stacking gel, remove the gel cassette sandwich from the casting stand.
2. Remove the casting frame from the gel cassette sandwich and place the sandwich against the gasket on one side of the electrode assembly, with the short plate facing inward (comb facing inward). Place a second gel cassette or a buffer dam against the gasket in the other side of the electrode assembly.
3. Slide the gel cassette sandwich and electrode assembly into the clamping frame.
4. Press down on the electrode assembly while closing the two levers of the clamping frame. Ensure you have and inner and outer chamber by either using two gels or a gel dam.
5. Lower the chamber into the electrophoresis tank.
6. Fill the space between the two gels with Tris-glycine electrophoresis buffer. This forms the upper chamber for electrophoresis.
7. Add SDS-PAGE running buffer to the outer (lower) chamber until the level is high enough to cover the platinum wire in the electrode assembly.

Electrode assembly

One gel is positioned on each side of the electrode assembly

Top of short plate fits snugly into notch in the green gasket of the electrode assembly

Electrode assembly with two gels is lowered into the clamping frame and clear plastic gates (rectangles) of frame are closed



Load and run samples on the SDS-PAGE gel

1. Carefully remove the comb from the spacer gel. If you wish, place the sample loading guide in between the two gels in the electrode assembly before loading your samples.
2. Using gel loading micropipette tips (tips have very long, thin points and fit P20s or P200s), load up to 15 µL of sample into each well. Load 5 µL of a molecular weight standard into one lane of the gel. Load samples slowly and allow the samples to settle evenly on the bottom of the well.
3. NOTE: Be sure to record the order of samples loaded onto the gel.
4. Connect the tank to the power supply. Fit the tank cover onto the electrodes protruding up from the electrode assembly. Insert the electrical leads into the power supply outlets (connect black to black and red to red).
5. Turn on the power supply. Run the gel at a constant voltage of 120-150 V. Run the gel until the blue dye front nearly reaches the bottom of the gel. This may take between 40-45 min.

Staining SDS-PAGE gels

1. Turn off the power supply.
2. Remove the gel apparatus from the tank. ***NOTE: Do NOT discard the buffer. Running buffer may be used multiple times.***
3. Open the clamping frame and remove the gel cassette sandwich. Carefully, pry the two plates apart with a spatula. With the spatula, remove the lower right or left corner of the gel to serve as an orientation marker. ***Be sure to indicate in your lab notebook whether the notched corner corresponds to lane 1 or lane 10 of the gel.*** You may also remove the stacking gel with the spatula, if you desire.
4. Place the gel in a small plastic tray and lab the tray with you initials on a piece of tape. To do this, fill the tray about halfway with deionized water. Gently free the gel from the glass plate, allowing it to slide into the water. The gel should move freely in the water. Place the gel and tray on a rocking platform. Rock the gel for 5 minutes.
5. Drain the water from the gel and then refill the tray about half-full. Rock the gel again for 5 minutes. Repeat this wash step one more time.
6. Drain the water from the gel and add ~20 mL of Simply Blue. Cover the gel container with saran-wrap and rock overnight.
7. In the morning, drain the Simply Blue stain into an appropriately labeled waste container in the hood of the lab room. Destain the gel by filling the container about half full with deionized water.
8. When individual bands are detectable, record your data. You may photograph the gel with your cell phone camera. Alternatively, place the gel in a clear plastic page protector and scan the gel.
9. After recording the data, dispose of the gel in the Biohazard waste container.

Chapter 14

References

Gelperin, D.M. White, M.A., Wilkinson, M.L., Kon, Y., Kung, L.A., Wise, K.J., Lopez-Hoyo, N., Jiang, L., Piccirillo, S., Yu, H., Gerstein, M., Dumont, M.E., Phizicky, E.M., Snyder, M., and Grayhack, E.J. (2005). Biochemical and genetic analysis of the yeast proteome with a movable ORF collection. *Genes Develop.* **19**, 2816-2826.

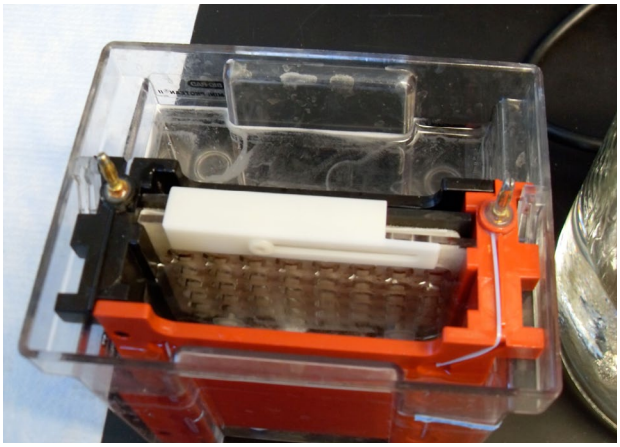
Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

Shapiro, A.L., Viñuela, E., and Maizel, J.V., Jr. (1967). Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. *Biochem. Biophys. Res. Commun.* **28**, 815–820.

Steinberg, T.H. (2009). Protein gel staining methods: An introduction and overview. *Methods Enzymol.* **463**, 542-563.

