Molecular Biology Laboratory Manual

P. Oelkers

(June, 2016 version)

Table of Contents

Welcome to the Molecular Biology Laboratory

Science can be defined in a variety of ways. Science may be the construction of rules and relationships that define a world that matches our world as closely as possible. Science may the acquisition of knowledge through information collection and analysis. In any case, science involves **learning**. It involves taking what is known and **expanding** and **refining** that knowledge. While science it rooted in work already done, its branches stretch out into a seemingly endless sky. Scientists are **explorers** who go beyond what it is currently known.

With these lofty ideals in mind, each group of two or three students will be conducting an independent research project during the course of the term. In the education field, this is called **experiential learning** or **inquiry-based learning**. A framework for the projects will be suggested. This framework was chosen so that a variety of techniques are employed, the logistics are feasible, and planning can be completed in two or three weeks. Projects involving molecular biology outside the proscribed framework are possible. Please discuss such projects with the instructor prior to initiation.

As in any project, a **goal-centered** approach and attitude will be required. To put it simply, **get it done, well.** If a certain experimental step in unsuccessful in achieving the experimental goal, that step will have to be repeated or an alternate strategy adopted. **Progress** will require **careful**, **expedient,** and **productive** work. Such a goal-centered approach and attitude are also required in many professional settings. Few professionals are paid to take tests. Accordingly, your evaluation will primarily be based on the progress of the project at the end of the term. To maintain a productive pace, substantial progress should be make each week. This will require **self-motivation** and **discipline**. In the words of a very successful basketball coach, John Wooden, "Move quickly but do not rush". This pace may benefit from one or more group members coming to the lab on days and at times beyond scheduled class meetings. Due to the use of the laboratory for the instruction of other classes, the possible days and times will be limited and require advanced scheduling.

All of this may seem daunting. Exploration involves the unknown. There is no getting around that. However, taking your knowledge and skills and applying them to a goal will hopefully be an enriching experience. Eleanor Roosevelt is quoted as saying, "You gain strength, courage and confidence by every experience in which you really stop to look fear in the face. You are able to say to yourself, 'I have lived through this horror. I can take the next thing that comes along.' You must do the thing you think you cannot do." Of course, the aim is for this course to stop well short of horror. The aim is also to have skills

to support the confidence for facing the unknown. When **confidence** and **competence** come together, great things may be achieved.

Please appreciate that this laboratory manual does not provide information in the order in which it will be needed (i.e. week 1, week 2 …). The manual is instead a collection of protocols for performing techniques that will likely be part of your project. The suggested schedule of experimental steps on the following page (also in the syllabus), will indicate what protocol is needed at any given time. The reasoning behind this manner of organization is that few professional references are custom tailored to a single user's needs. Instead, references are organized so that a myriad of users can readily find what they need.

Good luck! Please use the instructor as a resource whenever needed.

Overview of Transcriptional Regulation Project:
Promoter - Report Construct Strategy

Our goal is to better understand transcriptional regulation by designing and conducting experiments involving the promoters of one or more genes. *Saccharomyces cerevisiae* (Baker's or Brewer's yeast) will be used as a model system.

Many advantages provided by *S. cerevisiae* make it a convenient model experimental system Complete sequencing of the *S. cerevisiae* genome was achieved about 20 years ago (4) and a well-annotated, on-line genome compendium (www.yeastgenome.org) facilitates bioinformatics efforts. Growth media components are commonly inexpensive and strains readily available from principal investigators or the American Type Culture Collection (ATCC). Plasmid vectors with a range of origins of replication, promoters, and marker genes are available for cloning or other modification using recombinant DNA techniques. Rapid growth, high efficiency transformation, ease of environmental alteration, and well established protocols round out the pragmatic benefits of experimenting with *S. cerevisiae*.

While the power of yeast genetics potentiates screens to establish gene - function relationships, *S. cerevisiae* is also amenable to studies of transcriptional regulation. Nutrient availability, oxidation, temperature, osmotic environment, concentration of metals, radiation, agonists, and antagonists offer a variety of stimuli, some stressful, that yeast may experience in nature. Each of these has been found to initiate in yeast a regulatory response with a transcriptional component (1, 2). Transcriptional changes also occur in response to fermentation byproducts such as acetic acid and furfural generated during commercial ethanol production (3).

Analysis of the entire yeast transcriptome in response to environmental stimuli has been performed using comprehensive microarrays and RNA sequencing (1). These data are often available in the public domain linked to publications or within the annotated genome (www.yeastgenome.org). Accordingly, a good deal of information can be found on-line about the transcriptional regulation of any gene of interest.

One experimental strategy to examine transcriptional regulation of yeast gene(s) of interest is called the **promoter - reporter method**. This approach uses the **polymerase chain reaction** (PCR) to amplify the putative promoter region from *S. cerevisiae* genomic DNA. As for most PCRs, two specific **oligonucleotides** (i.e. **primers or oligos**) must be designed. The two oligos flank the region intended for amplification. Restriction

endonucleotide recognition sequences may be incorporated into the oligos to facilitate subsequent ligating into the reporter plasmid.

Following the PCR, the products are resolved by **agarose gel electrophoresis** and purified from the gel. **Restriction enzyme** digests then create "sticky ends" on the PCR product. Alternatively, vectors designed for ligating PCR products directly may be used. Identical digests of the reporter **plasmid**, pSF011 (5) that contains a β-galactosidase reporter gene allows for subsequent ligation of the PCR product and the digested pSF011. Upon successful ligation, the promoter now may control transcription of the neighboring β-galactosidase gene.

Following ligation, the reaction mixtures are incubated with carefully prepared lab strains of *E. coli* under conditions known to promote the uptake of exogenous DNA. These carefully prepared *E. coli* are known as **competent cells** and the process is called **transformation**. The transformation process is fairly inefficient such that the vast majority of bacteria in a transformation do not take up the DNA of interest. Transformation can also done with yeast but that process is orders of magnitude less efficient than going into bacteria first. To selectively allow only those bacteria which took up plasmid DNA to grow, the transformation mixtures are grown on agar plates that contain the antibiotic, ampicillin. pSF011 contains the **β-lactamase** gene that confers ampicillin resistance to cells that contain the plasmid. This way, the few bacteria that take up the plasmid will have a selective growth advantage.

Overnight growth on ampicillin containing plates allows cells that contain plasmids to proliferate and form a colony. Each colony presumable arose from a single cell that took up one of the plasmids from the ligation reaction. There is a possibility that colonies arise from digested plasmid (e.g. pSF011) ligating upon itself without insertion of the PCR product. To address this possibility, parallel "control" ligation reactions are often assembled which contain all the components without the PCR product. The number of colonies generated from transforming these control ligation reactions will establish the background. Transformation plates with ligations that contain PCR products should have at least twice as many colonies as plates with control ligations.

Even if the desired ratio is observed, the insertion of the PCR product into pSF011 has to be verified. Therefore, bacteria from a number of colonies (often eight) from the "plasmid + PCR product" transformation plate are used to inoculate 5 ml of liquid media with ampicillin.. This growth in liquid media is necessary to amplify the number of bacteria and thus the mass of plasmids (around 10 μg from 5 ml). Following the growth, plasmids will be isolated from the bacteria using the alkaline lysis miniprep protocol. This

plasmid DNA is then digested so to confirm its sequence. Planning this analytical digest requires choosing restriction enzymes that will generate distinctly unique numbers and / or lengths of plasmid fragments if pSF011 has the insert or not. Agarose gel electrophoresis in parallel to DNA size standards will allow the estimation of the digested fragment sizes. If the banding pattern matches the expected pattern of the vector + insert, a glycerol stock will be made of the bacteria so to allow future culturing of those bacteria and the generation of more plasmid DNA.

If time allows, the newly constructed plasmid will be submitted for DNA sequencing. One possibility that should be addressed is that during the PCR reaction the polymerase introduced a mispaired nucleotide into the product.

Following generation of the pSF011 / promoter plasmids, the plasmid will be linearized by restriction enzyme digestion and purified. This will allow recombination with the host yeast's genome and integration. Lab strains of *S. cerevisiae* (yeast) will then be **transformed** with these plasmids. Transformation requires that the yeast cells be made **competent** or induced to take up exogenous DNA. This process involves growing yeast into the logarithmic (i.e. log) stage of growth and suspending then in a mixture of ions and detergents including lithium acetate and polyethylene glycol. Transformations are plated onto specialized yeast agar plates that lack the nutrient uracil.

Only transformed yeast will grow in the absence of uracil. This is because the lab strains utilized have been selectively mutated so that the *URA3* gene is non-functional. Without the protein made from the URA3 gene, the cells cannot synthesize uracil *de novo* and thus require it in the environment. However, pSF011 contains a functional copy of *URA3* and confers the ability to grow in the absence of uracil. After two days of growth at 30 °C, colonies should be present on the transformation plates.

Transformed yeast are commonly streaked onto a new agar plate that lacks uracil. This "master plate" can seed cultures for experiments involving exposing yeast to various environmental conditions. 5 ml cultures of yeast are often used for each condition. After the planned length of exposure to the conditions (perhaps a time course will be performed), yeast can be harvested, lysed, and β-galactoside reactions performed.

Bar graphs are a common way the β-galactoside activities are reported. If time allows, the yeast can be cultured again, exposed to the conditions of interest, and the β-galactoside assays repeated.

References

(1) H. Taymaz-Nikerel, A. Cankorur-Cetinkaya, B. Kirdar (2016) Genome-Wide Transcriptional Response of Saccharomyces cerevisiae to Stress-Induced Perturbations. Front. Bioeng. Biotechnol.. 4, 17.
(2) N.C. Bauer, A.H. Corbett, P.W. Doetsch (2015) The current state of eukaryotic DNA base damage and repair. Nucleic Acids Res. 43, 10083-10101.
(3) Y. Chen, J. Sheng, T. Jiang, J. Stevens, X. Feng, N. Wei (2016) Transcriptional profiling reveals molecular basis and novel genetic targets for improved resistance to multiple fermentation inhibitors in *Saccharomyces cerevisiae*. Biotechnol Biofuels. 9,9.
(4) A. Goffeau, B.G. Barrell, H. Bussey, R.W. Davis, B. Dujon, H. Feldmann,F. Galibert , J.D. Hoheisel, C. Jacq, M. Johnston, E.J. Louis, H.W. Mewes, Y. Murakami, P. Philippsen, H. Tettelin, S.G. Oliver (1996) Life with 6000 genes. Science. 274, 563-567.
(5) Y.J. Joo a, J. Kim, J.H. Baek, K.M. Seong, J.Y. Lee, J. Kim (2009) Determination of the core promoter regions of the Saccharomyces cerevisiae RPS3 gene. Biochim. Biophys. Acta 1789: 741-750.

Outline of Transcriptional Regulation Project:
Promoter - Report Construct Strategy

I. Planning, stage 1 (to be completed by the beginning of the third class meeting)

A. Choose a cell or physiological function or process of interest.

B. Use prior knowledge and the scientific literature to identify three proteins in any organism that are components in that function or process.
   1. Be sure to keep a record of papers used. Save the pdf files if possible.
   2. Find a copy of the proteins' amino acid sequence
      - www.ncbi.nlm.edu and the <Protein> tab may help with this
      - finding an accession number in a paper will also help
      - establish a file with protein amino acid sequences
   3. OR, use YeastMine (http://yeastmine.yeastgenome.org/yeastmine/begin.do) to search for textwords related to the function or process in yeast gene descriptions

C. Use the blastp function at www.yeastgenome.org to search for a similar protein in the yeast proteome. Record the name of the yeast protein and the corresponding gene. If this process does not identify at least two yeast genes, repeat steps B and C until at least two yeast genes which encode for proteins with similar sequence to proteins in other organisms with established functions.

D. Search the literature and the www.yeastgenome.org site for information about the transcriptional regulation of the genes of interest. Make careful notes of the conditions which lead to the transcriptional regulation and the degree and direction of the regulation. Download pdf files of relevant papers when possible.

E. Using previous and gathered knowledge, generate three hypotheses or formulate three questions about the transcriptional regulation of each gene. Include a mechanistic rationale that supports the hypotheses or gives the question importance. Include detailed logistics such as specific media compositions and comparisons that will be made. Along with the background information gathered, formally structure this information into an experimental design that will be submitted and evaluated.

II. Planning, stage 2 (to be completed by two days after the third class meeting so to allow the ordering of oligonucleotide prior to and PCR on the fourth class meeting)

A. Determine the genomic (i.e. chromosomal) location of the open reading frames for the two genes of interest. Also determine the genomic location of the open reading frame of the nearest gene to the 5' of the start codon of each gene of interest. Make a table of these locations.

B. From the table made in (1), determine the genomic / chromosomal location (i.e. beginning and end) of the putative promoter region of each gene of interest.

C. Design oligos to amplify the complete, predicted promoter containing 5' flanking region of each gene of interest. Two, non-overlapping reverse oligos will be very close to the start codon of the gene of interest and two, non-overlapping forward oligos will be very close to the start or stop (whichever is closer) codon of the nearest neighbor.

D. [optional] Design additional forward oligos that will only amplify a portion of the putative promoter, thus producing a truncation.

E. At the 5' of each oligo, include sequence that is the recognition sequence for a restriction enzyme that has no sites in the sequence of interest and has one site in the polylinker of pSF011. Primers intended for use as pairs should have different restriction enzyme recognition sequences. The goal is to digest the PCR product with the respective enzymes, digest pSF011 plasmid with the same two enzymes, gel-purify the products, and perform a ligation. The 5' flanking sequence should have the same orientation in the plasmid than it had in the genome.

F. Add 8 nt of extra sequence to allow for optimal digestion with the respective restriction enzyme.

G. Post an Excel spreadsheet to your on-line lab notebook with a unique and informative name for each oligo and its sequence

III. Execute the experimental plan, stage 1 (PCR and subcloning)
- steps (1) are for generating and preparing the insert for the ligation
- steps (2) are for generating and preparing the vector for the ligation

A. Isolate genomic DNA and use this as the template with the appropriate oligo pairs in a PCR.

B. Resolve PCR products by agarose electrophoresis

C1. Verify that PCR products are the correct size and if so, purify PCR products from the agarose gel

C2. Isolate pSF011 plasmid DNA from bacterial cultures grown overnight

D1. Digest PCR products with the appropriate restriction enzymes so to create asymmetrical "sticky" ends.

D2. Digest pSF011 plasmid DNA with the appropriate enzymes (as in 4a)

E1. Resolve digested PCR products by agarose electrophoresis and purify.

E2. Resolve digested PCR products by agarose electrophoresis and purify

F. Set up a ligation with each sticky-ended PCR product with the respective, sticky-ended pSF011 DNA. Ideally, three ligations will be done for each. Two of these will contain both pSF011 and PCR product. They will differ in regards to the amount of PCR product. The third ligation will only have pSF011 and no PCR product. This is the negative control. Put ligation at 15° C (4 hours – 24 hours).

G. Transform ligations into competent *E. coli*. Place transformation plates at 37° C for overnight growth.

H. If the distribution of colonies is as expected, pick 8 colonies from the respective insert + vector transformations and inoculated 5 ml LB + ampicillin. Culture at 37° C overnight with shaking.

I. Extract plasmid DNA from bacterial cultures.

J. Perform diagnostic restriction enzyme digests and resolve by agarose gel electrophoresis. Compare the size of the bands in each lane to the expected sizes for the pSF011 alone and pSF011 with insert. If the bands match the expected for pSF011 with insert then make a glycerol stock of the corresponding bacterial culture.

IV. Execute the experimental plan, stage 2 (yeast transformation and culturing in stimulus conditions)

A. Digest the pSF011 / promoter constructs with NcoI to linearized and prepare the plasmids for transformation into *S. cerevisiae*

B. Culture normal S. cerevisiae strain into log phase, make yeast competent, and transform them with NcoI – digested pSF011 / promoter and plate onto SC-Ura agar plates Incubate plates for 2 days at 30° C. In parallel, transform cells with sterile water.

C. If transformation plates have colonies on the (+) DNA plates and no colonies on the (-) DNA plates, streak transformants onto a SC - ura agar plate for a master plate.

