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Uncooking Yeast: Cells Signaling a Rise to Inquiry

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We converted a three-hour confirmatory lab exercise on yeast (*Saccharomyces cerevisiae)* mating response into a scaffolded 5-week process of science unit on signal transduction. Week 1 of the curriculum begins with a guided exercise allowing students to gain familiarity with the assays and data forms, and G-protein mediated signaling concepts. In week 2, teams present a novel research proposal focusing on one aspect of the signaling pathway they seek to perturb. Teams present their proposal to their peers and instructors to solicit feedback on their biological rationale, hypothesis, methods, expected and alternative results, experimental implications, and questions they still have. Students collect and analyze data in weeks 3 and 4, write a research proposal and engage in peer review. The unit culminates in week 5 group formal presentations of research findings. This paper describes our curriculum and student learning experience, and discusses the challenges and rewards of creating inquiry-learning opportunities.

Keywords: cell signaling, yeast, inquiry-based learning

Introduction

How do cells communicate? This question is intriguing for students—and encompasses concepts of cellular signaling that integrate all cellular processes, from metabolism and transport to molecular genetics and the central dogma. The single-celled eukaryotic baker's yeast, *Saccharomyces cerevisiae,* is an excellent model system to study cell signaling. The yeast mating response has proven to be a particularly compelling system for studying signal transduction in an undergraduate lab course setting because yeast are easy to culture, are readily-observable under the light microscope, have intriguing phenotypic responses to environmental signals (e.g. the shmoo), and are well-described as a classic model system in the scientific literature (Engelberg et al., 2014).

We describe how we converted a confirmatory ('cookbook') yeast signaling lab exercise into an authentic, process of science (PoS) unit that is in alignment with the Core Competencies and Disciplinary Practices described in AAAS's Vision & Change in Undergraduate Education (2011). We also present undergraduate reflections on their practice of unique science skills during this scaffolded PoS unit. We encourage the ABLE community to adapt our approach as a model for transforming traditional lab exercises into opportunities for students to pursue novel questions of their own choosing.

The Biocore Program

Undergraduate students in the honors Biology Core Curriculum (Biocore) program at UW-Madison acquire authentic, independent research experimentation skills as they progress through 2-3 semesters of our lab curriculum (Batzli, 2005; see also Batzli *et al.* in this 2018 ABLE Proceedings). Biocore was founded in 1967 with the philosophy and pedagogy of a PoS approach- to teach science as it is practiced. In particular, our students repeatedly practice independent research experimentation and effective communication skills as they progress through 2-3 semesters of our lab curriculum. The evolution of the 5-week yeast signaling unit described here highlights key details of our process of science teaching approach, including our scaffolded curriculum, instructional materials supporting students' development of scientific communication and statistical reasoning skills, and reflections indicating student achievement of experimentation competencies.

Yeast Lab Origins in ABLE

In the proceedings of the 19th ABLE national conference, Hoopes *et al.* (1998) provide an excellent description of a lab exercise focused on yeast mating response and student identification of mutant strains. Initially we adapted the Hoopes lab as a two-week confirmatory lab exercise, in which students used their

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observations to distinguish wild type yeast from two unknown mutant yeast strains. While this confirmatory approach allowed our students to practice important skills such as making careful observations, practicing a genetic transformation and aseptic technique, and integrating multiple forms of evidence to make conclusions, we saw great potential to adapt this exercise in a way that allowed our students to experience authentic experimentation.

We therefore 'uncooked' this lab to create a three- week inquiry unit in which the original ABLE lab (Hoopes *et al.*, 1998) was done by students in week 1 of the second course in our 3-semester lab curriculum (Biocore II, Cell Biology lab; see Batzli *et al.* in this ABLE proceedings). After this initial week, we guided our students to work in teams to "*design an experiment to study some aspect of signal transduction, using the techniques that you learned in the first week of this unit*". Teams chose their own testable question, identified independent and dependent variables, generated an aligned hypothesis, proposed their experiment to instructors and peers via an informal 'feedback presentation,' and carried out their experiment. In subsequent years we expanded this yeast unit to five weeks so that data collection time doubled from one to two weeks (to allow for pilot and revision of protocols), and the toolkit was expanded to include Western blotting . Our current student learning goals for the yeast signal transduction unit include 1) working in a research team to design and carry out a novel experiment that helps elucidate protein interactions and gene expression in the yeast mating pathway, 2) combining evidence from β-galactosidase spectrophotometric assay, Western blot assay, and microscopy of cell shape to generate conclusions about hypotheses, 3.) practicing scientific reasoning and communicating research in both written and oral form.