Chapter 15

Western blots



Western blots are one of the most widely used techniques in cell biology. In a western blot, investigators take advantage of the exquisite sensitivity of antibodies to identify proteins of interest in complex samples. In this lab, you'll learn about the different kinds of antibodies used in western blots. You will use western blots to analyze Met protein expression in your transformed yeast strains.

Objectives

- Understand how monoclonal and polyclonal antibodies are produced.
- Understand the structural features of antibody molecules.
- Learn how to prepare a western blot.
- Understand how primary and secondary antibodies are used to detect proteins in western blots.
- Use western blots to analyze Met protein expression in yeast transformed with overexpression plasmids.

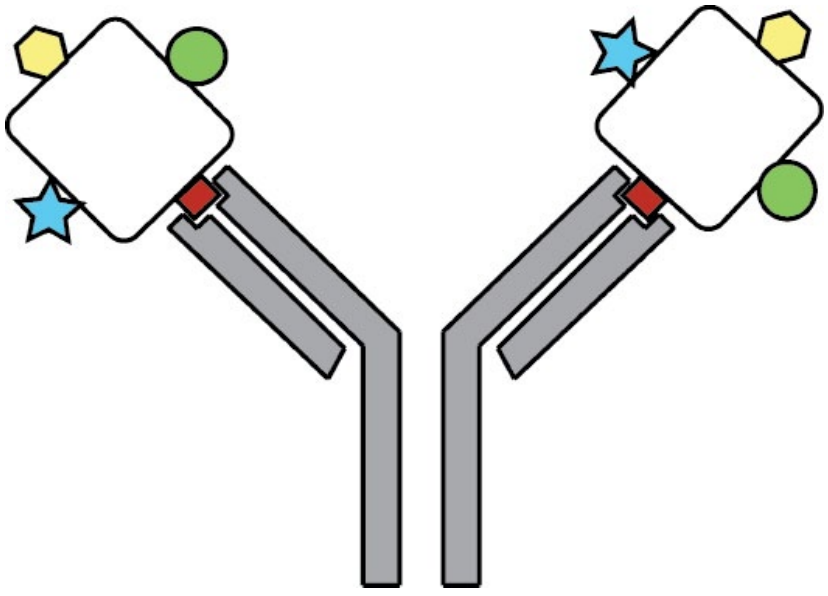
Chapter 15

Antibodies are produced by cells of the immune system

Western blots provide a method to find the proverbial "needle in a haystack." A typical cell expresses thousands of different proteins, and it is often difficult to detect changes in expression of your favorite protein (Yfp) without a probe that is capable of discriminating the Yfp against a large background of unrelated cellular proteins. Fortunately, antibodies provide highly specific molecular probes that can be used to detect the expression of proteins on western blots. To appreciate the sensitivity of western blots, it's helpful to have some understanding of the generation of antibody molecules during immune responses and an appreciation of antibody structure. (Disclaimer: The following paragraphs provide a highly abbreviated overview of antibodies and one segment of the complex vertebrate immune system. The Department offers an immunology course that will introduce you to the finer details of this fascinating system.)

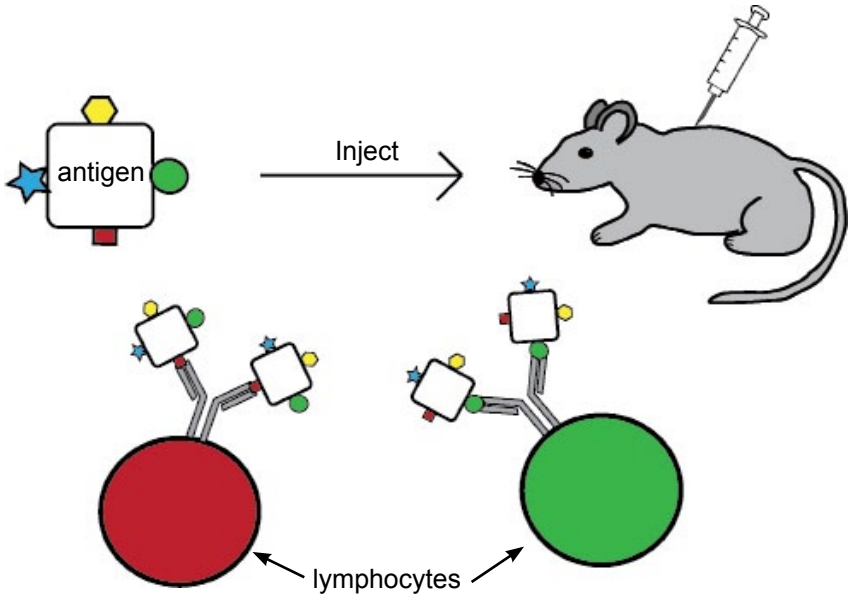
Antibodies are produced by lymphocytes in response to antigens

Antibodies are produced by vertebrates with adaptive immune systems capable of responding to foreign antigens. In the initial stages of the immune response, small numbers of immature B lymphocytes are able to bind foreign antigen molecules through antibodies that serve as receptors on the lymphocyte surface. Antigen binding stimulates the lymphocytes to proliferate and to differentiate into mature, antibody-secreting lymphocytes. Antigens act as selective agents. The lymphocytes that bind the antigen with the highest affinity receive the greatest growth signal and proliferate most rapidly, because a higher fraction of their surface antibodies are bound to antigen at any one time.