D. Culture yeast under conditions shown to regulate transcription.

E. Measure β-galactosidase activity in stimulated and control cells. Compare.

Agarose Gel Electrophoresis

Many techniques of molecular biology address the goal of studying a molecule of interest among a complex array of molecules such as is found in a cell lysate. The challenge soon becomes one of **specificity**. How to look at one type of molecule and set aside all others? One very simple approach is to **separate** or **resolve** the complex array of molecules by **size**. If the size of the molecule of interest is known, this provides an initial pass at establishing specificity. However, please appreciate that complex mixtures may contain two or more molecules of different structures but the same size. Subsequent analysis may allow for distinction among such **co-migrating** molecules.

Separating molecules by size can be achieved by **size exclusion chromatography**. This process uses force from gravity or a pump to force molecules through a sort of obstacle course. The course is commonly comprised of inert beads that contain indentations like cul-de-sacs. Smaller molecules may get transiently trapped in these and this slow their progression. Larger molecules will not fit so well into the indentations and will progress more quickly.

For separating protein, RNA, and DNA molecular species, **gel electrophoresis** provides a more commonly utilized alternative. The main component of the gel is usually either **acylamide** or **agarose**, both of which can form a three-dimensional lattice or matrix (i.e. spider web) when either cross-linked (acylamide) or heated and cooled (agarose). This matrix also forms an obstacle course of sorts. Smaller molecules will be able to migrate, perhaps tumble, through the pores of the matrix while larger molecules will take longer to navigate the same series of pores.

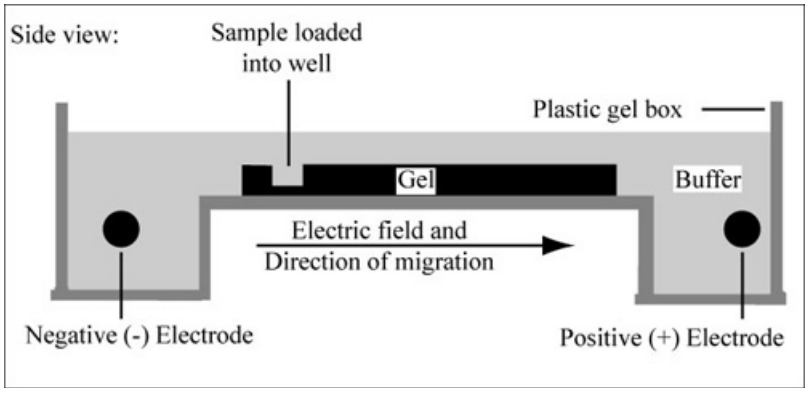
We will be working with agarose. It is a mixture of galactose-rich polysaccharides which are commercially obtained from sea weed (more specifically, a few species of red algae). Agarose is a refined form of the agar used to make microbiological media plates. It is a white powder that is fairly expensive.

In order to make a gel with agarose, an appropriate solvent is necessary. Not only must the agarose dissolve in this solvent but the solvent must be able to stably conduct an electrical charge. This is because electrophoresis does not rely on gravity or a pump but instead on an electrical differential, a **voltage**. With a positive electrode on one side of the gel, a negative electrode on the other, and the gel immersed in a solution (the **running buffer**) that can conduct electricity molecules are propelled through the gel based on their relationship with the two electrodes. Molecules with a positive charge will be propelled towards the negative electrode and molecules with a negative charge will be propelled towards the positive electrode. Consider in which direction proteins, RNA, and DNA will be propelled or **migrate**. One solvent or running buffer commonly used in agarose electrophoresis is called TBE: a mixture of Tris (aka Trima, THAM), boric acid, and the disodium salt of ethylenediaminetetraacetic acid, (Na_2EDTA). The respective

concentrations are often 89 mM, 89 mM, and 2 mM with pH = 8. At these concentrations, this is referred to as 1X TBE. 5X and 10X TBE are often made and stored to save time in solution preparation and space for storage. Generating the volume of 1X TBE needed for a gel then requires a simple dilution of the concentrated stock. FYI, acetate replaces boric acid in some recipes (e.g. TAE). On the plus side, acetate does not come out of solution as readily as boric acid. On the down side, acetate does not maintain the pH as well during a run. Instructions for pouring an agarose gel can be found below.

Pouring a gel takes a few steps. Most gels are poured as **slabs** which have long horizontal dimensions and a short vertical dimension. Setting up the form that will accept the hot agarose and have it cool in the shape of a slab will vary among manufacturers. Also varying will be the appropriate volume of agarose / 1X TBE for each form. 50 ml is a common volume for a gel with 10 lanes. If all else fails, set up the form and pour various volumes of water until you find one that gives a gel height of about 7.5 mm. This is not a bad idea since this "dry" run with water should detect any leaks in the gel form after it is assembled. Any leak will essentially ruin a gel and require clean up and another half hour or so of gel preparation.

Combs are placed into the gel form prior to pouring the gel so to create **wells** into which samples may be loaded prior to resolving in the gel. Please be advised that different wells have a different volume they may hold. Knowing exactly how many samples will be loaded onto the gel and how many wells are needed per sample, including a well per set for the size standard, is important in planning how large a gel and how many wells will be needed.



MIT OpenCourseWare.

Successful loading of a sample into a well requires that the sample be denser than the running buffer. This will prevent the sample contents from diffusing into the running buffer prior to the electricity being applied. Providing a slightly alkaline pH to the sample promotes the formation of negatively charged phosphate groups. Dyes also assist in

loading and monitoring sample progression. Adding **loading buffer** achieves these two goals by providing a mixture of glycerol, EDTA, and the dyes bromophenol blue and xylene cyanol. Loading buffer is often made in 5X or 6X concentrations. After adding the loading buffer to a sample, the components of the loading buffer should be at 1X. Consider developing an equation to allow you to calculate the amount of loading buffer that is appropriate for a given volume of sample.

Running agarose electrophoresis involves attaching the gel to a power source. Red wires usually the positive electrodes from the power sources to the red port on the gel box. Blacks wires are for the negative electrodes. The main thing is that the **negative** electrode is attached to the gel box on the end where the **samples are loaded**. Be sure that the gel box cover is closed prior to turning on the electricity. Recall that the running buffer will be an **open wire** that may transmit a current to an immersed structure such as a finger if given the chance. Voltages of about 100 V for small gels and 150 V for medium gels are common. The lower the voltage, the better the resolution. If the voltage is too high, the gel may melt.

Gel electrophoresis protocol

10X TBE

890 mM Tris
890 mM boric acid
20 mM disodium ethylenediaminetetraacetic acid (EDTA)
adjust pH to 8.0

5X loading buffer

50 % (v/v) glycerol
100 mM EDTA, pH = 8.0
0.1% (w/v) bromophenol blue
0.1% (w/v) xylene cyanol

I. Pouring

1. Determine the volume of gel necessary for the gel form you are using. 50 ml is a common volume for "small" gels that will have 10 lanes.
2. Weigh the proper amount of agarose. Consider that 0.8% (w/v) gels are proper to resolve DNA bands ~1.5 kb, 1.0 % (w/v) agarose gels for bands ~ 1.0 kb, 1.2% (w/v) for bands ~ 750 bp, 1.4% (w/v) for bands ~ 500 bp, and 1.6% (w/v) for bands less than 400 bp. There is flexibility in these numbers. Getting the gel percentage right is

particularly important when resolving two different molecules of similar size in the same lane.

3. Transfer the agarose into a clean 250 ml Ehrlenmeyer flask.
4. Measure the appropriate volume of 1X TBE in a grad cylinder and transfer to the flask. Swirl gently.
5. Place a small (just big enough to cover) piece of plastic wrap over the flask's opening.
6. Use a micropipet tip to poke a few holes in the plastic. The plastic is to prevent eruptive boiling from projecting very hot liquid out of the flask and onto an unsuspecting experimenter or nearby person. The holes are to prevent the heating of a closed system. Such systems unlikely stay closed for long. Please be aware that after heating, steam will emit from the holes and provide a **burning hazard**. **Work with care**.
7. Place the flask into a microwave and heat for 60 seconds for a 50 ml volume. Longer will be needed for a larger volume. At about 30 seconds of heating, open the microwave door and use thick, heat-resistant glove to swirl the flask. Keep skin, especially faces, away from the opening as steam may come out.
8. Heat for the second half of the time. Again, take out the flask and carefully swirl it. The solution should be clear, an indication that the agarose has dissolved. If translucent floating flecks are seen, heat for a few seconds more. Avoid overheating as that may cause eruptive boiling.
9. Set the flask in a safe area (i.e. not going to be knocked over or accidentally picked up) to cool for about 15 minutes. Sufficient cooling will allow touching of the glass with a latex-gloved hand to occur without pain. This temperature is called, "hand cool". Naturally, the initial checks should be quick touches.
10. While the gel is cooling, assemble the **gel form**. Most gel forms allow the **gel tray** to fit snuggly in the **gel box** perpendicular to the eventual flow of electricity. Place the combs near one end of the gel tray but not right at the end. Some gels can accommodate two combs. This will increase the number of samples that can be loaded but reduces the gel distance over which samples may resolve.
11. Once the flask with the agarose can be handled, add 5 μl of the non-toxic DNA binding dye, **gel red**. It is provided at a concentration of 10,000X. Alternatively, add 2 μl of **ethidium bromide (EtBr)** per 50 ml of gel. Ethidium bromide is a suspected **carcinogen** so please be very careful with it and discard the tips in the marked beaker. Being careful includes wearing latex gloves when touching gels or when there is any chance for ethidium bromide exposure to the skin.

    After swirling to promote even mixing of the dye into the agarose, pour the molten agarose into the gel tray. Visual inspection will confirm that there are no leaks, that the gel box is level, and the gel tray is level. Carefully make any necessary adjustments. Bubbles may be shepherded to the periphery with a micropipet tip.

12. Let the gel cool until it looks opaque. This often takes 15 – 20 minutes.
13. Gels can be poured in advance of need. Wrapping in plastic wrap and storing at 4 °C should keep the gel moist until needed. Please be aware that if such gels contain ethidium bromide they should **labeled, stored, and handled with care**.

II. Loading and running gels

A. Preparing the gel for loading

1. Move the gel form to your bench. Gently remove the comb.
2. Carefully lift the gel tray out of the gel box. Consider that the gel will readily slip out of the open ends of the gel tray. Position your fingers accordingly. Gels will burst into pieces if dropped.
3. Rotate the gel tray so that the wells are closest to the negative (black) electrode.
4. Pour 1X TBE or appropriate running buffer into the two reservoirs on either end of the gel. Continue to fill until the gel is just barely immersed and the two reservoirs match that level. In essence, the running buffer will create an open wire between the two electrodes. The gel should be in the middle of that wire so that the current runs through the gel. If sufficient running buffer has been added, the wells will be filled with running buffer.

B. Preparing samples and loading the gel (done while gel is cooling)

1. Add a sufficient volume of 5X or 6X **loading buffer** to bring the conc. of the loading buffer in the sample to 1X. Consider how to formulate an equation for calculating this volume. Depending on the dimensions of the well, there will be a maximum volume of sample / sample buffer that the well can hold. 20 μl is a common maximum volume. To run all of the volume for a particular sample, multiple wells may be needed. This should have been built into the **plan** about which samples go into which wells.
2. Use the appropriate micropipet to draw up the appropriate volume into a tip.
3. Carefully position the pipet over the designated well. Altering the angle at which you view the gel may aid in seeing the well. Placing contrasting, color paper beneath the gel may help, too.
4. Lower the tip into the running buffer just above the desired well. Slowly depress the micropipet's plunger so that the blue solution "falls" into the well. Avoid having the tip so far into the well that the liquid has nowhere to do. Also avoid pipeting air bubbles into the well as these will rise, lift sample, and disperse it into the running buffer. If you have extra lanes, consider a practice well or two with water mixed with loading buffer before any samples are loaded. Remember to

load your **size marker (i.e. DNA ladder)** into one well in each set of wells. 8 µl of this should be enough.

C. Running the gel

1. When done loading, carefully attach the cover and the electrode wires. Again, the negative electrode should connect to the gel box towards the wells with samples.
2. Turn on the power source, set the appropriate voltage (~100 volts for a small gel), and hit start. Very small bubbles should rise from the negative electrode.
3. After 20 minutes or so, you should see the blue dye of the samples migrate toward the positive electrode. This dye does not bind DNA; it is merely present to allow easily visualization of the sample's progress. Disconnect the electrode wires and, wearing gloves, carefully lift the gel / gel tray up. Again, consider that the gel may slip out of the gel tray.
4. Tilt the tray / gel to let most of the liquid drip back into the gel box.
5. Place the tray / gel onto a paper towel to avoid dripping and walk over to the dark room through the archway and to the left.
6. Afterwards, the gel may be placed back into the box and run for longer if needed.
7. Running the gel for too short a period of time will result in little resolution between DNA bands of different sizes. Running the gel for too long can either result in the positively charged dye running past your DNA, in the other direction, or the DNA running off of the gel.
8. Compare the migration distance of the DNA bands in the sample lanes to the bands in the marker lane. This will allow estimation of the lengths of the DNA bands in your sample. Record these values and compare to the lengths expected.
9. See a separate protocol for taking and recording a picture of the gel using the Kodak gel documentation system.

β-galactosidase Activity Assay in Permeabilized Yeast

(written by J. Stebbins, Triezenberg lab; edited by P. Oelkers)

Reagents (these will be made for you)

Appropriate synthetic complete (SC) liquid medium

Z buffer (Miller 1972):

Component	for 1 L	Final conc.
$Na_2HPO_4 \cdot 7H_2O$	16.1 g	60 mM
$NaH_2PO_4 \cdot H_2O$	5.5 g	40 mM
KCl	0.75 g	10 mM
$MgSO_4 \cdot 7H_2O$	0.25 g	1 mM

- pH to 7. Store at 4 °C
- right before using add 2-Mercaptoethanol to 50 mM (2.7 ml per liter Z buffer)

0.4 % ONPG (o-nitrophenyl-β-D-galactoside) stock solution:

4 mg/ml in Z buffer. Store at –20°C or make fresh each time to avoid storage issues.

1 M Na_2CO_3 (in dH_2O) for 500 ml

53 g in 500 ml dH2O

Chloroform

0.1% (w/v) SDS (in H_2O)

Disposable plastic cuvettes (1.5 ml volume)

Procedure

1. Grow yeast cells under the conditions dictated by the experimental design.

2. *Determine the cell density*. Pipet 1 ml of the culture into a spectrophotometric cuvette and read and record the optical density (i.e. absorbance) in a spectrophotometer set to a wavelength of 600 nm (OD_{600}). As the blank, pipet 1 ml of the media used to grow the cells. Log phase is commonly considered to be OD_{600} = 0.3 – 1.0. If the cells are past log phase, that is probably not a deal breaker but it is less than ideal. The cell concentration of a yeast culture can be calculated from the OD_{600}. If the OD_{600} = 1.0 then the cell concentration is about 2 x 10^7 cells / ml. If the reading is higher than 1.0, dilute the culture prior to reading the OD and then multiply the reading by the dilution factor. The cell density of each culture will be needed to harvest the same number of cells for each culture.

3. *Harvest about 1 x 10^7 cells*. If the cell density is 2 x 10^7 cells, pipeting 0.5 ml of cells into a 2.0 ml microcentrifuge (i.e. eppendorf tube) will harvest ~ 1 x 10^7 cells. Using more cells should not be a problem but be sure to utilize the same number of cells for each sample. If harvesting more than 2 ml, use a 15 ml conical tube (i.e. Falcon tube). Harvesting involves transferring cells to an appropriately sized tube and performing a centrifugation (i.e. spin) for about 2 minutes at ~4000 x g. Discarding the supernatant will leave a cell pellet for subsequent work. [note: Save the remaining cell culture (i.e. cells not spun down). If step 5 shows that the reaction is slow or yields little product, a greater volume of cell culture may be used for harvesting cells for a second attempt.]

4. *Lyse cells*. Resuspend the cells in 700 μl of Z buffer. Transfer to a 1.5 ml tube. In parallel, generate a "blank" tube with only 700 μl Z buffer (no cells). In the fume hood, use a Pasteur pipet or p1000 to add 3 drops of chloroform to each tube, including the blank. With a fresh pipet, add 2 drops of 0.1% (w/v) SDS to each. Vortex at top speed for 10 seconds.