5-Week Curriculum Scaffold

The 5-week yeast unit described here exemplifies the scaffolded PoS curriculum structure that is used repeatedly in all 3 Biocore lab courses (see Table 1). The curriculum begins with a guided exercise the first week allowing students to gain familiarity with the assays and forms of data, as well as the concepts of G-protein mediated signaling that explain the mating response pathway associated with *Saccharomyces cerevisiae* Type A cells treated with alpha factor pheromone. In week two, research teams present a novel research proposal of their own design focusing on one aspect of the signaling pathway they seek to perturb via an experimental treatment. Teams present their proposal to their peers and instructors to solicit feedback on their biological rationale, hypothesis, methods, expected and alternative results, implications for their experiment and questions they still have. Students carry out their experiment, collect data, and analyze data in weeks 3 and 4, while also writing a research proposal paper and engaging in peer review. The unit culminates in week 5 group formal presentations of research findings.

Examples of Student Research Questions

Since we converted our yeast lab exercise into an authentic experimentation unit, student teams have pursued an impressive variety of novel research questions. Students have investigated how yeast cell signaling is affected by different abiotic (e.g. temperature) and chemical (MAPK and G-protein inhibitors), agonists/stimulants of alternative cross-talk pathways (e.g. nutrient deprivation, high osmotic agents, filamentous growth pathway), altered pheromones, and much more. Appendix 5 provides a more comprehensive list of independent variables chosen by our students. When teams struggle to find a research direction, we call their attention to some helpful yeast mating pathway review articles in the 'References Cited' section of their Lab Manual Part A.

Balancing Challenge and Support

Using a process of science approach requires instructors to continuously navigate a balance of curricular rigor with appropriate instructional support. The 5-week scaffold shown in Table 1 depicts how the unit evolves from being instructor-driven in week 1, to total student ownership by week 5. We often describe our roles as 'science coaches', particularly in the latter weeks of each unit when each team requires unique guidance. The informal feedback presentations that teams give in week 2 are excellent formative assessment opportunities, as we can quickly gauge students' understanding of concepts and their grasp of experimental design principles. We also rely heavily on supporting instructional materials (see Appendices). Our Biocore Writing Manual is a key resource providing guidance and defining expectations with a complete set of rubrics for research papers, feedback presentations, and formal oral presentations. Our Biocore Statistics Primer supports students' data analysis and interpretation (materials are available online www.biocore.wisc.edu/bioresources).

Appropriate for Intro Students? Yes!

We are often asked whether our scaffolded, process of science approach is appropriate for non-honors students, particularly at the introductory level. While implementation in different institutions and courses will of course differ from our approach, we believe that the fundamental process of science components are quite transferable to a variety of settings. Further, reflections from our own students on key curricular components of the yeast cell signaling unit indicate that students highly value the learning they experience in this authentic, rigorous independent research environment (see Table 2).

Table 1. Five-week scaffolded Process of Science unit, using yeast mating response as a study system. Students attend a required 50 minute discussion section 1-2 days prior to attending a 3 hour lab section each week. Bolded items are graded assignments. Asterisks indicate team assignments. TA = graduate teaching assistant. PPT = PowerPoint slideshow.

	TA led Discussion (50 min weekly)	Instructor and TA led Lab (3h weekly)
Week 1	Introduction to yeast mating pathway (Students assigned to groups of 4-6) • Team concept map of key terms	'Cookbook' lab, to get familiar with protocols • Beta galactosidase assay • Cell shape assay • Guided tour of Western Blot stations/equipment
Week 2	Teams prepare informal (non-graded) feedback PPT presentations **Yeast Signaling Prelab**	Informal team feedback presentations followed by Q&A
Week 3 (Open lab)	Formal Peer review- student peer review partners meet to discuss their yeast proposal papers	Teams do pilot studies/experiment • Beta gal assay • Cell shape assay • Western blot (~7 hours) • **Research Proposal paper (individual)**
Week 4 (Open lab)	Data consultations with instructors	Teams repeat/adjust experiment • Beta gal assay • Cell shape assay • (if needed) Western blot
Week 5	Teams practice formal presentations with undergraduate TAs	***Team formal PPT presentations** **Response to reviewers Part 1 (individual)**
Finals Week	***Response to reviewers Part 2 (team)**	

Table 2. Undergraduate student reflections on key curricular components of the yeast cell signaling unit.