Antibody molecules have two identical binding sites for antigens. The antigen molecules in the cartoon above have four epitopes, shown as different shapes on the surface of the antigen (white rectangle). An antibody molecule (grey) binds a single epitope on the antigen.

In the initial stages of an immune response, the antibodies on the surfaces of B lymphocytes bind antigen weakly. An amazing transformation occurs as B lymphocytes mature in response to antigen. Antigen binding stimulates responding lymphocytes to rearrange segments of their antibody-encoding genes, producing new potential antigen-binding sites. The scope of antibody diversity is immense - vertebrates are capable of producing billions of antibody molecules with distinct specificities. Most rearrangements are unproductive, but some rearrangements generate antibodies with greater affinity for the antigen. In the latter stages of differentiation, somatic hypermutation further increases the range of potential antibody sequences. Mature B lymphocytes, also known as plasma cells, have survived the selection process. Each plasma cell secretes a single antibody with high affinity for antigen. Plasma cells are virtual antibody factories that can be identified in electron micrographs by their extensive rough endoplasmic reticulum. (See the inside cover for a graphical depiction of this process.)



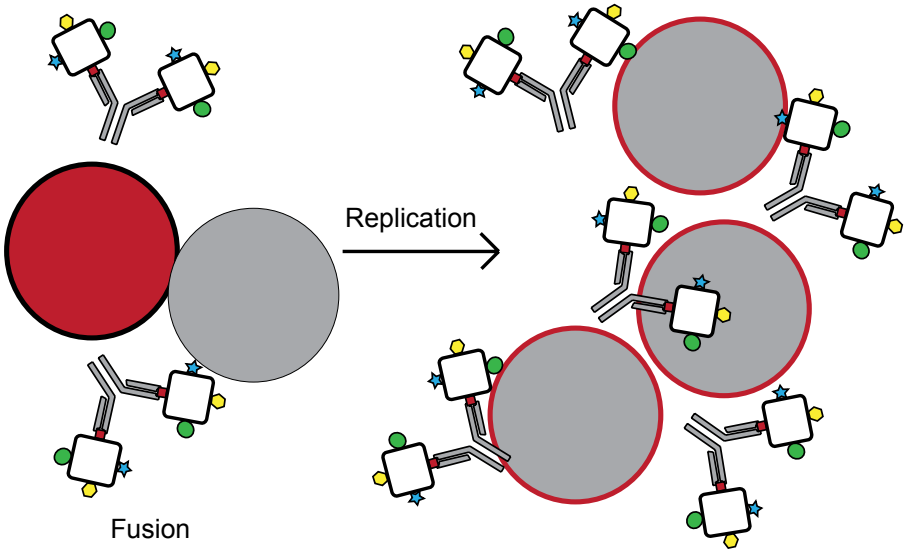
Polyclonal antibody production. Injection of an animal with an antigen stimulates the growth of lymphocyte clones.

Polyclonal vs. monoclonal antibodies

An animal's response to antigen is polyclonal, because antigens stimulate the proliferation of multiple lymphocyte clones, each of which produces a different antibody to the antigen. Consequently, serum collected from an immunized animal contains a mixture of antibodies that bind an antigen at distinct sites. These antibody-binding sites on antigens are known as epitopes.

In contrast to polyclonal antisera, which are harvested from the serum of living animals, monoclonal antibodies are produced from cultured hybridoma cells. Hybridoma cells are generated by fusing lymphocytes from an immunized animal, most commonly a mouse, with myeloma cells, cancerous cells that are capable of dividing indefinitely in culture. Standard culture techniques are used to isolate individual hybridoma cell lines from the fusions. Each hybridoma cell line secretes a unique antibody that recognizes a single epitope on an antigen. Hybridoma

technology has revolutionized biomedical research since its description (Kohler and Milstein, 1975), because monoclonal antibodies recognize well-defined epitopes and because monoclonal antibodies can be produced indefinitely by cultured hybridoma cells. Investigators often use both monoclonal and polyclonal antibodies at different steps in western blots.

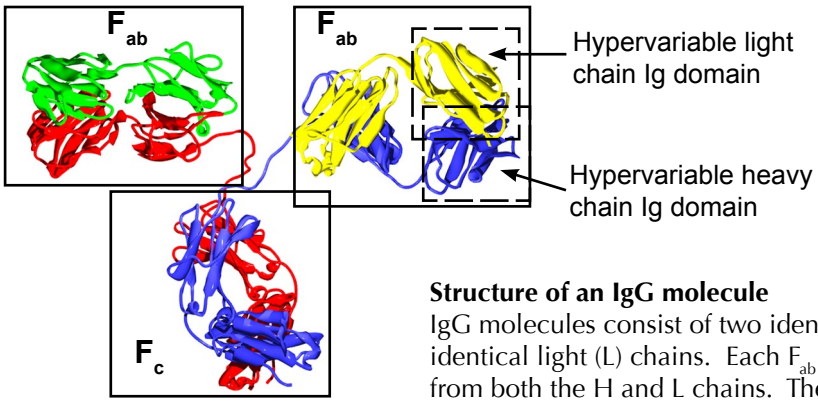


Fusion of an antibody-secreting lymphocyte with a myeloma cell generates hybridoma cell lines, which can be grown in large quantities.