5. *Provide colorimetric substrate to the lysate and measure color development*. Preincubate the samples in a water bath at 28 °C for 5 minutes. Add 200 μl of 4 mg / ml ONPG (a lactose analog) and record the time. Incubate the samples at 28 °C until a pale yellow color develops. When it does, record the time again (to the second, if possible) and directly proceed to the next step to stop the reaction. Be quick. A lack of quickness will lead to some, if not a lot of, error in the time component of the velocity. Not all samples need to be stopped at the same time.

6. *Stop the reaction*. Add 500 µl of 1 M Na_2CO_3 to stop the reaction. Vortex for 10 seconds. Microfuge at full speed, 10 minutes, at room temp. to pellet the cell debris. Use a p1000 to carefully transfer the supernatant to a clean, spectro-photometric cuvette.

7. *Measure the OD_{420} of the reactions*. Use the "blank" solution to blank or zero a spectrophotometer set to 420 nm. Read and record the OD_{420} for the cell lysate reactions.

8. *Express the β-gal activity as moles / min. / 10 million cells.*
   a) Convert the OD_{420} data to a molarity using Beer's law (c = A /(e x l)). The absorption coefficient (e) for ONPG is 4500 M^{-1} cm^{-1}. The path length (l) is 1 cm.
   b) Convert the molarity to moles by multiplying the molarity by the volume of the reaction (about 0.0018 L)
   c) Divide the moles of product formed by the time of the assay to calculate bgal velocity in moles (use a prefix so that the reported value is between 0.5 and 500 and scientific notation is avoided) per minute per / the 10 million (1×10^7) cells. Adjust the number of cells if a different number was used. More conventionally, we would perform a protein quantification assay on the cell lysate to allow reporting the activity as **relative specific activity**: moles of product / assay time / mass of cell lysate protein. Just a reminder, **specific activity** is when a velocity is expressed as moles of product / min. / mg of pure enzyme. However, normalizing to the number of cells instead of mg of cell lysate should suffice.

Safety notes

Chloroform is volatile and should be used in a fume hood. Chloroform is irritating to the skin, eyes, mucous membranes, and upper respiratory tract. It should only be used in a chemical hood. Gloves and safety goggles should also be worn. Chloroform is a carcinogen and may damage the liver and kidneys.

References

1. Bradford, M.M. (1976) A dye binding assay for protein. *Anal. Biochem.* **72**:248-254.
2. Guarente, L. (1983) Yeast promoters and *lacZ* fusions designed to study expression of cloned genes in yeast. *Methods Enzymol.* **101**:181-191.
3. Miller, J.H. (1972) *Experiments in molecular genetics.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
4. Rose M. and Botstein, D. (1983) Construction and use of gene fusions *lacz* (β-galactosidase) which are expressed in yeast. *Methods Enzymol.* **101**:167-180.

BACTERIAL TRANSFORMATION

Background

Transformations are procedures for inducing microbes to take up exogenous DNA from their environment. This protocol uses "**chemically competent**" *E. coli*. Chemically competent bacterial cells have been made more permeable by washing the cells in cold solutions of salts. These cells are then incubated with a detergent (polyethylene glycol) and undergo heat shock (change from ice bucket to room temp) to further increase the cells' permeability and promote the uptake of exogenous DNA. Such a procedure can be very efficient, yielding up to 1 x 10^7 transformants (i.e. cells that have taken up the DNA) per μg of plasmid DNA. Even so, during the procedure, the vast majority of cells will not take up any DNA. To only have the cells with the plasmid grow, a growth environment must be used in which only cells with the plasmid can grow.

Ampicillin is an antibiotic that belongs to the same family of antibiotics as penicillin. These antibiotics contain a four atom, β –lactam ring. Their ability to limit bacterial growth is based upon their ability to inhibit an enzyme important in the formation of crosslinks in bacterial cell walls. Inhibition profoundly affects cell viability. Bacteria with the β – lactamase gene can produce and secrete the β – lactamase enzyme which can cleave the antibiotic near the cell and leave cell wall crosslinking uninhibited. For simplicity, the β – lactamase gene is often referred to as Amp^R for **ampicillin resistance** gene.

Such a system is created if the plasmid contains an antibiotic resistance gene and after the transformation, the cells are grown on agar plates that include the corresponding antibiotic (e.g. LB agar + Ampicillin (Amp) plates). After 18-24 of growth on LB + Amp plates, single cells which took up the plasmid (i.e. transformants) will divide enough times to form a mound of cells called a colony.

Colonies are often a few millimeters in diameter and are raised from the plate. These can be seen with the naked eye. The next step, addressed in a separate protocol, often involves transferring some of the cells from each colony into 5 ml of LB + amp liquid media for over night culturing and subsequent plasmid isolation (i.e. miniprep). Selection pressure is necessary so that only bacteria containing a plasmid with the β – lactamase gene grow

Equipment

Sterile, glass test tubes or sterile, 1.5 ml eppendorf tubes
Bucket with ice
Bunsen burner
Plate spinner (if available)
Cell spreader (glass rod bent into hockey stick shape)
95% ethanol, about 2 cm high, in a beaker that can accommodate the cell spreader
Incubator set at 37° C

Reagents (will be made for you)

30% (w/v) polyethylene glycol 3350 (PEG) 1 ml
3350 PEG 0.3 g
- place the 0.3 g in a 1.5 ml eppendorf tube
- bring volume to 1.0 with dH_2O using markings on side of tube
- use vortexing and incubation in warm water bath to dissolve the solute
- filter sterilize using a syringe filter
    - aliquot 5 x 200 ml into sterile, 1.5 ml eppendorf tubes
- store at 4° C (1 tube per group)

10X KCM 1 ml
1.0 M KCl (M.W. = 74.55) 74.5 mg
0.3 M $CaCl_2$ (M.W. = 110.98 anhydrous) 33.3 mg
0.5 M $MgCl_2$ (M.W. = 95.2 if anhydrous) 47.6 mg
- prepare as described for 3350 PEG
- if the available salts are not anhydrous, the necessary mass will need to be increased based on how hydrated the salt is

LB (Lauria broth) + ampicillin agar plates 500 ml
1.0% (w/v) tryptone 5.0 g
0.5% (w/v) yeast extract 2.5 g
1.0% (w/v) sodium chloride 5.0 g
1.5% (w/v) agar 7.5 g
- begin with 400 ml deionized water (~80% of target volum)
- while mixing on a stir plate, add first three components to water and dissolve
- bring volume up to 500 ml
- transfer to a 500 ml bottle (clean, not necessarily sterile)
- weigh agar and transfer to the bottle along with a large stir bar
- autoclave for 20 minutes, liquid cycle

- cool on stir plate
- when hand cool, add powdered or resuspended ampiclllin to bring the final concentration to 50 μg / ml.
- pour plates (about 20) using sterile technique

Procedure:

1) Make the following 100 ml mixture in a sterile, small, glass culture tube on ice (sterile eppendorf tubes may substitute):

   5 μl 10X KCM
   4 μl 30% (v/v) PEG
   x μl ligation or plasmid DNA (usually 4 ml of ligation or 1 ml of dilute plasmid)
   y μl H_2O
   50 μl freshly thawed competent cells (XL-1 Blues)
   100 μl

2) Incubate for 20 minutes on ice.
3) Remove from ice and wipe residual ice off.
4) Incubate for 10 minutes at room temp.
5) Use the rotating plate and glass spreader to plate all 100 μl onto pre-warmed, pre-labeled LB + Amp (with IPTG and Xgal added if vector has blue / white selection; pSF011 does not) plate.
6) After allowing a few minutes to dry, place plates agar-side up in a 37° C incubator over night. Do not let cells grow more than 30 hours. Satellite colonies may begin to grow where transformants have degraded the ampicillin.

Designing a Scientific Project

Planning is a critical part of science. Such planning often involves the **scientific method** which can be boiled down to five steps.

1) Make observations of the world either directly or through the work of others.
2) Generate a **hypothesis** (a declarative statement) of how some aspect of the world functions based on the observations in (1).
3) Predict events that will occur if the hypothesis from (2) is true.
4) Plan and execute experiments to determine if the events in (3) occur.
5) Declare the hypothesis as supported or not supported. Rarely are hypotheses proven or disproven with certainty.

Narrowly focused, specific, unambiguous hypotheses are generally considered a hallmark of good science. For example, "Daily consumption of broccoli will slow the progression of pancreatic cancer." is better than, "Broccoli promotes good health". One drawback is that the investigator may have bias toward the hypotheses that he/she develops being true. Therefore, an alternative to a hypothesis is a **question** to ask. If the question is carefully crafted so that there are only a few possible answers, that can also be considered good science. For example, "Does the consumption of broccoli slow the progression of pancreatic cancer?" Broad questions with many possible answers are often criticized as "fishing expeditions". For example, "Does eating broccoli promote good health?" is likely too broad a question. Ideally, posed hypotheses included a **proposed mechanism**. Much like the inner workings of a watch that relay the force of a spring to the rotating of hands, cellular components specifically interact to perform the functions of the cell. For example, if broccoli contains a relatively high amount of folate and if pancreatic cancer cells have been shown to down regulate enzymes that use folate as a coenzyme, then the proposed mechanism is that increasing folate intake will facilitate the activity of enzymes in pancreatic cancer cells that limit the ability of those cells to grow and divide. Even better would be the naming of those enzymes. Please appreciate that this is a spontaneously derived example.

To be clear, if you propose that yeast grown at a cooler temperature will result in the down regulation of gene X, your hypothesis should be followed by a statement proposing a mechanism linking cool temperature with a reduced needed of protein X (presuming X is a protein - encoding gene). In other words, why is less protein X needed when cells are cooler?

Coming up with a hypothesis to test or question to ask may take months of reading, thinking, mulling, hemming and hawing. I have heard it said that three hours in the library can save three months in the laboratory. Due to the time constraints of the semester, we must accelerate the planning process. To do so, a general framework for experiments will be provided. Please feel free to go outside of this framework as long as your hypothesis or question addresses a topic in molecular biology (i.e. DNA, RNA, proteins).

Even though molecular biology focuses on DNA, RNA, and proteins, molecular biology projects often in the context of the very broad question, "How does life work?" Accordingly, when planning molecular biology experiments, it is often best to begin with choosing an aspect of life to better understand. One approach is to first choose a **cellular or physiological function or process**. Examples are, "Monocytes develop into macrophages", "Wheat plants adjust to increased soil salinity?" and "*Mycobacterium tuberculosis* (a pathogen that causes tuberculosis) avoids the host's immune response". Even though we will study transcriptional regulation in yeast, there is a long history of using this single-celled eukaryote to understand molecular mechanisms in higher (i.e. multicellular) eukaryotes.

Identifying proteins that have a role in the chosen function or process comes next. These proteins can be in a higher, multicellular organism such as humans or any other you find interesting.
. Searching the scientific literature will likely be necessary for that step. The instructor will help with the literature searches upon request. Thirdly, use **bioinformatics** to determine if the *S. cerevisiae* genome contains a **homolog** of the gene(s). Such bioinformatics will take some time to explain. Crafty use of scientific literature searching may be used to combine steps two and three.

The suggested framework for projects is for each group to **choose a gene** or genes in the *Saccharomyces cerevisiae* genome whose transcriptional regulation fits with some physiological process. Propose environmental conditions which will increase or decrease the rate of transcription and consequently the mRNA concentration for that gene or those genes. For example, amino acid transporters may be upregulated in response to a particular amino acid being presen in the environment so to promote the harvesting of that amino acid from the environment. Alternatively, those transporters may be down regulated since there is so much solute to transport, fewer transporers are needed. In either case, beginning to study the transcriptional regulation of such a transporter starts with either a clearly stated hypothesis or question to be asked. Consider how you would phrase a hypothesis or ask a question regarding the amino acid transporter.

After a gene or genes have been chosen, the next goal is to propose **environmental conditions** that may alter the transcription of that gene. As you may imagine, it is best to base the proposal on either previous work (more searching of the literature) or purely creative thought. For any condition, consider that the dose or intensity of the treatment as well as the length of the treatment may influence the transcriptional response. Please also consider that not all condiions can be logistically achieved. Consulting with the instructor prior to selecting conditions is advised.

Lastly, the **experimental method** for detecting changes in the rate of transcription of the gene(s) should be determined. One method will be the generation of a **promoter / reporter plasmid**. This will require PCR amplificaiton of the 5' flanking region of the gene which likely contains the gene's promoter, digestion of that PCR product with restriction enzymes, ligation of that digested PCR product into a similarly digested yeast reporter plasmid (e.g. pSF011), and transformation into bacteria. Isolation of the successfully constructed promoter / reporter plasmsid will allow transformation into wild type *S. cerevisiae*. These transformed yeast can then be grown in the environmental coditions hypothesized to alter transcription of the gene of interest, as well as **control conditions** for comparision. Activation or inhibition of the promoter should result in greater or lesser transcription of the reporter gene (e.g. **b-galactosidase**) compared to the control. Presuming that altered transcription will yield a proportionate alteration in the abundance of the b-galactosidase enzyme, assaying cell lysate b-galactosidase activity using a **colorimetric assay** using the ONPG substrate allows ready estimation of the rate of transcription. Please appreciate that mechanisms besides changes in transcription rate can also alter RNA abundance. Please also appreciate that a strength of this approach is that **truncated promoter constructs** can be generated so to map the location of likely transcription factor binding sites within a promoter.

Alternatively, instead of generating a promoter / reporter construct, **real-time PCR** may be employed. This method avoids the production of a promoter/reporter plasmid. Instead, the transcriptional rate of a gene is measured by direct estimation the abundance of the RNA transcript. This procedure involves growing yeast in the environmental conditions of interest. Again, it is important to consider control conditions for comparison. After growth, RNA is isolated from the yeast culutre. **Reverse transcription** is then used to copy the RNA into complementary DNA (cDNA). PCR is then used to amplify a small, 3' part of the cDNA generated from the gene of interest as well as **control genes** whose abundance is likely similar among all samples. This PCR is performred in the prensce of a double-strand specific fluorescent dye which allows for

determination of PCR product abudance via measurement of light emission. This procedure will be explained in more detail in a separate document. Strenghts of this method include that the gene is being studied in its native position in chromatin. The effect of promoter elements distant from the gene's start codon as well as the effects of chromatin structure will be detected. This is not true of the promoter / reporter method. Weaknesses include the challenge of working with RNA (e.g. RNAses are present on human skin) and precise pipetting is required for real-time PCR results to be reproducible.

At a date about four weeks after the term begins, the instructor will request that each group submits an experimental design for its project.

Those experimental designs should include:

a) the hypothesis
b) background information
- the physiological function(s) involved
- how the product(s) (e.g. proteins) of the gene(s) of interest relate to the physiological function
- information about the stimuli chosen that will likely increase or decrease transcription of the gene(s) of choice

c) experimental detail
- this should be a sequence of events with a good amout of detail. The composition of every buffer need not be listed but including details and perhaps a time line will help give the project structure.

d) data analysis
- what data will be collected, including controls, how will it be presented (e.g. bar graph), and what values will be compared for statistical significance?

DNA Extraction from Agarose Gel

Reagents:

TE buffered phenol
- there is often an aqueous layer above the phenol / TE

TE buffered diethyl ether
- the ether is the upper layer

3M sodium acetate (NaOAc), pH = 5.2
95% ethanol
70% ethanol

note: please discard all organic waste (phenol and ether) into an appropriate waste container

Procedure:

Note*: Perform all work with **phenol** and **ether** containing solutions in the fume hood. Phenol is a **volatile organic solvent** and a **neurotoxin**. Ether is **noxious** and very **combustable**. Please wear gloves and have arms covered when working with these solutions. Please discard phenol and ether containing liquid and solid waste in the appropriate beakers in the fume hood.*

1) Run out DNA on agarose gel. Be sure to place lanes of same DNA consecutively.
2) Using a razor blade, create a tight-as-possible rectangle of agarose that contains your band(s) of interest. Be sure to only cut straight down. Do not drag the blade since it will likely scratch the underlying plastic. Use tweezers to transfer the gel slice to a 1.5 ml eppendorf tube.
3) Using a sterile 18 gauge needle for each slice, carefuly chop until the slice is in very small pieces. This can be done by squeezing the gel slice between the needle and the side of the tube.
4) Estimate the gel volume using markings on side of tube. Bring volume to 300 μl with TE if the gel slice volume is below 300 μl.
5) Working in the fume hood (phenol being a volatile organic solvent) add an equal volume of phenol buffered with TE. Do not substitute phenol / chloroform or unbuffered phenol. Please notice that phenol buffered with TE has a covering (~ 1 cm thick) layer of aqueous solution through which a pipet tip must pass before reaching the phenol.