Curricular Component	**Student Reflections**
Week 1 iClicker questions on background information	• Similar to Biocore lecture style of learning • Opened up class discussion on main concepts/ points of confusion • Allowed small group discussion
Week 1 Team concept map	• Challenged students to draw the mating pathway from memory • Most effective when proctored (by instructors) to ensure everyone is participating • Allows small group to work together for the first time and use strengths
Week 1 Practicing protocols	• Develop uniform knowledge- some students had background from other classes/working in a lab and others had no experience • Challenged students to think about what was actually going on in the test tube while completing protocol

	● Group can divide and conquer different tasks (e.g. 2 students learn cell shape assay and 3 learn beta-gal assay) ● Gives opportunity for group to work together before discussing research topics
Week 2 Feedback presentations	● Helps teams begin to focus in on ideas for projects ● Teaches students how to give and receive constructive criticism and clearly explain ideas ● Peer feedback could be as valuable as instructor feedback because peers likely think of the material in the same way presenters do- clarity ● Important to include all ideas/aspects of background and biorationale, to get feedback on this material before each student puts it into a paper ● Group learns to work together to make changes/additions to experiment ● Sometimes groups must change entire experiment after the feedback presentation
Week 3 Proposal papers	● Start writing once experiment is approved by instructors ● Have undergraduate teaching assistant (uTA) and peers review sections ● Introduces students to the format and style of a scientific paper ● Tests students' abilities to understand the study system and experiment on a deep level individually and written in their own words ● Helps students practice explaining a biological rationale and the experiment in a concise, clear manner
Weeks 3 & 4 Data collection - trial and repeat	● In this experiment students have the most freedom for designing their experiment and methods ● Team decides how to best collect data ● Teams usually run into some issues with some of their first data collection and so a repeat is needed
Week 4 Data analysis with instructors	● Allows team to consider multiple interpretations of data and discuss potential flaws ● Team works together to find new literature to discuss outcome of experiment
Week 5 Formal presentations	● Time constraint (presentation is limited to 15 min.) is an obstacle for groups to give most important information and focus on most important sections ● Q&A session challenges students to think on the spot about project, understand all aspects thoroughly

Student Outline
Yeast Lab Manual Part A
Signal Transduction and Control of the Cell Cycle in Yeast (*Saccharomyces cerevisiae)**

*This lab was adapted by David Hall and Michelle Harris from a lab designed by Barbara Hoopes, Nancy L. Pruitt, Kathleen Baier, and Sherry Brooks at Colgate University (Hoopes *et al.*, 1998).

Overview

During these last 5 weeks of lab you will conduct an independent investigation of cellular signal transduction using yeast cells as a model system. Specifically, you will examine yeast response to mating pheromone.
Researchers have elucidated much of the yeast mating response signal transduction pathway by generating mutant strains and screening them for particular behaviors. In the first of five lab periods you will learn techniques that will allow you to determine how wild type yeast responds when exposed to the mating ligand α-factor. Based on your observations and background information you gather, your team will generate a question relating to the signal transduction pathway and propose an experiment during week 2. Your team will carry out your pilot studies and experiment in weeks 3 & 4 and communicate your results using an oral PowerPoint presentation in week 5.

Learning Objectives

Concepts - By the end of this unit, you should be able to:

- use your understanding of G-protein mediated signal transduction pathways to design and carry out a novel experiment that helps elucidate the yeast mating pathway.
- integrate evidence from various assays to make logical conclusions about the yeast mating signal transduction pathway.
- work with your team to design and present an oral research PowerPoint slideshow summarizing your experiment.

Techniques – By the end of this unit, you should be able to:

- use yeast cell shape to detect signal transduction induced mating morphogenesis and cell-cycle arrest.
- detect *fus1* mating gene expression in genetically transformed yeast utilizing a β-galactosidase reporter assay, SDS- PAGE, and Western blot techniques.
- perform and interpret a Western blot and make logical conclusions from the resulting blot data. Specifically, students will:
    - understand and explain how detergents and β-mercaptoethanol affect the structure of proteins.
    - explain how denatured proteins migrate through a polyacrylamide gel matrix and describe the method for their detection.
    - use Western blotting and imaging software to analyze the relative amount of the β-galactosidase reporter protein in yeast under different conditions.