The immunoglobin molecule

The availability of hybridoma cells that secrete large quantities of antibodies with a single specificity has greatly facilitated structural studies on antibodies. Researchers are able to harvest antibody molecules secreted by hybridoma cells and to prepare crystals for X-ray diffraction. Based on a large number of crystallographic studies, we now understand the basic architecture of antibodies, more properly known as immunoglobins. Immmunoglobins (Igs) are Y-shaped molecules with three clearly distinguishable regions that are readily apparent in figure below. The two F_{ab} regions (antibody-binding fragments) forming the arms of the "Y", are hypervariable regions involved in binding antigen. The F_c region (crystallizable fragment) that forms the base of the "Y" is receognized by non-immune effector cells, such as mast cells and macrophages, which process antigen-antibody complexes. The number of different F_c regions is similar to the different kinds of effector cells. Each Ig class has a characteristic heavy chain, which gives the class its name. We are using antibodies from the IgG class of immunoglobins, which have gamma heavy chains. (IgGs are also known as gamma globulins. IgA molecules have alpha chains, etc.)

X-ray crystallography has given us a detailed view of the IgG structure. IgGs are tetramers consisting of two identical light chains and two identical gamma heavy chains. Both the light and heavy chains are comprised of smaller immunoglobin (Ig) domains, in which two beta



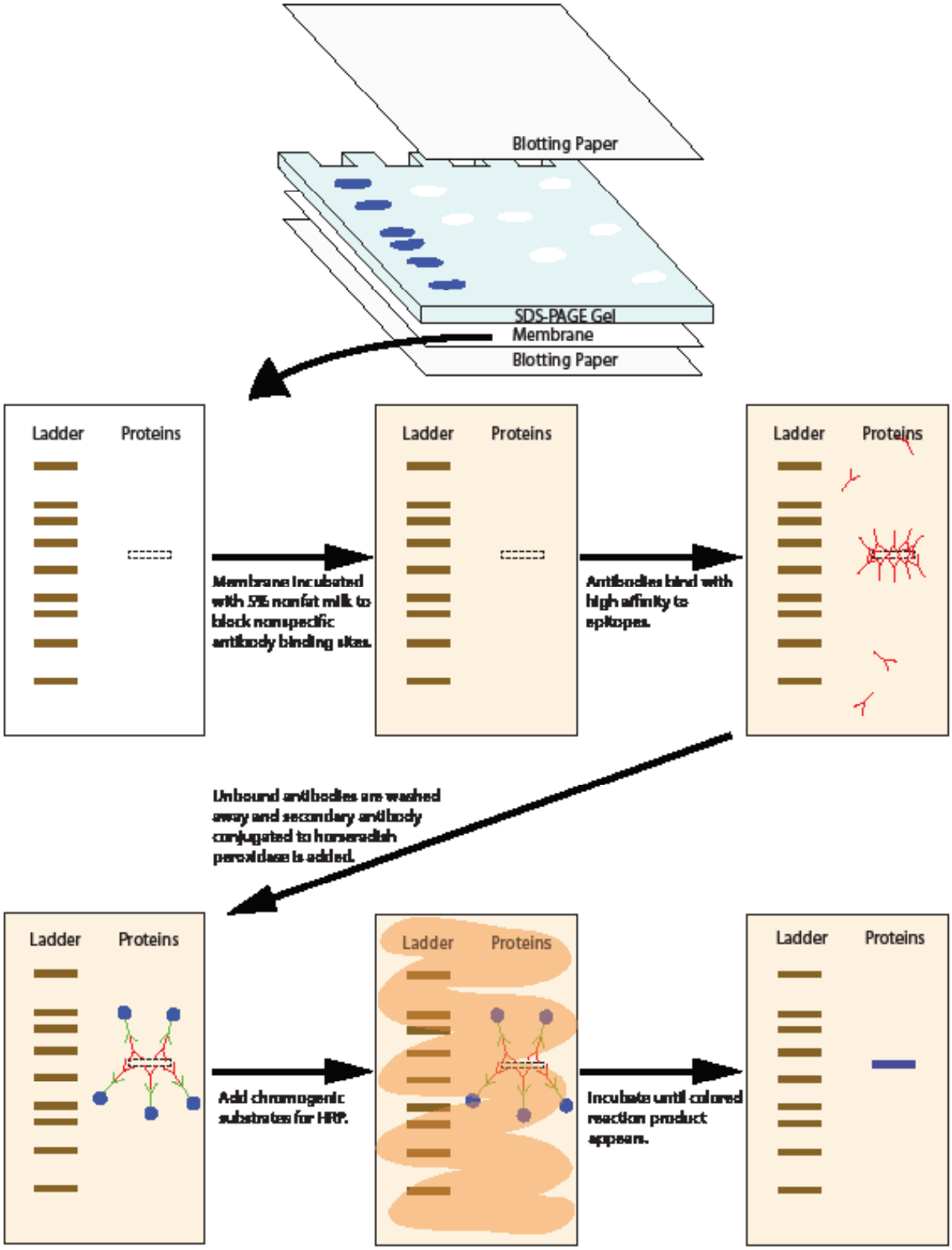
Structure of an IgG molecule
IgG molecules consist of two identical heavy (H) chains and two identical light (L) chains. Each F_{ab} fragment contains two Ig domains from both the H and L chains. The N-terminal hypervariable domains form the antigen-binding sites. F_c fragments are formed by two Ig domains from each of the H chains. This figure is derived from PDB entry 1IGT (Harris *et al.*, 1997).