6) Vortex well, being careful to not let the top come open. Even after vorexing, you may need to flick the tube with a fingernail to insure that the phenol and agarose pieces are completely mixed at the bottom.
6) Freeze at -70° C for at least 15 minutes.
7) Microfuge for 15 minutes at room temperature at full speed.
8) Using a p200, carefully transfer top, aqueous phase to new tube. Be careful not to transfer any of the interphase. In general, sacrifice yield for purity.
9) To this, add an equal volume of phenol buffered with TE
10) Vortex well. Microfuge, 2 minutes, room temp., full speed.
11) Carefully transfer the upper aqueous phase to a fresh tube.
12) To this, add an equal volume of TE buffered diethyl ether using a Pasteur pipet.
13) Vortex well. Microfuge, 30 seconds, room temp., full speed. Remove and discard the upper, ether layer using a Pasteur pipet. Go just below the interface so to not have any ether left in the lower aqueous phase.
14) Let the tube sit open in the fume hood for about 5 minutes to allow residual ether to evaporate.
15) Ethanol precipitate. First, determine the volume of the sample. Then, add 2.5 volumes of 95% ethanol and 0.1 volume of 3M sodium acetate. Vortex well and incubate either at -20° C for at least 2 hours (days is OK) or at -70° C for at least 30 minutes.
16) Microfuge sample (ideally at 4° C) for 15 minutes. Carefully pour off the supernatant, being careful not to lose the pellet.
17) Remembering the volume of 95% ethanol that you added, add this much 70% ethanol to the pellet. Vortex.
18) Microfuge sample (ideally at 4° C) for 10 minutes. Carefully pour off the supernatant, being careful not to lose the pellet.
19) Dab tubes on a clean piece of paper towel to remove ethanol on the rim and let sit horizontally with tops open until the pellet is dry. This may take 20 – 30 minutes.
20) Resuspend the DNA in a desired amount (often 10 – 20 μl) of TE.

Laboratory Notes and Setting Up a Google Site

Keeping careful laboratory notes is critical to successful experimentation. Detailed notes can be relied upon for accurately communicating results in a **manuscript** (the goal of most science) months after the experiments were completed. Solid experimentation involving earnest effort will likely go to waste if it is not carefully recorded. Additionally, carefully record keeping will allow experiments to repeated using the same steps, greatly increasing the likelihood that the results will be reproducible. Recording failures as well as successes is useful since it will help avoid trying the same strategy in the future. At the very least, in a professional setting, a careful set of records provides proof of effort. If experiments are not going well, you want to at least be able to document that you are trying.

With that in mind, consider laboratory notes to be a **functional reminder to your future self** and not some triviality that a persnickety professor is forcing you to do. In a professional setting, laboratory notes should be **understandable to an uninvolved third party**. Please take this audience into consideration when keeping laboratory notes. Also, consider that within a day or two, information stored in short-term memory is replaced by new information. Therefore, notes are best taken as the experiment is being performed and not days later. Within 24 hours of every class meeting, each group will be expected to write their lab notes for the week and post them in an electronic portfolio described below.

Laboratory notes also play an important role in experimental design. Carefully laying out the plan of an experiment prior to any bench work should allow for efficiency of effort. In science, you are often both the architect and the builder. Architecture comes first. Describing an experimental plan should begin either with the question being asked or the hypothesis being tested. After that often comes a description of the experimental system (e.g. *S. cerevisiae* and iron uptake) and an explanation of why that system is appropriate. The first day of notes for a particular project often involves much more text than subsequent days. Even so, planning each day's experiments ahead of time in written form allows you to check off actions as they are taken. This is the best way to do science.

Keeping lab notes on paper provides some potential pitfalls. What if they are damaged or in a bag that is stolen? What if one lab member has the lab notebook but decides to drop the class and join a band? What if each person keeps his / her own notes so that there is no organized and thorough accounting? To avoid these pitfalls, each group will be asked to set up a single **Google Site** for cataloging lab notes. Please only set up one site per group. One person will be the initial owner and he/she will then share

access with the other members and the instructor with parallel, owner status.

Steps for setting up a Google Site with the appropriate template

1) With your personal UMich Email page open, click on the nine-box "grid" next to your name in the upper right.
2) Click on the "Sites" symbol.
3) Click on the red "CREATE" button.
4) Click on the "Browse the gallery for more" box
5) In the search box, type "molecular".
6) Click on the icon for "UM-Dearborn Molecular Biology Lab Template"
7) Click on the blue "Select" box at the bottom of the page.
8) Click once on the "UM-Dearborn Molecular Biology Lab Template" box to highlight it. The next part is to name your group which takes a little explaining. Please begin the name of your group with "474L W16 X-Y" so that all of the class groups align. If you are in section 1 and are group #3 (numbers will be assigned in class), then please name your group "474L W16 1-3 ….". If you want to include a moniker in your group name, such "Group Awesome", your name will be "474L W16 1-3 Group Awesome".
9) Look slightly lower in the page for the "select a theme". Choose a color scheme that fits your taste.
10) Making sure that the proper template box is still outlined in blue, click on the red CREATE button on the upper left

Congratulations! You now have a Google Site. There are still a few more things to do.

On the top right, there is a blue "share" box. Click on that. On the page that opens, in the "Invite people" section, change "can edit" to "is owner". In the text box, type in the UMich email address of a group member. Clicking on the blue "Send" box will enter that person as an owner for the site. Repeat this process so that all group members and the instructor (poelkers@umich.edu) are listed as owners. Joining will add the site to those which you can access from the "SITES" folder which can be accessed from your email front page after clicking on the cluster of boxes icon in the upper right of your email page.

Now that your site has been established, you can begin to use it. For one, the front page should be edited to include the name of the group members. This can be done by clicking on the pencil icon in the tool bar. This will allow you to write in the front text box. Clicking on the blue SAVE button will save you work.

Next is the beginning of the lab notebook. If you click on "A. Lab notebook" on the left

side, a new page will open. Please do not use option 1 for posting notes. Uploading files makes editing and critiquing cumbersome. Please use option 2. To create a page for entering text, click on the icon next to the pencil in the toolbar. This is the "Create page" icon (a plus sign on a folder sheet of paper). When the next page asks you to name the page, I suggest using the date for ease of organization. You might use the name (1. Jan. 12) so that when "Feb" pages are added, then are not placed earlier due to alphabetical orering. I also suggest changing the "Select a location" to "Put page under A. Lab Notebook". Click on the red CREATE box at the top left when you are done.

Alternate to having a separate page for each week is to have one page for the entire term with the dates of entries as separators. Either is fine.

Just to be clear, the benefit of using Google documents is that it provides a "living" document. Multiple users can readily edit it remotely. Instructor comments can be written directly into the text (usually in blue). Thereafter, edits can be repeatedly made. The site has other features that you will in all likelihood learn by doing a little exploring. Uploading ("adding files") pdf files for any relevant references into the papers section will likely be useful. Please also upload all data, just as .jpg files of gels, into the data section.

Ligation

DNA molecules with complementary sticky ends may undergo a process called **ligation**. Ligation involves the creation of **phosphodiester bonds** between the sugar backbones of two or more DNA fragments. It is a bit like a magic trick where the magician takes two pieces of rope and makes a single piece of rope. In this case, both ends of the rope pieces are joined so to make a single, circular piece of rope. If each piece of DNA has two different sticky ends, perhaps generated by restriction enzymes A and B, then there is only one orientation in which the DNA fragments may be joined (A to A, B to B). This is referred to as **directional cloning**. If both DNA fragments have two identical sticky ends, then the fragments may be joined in two different orientations. This is referred to a **bidirectional cloning**. Such cloning is particularly challenging because the two ends of each DNA piece may ligate to each other. To extend the magic trick analogy, the product could be two loops of rope instead of one combined loop of rope. This strategy is generally avoided if at all possible.

Ligation is achieved by incubating the two DNA fragments of interest, in our case a digested pSF011 plasmid and our digested PCR product, in a test tube with the bacterial enzyme **T4 DNA ligase** and the appropriate reaction buffer. Please be reminded that almost all enzymes have a unique set of optimal conditions for reactivity. Such conditions include the chemical environment, substrates, and temperature. These are recreated, or at least attempted to be recreated, *in vitro* by providing a **reaction buffer**. Such buffers are often supplied at ten-times concentrated solutions by the commercial supplier of the enzyme.

Ligation reactions, or ligations, have similar considerations to restriction digests. In fact, there is an even stronger emphasis on small volumes so that the volume is commonly 10 ml. An optimal molar ratio of insert DNA to plasmid (or vector) DNA is 10:1. This ratio can be achieved either by knowing the concentration of the solutions of insert DNA and plasmid DNA or by estimations and trying a few ratios. A 10 X buffer is also routinely provided. Control ligations are often performed. These may contain plasmid DNA with no insert DNA and insert DNA without plasmid DNA. In either case, no successful generation of a circular, plasmid DNA should occur.

For example (values are volumes in ml):

	Rxn. 1	Rxn. 2	Rxn. 3	Rxn. 4
Deionized water	6	3	7	7
10 X ligase buffer	1	1	1	1
plasmid DNA(~ 0.1 μg)	1	1	1	0
insert DNA (~0.2 μg)	1	4	0	1
ligase	1	1	1	1
	10	10	10	10

Protocols differ on the suggested temperature and time. Incubating overnight at 15 °C is common. This temperature is commonly achieved by placing a water bath put at a low setting into a cold room. After ligation, the next step is bacterial **transformation**. That is the process of inducing bacteria to take up DNA from the environment and to selecting for those bacteria that took up closed-circular DNA (i.e. plasmid with insert successfully ligated). Proceeding from the ligation to transformation without sample storage is preferable.

Microcentrifuge

As the name implies, microcentrifuges are smaller than most centrifuges. 1.5 ml conical tubes (called microcentrifuge tubes), often made of polypropylene plastic, fit into a rotor that often has 24 – 40 positions. Like any centrifuge please make sure that all samples are balanced across the center of the rotor. Visual inspection of the paired samples is sufficient to establish proper balancing. If an odd number of samples are being processed, use a balance tube with an appropriate amount of water.

If the sample should remain cold during the spin, there are two choices. You can either use a refrigerated microcentrifuge or place a regular microcentrifuge in a cold environment such as a cold room.

After placing the samples in the microcentrifuge, put on the rotor lid if there is one. Lids reduce the air drag as the centrifuge spins and limit noise. Close the lid and set the time (usually in minutes) and speed (usually in revolutions per minute (rpm)). If you set the timer for longer than intended, please do not force the time to a shorter time. Some microcentrifuges can accommodate such changing of the time. Others have broken timers and are accordingly less convenient to use. The speed can be changed at any time.

Most microcentrifuges have a "quick spin" feature. In that case, the rotor will spin as long as a particular button is depressed. These are usually 5 – 10 second spins.

Most microcentrifuges will automatically lock upon the spin starting. This is a safety feature since opening the centrifuge lid during a spin may expose the user to debris moving at high speed. After the run is complete, a button commonly needs to be depressed to release the lock and allow the samples to be removed.

Micropipeting: Moving Very Small Volumes Very Accurately

Many, if not all, procedures in molecular biology require the mixing of precise volumes of solutions. Accurately transferring solutions from some source to a reaction or sampe tube is therefore a central skill. Micropipets allow for such accurate transfer with percent errors often less than 2% with volumes down to 2 μl (2×10^{-6} L). Consider that a drop of rain has an estimated volume of 100 μl. Commonly used micropipettes, such as the variable volume model from Gilson (below) operate using air displacement. When working with any apparatus, if is often beneficial to understand the basic mechanisms of that apparatus.

Air displacement means that upon depressing the white knob or plunger with the thumb, air is pushed out of the barrel and subsequently, out of the disposable tip attached to the end of the barrel. Pushing should cease when distinct resistance is felt. This is described as the "first stop". Air should be pushed out of the tip prior to immersion in the source solution.

With the plunger held down, immerse the tip just below the surface of the source solution is followed by gradual release of the plunger to its original position. This draws air back into the barrel and creates a vacuum inside the tip, drawing in liquid. Letting the plunger spring back should be avoided as it will result in inaccuracy. The extent of depression and subsequent return is controlled by a spring which is controlled by the volume setting. Most micriopipets have a three digit setting. The units that each digit represents depends on the maximum volume the micropipette can accurately dispense. P1000s can accurate dispense between 200 and 1000 μl. P200s can accurately dispense 20 – 200 μl and P20s dispense 2 – 20 μl.

P1000		P200		P20	
thousands	☐	hundreds	☐	tens	☐
hundreds	☐	tens	☐	ones	☐
tens	☐	ones	☐	tenths	☐

Trying to set a micropipette to a volume outside of its proscribed range will likely damage it, render it inaccurate and require repair. As the old carpenter saying goes, "Be good to your tools and your tools will be good to you."

The last step is push out the volume from the tip into a tube of choice. This is achieved by placing the tip either above the solution one the side of the tube above the solution. or into the solution in the tube and pushing down on the plunger until the "first stop" is reached. Be wary of drops that essentially go sideways onto the wall of the tube. Such volume must be induced to join and mix with the volume in the tube. If a significant volume remains in the very end of the tip, further pushing of the plunger or "blow out" will push out a little extra air to remove the last bit of volume from the tip. Be careful to raise the micropipette out of the solution prior to gradually relaxing your thumb and allowing the plunger back to its resting position. It is a good idea to visually confirm that the tip is empty prior to ejecting the tip into the waste beaker. Pipetting small or viscous solutions often involves rinsing the solution in the tip during transfer. Swirling with the tip also serves to disperse a small volume in a much larger one.

Whether the tip is first immersed in the solution in the accepting tube prior to transfer or held above and allowed to emit a drop into the tube is determined by a few factors. Small volumes may not form a sufficiently large drop to leave the tip. In that case, direct transfer to the solution already in the tube is best. If the same solution is going to be transferred to multiple tubes, dropping the solution from above allows the same tip to be used for multiple samples. Please be aware that good chemistry laboratory practice rules apply. Do use a pipette tip for multiple samples if that use runs the risk of introducing solutes to solution that do not belong (ala double dipping a chip into the Super Bowl guacamole). Please also be aware that what happens in a tube is dictated by what solutes actually intermingled in the solution and not by what solutes we intended to intermingle. Mixing with a vortex is one way to have solutions mix. However, this can kick small droplets up onto the side of the tube. In that case, a quick spin in a microcentrifuge may force all solutions down into the tube.

If you have any doubts about how accurate a micropipet is or how precise your technique is, investing a few minutes to pipet deionized water onto a fine balance is a good idea. If you are not familiar with the operation of a fine (i.e. analytical) balance, please ask. Using a range of volumes for each micropipet should be performed in such exercises.

Oligonucleotide (e.g. primers, oligos) Design for PCR

A. Import the sequence of the template into a DNA analysis software such as ApE (freeware).

B. Design the **forward primer**.

Highlight about 25 nt in the area of the intended "upstream" end of the PCR product and determine if that sequence meets the criteria in part (C). You may need to move the highlighted sequence one way or another so to meet the criteria. It may come down to a decision between compromising the intent of the experiment by moving the targeted sequence or using oligonucleotides that are predicted to be logistically troublesome but are located exactly where intended.

C. Follow general rules about designing primers for PCR.

1. The calculated **melting temperature (T_m)** should be between 50 and 65° C. Melting temperature is a reflection of the amount of heat needed to separate half of the primers from a template DNA with a perfectly complementary (i.e. Watson – Crick pairing) sequence. Calculating the melting temperature can be done in a variety of ways. One simple way is to count the number of each nucleotide and fill the values into the following formula: T_m = (C+G)x4 + (A+T)x 2. There are also a variety of software programs that use more complex algorithms to estimate T_m. ApE includes this functionality. If you highlight a putative primer sequence, the length of the highlighted sequence as well as the calculated T_m will be shown in the upper tool bar. You will notice that the longer primes are, the higher the annealing T_m becomes. Relatively more C-G pairs will also increase the T_m. It is best if you choose one method for calculating T_m and only use that method since the different methods can give quite different values.