Background

All cells have ways of sensing and responding to their environment. For instance, bacteria detect the presence of nutrients or noxious substances and move toward or away from them in a process called chemotaxis. When you cut your finger, skin cells start dividing rapidly to replace damaged cells and close up the wound. Both of these responses of cells have the same general components: (see also Fig. 1)

1. **Signal** (molecules that promote cellular signals are called **ligands**). Many different ones are possible; in the case of our examples above, the signals would be:
   - nutrients or noxious substances in the media (for the bacteria)
   - factors released by the damaged cells (for the cut finger)
2. **Receptor for the signal** proteins in the cell membrane that bind the nutrients or factors
3. **Transducers or second messengers** proteins within the cell that change as a result of the binding of the signal to the receptor and pass the signal along to other proteins
   *ResponseA change in the behavior or metabolism of the cell, such as the number or types of genes transcribed or the activity of an enzyme.

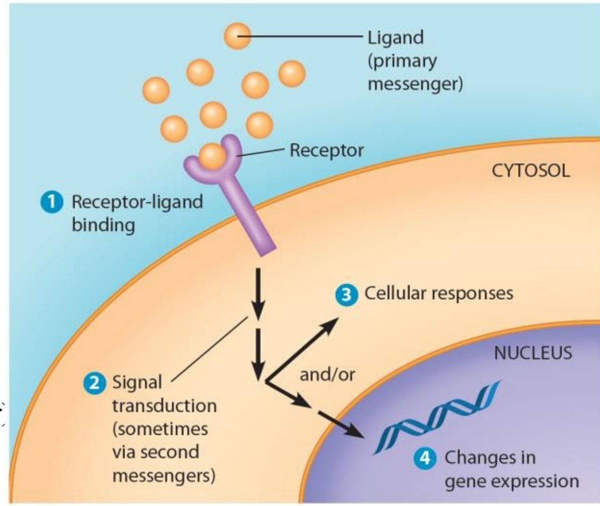


Figure 1. General diagram of signal transduction (Fig. 23-2 in Becker's World of the Cell 9e, Hardin et al. 2016).

*Note: in this lab we will document the physical manifestation of the signal transduction response (cell shape change observations) as well as the gene expression mechanisms behind this response resulting in changes in protein transcription (Beta-gal assay and Western Blot assay).

In the case of the bacterial cell, the binding of nutrients to receptors leads to a change in the beating of the flagella that move the bacteria through the medium. The flagella allows the bacteria to move forward, toward nutrients. In the case of a cut finger, the binding of factors released by damaged cells leads to the transcription in the surrounding cells of particular genes whose products affect the cell cycle. The transcription of these genes produces proteins which lead skin cells in the area of the wound to begin dividing more rapidly.

The response of cells to cues in their environment is called ***signal transduction***. Signal transduction occurs in single-cell and multicellular organisms and is what converts extracellular signals into cascading actions within the cell, leading to a host of biological processes such as development, reaction to stimuli, intracellular communication, or when communication between cells fails, cancer.

Yeast Life Cycle

One pathway that has been extensively studied as a model for understanding signal transduction is the mating pathway in baker's or brewer's yeast, *Saccharomyces cerevisiae*, a **eukaryotic** fungus. The yeast life cycle can alternate between haploid and diploid phases. Haploid spore cells result from meiotic division of diploid cells. Note that both haploid and diploid cells can produce a daughter cell from a mother cell through a mitotic process called budding. Our interest lies in the haploid phase where yeast cells differentiate into a or α type cells, which are both necessary for mating (sexual reproduction) to occur.

In the absence of the other cell type, each type will divide mitotically (asexual budding), proceeding through one full cell cycle in about an hour During this asexual process, the cell allows another cell to bud off of it, resulting in the formation of a new cell (Fig. 2).