sheets form a kind of sandwich held together by several disulfide bonds. Light chains contain two Ig domains, while heavy chains contain four Ig domains. The Ig domains closest to the N-termini of the heavy and light chains are hypervariable domains that have undergone rearrangement during lymphocyte maturation. These hypervariable domains form the antigen-binding site on the antibody. The other IgG domains in the light and heavy chains are constant domains that are also found in IgG molecules with different antigen-binding specificities.

Western blot overview

In a western blot procedure, proteins are first separated on an SDS-PAGE gel and then transferred to a membrane. This membrane replica is treated with antibodies that specifically recognize a protein or epitope of interest. Additional processing steps generate a signal at the position of the bound antibody. A typical western blot consists of multiple steps, which are discussed in greater detail below:

- Electrophoretic transfer of proteins from an SDS-PAGE gel to a membrane
- Blocking of nonspecific protein binding sites on transfer membranes
- Incubation of the membrane with a primary antibody specific for the epitope of interest
- Incubation with a secondary antibody specific for the primary antibody
- Detection of the primary antibody-secondary antibody complex


Blotting Paper
SDS-PAGE Gel
Membrane
Blotting Paper
Ladder
Proteins
Membrane incubated with 5% nonfat milk to block nonspecific antibody binding sites.
Antibodies bind with high affinity to epitopes.
Unbound antibodies are washed away and secondary antibody conjugated to horseradish peroxidase is added.
Add chromogenic substrates for HRP.
Incubate until colored reaction product appears.

Electrophoretic transfer of proteins from an SDS-PAGE gel to a membrane

The first step in a western blot is to generate a replica of the SDS-PAGE gel by transferring proteins electrophoretically to a synthetic membrane. Theoretically, antibody visualization could be done with a polyacrylamide gel, but membranes offer some distinct technical advantages. Polyacrylamide gels are mechanically fragile and can be easily torn during prolonged manipulations. In addition, the thickness of a polyacrylamide gel, which is 1 mm in our case, is greater than that of a membrane. Antibodies must also diffuse through the pores of the gel before they are able to bind their target molecule. By contrast, proteins bind quickly to the surface of the membrane, so antibodies don't need to diffuse between pores as they seek out their target epitopes.

The membranes used in western blots have a high protein binding capacity. A number of different types of membranes, including nitrocellulose, nylon and polyvinylidine fluoride (PVDF), are used for western blots. In our experiments, we will use PVDF membranes. PVDF is hydrophobic and doesn't wet properly with water. (PVDF is similar to Teflon™.) Therefore, PVDF membranes are first wet with methanol, then rinsed with deionized water, and finally rinsed with transfer buffer. PVDF membranes can be prepared while the SDS-PAGE gel is running and stored in transfer buffer until they are used. They must not be allowed to dry out during the transfer and immunoblot procedures. If they do dry out, they must be re-wet with methanol and rinsed with water before proceeding.

During the transfer process, the gel and membrane are placed directly against each other within a "sandwich" of filter papers and foam pads, as described below. All of the filter papers and fiber pads are pre-wet with transfer buffer before the transfer cassette is assembled. The membrane is cut to just about the same size as the gel. During the transfer, current should flow evenly across the entire surface area of the gel. It's important, therefore, that air bubbles are not trapped between the gel and membrane.

With the miniblot system, proteins are transferred for either an hour at 100 volts or overnight at 20 volts. Considerable heat is generated in the process. Therefore, gels transferred at 100 volts are done with an ice pack in the transfer chamber. (Most of the ice will melt during the transfer.) Gel transfers at 20 volts are usually carried out in a cold room. After the transfer is complete, membranes with the transferred proteins can be allowed to dry out and stored for later visualization with antibodies.

Blocking of non-specific protein binding sites on membranes

The transfer membranes used in western blots are designed to have a high protein binding capacity. The membranes also bind proteins nonspecifically, which is why they work so well for transfers. Before the membranes are incubated with specific (and expensive) antibodies, they must be pretreated with blocking solutions that contain high concentrations of abundant (and cheap) proteins to saturate non-specific binding sites. If the transfer membranes are not adequately blocked before the antibody is applied, the nonspecific sites on the membranes will

absorb some of the primary and secondary antibodies. Antibody binding to these nonspecific sites both reduces the amount of antibody available to bind the target proteins and increases the blot background. Effective blocking of these nonspecific sites increases both specific antibody binding (the signal) and the signal to noise ratio. Blocking proteins are also included in the antibody solutions used in western blots. Because the concentrations of blocking proteins are orders of magnitude greater than the concentrations of antibodies, they will out-compete the antibodies for binding to the many nonspecific sites on the membranes. The antibodies have a much higher affinity for their specific targets than for nonspecific sites on the membrane, so they will concentrate at sites with the target proteins.