2. The content of Cs and Gs in the primer should be between 30 and 70%. Near 50% is usually best.

3. Primers should have **little self-complementarity** so to avoid the formation of primer-dimers or hairpins during the PCR reaction. Computer algorithms assist in this. Most companies that sell oligonucleotides provide use of an algorithm upon registering. One such company is Sigma (http://www.sigma-genosys.com/calc/DNACalc.asp). At times, registering can be a pain.

An alternative to using an algorithm is to visually inspect each oligo for regions of self-complementarity. This involves writing out the sequence of the oligo, 5′ to 3′ (left to right), on a piece of scrap paper. Then, below it, write the oligo sequence again but this time 3′ to 5′ (left to right). Look for regions where a series of nucleotides in the 5′ to 3′ sequence will bind to a series of nucleotides in the 3′ to 5′ sequence.

For example:

5′ TGGTTCAGTCGTCGATGCAATGC 3′

3′ CGTAACGTAGCTGCTGACTTGGT 5′

One eyeball alignment yields:

```
5′ TGGTTCAGTCGTCGATGCAATGC 3′
        - -       - - - - -     - -
3′ CGTAACGTAGCTGCTGACTTGGT 5′
```

The dashes in between the sequences indicate where hydrogen bonds might form. Please appreciate that sliding each oligo left and right and looking for complimentarity is required for a full analysis. In my experience, the above amount of similarity will not yield primer dimers.

4. The primers should either end (3′) in CC, CG, GC, or GG (a C-G clamp) or have 3 of the final 5 nucleotides be C or G. This is called a **G-C clamp**. The reason being that C-G pairs share three hydrogen bonds while A-T pairs share two. Polymerases require a distinct double stranded template to bind to prior to extending the primer based on the sequence of the single-stranded template. In other words, the ability of the polymerase to extend the primer requires a firm association of the primer with the template, especially near the 3′ end.

D. Design the **reverse primer** or down stream primer following the rules in C.

This is a little trickier. The reverse primer will anneal to the upper strand (i.e.Watson in yeasat) so it should contain the same sequence as the lower strand. In other words, the reverse primer will be the **reverse complement** of the sequence at the far end of the sequence targeted for amplification. This idea of reverse primers is often not intuitive.

Writing out the sequences of the target and primer sequences on scratch paper may help you to picture how the primers will bind.

It is useful to keep in mind is that polymerases, without exception, can produce only polymers by adding nucleotides to the 3′ end of a previous nucleotide In other words, polymerases progress in a 5′ to 3′ direction. If two primers are going to amplify a region of DNA, the 3′ ends of those primers must oppose or face each other when bound to the template.

The guidelines for designing reverse primers are the same as for designing the forward primers. Please appreciate that when oligonucleotides are ordered from a company, the sequence is always provided in a 5′ to 3′ direction.

One strategy for designing reverse primers is to make a second, separate ApE file for the template sequence. This can be achieved by beginning with an ApE file containing the putative promoter sequence. Highlight all of the sequence and use the reverse-complement function (the symbol with two opposing arrows found along the tool bar at the top of the screen) to now view the reverse complement. This is essentially the bottom strand, viewed 5′ to 3′. Using this sequence exactly how you used the sequence to design the forward primrer should result in a properly designed reverse primer. Be sure to save the ApE file using a name to indicate that it is the reverse complement of the sequence copied from the genome database.

When you are finished, please generate an Excel file with the following headings and the appropriate information in the boxes below. Consider a systematic naming system with the idea that you do not know how many other oligos you may order for this gene in the future. In other words, if the first two oligos ordered for a gene are ABC1for and ABC1rev, what will the third oligo be called?

If you have questions about how to determine the locations needed in columns 3 and 4, please ask. Please post this Excel file on your Google doc site. Oligo orders will likely be placed on Wednesday evening to insure delivery on Monday for use on Tuesday. Please limit the number of oligonucleotides requested per group to ten.

Oligo name	Oligo sequence	Location in yeast genome	Location in reference to gene's start codon

When designing primers for PCR, the user should consider if the PCR product will be subsequently **digested** for eventual insertion into a plasmid via ligation. If this is the case, additional sequence may be included on the 5' end of the primer. This sequence may include a **restriction enzyme recognition site** and additional sequence so that the restriction enzyme site is not at the absolute end of the PCR product. It is important that the reasons for only being able to modify the 5' end and the need for additional sequence are understood.

After primers anneal to template DNA during the annealing phase of PCR (the second step), the polymerase will begin to attach nucleotides to the 3' end of the primer during the extension phase (third step). The sequence of these nucleotides will be dictated by the sequence of nucleotides in the template. If the primer / template combination does not provide a double stranded template for the polymerase, no addition of nucleotides will occur. Therefore, the 3' end of the primer must precisely be the reverse complement of the template. This is the same reason that C and G bases are favored at the 3' end of primers; to give that double strand template a more stable nature compared to A and T bases which pair more loosely.

Choosing which restriction enzyme recognition site to include in the 5' end of your primers takes a little strategy. To be clear, each primer in a pair of forward and reverse primers should have different restriction enzyme sites. This allows for unidirectional (sometimes just called directional) cloning into the plasmid.

Criteria #1) The restriction enzyme recognition site **is** found in the **polylinker / multiple cloning site** of the **plasmid** into which you intend to ligate the PCR product, pSF011 in our case.

Criteria #2) The restriction enzyme site is not found within the PCR product. The idea is, after the PCR, to purify the product and digest it with enzymes that will cleave the sites included in the 5' ends of the primers so to create sticky ends but NOT cleave the PCR product internally. ApE can be used to check for the presence of restriction sites. Please recognize that an acceptable enzyme for use with one gene may not be acceptable for another gene.

Criteria #3) The restriction enzyme leaves a predictable **sticky end**. Some restriction enzymes have an "N" in their recognition site which means that there are multiple sticky ends possible. Some restriction enzymes also cleave downstream of the recognition sequence which also leaves the possibility of different sticky ends. Such enzymes are not useful for cloning.

Criteria #4) The **orientation** of the ligation of the PCR production into the plasmid is considered. In our case, *the part of the putative promoter closest to the start codon of the gene of interest should wind up closest to the start codon for the b gal (lacZ) gene.* In that case, once you have chosen two restriction enzymes to use, find out which one is closer to the bgal gene on pSF011. That enzyme's site is the one you should include in the primer closest to the start codon of the gene of interest. You might make a drawing of your gene of interest (including both DNA strands), where each primer will bind, and the pSF011 plasmid with the polylinker drawn to make sure all of the pieces will fit together as you intend. Things can get a little tricky when your gene of interest is on the bottom strand of the yeast genome. Even so, you can do this.

Criteria #5) Consider that if you choose two restriction enzymes that have sites very close to each other in the pSF011 polylinker, that is less than ideal. The reason being that prior to the ligation, pSF011 has to be digested with the same enzymes as the PCR product. Consider that one enzyme will likely cut first, then the other. Which one cuts first will likely be random and determined by which enzyme bumps into the DNA first. Once the first enzyme cuts the plasmid DNA, the DNA will now be linear. If the second enzyme site is now very near one of the ends of this linear DNA, that enzyme may bind and cut inefficiently. That is not good. Therefore, choosing restriction enzymes whose sites are at least 15 base pairs apart in the plasmid should address this concern.

Criteria #6) It is possible that with all of this planning, one of the enzymes you choose is exceedingly expensive or not easily obtained commercially. However, polylinkers rarely include such enzymes. New England Biolabs (www.neb.com) and Promega (www.promega.com) are large suppliers of restriction enzymes. Their websites can be searched for availability and price. It is not uncommon for the smallest size of a restriction enzyme to cost $60. This is not expensive. As a side note, restriction enzymes are often sold in terms of units of activity, simply called "units", rather than mass. Consider the possibility of 1 gram of a poorly purified enzyme which contains many proteins besides the enzyme of interest, compared to 1 mg of a pure preparation of the enzyme. Agreed upon definitions of a "unit" of enzyme, such as the ability to create 1 nmol of sticky ends in 1 hour under standard conditions, can be found at manufacturer's websites.

Now that you have chosen your restriction sites for each primer, you are almost done. Presuming the restriction site has a recognition sequence that is six nucleotides long, these size nucleotides, in order 5' to 3', can be added to the 5' end of your primer sequence. For instance, if you choose EcoRI which has a recognition sequence of

GAATTC, and your primer has sequence 5' GGCTCAGGGTTTCCCAA ', then your primer + restriction enzyme site is 5' GAATTCGGCTCAGGGTTTCCCAA 3'.

The last step is to include eight nucleotides of "random" sequence to the 5' of the restriction enzyme site. This addresses the concern in criteria #5 above. While this sequence can be considered to be spacer sequence that is only needed to keep the restriction site from being so close to the end of the PCR product, the actual sequence requires some consideration. The reason is that if poorly chosen, the sequence will result in the primer contain self-complementary sequence that may result in secondary structural (i.e. the primer folding upon itself like a horseshoe) involving Watson-Crick pairings The ability of the primers to form primer dimers (i.e. annealing to each other and thus forming a double stranded template for the polymerase during the PCR) is also a concern.

Both of these concerns can be addressed by analyzing the entire primer sequences using a primer design website or visual inspection using pen and paper. Trial and error may be required before each pair of primers is designed in a way that optimizes your chances of successfully cloning the putative promoter of interest into pSF011.

The above contains a lot of information. Please ask the instructor for clarification or to answer questions as needed. For each primer, please include a name, the sequence (always 5' to 3'), the genomic DNA location for the primer (using the same numbering system used by the Saccharomyces Genome Database), the predicted melting temperature (T_m) (only considering the part of the primer that will anneal to the target genomic sequence), and the method of estimating the T_m. In my own notebook, I often include the drawing from Criteria #4. However, that is not so easy to do electronically.

Oligonucleotide Handling

Now that you have designed your oligonucleotides (aka oligos, primers) and ordered them from your supplier of choice, an express mail envelope will arrive in a few days. In the envelope will be your oligos, each in a tube labeled with information such as the sequence, estimated melting temperature, and the number of molecules in nanomoles (nmoles). Please note that since the oligos are sent in a desiccated (i.e. dried) form, units of molarity cannot apply. Our first charge is to resuspend the oligos to a convenient concentration.

Resuspending or dissolving DNA is commonly done in a slightly alkaline solution since the phosphodiester bonds are more susceptible to hydrolysis under acidic conditions. A mixture of 10 mM Tris base (aka Trizma, THAM. Tris(hydroxymethyl)amino methane) and 1 mM ethylenediaminetetraacetic acid (aka EDTA), with a pH ranging from 7.4 to 8.0, is a commonly used solvent. This is mixture is called **TE**.

Before adding the TE to the dried oligos we have to do two things. The first is to calculate the volume of TE to add so to bring each oligo to a concentration of 100 μM. This will be called the **storage stock.** 100 μM is chosen because it is a round and convenient number. Also, oligos are more stable over time when stored at a relatively high concentration such as 100 μM. Just to be clear, the storage stock will remain in the tube sent by the supplier.

Calculating the necessary volume should be straightforward so you will be left to your own mathematical devices for that. Now, before opening the tube to add the appropriate volume of TE, give the tube of oligos a quick spin in the **microcentrifuge**. This will insure that none of the desiccated oligos are in the cap of the tube so that they fall out upon opening the tube. Please appreciate that the tube my have been upside down during shipment. If the use of a microcentrifuge is something you do not have experience with, please see the microcentrifuge instructions.

Be sure to **label** the top of your tube so that it can be easily found from above when searching through a box. General guidelines for labeling any chemical apply: name of chemical (use a logical system for labeling the oligos) and concentration. The date and initials of the operator will not fit on the cap but can be put on the side of the tube.

From this storage stock, make a 20 μM **working stock** of each oligo. Having working stock addresses the hazard of PCR that is contamination. DNA besides the template may find its way into any of the PCR reagents. This may cause products besides the desired to

be generated. If contamination is found, one course of action is to discard all solutions and make fresh. Having working stocks allows for the discarding of such solutions and making fresh without having to place a new order for oligos. Please use the $\mathbf{C_1V_1 = C_2V_2}$ equation to make 100 ml of the 20 mM working stock. TE is still the appropriate solvent. Both of these should be **stored at -20 °C** (i.e. in a freezer) between uses. They may be thawed at room temperature prior to use but put on ice as soon as possible after thawing.

Polymerase Chain Reaction

Background

The **polymerase chain reaction (PCR)** allows for the selective copying (i.e. amplification) of a defined section of DNA within a DNA sample called the **template**. Commonly used templates are genomic DNA, mitochondrial DNA, and complementary DNA (cDNA). What cDNA is will be explained in the future. For example, of the 3.4×10^9 base pairs in the human genome, it may be of interest to copy the ~150 bp which comprise exon 5 of the alpha subunit of the insulin receptor. Most PCR protocols involve two **primers** (single stranded DNA oligonucleotides of about 25 nucleotides long) which, upon annealing to the template, dictate which portion of the template is amplified. Also included in PCR reactions are the **DNA nucleotides** (i.e. deoxynucleotide triphosphates (**dNTPs**)) and appropriate solutes which often include a slightly alkaline buffer and **magnesium**.

In terms of experimental applictions, subsequent uses of the **PCR product** (i.e. **amplicon**) include sequencing, ligating into a plasmid, and/or using as a probe in a hybridization experiment. In other words, PCR can be **analytical** (some answer is provided by analysis of the PCR product and the product is consumed in that analysis) or **preparative** (the PCR product is purified for subsequent use). The array of applictions provides powerful tools for use in the fields of molecular biology, genetics, biotechnology, and forensics to name a few.

If you have learned the process of DNA replication in some detail, the following explanation should help make sense of PCR. If not, you may skip the next two paragraphs.

For the most part, the procedure for PCR parallels the process of DNA replication. DNA replication can be simplified to a few steps: 1) origin recognition 2) activation of the helicase to denature the double stranded DNA 3) formation of the initial complementary sequences (called a oligo) on each template strand 4) extension of the 3' end of each oligo by a polymerase using the order of nucleotides in the single stranded DNA as a template 5) termination when replication forks meet or the end of the chromosome is reached. Recall that replication of DNA occurs at replication forks which have both a leading and lagging strand since DNA replication always occurs in a 5' to 3' direction, using the numbering on the strand being generated

In PCR, there are essentially two origins per reaction but only the leading strand is made. Each origin is defined by where the respective oligonucleotide (i.e. oligo, primer) binds. Heat (~ 94 °C) replaces the need for helicases to denature DNA. Oligos bound to the template provide the double stranded substrate with the free 3′ end onto which the polymerase (necessarily thermal stable to withstand the high temperature needed for denaturing) will add nucleotides that form Watson-Crick pairing with the template. Finally, since there are no replication forks, only two leading strands generated from each "origin", the process has no set point of termination, at least initially.

In practice, PCR involves three basic **steps**. 1) DNA **denaturation** using heat (~94 °C) to compromise the hydrogen bonds which keep the opposing strands together. After an initial "hot start" of about three minutes, denaturation steps often only require 5 - 30 seconds. 2) Oligo **annealing** involves a cooler temperature whereby the oligos may form sufficienet hydrogen bonds to maintain an association with the template if they "bump" (recall that all of this is happening in a small tube with a small volume where molecules are diffusing) into a portion of the template that has an exactly, or almost exactly, complementary sequence. Depending on the length of the oligo, this step often occurs between 50 and 65 °C for 30 - 120 seconds. 3) **Extension** of the bound oligos involves the thermal stable DNA dependent DNA **polymerase**, often originating from a microbe that exists in very warm habitats (e.g. Thermus aquaticus) binding to the 3′ end of the oligo and beginning to incorporate nucleotides dictated by complementation with the template. Polymerases always add onto 3′ ends. This is referred to the **5′ to 3′ direction** since the 3′ end gets added on to. Extension routinely occurs at 68 - 72 °C for one minute per kilobase (kb) of template being amplified. Prior to performing a PCR, the experimenter should know the expected length of the amplicon.