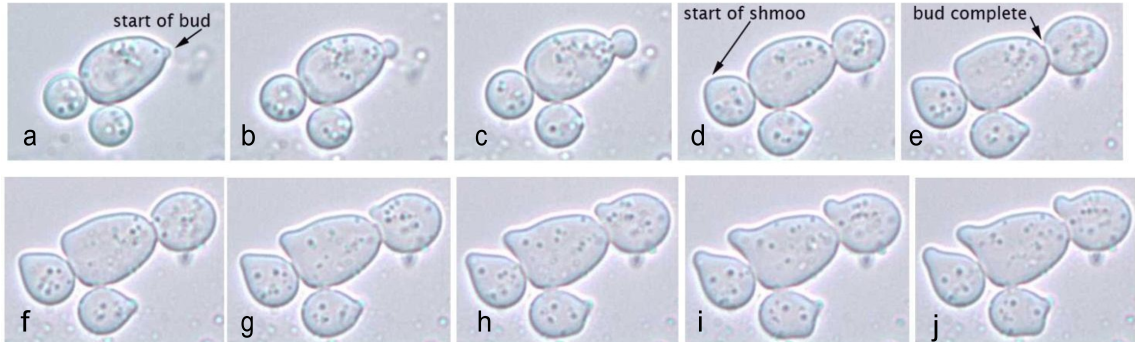


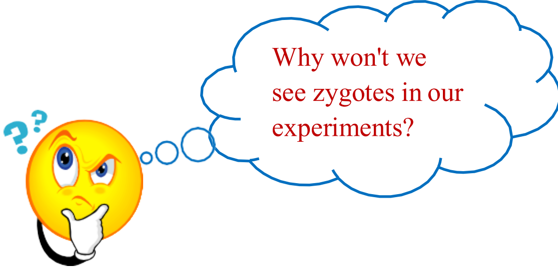
Figure 2. Time series of haploid type a yeast cells under phase-contrast microscopy (1000x) with images taken at ~15 min intervals. α factor added in image a. Images a-c: Budding yeast cell in upper right; notice the small bud forms and enlarges over time while constantly maintaining its spherical shape. Budding begins in the S phase of the cell cycle and is completed after mitosis with the separation of the mother and daughter cells (image e). When a bud is at least 50% the size of the parent cell, it is considered an independent cell – aka unbudded. An early shmoo (image d) is detectible by the morphing parent cell forming a raindrop shape. The haploid cells in this image all eventually become shmoo-shaped (images f-j). Note the double shmoo in image j.

In sexual reproduction, when the two cell types **a** and **α** are in close proximity, they can mate by fusing to form a diploid cell (which subsequently goes through meiosis). Mating between two cells requires the following to occur simultaneously in both cells:

a) arrest of the cell cycle in the G_1 phase (before DNA is synthesized)

b) changing cell shape (called "shmooing" or mating morphogenesis; see Fig. 2 f-j) to bring the cells closer together. During this process, both cells elongate in order to get close together so that they can fuse and form a diploid cell. Diploid cells then go through meiosis to form new haploid yeast cells.

c) increasing transcription and translation of genes involved in cell fusion.



G-protein Signaling in Response to Pheromone

How does an **a** cell “know” there is an **α** cell in the vicinity with which it can mate? Yeast mating response, like all signal transduction pathways, converts extracellular signals to cellular responses via a series of molecular interactions involving intracellular second messengers. **G- protein** (guanine-nucleotide binding protein) **pathways** all use protein receptors embedded in the plasma membrane to bind extracellular ligands such as growth factors, photons of light, and hormones like yeast mating pheromones. Conformational changes in the G-protein receptors activate G- proteins, which in turn activate second messenger molecules such as calcium and cyclic AMP. Second messengers amplify the original signal and transmit it to various intracellular targets, usually via a series of enzymatic phosphorylation events or “enzyme cascades.” In yeast, the **α** cells release a 13-amino acid peptide "factor" called **α-factor, which acts as a ligand**. The yeast **a** cell response to **α** -factor is an example of a G-protein pathway involving a **MAP (mitogen-activated protein) kinase cascade.** The α-factor receptor belongs to the G-protein linked family of cell surface receptors containing 7 transmembrane alpha helix segments as shown in Figure 3.