Casein proteins from milk are a popular blocking reagent. Caseins are available in various degrees of purity. When a target protein is expressed at a very low level, it's best to use a purified formulation of caseins. For most purposes, however, nonfat dried milk (direct from the grocery store!) provides a very adequate and inexpensive casein preparation. In addition to high concentrations of proteins, blocking solutions commonly contain a non-ionic detergent. Nonionic detergents reduce nonspecific binding to membranes, but they do not denature proteins. Our blocking solution consists of 5% nonfat dried milk dissolved in a buffer (TBS-T: Tris-buffered saline) with 0.05% Tween-20, a nonionic detergent.

The timing of the blocking step is not particularly critical. Nonspecific sites on the membrane are rapidly saturated with proteins, but it is a good idea to incubate the membranes with blocking solution for at least one hour. Blots can be left in blocking solution for prolonged periods of time, if necessary. In this latter case, blots should be incubated in the cold to prevent microbial growth on the proteins in the blocking solution.

Primary antibody binding

Either polyclonal or monoclonal antibodies can be used as the primary antibody on western blots. Antibodies can be directed toward a naturally-occurring protein or toward an epitope that has been added to a naturally-occurring protein using recombinant DNA technology. Increasingly, researchers are introducing epitopes into proteins to facilitate their detection on western blots, because antibodies against naturally-occurring proteins are expensive and time-consuming to prepare. By contrast, an antibody directed against an epitope can be used to detect many different proteins carrying that same epitope. In our western blots, we'll use monoclonal antibodies directed against viral epitopes that were added to the C-termini of Met proteins during the cloning process. The viruses that produce the epitopes are not indigenous to yeast. Virologists have previously shown that these particular epitopes, which are short amino acid sequences in viral coat proteins, elicit strong immune responses during viral infection. The pBG1805-based vectors that we are using to overexpress *S. cerevisiae* proteins encodes a hemagglutinin (HA) epitope tag from human influenza virus (Sleigh et al., 1981). The pYES2.1-based plasmids that we will use to overexpress *S. pombe* proteins contains a V5 epitope tag from simian virus 5 (Southern et al., 1991). Commercial suppliers have taken advantage of this information to produce very potent (and expensive!) antibodies directed against these amino acid sequences.

An important consideration in western blots is the concentration of antibody solution required to detect the protein of interest. Antibody production ultimately depends on the immune response of an animal, which can show significant variability in the strength of the reaction and the spectrum of antibodies produced in response to the antigen. Commercial antibody preparations often contain additional proteins other than antibodies. Polyclonal antibodies are partially purified from animal serum and contain the whole spectrum of immunoglobins in the animal's blood. Monoclonal antibodies are purified from the culture medium of hybridoma cells and may also contain other proteins. Consequently, antibody suppliers measure the effective antibody concentration, or titer, of each antibody preparation. Researchers then use this information to plan their western blots. The primary antibodies that we are using in our experiments are monoclonal antibodies that will be diluted several thousand-fold. Because the primary antibody is monoclonal, a maximum of one antibody molecule will bind to each epitope-tagged protein. It is unlikely that all of the immobilized epitope-tagged proteins will bind antibody, since some are probably be inaccessible to the antibodies on the membranes.

Secondary antibody binding

Polyclonal antisera are commonly used as the source of secondary antibodies in western blotting. The antisera are prepared by injecting a second species with F_c fragments of IgGs from the species used to generate the primary antibody. Antisera designed to be used on western blots are then covalently linked to a reagent that aids in visualizing bound antibody. In some cases, secondary antibodies are bound to a fluorescent dye, so that blots can be analyzed directly with a fluorescent scanner. In other cases, the antibodies are linked to an enzyme, such as horseradish peroxidase or alkaline phosphatase, that can be used for histochemical detection of bound antibody in an additional step. The secondary antibody-binding step plays an important role in amplifying the signal on western blots. Because the antibody is polyclonal, secondary antibodies will bind to multiple sites in the F_c fragment of the primary antibody. If an enzyme is attached to the secondary antibody, the histochemical detection step will provide additional amplification of the signal.

Immunochemical detection of bound antibody

In this final step of the procedure, the western blot is incubated with substrates for the enzyme that has been conjugated to the secondary antibody. Because enzymes typically convert thousands of substrates to products every minute, this step can provide tremendous sensitivity to western blots. In our experiments, we will use a secondary antibody that has been conjugated to horseradish peroxidase (HRP). HRP is a hardy enzyme with a high turnover number that reacts with hydrogen peroxide and 3,3',5,5' - tetramethylbenzidine (TMB), to generate a dark blue-grey reaction product that precipitates at the reaction site on the western blot. The amount of reaction product at a particular site on the transfer membrane is directly proportional to the number of HRP molecules bound to the same site. Colored reaction product accumulates with time until the reaction is stopped by washing away unreacted substrate. The reaction should be terminated before nonspecific antibody binding becomes problematic.