A completion of these three steps is called a **cycle**. Repeated cycles allows geometric amplification of the targeted region of the template. The total number of cycles often ranges from 25 to 35 with 30 as a common middle ground.

In order for this to work, one oligo must be complementary to the bottom strand of the "upstream" sequence. This may be called the "forward" oligo. The other oligo is complementary to the top strand of the "downstream" sequence and is the "reverse" oligo. One way to try to understand how this all comes together to allow PCR to produce a double stranded DNA product that includes the two oligos and the intervening sequence is to view an on-line tutorial, such as can be found at: http://www.dnalc.org/resources/animations/pcr.html. This involves downloading an application. Downloading applications is certainly to be done with caution but that has been down without issues by the instructor.

One concept to appreciate regarding PCR is that the first few cycles and the later cycles are qualitatively different. Try to imagine what is happening during the first cycle. After the denaturing and oligo annealing to however many of copies of template are in the reaction (thinking about just one template may be simpler for now), the polymerase binds the each oligo and begins to generate a DNA strand complementary to the template. In a way, the extension of the "forward" oligo is going "downstream" towards the "reverse" oligo and the extension of the "reverse" oligo is going "upstream" towards the "forward" oligo. Just to be clear, the opposing polymerases will not collide because each is on a different, single stranded template. In fact, the polymerases will likely go past the sequence to which to opposing oligo was designed and keep generating a complementary strand until either it falls off or the temperature changes. There is no STOP sign.

During the second cycle, the same events as occurred in the first cycle may occur. However, the products of the first cycle may also serve as templates to which oligos will bind. Consider the product made during the first cycle with the forward oligo. It has generated a copy of the "top" strand of the target sequence. This single stranded DNA may be bound by the "reverse" oligo. After the polymerase binds this oligo and begins to generate a "bottom strand", extension will continue until the far end of the template is reached. It is no coincidence that the far end of the template is comprised of DNA that includes the "forward" oligo. At this point, the polymerase falls off, leaving a "bottom" strand of the targeted region with oligo sequence on each end.

We are almost done so hang in there. In the third cycle, the same events that occurred in the first two cycles may occur. However, the exact copy of the "bottom" strand that we described may serve as a template for a "forward" oligo. After binding, the polymerase will synthesize a "top" strand and fall off upon reaching the end. That end is the former "reverse" oligo. These double stranded products of defined length are what we are after.

In cycles four through thirty (or thereabouts), the same events that occurred in the first three cycles can all happen. However, as more and more double stranded products of defined length are generated, those will become more and more likely to be used as template. This will allow those defined length products to predominate and be the vast majority of DNAs produced from the PCR.

Taking some scrap paper to write out the events that occur during the first few cycles of a generic PCR is a worthwhile exercise. Considering using black and blue (or whatever

color pens you have) for the "top" and "bottom" strand DNA sequences. Again, the tutorial referenced above does a nice job of displaying the events of the first few cycles and relating the geometric amplification of the targeted region.

Protocol:

PCR reactions are often performed in parallel with only the template or oligo pairs varying among the reactions within a set. To avoid having to pipet each component into each reaction tube, a cocktail or master-mix is often generated. This cocktail includes the components which are found in all reactions in the set. Components appropriate to some reactions but not to all are added individually. For example, for our experiments, we can include the water, PCR buffer, deoxynucleotides triphosphates (dNTPs), Taq polymerase, and template DNA to the cocktail. Oligos will vary among the reactions so they may be added uniquely to each reaction. Cocktails are often made in excess to allow some error or variation in pipeting. For each ten reactions, another reaction-worth of cocktail is often made so to have all the samples have the same volume. Many PCRs have a final volume of 50 ml but that is a flexible number.

The amount of the various components varies among protocols. Most companies that sell Taq, or other thermal-stable polymerases, provide a 10X buffer. This means that the tube contains the necessary salts and buffers at ten times the concentration needed to create a functional environment for the enzyme. In other words, 5 μl of the 10X buffer should be included in each 50 μl reaction. Magnesium chloride ($MgCl_2$) is an important component of this salt and buffer mixture. Some companies include $MgCl_2$ in the 10X buffer and some provide a tube of 25 or 50 mM $MgCl_2$ separately to allow users to customize the concentration. We commonly order the Taq from New England Biolabs which often includes the $MgCl_2$ in the 10X buffer.

In addition to the buffer, PCRs incude:

Deoxyribonucleotides (final conc. in rxn. of 0.2 mM)
Taq polymerase (2.5. units per reaction is often plenty)
Oligos (2) (final conc. of each in rxn. of ~ 0.8 μM)
Template DNA (a few ng is often plenty)
- adding too much template can be problematic

A sample PCR reaction set up is below:

Component	Vol per reaction (μl)	7 X cocktail (μl)
dH_2O	33.5	234.5
10X Taq pol. buffer	5	35
2 mM dNTPs	5	35
Taq DNA pol.(5 U / μl)_	0.5	3.5
Yeast gDNA	2	14
total volume	*46*	*322*

Please appreciate that the first numerical column is for calculation purposes only and those volumes will *not be pipeted*. In this case, the volumes for the 7X cocktail would be pipeted into a 1.5 ml eppendorf tube and vortexed. Be particularly careful when pipeting the Taq DNA polymerase. Enzymes are often stored in glycerol to promote protein stability.

46 μl of the cocktail would then be pipeted into each of six PCR tubes. PCR tubes often have a maximum volume of 0.2 ml and have thin walls to allow rapid temperature changes. To each PCR tube, 2 μl each of 20 μM stocks of oligo #1 and oligo #2 would be added. While a negative control or water control is not necessary for the **preparative PCR** we are setting up, **analytical PCRs** would often have one reaction that does not include template. Obviously, in analytical PCRs, the template would not be included in the cocktail. PCRs are generally kept on **ice** during assembly to prevent the reactions from starting prior to intention.

Once the PCRs are assembled in the reaction tubes, it is time to program the t**hermal cycler** (i.e. PCR machine). In fact, to be particularly efficient, you can turn the thermal cycle on and program it while the PCR components are thawing. Every thermal cycler has its own software conformation but in general, programming the cycles can usually be achieved with a little effort. The precise temperature and times of the cycles will vary by application and laboratory.

Two important variables are the temperature of the annealing step and the length of the extension step. The annealing temperature is determined by the lowest estimated oligo melting temperature minus 3 degrees. Estimating the melting temperature for oligos will be covered in a separate section dedicated to the design of oligonucleotides. It is ideal if the oligos used have similar melting temperatures.

While calculations may prodict a useful annealing temperature, some experimentation may be required to determine the optimal annealing temperature. If the annealing temperature is too low, the oligos may bind to site(s) on the template beside those targetted. Such non-specific priming may reduce the efficiency of the reaction and / or generate non-targeted products. On gels, these may be called **"ghost bands".** Self-annealing of oligos and oligo dimers are also more likely to form at lower annealing temperatures. If the annealing temperature is too high, an insufficient number of oligos will bind long enough to the template to allow extension to occur. This will also reduce the efficiency of the reaction, perhaps to the point of no product being observed.

Determining the extension time is much more straightforward. Most polymerases used in PCR can extend at a rate of 1 kilobase (kb) per minute. Erring on the high side (e.g. extending for 1 min 30 sec for a 1 kb expected product) is generally a good idea. Extending for far longer than necessary may also generate ghost bands.

Generic cycling onditions for a PCR are:

94 °C 3 minutes 1 cycle (this is a "**hot start"** to fully denature the template)

94 °C 30 seconds
55 °C 45 seconds } 30 cycles of these 3 temperatures
72 °C 1 min 30 seconds

72 °C 5 min 1 cycle
4 °C hold (99 min 59 sec often is interpreted as infinity)

If possible, PCRs are not run overnight but rather placed in a refrigerator (4 °C) until time allows for use. That will prevent the machine holding 4 °C for hours which may strain the machine and create condensation which can leaking into the machine and damage it.

After the PCRs are done, one common step is to run them on an agarose gel. That procedure is addressed in a separate section.

Restriction Enzyme Digestion

Restriction enzymes are produced by bacteria to degrade exogenous DNA such as inserted by infecting viruses, also called phages or bacteriophages. This serves as a sort of innate immune system referred to as the **restriction – modification (R-M) system**. Loenen et al. have recently (2014) written a nice review of the history of research of restriction enzymes. Of the four types of restriction enzymes, Type II has proven to be the most useful in molecular biology and biotechnology. Among the reasons are that in Type II enzymes, the endonuclease activity can be separated from the methyltransferase activity and that the endonuclease activity occurs within defined DNA sequence (i.e. **restriction sites**) and often leaves defined, single-stranded **overhangs**. For example, one of the first Type II restriction enzymes characterized was Hind III, purified from the bacterium, *Haemophilus influenzae serotype d.* Subsequently identified restriction enzymes have been named using the genus and species of the producing bacterium. The restriction site of HindIII is 5′ AAGCTT 3′. You may notice that the "bottom" strand, written 5′ to 3′, has the same sequence. This palindromic nature is common to many but not all type II restriction enzymes. HindIII always cuts between the two As, leaving the following two "sticky" ends (N is any nucleotide):

(5′)…NNNNA AGCTTNNNN… (3′)
(3′)…NNNNTTCGA ANNNN… (5′)

Not all enzymes have a restriction site of six nucleotides. The ones that do are called "**six cutters".** Four and six nucleotides are the most common lengths of restriction sites although odd-numbered sites are also found. With any restriction site, the likelihood of finding it in a DNA sequence involves a quick calculation of $1/4^{(\#\text{ of nucleotides in restriction site})}$. This does presume that all four nucleotides are in equal abundance in the DNA sequence of interest which is not necessarily true.

HindIII leaves a **5′ overhang** since the single stranded portions that remain have a free 5′ end. Other enzymes, such as KpnI (restriction site = GGTACC), leave a **3′ overhang**.

(5′)…NNNNGGTAC CNNNN… (3′)
(3′)…NNNNC CATGGNNNN… (5′)

Some enzymes cleave after the 2nd nucleotide in a six-nucleotide restriction site, leaving a 2-nucleotide sticky end. Yet others cleave after the 3rd nucleotide in a six-nucleotide restriction site or after the 2nd nucleotide in a four-nucleotide restriction site, leaving ends

without any single strand DNA overhangs. These are called **blunt ends** and the enzymes are called **blunt cutters**. PvuII and SmaI are examples of such enzymes.

Some enzymes have some elasticity or degeneracy in the restriction site. For example, SmlI has a recognition site of CTYRAG where Y is any pyrimidine and R is any purine. The overhangs left by these enzyme are not entirely predictable and therefore, not all ends generated by SmlI digestion will readily form hydrogen bonds with each other.

The discovery of the type II restriction enzymes and the symmetrical, sticky ends they may generate opened an almost limitless ability to manipulate DNA. DNA from one source could be digested, isolated and incubated with DNA from a second source that was similarly digested. Through Watson–Crick base pairing, complementary sticky ends can provide sufficient hydrogen bonding to place the respective, free 5′ and 3′ ends in close proximity. This proximity allows the enzyme ligase, used physiologically in DNA replication (recall Okazaki fragments) and repair, to create new, phosphodiester bonds between these 5′ and 3′ ends so to create a new, contiguous strand of DNA. This process is called **ligation**. Ligase from the T4 bacteriophage is commonly used for such purposes. Due to the universal (barring retroviruses) use of DNA as the genetic material in living things, this allowed combining DNA from different species into a single strand. This process became known as **recombinant DNA technology**. This technology is used for purposes such as cloning genes into plasmids and generating genetically modified organisms (GMOs).

Cloning genes into plasmids was originally performed using naturally occurring restriction sites that were found to flank a gene or region of interest. If a restriction site for enzyme A lay upstream of a gene and restriction site B lay downstream, digestion with A and B and subsequent ligation into a plasmid digested with A and B allowed for a copy or **clone** of that gene to be maintained in bacteria. Plasmids often have a cluster of unique (only found once in the plasmid) restriction sites near a promoter. These are called **multiple cloning sites** or **polylinkers**. This allows for an array of possible DNA fragments, perhaps including genes, to be ligated with the plasmid in close proximity to the promoter. Using two enzymes, A and B, allows for **directional cloning** as the "insert" DNA will only ligate with the plasmid in one orientation. As well, the insert and vector will be prevented from binding to themselves.

In practice, using restriction enzymes is straightforward. Biotechnology companies have purified a vast collection of restriction enzymes and provide these for sale. Due to the differing pH optima and cofactor needs for the various enzymes, the companies also provide a 10X buffer with each enzyme that will supply an environment for optimal enzyme activity when diluted 10-fold. To avoid having a custom buffer for each enzyme,

most companies have assembled a collection of four of five buffers for use with their enzymes. All of their enzyme will digest optimally in at least one of the buffers. When digesting with two different restriction enzymes at once (a **double-digest**), finding a buffer in which both cut optimally takes a little scanning of the company's information, often provided on-line. New England Biolabs (NEB) has engineered many of their enzymes to be "high fidelity" (HF). This means that all "HF" enzymes cut optimally in the "Cutsmart" buffer, simplifying the planning of double-digests.

Restriction enzymes are notoriously **thermal labile** so it is good laboratory practice (GLP) to keep enzymes **cold at all times**. This cannot be emphasized enough. A denatured enzyme may yield results that suggest a particular DNA does not contain a restriction site when in fact that site is present. Also, restriction enzymes are provided in quantities referred to as "**units of activity**" (U) and not in units of concentration like moles or % (w/v). Commonly, one unit of restriction enzyme is sufficient to digest 1 mg of DNA at 37 °C in one hour. Most restriction enzyme are provided in concentrations of about 20 U / μl. Therefore, one μl is sufficient for most digests, even with the consideration of using a four-fold excess of enzyme above that indicated by the unit definition and the mass of DNA being digested.

To allow storage at -20 °C without freezing, restriction enzymes are routinely provided suspended in glycerol. Glycerol acts as a sort of cryoprotectant, keeping the protein folded and in solution at cold temperatures. Since glycerol can alter the activity of enzymes, the final concentration of glycerol in any reaction should not exceed 10% (v/v). Therefore, enzymes should not comprise more than 10% of a reactions volume. Aiming for less than 10% is a good idea. Due to its viscosity and thus likelihood to stick the outside and inside of pipet tips, glycerol makess accurately dispensing volumes difficult.

Restriction enzyme digest reaction volumes are often kept small, 10 – 30 μl, to a) keep the enzyme and substrate in close proximity and b) to limit the number of wells needed to resolve the digested products afterward. Once a reaction's volume is determined, one-tenth of that volume is routinely comprised of the appropriate 10 X reaction buffer, one tenth of 1 mg / ml **bovine serum albumin (BSA)** and one-tenth with enzyme(s). DNA, often about 3 μg but mass varies by application, and water comprise the balance of the reaction volume. For example, a double digest of pSF011 with HindIII-HF and KpnI-HF might include:

	1 rxn. (vol. in μl)
deionized water	15.0
10 X Cutsmart buffer	2.5
1 mg / ml BSA	2.5
pSF011 (1 mg / ml)	3.0
HindIII-HF	1.0
KpnI-HF	1.0
	25.0

As with any mixture, components are often added in order of increasing importance. This limits waste of valuable assets if a mistake is made. Starting with water if often a good idea since it prevents any solution components from interacting at high concentration. Similar to PCR, if many different DNAs are going to be digested in parallel, setting up a cocktail for the reaction mixture without the DNA may simplify the set up.

Digests are often resolved on an **agarose gel** to separate the fragments or bands of interest from the other bands / fragments. These are preparative gels. Subsequent isolation of the DNA fragments allows the assembly of ligation reaction with carefully controlled DNA components.

Scientific Literature Searching

Since we live in the information age, being able to efficiently find information, mostly on-line, is a very applicable and marketable skill. Manuscripts which have met the criteria of peer-review (i.e. critical evaluation by scientists in the field) and become **published papers** are the most valuable source of scientific information. **Primary papers** describe experiments performed by the authors. **Review papers** summarize the work of many different authors. For many purposes, beginning a literature search by first finding relevant review papers is best. Reviews, especially those written within the last two years or so, have essentially done the "leg work" of finding and sorting through all the papers written on a topic. **Citations** in review papers allow readers to easily track down the primary papers which published particularly interesting work. Searching the scientific literature for primary or review papers is often done using **search engines**.