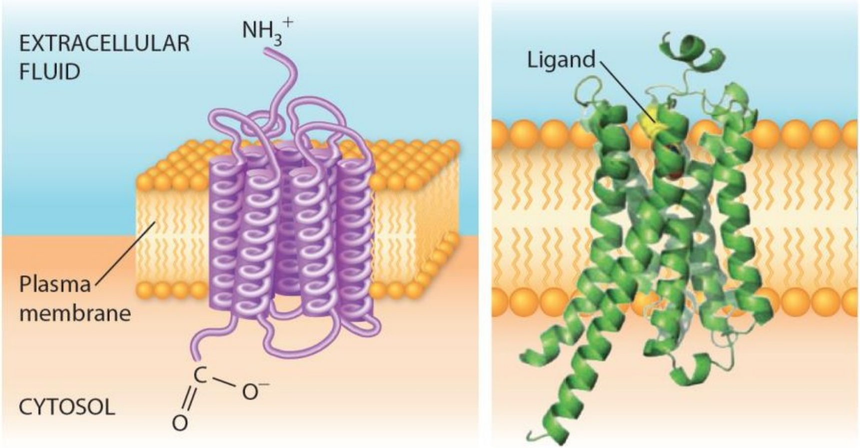


Figure 3. a) schematic image of a seven transmembrane alpha helix G Protein-Coupled Receptor (GPCR). (b) β -adrenergic GPCR with alpha helices shown as spiraling ribbons (Fig. 23-6 in Becker's World of the Cell 9e, Hardin et al. 2016).

When **α -factor** binds to the receptor on the surface of an **a** cell (indicating the proximity of a potential mate), a trimeric G-protein complex within the cell is activated resulting in dissociation of the alpha subunit from the beta-gamma subunits. The activated beta-gamma (ß-γ) subunit of the G-protein complex subsequently turns on a pathway resulting in actin microfilament skeleton changes producing polarized cell growth (mating morphogenesis), or the "shmoo" cell shape (Matheos *et al.* 2004; see Fig. 4 a). The activated ß-γ subunits also activate a scaffolding complex, composed of a Ste5 scaffolding protein and several kinases that ultimately activate a MAP kinase (MAPK), which has the ability to phosphorylate other cellular proteins (Rensing & Ruoff, 2009; see also Fig. 4). Among the targets of the MAP protein kinases are proteins that affect cell cycle arrest and a transcription factor that is specific for genes involved in mating. Both the cell cycle arrest factor and the transcription factor lie dormant in the cell until they are activated by the addition of phosphates by protein kinase in response to the binding of α-factor at the cell surface. At the same time, **a** cells also produce **a factor**, which binds to an **a factor receptor** on **α**cells. In this experiment you will examine the effect of **α-factor** on **a** cells.

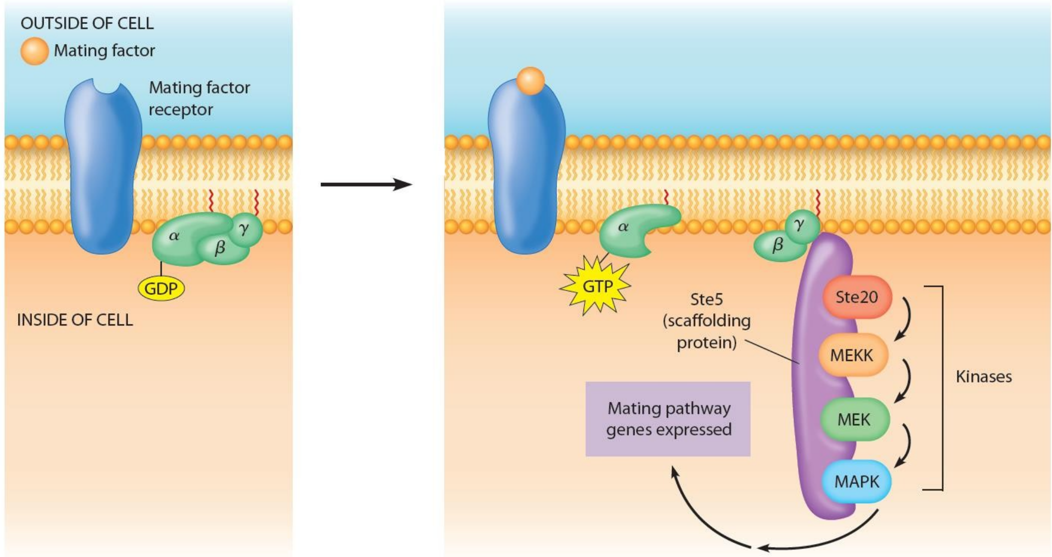
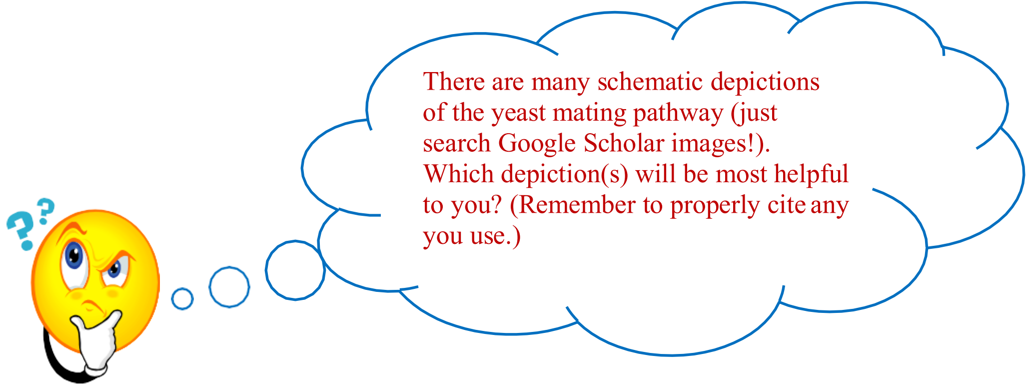