Chapter 15

Western blot procedure

Separate proteins on an SDS-PAGE gel

1. Separate the proteins that will be analyzed on western blots by SDS-PAGE.
2. Remove the electrode apparatus and holder from the tank, and remove the gel from the holder. Do not remove the gel from the plates until you are ready to assemble the transfer cassette (see below).
3. Pour as much of the SDS running buffer as possible back into the SDS running buffer flask, so it can be used again. Dispose of any remaining buffer down the sink. Rinse out the buffer tank with deioinized water to remove residual SDS, which can interfere with the transfer process.

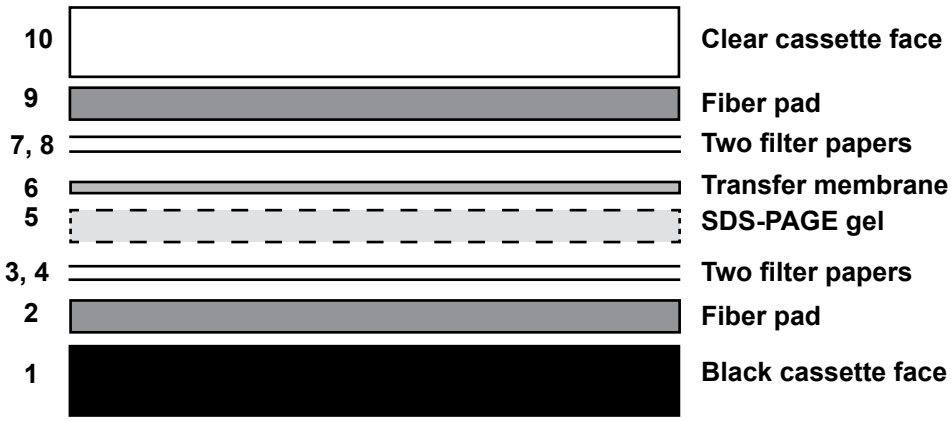
Prepare the transfer membrane

NOTE: DO NOT touch transfer membranes with your fingers. Wear gloves and use filter forceps when you handle transfer membranes.

1. While the gel is running, gather the PVDF membrane and four pieces of thick filter paper, such as Whatman 3MM™. The PVDF membrane and filter papers should be cut to a size that is slightly larger than the SDS-PAGE gel. You will also need a transfer cassette and two fiber pads.
2. Prepare the PVDF membrane. Using pencil, place an orientation mark in a corner of the PVDF membrane for later identification. Wet the membrane by placing it in a small tray containing methanol for ~30-60 seconds with gentle agitation.
3. Dispose of the methanol in the waste container and add deionized water to the tray. Gently agitate for ~1 minute.
4. Replace the deioinized water with transfer buffer. Store the membrane in transfer buffer until you are ready to start the transfer.

Assemble the transfer cassette

1. Fill the buffer tank about half way to the top with transfer buffer. Wet the fiber sponges and filter papers for the transfer cassette by placing them into the transfer buffer in the tank. (Transfer buffer contains Tris and glycine, as well as 10% methanol. Unlike SDS-PAGE running buffer, the transfer buffer does not contain SDS.)
2. Using a spatula or a green plastic wedge, remove the small glass plate from the gel. The gel will remain attached to the large glass plate. With a spatula, remove the lower right corner of the gel to serve as an orientation mark. (This correponds to the first lane of your gel.)
3. Assemble the transfer cassette as shown on the following page. Be sure that all parts of the transfer "sandwich" remain moist at all times.

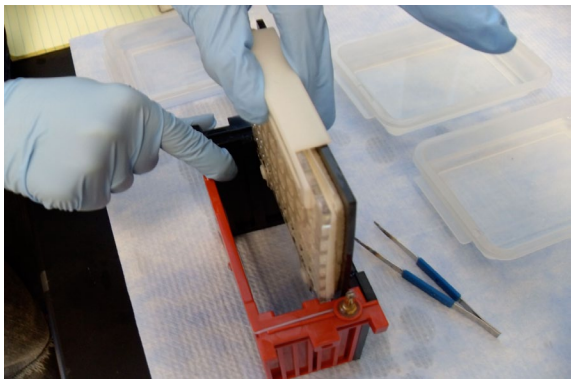


Assembly of the transfer cassette.

- Place a wet fiber pad on top of the black cassette face.
- Add two pieces of filter paper.
- Position the gel on top of the filter paper while it is still attached to the glass plate. Use a spatula to carefully release the gel from the plate. You may find it easier to remove the gel by beginning at the bottom edge near the dye front.
- Place the PVDF membrane blot on top of the gel. Orient the gel so that the pencil mark on the membrane corresponds to the clipped corner of the gel. Using a pencil or spatula, carefully roll out any trapped air bubbles between the blot and the gel.
- Add the remaining filter paper(s) and the fiber pad.
- Fold the clear cassette face over the gel assembly and slide the clamp into place.

Electrophoretic protein transfer

1. Place the transfer sandwich into the cassette holder with the black face of the transfer cassette aligned with black side of the cassette holder and the clear face aligned with red side of the cassette holder (Figure 3). NOTE: Each cassette holder can hold two transfer cassettes.
2. Place the cassette holder and assembled cassettes into the electrophoresis tank. If the transfer will be done at 100V (see step 5 below), the electrophoresis tank should contain an ice pack.
3. Fill the electrophoresis tank to the top with transfer buffer.
4. Place lid on tank by aligning black with black and red with red.
5. Run the transfer at 100V for 1 hour at room temperature or at 20 V overnight in the cold room.
6. When the transfer is complete, remove the transfer cassette from the tank. Pour the transfer buffer back into its original bottle so that it can be reused.