Search engines, often provided free of charge on-line, use unique algorithms to use text (i.e. keywords) provided by the user to find relevant papers. Mostly, these algorithms use string searches to find identical text in the titles, abstracts, or listed keywords of papers in a particular collection. Different search engines search in different databases (i.e. collections of scientific journals). Falagas, M.E. *et al.*, 2008 reviewed four commonly used search engines and generated the table below.

.

TABLE 1. *Characteristics of databases*

Characteristic	Pub Med	Scopus	Web of Science	Google Scholar
Date of official inauguration	06/1997[a]	11/2004	2004[b]	11/2004
Content				
No. of journals	6000 (827 open access)	12,850 (500 open access)	8700	No data provided (theoretically all electronic resources)
Languages	English (plus 56 other languages)	English (plus more than 30 other languages)	English (plus 45 other languages)	English (plus any language)
Focus (field)	Core clinical journals, dental journals, nursing journals, biomedicine, medicine, history of medicine, bioethics, space, life sciences	Physical sciences, health sciences, life sciences, social sciences	Science, technology, social sciences, arts and humanities	Biology, life sciences and environmental sciences, business, administration, finance and economics, chemistry and materials science, engineering, pharmacology, veterinary science, social sciences, arts and humanities
Period covered	1950–present	1966–present	1900–present	Theoretically all available electronically
Databases covered	Medline (1966–present), old Medline (1950–1965), PubMed Central, linked to other, more specialized, NLM databases	100% Medline, Embase, Compendex, World textile index, Fluidex, Geobase, Biobase	Science citation index expanded, social sciences citation index, arts and humanities citation index, index chemistry, current chemical reactions	PubMed, OCLC First Search

Falagas, M.E., E.I. Pitsouni, G.A. Malietzis, G. Pappas (2008) Comparison of PubMed, Scopus, Web of Science, and Google Scholar: strengths and weaknesses. *FASEB J.* 22, 338–342.

a) Pubmed (www.ncbi.nlm.nih.gov)
b) Google scholar (scholar.google.com)
c) Scopus (www.scopus.com)
d) Web of Science
Access requires a subscription which the U.M. Dearborn currently has. The website can be accessed through the Mardigian Library website

Choosing keywords that find all the relevant papers and limit the finding of papers not directly relevant is a skill in itself. For example, when searching for papers about *Saccharomyces cerevisiae*, I suggest using "*cerevisiae*" as a keyword. "Yeast" could also be used but there are many genus and species under the taxonomic heading of yeast. Also, in the interest of brevity, some abstracts may only write "*S. cerevisiae*". Keywords that are essentially redundant, such as *Saccharomyces* and *cerevisiae*, are generally best avoided. Using "review" as a keyword will limit the search to review papers.

Results are often provided in a numbered list with only the article title, author list, journal, volume, and page number. Properly descriptive titles allow users to scroll through the list and identify papers that likely contain relevant information. What exactly to look for in a title is not easy to explain. Ideally, authors have carefully chosen the wording of the title so to clearly indicate the focus of the paper. Clicking on a title of interest often opens a new page that contains the abstract.

If after reading the abstract the user decides that paper contains relevant information (emphasizing the importance of a clearly worded abstract), a portable document format (pdf) file of the manuscript may be able to be downloaded. Most search engines have link "boxes" at the upper right hand corner of the abstract. These boxes often contain a symbol unique to the journal. Clicking on that box opens a link to the publisher's website that should show the manuscript text. Somewhere on that page, often in font far smaller than it should be, is wording "PDF" or "download PDF". This service is only available or "open access" journals and journals for which the U.M. Dearborn has an electronic subscription. Clicking on that text will allow the user to place a PDF file into a folder of choice. Alternatively, a page will appear that requests payment for accessing the pdf. This block can sometimes be circumvented by accessing the journal through the Mardigian Library website. Alternatively, the library's interlibrary loan system can be used

to request the paper. In that case, be sure to record the pertinent information of the paper: authors, year of publication, journal name, article title, journal, title.

Keeping downloaded articles well organized is a worthwhile goal. Consider naming the files with the format: [first author's late name, first author's first initial, year of publication, a 3 - 8 word summary of the paper.pdf]. Placing the PDF files into folders named for topics will facilitate finding the files in the future. Any time you can do something so that it does not need to be done again in the future provides for efficiency of effort.

Yeast Culturing

Saccharomyces cerevisiae (*S. cerevisiae*) is a unicellular eukaryote belonging to the kingdom, Fungi. Yeast is a common term for any, unicellular, oval fungi. However, since *S. cerevisiae* is such an actively research model organism, sometimes the term yeast is used to indicate *S. cerevisiae*. In nature, S. cerevisiae primary exits on rotting fruit and primarily exists in the **diploid** (2N) form. Upon experiencing harsh environmental conditions, diploids may undergo **meiosis** to form four **haploids** (1N). These haploids are ensconced in an ascus, a special version of a cell wall that prevents the haploids from undergoing dessication. When conditions improve, the ascus is broken down and the four haploids are released to either live independently or fuse with a yeast halpoid of the opposite mating type (either a or alpha (a)) to reestablish the diploid state. The ability for yeast cells to exist in both the haploid and diploid state is an interesting situation regarding gene dosage.

S. cerevisiae, like many microbes, when grown in **liquid culture** will grow rapidly. Sometimes this is called planktonic growth since the cells are floating around a liquid. One particularly rich media (i.e. broth of nutrients) that allows for rapid growth (generation time of about 1.5 hours) is comprised of **yeast extract, peptone, and dextrose**. It is called **YEPD or YPD**. The dextrose provide a "carbon source" to fuel catabolism, peptone is a source of amino acids and nitrogen for amino acid and nucleic acid synthesis, and yeast extract has a wide variety of nutrients including cofactors (inorganic ions utilized in reactions) and coenzymes (small, organic molecules that assist in reaction catabolism). It may seem odd to feed extracts of yeast to yeast but there you have it. Since the exact molecular composition of yeast extract will likely vary on the yeast grown and how it was grown, YPD is not a well-characterized or controlled media.

Conversely, **synthetic complete** (SC) media is a carefully synthesized, one pure molecule at a time, formulation of organic and inorganic molecules that provide the necessary nutrients for *S. cerevisiae* to live. Specialized "**drop out**" media, such as SC – uracil (ura), include all the usual components of SC except one or more nutrients. Using drop out SC media allows the experimenter to add back the missing nutrient(s) at a range of concentrations, thereby assessing the affect of nutrient availability on cell function.

Drop out media also allows for **autotrophic** selection. In other words, only cells which can make the missing nutrient(s) will grow in this media. These are **autotrophic** yeast in regard to this media environment. Yeast which cannot synthesize this nutrient are **auxotrophic**. SC media also commonly has a carbon source added such as glucose (e.g. SCD). Generation times in SCD are often around 2.0 hours.

Culturing can also be performed on **solid media**, such a agar-containing Petri dishes, often referred to as **plates**. These can be made in YPD and SCD mixtures. Solid media allows for individual cells to start a new population at a single, discrete location. This

location can be a colony or a streak. One limitation for culturing on solid media is that cells pile on top of one another and lose contact with the nutrient-rich media. This limits the vertical aspect of colony growth so that growth is more two-dimensional (i.e. along the surface of the plate). Generating more cells so that more cell lysates can be generated for experimentation is greatly facilitated by culturing discrete strains of *S. cerevisiae* in the three-dimensions of liquid culture.

Growth in liquid media may be limited by the supply of nutrients provided. As you may imagine, when nutrients begin to wane, the ability of cells to generate the necessary ATP and building blocks necessary for cell division decreases. Consequently, when a small number of yeast are inoculated into a liquid culture, the number of cells will increase over time. Initially, due to the small number of cells, the population will increase fairly slowly. This is the **lag phase** of growth. Once the population becomes more substantial, each new generation and thus doubling of the population results in "leaps" in population number. This is the **log** or **logarithmic phase** of growth**.** This phase is followed by discrete, physiological changes by the cells as they adjust to decreasing nutrient abundance. Eventually, the cells enter a **stationary phase of growth** where cell division is very slow. Put together, when graphing time on the x-axis and cell number on the y-axis, the yeast growth curve, including all three general phases, resembles a flattened "S" and is therefore called, **sigmoidal.**

Cell number may be approximated by measuring the absorbance of the yeast culture. The more cells there are, the less light gets through. 600 and 660 nm are common wavelengths for this analysis. Roughly speaking, if the Abs_{600} = 1.0, the *S. cerevisiae* cell density is 2 x 10^7 cells / ml. [side note: Will this value be higher for bacteria or yeast?] Similarly roughly speaking, if the Abs_{600} is below 0.2, the cells are in lag phase, between 0.2 and 0.9, log phase, and above 0.9, stationary phase. The cut-offs may vary among strains.

When performing experiments with *S. cerevisiae*, it is important to consider the **growth phase** of the cell population(s) under study. You may imagine that if one population of yeast is in log phase and not exposed to a stress while a second population is in stationary phase and exposed to a stress, then the differences between the populations, say in a certain mRNA abundance, may be due to the difference in stress or growth phase. You may also imagine that a stress may slow a population's growth, keeping it in log phase while a negative control, non-stressed population reproduces its way into stationary. As you may have divined, shorter time frames of stress may avoid such differences.

So, what does all of this have to do with our experiments? If your yeast transformations were successful in yielding colonies and your water control plates are free of colonies, you have likely progressed to having **master plates**. We still need to cover the ground, so to speak, between having master plates and assaying for transcriptional

regulation, as determined by b-galactosidase activity, in response to an environmental stress or variation. The same process can be followed for culturing yeast for an experiment involving RNA isolation and **real-time PCR** analysis.

Step one is to create what is called a **starter culture**. This is a small (~3 ml), liquid culture in a sterile 6 ml culture tube that has been grown to stationary phase. This growth often occurs over 18 - 24 hours. Most *S. cerevisiae* strains will hit a similar cell density in stationary phase in the neighborhood of 8×10^7 cells / ml. This will vary with media. From these starter cultures, small volumes may be pipetted (or seeded) into a liquid media of interest so to provide a well-controlled number of cells to begin a culturing. Based on a little experience, a certain dilution of cells, perhaps a 1:1000 dilution of a starter culture into the media of interest, will reach log phase after a certain amount of time. In YPD, it often takes about 14 hours for a 1:1000 dilution in YPD to result in a liquid culture with an $Abs_{600} = 0.6$. It takes more like 20 hours for a 1:1000 dilution into SCD to reach this OD. Such culturing usually occurs at 30 °C with agitation, to promote gas exchange and nutrient distribution, over night. **Ehrlenmeyers (i.e. culture) flasks** affixed to a shaking platform in an incubator are ideal for this.

SCD – ura is the relevant media for experiments if cells contain a plasmid harboring the URA3 gene. If such yeast cells were grown in YPD, there would be no advantage for the cells to harbor the plasmid since yeast extract contains uracil. With some cells randomly not providing the plasmid to daughter cells, later generations would "lose" the plasmid.

In practice, for an experiment to be performed on Tuesday, a starter culture should be grown in the previous week and stored at 4° C. As you may imagine, sterile technique is required for all steps involved in culturing the yeast so to avoid any environmental microbes entering and thus contaminating the nutrient-rich media. Be sure to label your tubes carefully. You will be making your own media so that also needs to be made the week before the experiment.

Planning how to set up liquid cultures, inoculated from the starter cultures, will be up to each group. Ideally, those will be grown over Monday night into Tuesday for use in Tuesday's lab. Beginning the culture on Tuesday morning for use later on Tuesday is another option. Consider what culture volumes will be needed to supply sufficient cells for the b galactosidase assays. Those assays are described in a separate protocol. Please also consider the benefits of performing replicates of experiments so that averages can be taken and statistical tests such as T-tests performed to determine if two averages (i.e. b-gal activity in two growth conditions) show a statistically significant difference.

It will be wise to find and read papers that study transcriptional regulation in yeast. These resources will provide precedents for growth conditions and maybe provide ideas about how to plan your own experiments. Feel free to run ideas past the instructor.

Good luck!

Yeast (*S. cerevisiae*) Genomic DNA Isolation

Overview

Isolating a molecule of interest from a natural source is akin to finding a friend in a crowded train station and neither one of you has a cell phone. The analogy is not quite right since biochemists have a variety of tools at their disposal to separate molecules based on their physical characteristics. It is not so easy to get a train station full of people to line up according to height. The correct tool or sequence of tools will not only allow the purification of the molecule of interest but also not damage the molecule.

Along those lines, when isolating genomic DNA from any source, the main considerations are a) remove protein, lipids, and carbohydrates and b) maintain the integrity of the DNA. Depending on the application, it may also be important that no environmental DNA, or DNA from a separate sample, contaminate the sample of interest. A unique consideration when working with yeast is that the cells have a cell wall which makes the cells resistant to lysis. Breaking organs and tissue into the composite cells and then lysing cells into their composite molecules is often the first step in a purification process.

The protocol below results in a fairly crude prepation of genomic DNA from yeast. However, it has been establish by experimentation (i.e. empirically) that this incomplete and simple purification is sufficient for generating genomic DNA which can be amplified by PCR. The one downside is that the DNA is best used within seven days of the preparation. Purer preparations are likely stable for longer.

Lysis is achieved by a combination of mechanical and chemical means. Vortexing yeast cells in the presence of glass beads will result in collisions between the beads with cells in between, resulting in the bursting of those cells. These collisions may also sheer the genomic DNA into pieces so this method is not appropriate if very long strands of genomic DNA are needed. The vortexing and subsequent collisions also create heat which would be more of a problem if we were trying to extract a temperature labile protein rather than DNA which is fairly resistant to denaturing. Yeast will also be lysed chemically by incubation in a mixture of detergents (sodium dodecyl sulfate (SDS), Triton X-100) and organic solvents (phenol / chloroform / isoamyl alcohol). The detergents will dissolve the proteins and the organic solvents will dissolve the membrane lipids. Upon centrifugation, the lower organic phase should contain hydrophobic molecules such as lipids. The interface should contain a whilte precipitate which is the complex of proteins and SDS. The aqueous upper phase will contain the hydrophilic molecules including genomic DNA.

Further purification could be achieved by performing an ethanol precipitation to create conditions where DNA is no longer soluble but other cell components such as

carbohydrates are. In that case, centrifugation would allow the pelleting and isolation of nucleic acids. Ridding the sample of RNA can be achieved with an RNAase. Since the subsequent use of the DNA in this case is polymerase chain reaction (PCR), the presence of RNA is not a problem since RNA is a not a substrate for PCR and does not interfere.

Solutions

Yeast Breaking Buffer
2% (v/v) Triton X-100
1% (w/v) sodium dodecyl sulfate (SDS)
100mM NaCl
10mM Tris-HCl, pH 8.0
1mM Ethylenediaminetetraacetic acid (EDTA), pH 8.0

Tris / EDTA (TE)
10 mM Tris-HCl, pH 7.4 – 8.0
1 mM EDTA

Phenol / chloroform / isoamyl alchohol (25:24:1)
Phenol is a neurotoxin. Take care to avoid any exposure to eyes and skin.

Procedure

1. Harvest cells from an overnight culture (~ 1 ml) by transferring the cells to a 1.5 ml eppendorf tube and centrifuging at 8,000 rpm for 30 seconds. Pour off the supernatant.
2. Wash cells with ~ 1 ml water to remove any residual media that is on the cells.
   - washing cells involves adding water to the cell pellet, vortexing the cells into suspension, centrifugation at 8,000 rpm for 30 seconds, and discarding the supernatant
3. Add 200 μl yeast breaking buffer and vortex until cells are suspended.
4. Add 0.25 g (~ 200 μl worth) of glass beads.
5. [work in fume hood, wear gloves] Add 200 ml phenol/chloroform/isoamyl alcohol.
   - recall that this mixture of organic solvents is shipped with a thin (~ ½ cm) layer of aqueous solution on top. Be sure to place your pipet tip below this layer so to only draw up the organic solvent mixture. Also, be careful not to knock the bottle an have that aqueous solution mix with the organic solvent.