Figure 4. Mating factor bind to GPCR, causing $G_{\beta\gamma}$ to dissociate from G_{α} subunit and then activate a scaffolding protein complex. (Fig. 23-23b in Becker's World of the Cell 9e, Hardin et al. 2016).



α -factor and G-Protein Structure & Interactions

The α -factor receptor (named Ste2) has never been crystallized because it is very difficult to purify and crystallize integral membrane proteins like G protein-coupled receptors (GPCR's). In 2017, however, Robles et al. described a hypothesized model of Ste2 based on crystallized fragments and 2D biochemical model data. Cevheroglu et al. (2017) used biofluorescent assays to show that newly synthesized Ste2 receptors are brought to the plasma membrane as monomers, but form dimers (and perhaps even oligomers) in the plasma membrane where they bind alpha factor.

Ligand binding causes G-protein receptors such as Ste2 to undergo conformational changes, including alpha helix movements, which are propagated to intracellular loops. This ultimately results in dissociation of the trimeric G-protein complex on the inside of the cell (Taslimi et al., 2012; Robles et al., 2017).

Binding of -factor cell triggers a ~10-fold increase in the rate of receptor-mediated endocytosis of the -factor receptor (Hicke *et al.*, 1998). Both the -factor and its receptor are transported to lysosomal-like vacuoles where they are both degraded; i.e., the receptor is not recycled (Hicke *et al.*, 1998; Mulholland *et al., 1998).*

In the absence of **α** -factor, Ste2 is regularly endocytosed from the surface of the a cells and broken down in lysosome-like vacuoles. New Ste2 receptors are also constitutively made, brought to the cell surface by actin-dependent secretory vesicles, and distributed uniformly in the plasma membrane (Suchkov *et al.*, 2010).

Experimental System

In order to develop a deeper understanding of the mating transduction pathway in Baker's yeast, three assays will be used, each providing a different line of evidence that the yeast are, indeed, going through the processes involved in mating. A **cell shape assay** is used to determine if yeast cells are undergoing the process of asexual or sexual reproduction based on proportions of budded, unbudded and shmoo phenotypes. A **beta-galactosidase assay** is used as evidence of mating gene transcription in the pathway. Finally, a **Western blot** is used to detect the amount of β –galactosidase protein in genetically transformed yeast as additional evidence of mating gene transcription, to support data from the beta-galactosidase enzymatic assay.

The yeast strain (strain LM23-3a) you will use for your investigation contains a mutation in the URA3 gene (*ura3*$^-$ strain). This gene codes for an enzyme required for the synthesis of uracil. Strains with mutations in URA3 cannot grow on media lacking uracil. This mutation allows you to select for yeast that have acquired the plasmid described below. This yeast strain has also been genetically engineered to prevent mating type switches from **a** cells to **α** cells.

Transformation of Yeast with Plasmid

Plasmids are small circular DNA molecules that are capable of replicating in a cell. The pBH315 plasmid was made by recombinant DNA techniques and contains the promoter from a gene involved in cell fusion during mating (the *fus1* gene) attached to a gene whose activity is easy to assay (a "reporter gene"). The plasmid enters the yeast cell nucleus, but does not recombine with nuclear DNA. In addition to directing the

transcription of the mating gene (*fus1*) from nuclear DNA, the promoter also directs the transcription of the reporter gene, in this case the enzyme β-galactosidase (*lacZ* gene) in the plasmid. When **α-factor** is detected by the cell, the *fus1* promoter turns on the transcription and translation of β- galactosidase. Therefore, the activity of β-galactosidase is an indirect measure of the amount of transcription directed by the *fus1* promoter and thus an indicator of mating gene transcription. β-galactosidase activity can be measured using a simple enzymatic assay.