Inserting the transfer cassette into the cassette holder.

7. Disassemble the transfer cassette. Depending on your schedule:
- If you will be continuing with the western procedure, skip the rehydration step (step 1) below and continue with the blocking step (step 2). Be careful that the membrane remains moist!
- If you will be processing the membrane at a later time, allow the membrane to dry out. Wrap the membrane in plastic wrap and save it for a later lab period.

Immunoblotting - This is a multi-day procedure. Timing may vary for different classes.
Membranes are rehydrated and treated with blocking reagents
1. Wearing GLOVES, unwrap the dry blot from the plastic wrap. Use the prestained standards to identify the side of the membrane to which the proteins are bound. Submerge the membrane in methanol with this side facing up. Gently agitate the membrane by hand rocking for 30-60 seconds until the membrane has been uniformly wet with methanol. Decant the methanol into the appropriate container and fill the tray half way full with deionized water. Gently agitate the membrane for an additional minute.
2. Decant the water and replace it with sufficient TBS-T (Tris buffered-saline containing 0.05% Tween 20) to cover the blot. Place the blot on a rocking platform. Equilibrate the blot in TBS-T for 5 minutes with slow rocking. At the end of 5 minutes, drain the TBS-T into the waste container.
3. Pour 50 ml of blocking solution (5% nonfat milk in TBS-T) onto the blot and cover the tray with a small piece of plastic wrap.
4. Place the tray on a rocking platform in the cold room (Higgins 377). The blot should float freely in the tray so that both sides are washed. Incubate the blot for at least an hour or up to 24 hours at 4˚C.

Membranes are washed and incubated with primary antibody (~30 minutes)
1. Locate your blot in the cold room and bring it back to the lab room.
2. Remove the plastic wrap from the container holding the blot and pour off the blocking solution in a waste container. SAVE the plasticwrap! You will need it to cover the container again!
3. With a graduated cylinder, measure 30 ml of TBS-T solution. Pour the TBS-T solution onto the blot and place the container on the rocking platform. Rock for 5 minutes.
4. Pour off the TBS-T and add a fresh 30 ml of TBS-T. Wash, with rocking, for 5 minutes.
5. Repeat step 4, for a total of three washes.
6. Pour off the TBS-T. Add 15 mL of primary antibody diluted in blocking buffer.
7. Cover the container with the same piece of plastic wrap and place the tray on the rocking platform in the 4˚C cold room. Make sure that the blot floats freely in the tray and that the standards are on the top face of the blot. Incubate overnight at 4˚C with slow rocking. NOTE: The timing of this step is the most critical in the procedure. Shortening the incubation time with primary antibody may reduce the sensitivity of the western blot.

Secondary antibody binding and detection (1.5-2 hours)

1. Locate your blot in the cold room and bring it to your lab classroom.
2. Carefully drain the antibody from the blot into the test tube marked "Used primary antibody". (Antibodies are expensive. Fortunately, the solutions can be re-used.)
3. Fill the tray with the blot about half-full with TBS-T. Place the tray on a rocking platform and wash the membrane for 5 minutes to remove unbound primary antibody. Drain the TBS-T when the wash is complete.
4. Repeat step 3 twice, for a total of three washes.
5. Incubate the membrane with 15 ml of secondary antibody solution for 1 hour with gentle rocking at room temperature. The secondary antibody, which is conjuated to horseradish peroxidase (HRP), has been diluted in blocking solution.
6. Carefully drain the antibody from the blot into the test tube marked "Used secondary antibody."
7. Wash the membrane 3 times for 5 minutes each with TBS-T, as in step 3.
8. Drain the TBS-T from the blot. Using a P1000 micropipette, cover the blot with 1 mL of 3,3'5,5'-tetramethyl benzidine (TMB), a colorigenic substrate for HRP. Let the color continue to develop until distinct bands are apparent. Bands will probably become apparent within minutes. Do not allow the blot to over-develop, when nonspecific bands become apparent.
9. Stop color development by diluting the substrate with an excess of deionized water. Drain the diluted substrate into the waste container.
10. Allow the blot to dry on a piece of filter paper. Record your data with the scanner.

Chapter 15

References

Harris, L.J., Larson, S.B., Hasel, K.W., and McPherson, A. (1997). Refined structure of an intact IgG2a monoclonal antibody. *Biochemistry* **36**: 1581-1597.

Kohler, G., and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefinied specificity. *Nature* **256**: 495-497.

Sleigh, M.J., Both, G.W., Underwood, P.A., and Bender, V.J. (1981). Antigenic drift in the hemagglutinin of the Hong Kong influenza subtype: Correlation of amino acid changes with alterations in viral antigenicity. *J. Virol.* **37**: 845-853.

Southern, J.A., Young, D.F., Heaney, F., Baumgartner, W.K., and Randall, R.E. (1971). Identification of an epitope on the P and V proteins of simian virus 5 that distinguishes between two isolates with different biological characteristics. *J. Gen. Virol.* **72**:1551-1557.