6. Be careful to tightly secure the lid of the eppendorf tube. If any glass beads get between the lid and the tube, solution will leak out when you vortex this tube. Having organic solvent leak onto your hands or worse, splatter, is to be avoided.
7. Vortex maximum speed for 2-3 min. Doing this in the fume hood is recommended given the possibility of organic solvent leaking from the tube.
8. Centrifuge at full speed for 5 min at room temperature.
9. Carefully transfer ~ 50 μl of the top aqueous layer to a new, labeled tube. Avoid transferring any material from the interface.
10. 2 μl of this solution is often suitable for use in a PCR

Yeast RNA Purification

Background

Purifying total cellular RNA from *S. cerevisiae* begins with culturing a strain of interest under standard or experimental conditions. The RNA yield from ten milliliters of yeast culture is often sufficient for applications such as cDNA synthesis. Due to having a cell wall, yeast require a more vigorous lysis procedure than animal cells. The procedure outlined below includes organic extractions. The protocol below is based on information in Aaron D. Hernday, Suzanne M. Noble, Quinn M. Mitrovich, and Alexander D. Johnson. *Methods in Enzymology* (2010), 470: 737 - 758.

Notes (P.O)

a) Recall that RNA is hydrophilic and will partition into the aqueous layer during these organic extractions.

b) The method involves phenol which is a volatile organic solvent that is also a neurotoxin. Be sure to work in the fume hood and wear gloves when working with phenol or any tube that contains phenol.

c) Be sure to use the 15 ml conical tubes made of polypropylene plastic. This plastic is a bit cloudy. The other plastic that conical tubes are often made of is polystyrene. It is clear and will be degraded by organic solvents such as chloroform and phenol. Simply put, *avoid the clear conical tubes.*

Protocol

1. Grow a 10 ml liquid culture of *S. cerevisiae* cells to an appropriate concentration (e.g., OD_{600} of 0.5 - 1.0). For other volumes and cell densities, all steps may be scaled proportionally.

2. Collect cells by centrifugation (2000 g, 5 min, 4 °C) in a 15-ml polypropylene conical tube. Remove as much liquid as possible, and freeze by immersing tube in liquid nitrogen or placing in a freezer. Store frozen cell pellet at -80 °C.
   [note: This protocol was likely written to allow samples to be collected at different times and then processed at the same time. If you are performing a time course assay, freezing samples is probably a good idea. If all of your samples are collected at once, you can skip to step 4.]

3. Transfer frozen tube containing cell pellet to ice, working quickly to avoid thawing prior to the addition of phenol.

4. To frozen pellet, first add 2 ml unbuffered phenol and 2 ml extraction buffer (50 mM sodium acetate [from pH 5.3 stock], 10 mM EDTA and 1% SDS). The use of acidic (pH = 4.5) rather than neutral phenol will reduce, but not eliminate, DNA contamination. While working with phenol and chloroform, use appropriate protective equipment (goggles, gloves, lab coat, fume hood) and dispose of hazardous waste appropriately. Also, unbuffered phenol is solid at room temperature so it must be warmed to 55 °C prior to use and worked with quickly after being taken out of the water bath.

5. Ensure that each tube is well-capped, then vortex 20 seconds. Transfer tube to 65 °C water bath. Incubate for 10 min,. Remove tubes to vortex vigorously every minute or so.

6. Transfer tube to ice for 5 min. Keep samples on ice for all subsequent steps, except where noted.

7. Add 2 ml chloroform to tube, cap securely, and vortex well (~ 20 seconds).

8. Spin tube in tabletop centrifuge (2000 g, 5 min, 4 °C) to separate phases.

9. Carefully transfer aqueous (top) phase to a fresh 15 ml tube. Add 2 ml phenol:chloroform:isoamylalcohol (25:24:1). Cap tube and vortex well (~20 seconds).

10. Spin tube in tabletop centrifuge (1500 g, 5 min, 4 °C). Carefully transfer aqueous (top) phase to a fresh 15 ml tube.

11. Add 2 ml chloroform, then shake and spin as before. (This step removes residual phenol from the sample.)

12. From this point on, ensure that all reagents and containers are free of RNases. Transfer aqueous phase into a fresh 15 ml tube. Add 200 μl 3 M sodium acetate (pH 5.3) and 2 ml isopropyl alcohol. Shake or vortex briefly, then incubate at room temperature for 10 min.

13. Pellet RNA in tabletop centrifuge at maximum speed (20 min, 4 °C). A substantial white pellet of RNA should be visible. Pour off supernatant and let drain briefly with tube inverted on a Kimwipe.

14. Use 800 μl 70% ethanol to transfer pellet by pipet to a 1.5-ml microfuge tube, breaking up pellet if necessary. Spin in 4 °C microcentrifuge at maximum speed for 5 min.

15. Carefully remove as much liquid as possible from pellet with pipet tip, then air dry for a few minutes. Do not allow RNA pellet to become too dry, as this will make resuspension difficult.

16. Resuspend RNA in 100 μl RNase-free water. Pipetting up and down will help to resuspend the RNA; if necessary, the tube can also be incubated at 50 °C for 10 min.

17. To determine the concentration of RNA in solution, measure its absorbance in an ultraviolet spectrophotometer. If using a NanoDrop (Thermo Scientific), 2 ml of solution can be measured directly. Otherwise, dilute 1:100 to measure. The concentration of the measured solution (in ng/ml) is given by the absorbance at 260 nm multiplied by 40. Expected yield is roughly 400 mg.

Yeast Transformation

Introduction:

Similar to the transformation of bacteria, transforming yeast (often *S. cerevisiae*) involves inducing the yeast to take up exogenous DNA. This induction is optimal in yeast grown into the log phase of growth (please a separate protocol detailing how to culture yeast) and suspended in lithium acetate. These are **competent** yeast. Incubating these cells in a mixture of lithium acetate, TE buffer, and polyethylene glycol (a detergent) along with the DNA (often a plasmid) of interest primes the yeast for transformation. Heat shock, changing the cells from from 30 °C to 42 °C, temporarily permeabilizes the cells and results in some of the cells taking up the exogenous DNA. All of this is done in small volumes (<1.5 ml) in microcentrifuge tubes. As in bacteria, this process only results in a very small percent of cells taking up the DNA. Identifying those cells is the aim of the step following transformation. Please appreciate that all steps of yeast transformation should use **sterile technique**. Environmental microbes may readily grow in the nutrient-rich media used. Such contamination requires the discarding of all samples and starting again. Please see a separated protocol for sterile technique.

Cells are then harvested by centrifugation and washed to remove the growth media. A mixture of lithium acetate and polyethylene glycol (PEG) buffered with TE (a mixture of Tris and ethylenediaminetetraacetic acid (EDTA)) is then added to the cells. PEG is a lipid polymer found in antifreeze with contributes to the weakening of the plasma membrane. To this mixture is added the plasmid DNA along with carrier DNA. Carriers are molecules that are similar to molecules of interest in a solution and prevent the molecules of interest from being lost. It is analogous to protecting a fish of interest by surrounding it with a school of similar, but different, fish. Heat shocking follows. The conditions for heat shocking varying among protocols. Commonly, incubation at a modest temperature such as 30 °C for about 30 minutes is followed by a shorter incubation at a warmer temperature, such as 42 °C. This rapid change in temperature also serves to permeabilize the plasma membrane and promote update of exogenous molecules such as DNA. Even all of this manipulation, only a very small percent of the yeast will actually be transformed or take up the plasmid. Therefore, a **selection** must occur so that only the yeast that take up the plasmid survive. Without going into all the details, suffice to say that plasmids confer an ability for growth in media that is lacking in cells that do not have the plasmid.

Before going further into the process of transformation, it is worthwhile addressing the nature of plasmids transformed into yeast. Plasmids designed for use in yeast often either have a two micron (2μ) **origin of replication** or a CEN origin of replication. These origins will target the plasmid for replication when the cell replicates. 2 μ origins allow for a high copy number (number of plasmids in each cell) in the range of 50 – 200. CEN origins allow for a low copy number in the range of one to three. Whether the experimental design is best served by a very high amount of expression from the plasmid, sometimes called **super physiological**, or a closer to physiological amount of expression will dictate which origin is best. Plasmids designed for expressing a gene in yeast will have a yeast **promoter**. This may be the same promoter as the gene being expressed or one of a series of commonly used promoters. Transcription under the control of commonly used promoters is often affected by the culture conditions provided, allowing the gene to essentially to be "turned on" or "turned off". However, some promoters provide constitutive expression. These plasmids also commonly have a selectable marker gene (e.g. Amp^R) for growth in bacteria and a selectable marker gene for growth in yeast. The second class of marker genes confers autotrophy which is described later.

After the transformation, the next step is to spread the yeast onto agar plates. This spreading allows only those yeast which took up the plasmid to selectively grow and divide. After about two days of growing and dividing at 30 °C, enough cells will be present to be view. This "pile" of cells is called a **colony**. Each colony should be **clonal**, that is, derived from a single cell. After a typical yeast transformation, there are about 40 colonies on a 100 mm diameter plate. Is it possible that two transformed cells stuck to the plate at exactly same spot and grew a heterogenous colony that appears to be a clonal colony? Yes, it is.

Selective growth can by conferred by the presence of an antibiotic resistance gene within the plasmid. This works the same as the Amp^R gene in bacteria. Kanamycin resistance is conferred by the Kan^R gene. More commonly, selective growth by cells that contain the plasmid is conferred by an **autotrophic marker gene** within the plasmid. Autotrophy takes a little explaining.

Yeast in the wild (i.e. **wild-type** yeast) can synthesize nucleotides and all the amino acids used to synthesize proteins if given access to a source of organic atoms, especially nitrogen. Ammonia, or the ammonium ion, is a common "simple" source of nitrogen. The synthesis of each nucleotide and amino acid requires a sequence of enzyme-mediated reactions to essentially build the corresponding molecule from scratch. If a yeast strain harbors a loss-of-function mutation in a gene that encodes for one of these anabolic enzymes and if the yeast genome does not contain another gene that encodes

for a redundant or over-lapping functionality then such a strain will be unable to synthesize the corresponding nucleotide or amino acid. In such a case, the strain will be **auxotrophic** for that nucleotide or amino acid.

Yeast used in laboratories (i.e. **normal yeast**) are often engineered to contain targeted mutations in genes that encode for anabolic enzymes in nucleotide and amino metabolism. By doing so, the cells are rendered auxotrophic for certain nutrients. Auxotrophy for adenine, histidine, leucine, tryptophan, and uracil is commonly conferred by mutations to the *ADE2*, *HIS3*, *LEU2*, *TRP1*, and *URA3* genes. In yeast, alleles for genes that have no mutations are written in all capitals and in italics. Mutant alleles are written in lowercase and italics. For example, a common normal yeast strain is **W303b** which has the genotype *ade2 his3 leu3 trp1 ura3*. One question you might ask is, why go through the trouble of making strains that cannot synthesize basic building blocks of macromolecules?

The answer lies in yeast transformations. A normal strain such as W303b can be initially grown in media such as YEPD (containing yeast extract (yes, cannibalism), peptone (a source of nitrogen), and dextrose (i.e. glucose)). This medium contains an abundance of nucleotides and amino acids, negating the need for yeast to synthesize them. If these yeast are transformed with a plasmid that not only contains DNA of experimental interest by also the *URA3* gene, then successful transformants can grow in media that lacks uracil. Media with well-defined components is called **synthetic complete (SC)** media. In SC, the components are included in a purified form, one at a time. There are no imprecise or ill-defined components such as yeast extract. **Dropout** media is SC media that is missing one or more commonly included nutrients. SC + Dextrose minus uracil (SCD - ura) is an example.
Now, consider a strain of W303b yeast transformed with a plasmid that contains the URA3 gene. Successful transformation will result in the **transformant** (i.e. the transformed yeast cell) becoming **autotrophic** for uracil. Autotrophy is the ability to grown in the absence of a particular nutrient. Consider that autotrophy cannot be used to identify successful transformants if the starting strain is not auxotrophic for that nutrient in the first place.

In terms of the logistics, after transformation, it commonly takes two days for colonies to form on the dropout media. The next step is to form a **master plate**. This is simply a SC-X plate (same media as used after the transformation) that has a grid of positions containing a streak (a ~1 cm line of cells dragged from a colony from the transformation plate). Such streaks may be make using a cloning loop or sterilized wooden toothpicks. Another day of growth is required for these streaks to grow up. Such streaks expand the population of the colony and allow a variety of experiments to be initiated from a single

transformant without depleting the colony. From the master plate, a **starter culture** is often created. Starter cultures often contain about 3 ml of the appropriate SC – X media and are grown overnight. The yeast will likely reach a dense, "saturated" population over night due to depleting many of the nutrients in the media. These starter cultures are routinely used to begin subsequent **experimental cultures** which are then used in experiments, such as testing the cellular response to oxidation damage induced by hydrogen peroxide exposure.

Planning yeast transformations and the subsequent use of the transformants takes some time but will yield a good deal of efficiency. One approach is to consider Day 0 to be the inoculation of the starter culture used in the transformation and count days from there with a predicted series of events for each day.

Reagents:
(these will be made for you unless you insist on making your own)

Media for growing yeast
YEPD (yeast extract, peptone, dextrose)
1% (w/v) yeast extract
2% (w/v) peptone
2% (w/v) dextrose (glucose)

SCD – ura + Dex agar plates
- use to select for the successfully transformed cells via autotrophy for uracil
0.17% (w/v) yeast nitrogen base
0.5% (w/v) ammonium sulfate
0.077 % (w/v) synthetic complete minus uracil
2% (w/v) glucose
1.5 % (w/v) agar

100 mM Lithium Acetate (LiAc)

1 M LiAc

50% polyethylene glycol (PEG) – 3350 (filter sterilized)

10X TE
100 mM Tris
10 mM EDTA - pH to 8.0

PEG / LiAc / TE (make fresh)
40 % PEG (8 parts of 50%)
100 mM LiAc (1 part of 1M)
1X TE (1 part of 10X)

single stranded carrier DNA (e.g. herring sperm)

sterile water

Procedure:

1) Day 1. Inoculate 8 ml of YPD per transformation (i.e. 4 plasmids plus 1 H_2O control - innoculate 40 ml) with the yeast strain to be transformed. You can either inoculate from a saturated liquid culture (preferred) or from a plate. [this will be done for you]
2) Day 2. Monitor the OD_{600} (OD_{660} is OK too) of the cultures. When it is 0.5 to 0.9, the cells are in mid-to-late log phase - where you want them. Pour the cultures into a 50 ml Falcon, screw top, plastic tube. Note the volume of the culture for step 9.
3) In a clinical centrifuge with a swing bucket rotor, centrifuge at 3/4 speed (about 2000 rpm) for 5 minutes.
4) Discard the supernatant. Add 20 ml sterile water to the cells, and resuspend the cells using medium-intensity vortexing.
5) Centrifuge as in step 3.
6) Discard the supernatant. Resuspend the cell pellet in 800 μl sterile water and transfer to a 1.5 ml microcentrifuge tube.
7) Microfuge cells at top speed for 7 seconds. Discard supernatant.
8) Resuspend cells in 800 μl 100 mM LiAc. Microguge at full speed for 7 seconds. Discard supernatant.
9) Accounting for the volume of the cells from step 2, resuspend the cells in 50 μl of 100 mM LiAc for every 5 ml of original culture. (i.e. for a 40 ml culture, add 350 μl to a 50 μl pellet)
10) Incubate cells at 30° C for 15 minutes with occasional light vortexing.
11) During the above incubation, boil carrier DNA for 10 minutes. Quick ice. In a1.5 ml microcentrifuge tube per transformation (remember a water control) mix the following:
    3 – 5 μl of carrier DNA (10 mg / ml)
    2 - 5 μl plasmid DNA (> 100 ng total)
    50 μl of the cell suspension
    300 μl PEG / LiAc / TE
12) Vortex, medium intensity, about 5 seconds.
13) Incubate at 30° C for 30 minutes.

14) Incubate at 42° C for 20 minutes (heat shock).
15) Microfuge, top speed, 10 seconds. Discard supernatant.
16) Resuspend cells in 1.0 ml sterile water. Plate 150 μl onto SC-ura plate.
17) Place plates agar side up in a 30° C incubator. Colonies should come up in 2 or 3 days.