This plasmid also contains a functional copy of the URA3 gene. Thus, yeast that take up this plasmid, even those missing an enzyme required for the synthesis of uracil (*ura3*$^-$), can grow on medium lacking uracil. This enables us to identify those *ura3*$^-$ yeast cells that have taken up the plasmid.

To transform yeast with a plasmid, one mixes the plasmid and yeast under conditions that render the cells partially
permeable and then selects for cells that have incorporated the plasmid by putting the mixture on appropriate plates. You will work with cells that have been transformed with plasmids containing the *ura3* gene and that have been plated on medium lacking uracil. Only the cells that have taken up the plasmid will be able to grow on these plates.

Yeast Cell Shape Assay

Microscopic observations of yeast cell shape can be used to approximate the cell cycle stage of each cell. Thus yeast cell shape observations are also known as **cell cycle progression assays**.

The cell cycle is the stages through which a cell passes from one cell division to the next. The cycle is divided into the M phase (which includes mitosis and cytokinesis) and interphase. Interphase is subdivided into three stages: a growth period (G_1) prior to and in preparation for DNA replication, a DNA synthesis stage (S), and a second growth stage (G_2) that precedes cell division (Fig. 5).

S. cerevisiae haploid cells divide asexually by growing a bud on the mother cell (see Fig. 2). This budding process begins when yeast enter the S phase). As they progress through the cell cycle, the bud grows until, at the end of the G_2, the cell looks like an asymmetrical "dumbbell." During the M phase the bud or "daughter cell" separates from the mother cell. When cells are in G_1, they do not contain a bud.

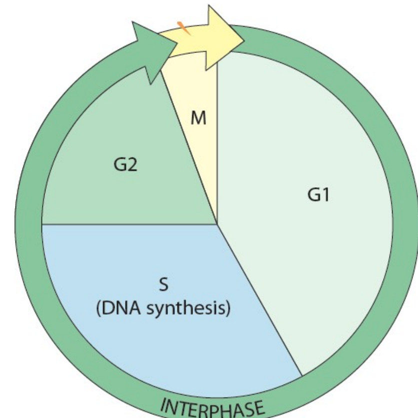


Figure 5. Eukaryotic cell cycle. (Fig. 24-1b in Becker's World of the Cell 9e, Hardin et al. 2016).

We use cell shape assays to detect cell cycle arrest as well as shmooing. In a given yeast cell culture that has been exposed to mating pheromone, the proportion of unbudded cells is a measure of the number of cells *not* progressing through the cell cycle but arrested in G_1. (See "Yeast Life Cycle" section.) This proportion should be compared to the proportion of unbudded to budding cells in a culture that has not been exposed to mating pheromone, to confirm that cells are indeed arresting in G_1.

Yeast sensitivity to α-factor pheromone is dependent on cell culture density. Under optimal conditions, yeast cell density is directly proportional to absorbance @600nm (Fig. 6). Therefore, OD readings at 600nm are used to estimate yeast cell density. It takes yeast about 1.5 hours to go through their complete life cycle, so cell densities increase quickly if nutrients are abundant. This period of rapid cell division at an exponential rate is called the Log Phase of growth (Fig. 7a). When cell densities become so high that nutrients are limiting, cell division slows and will eventually stop; this is called the Stationary Phase.

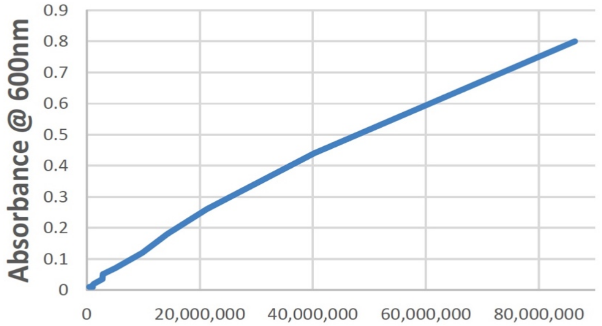
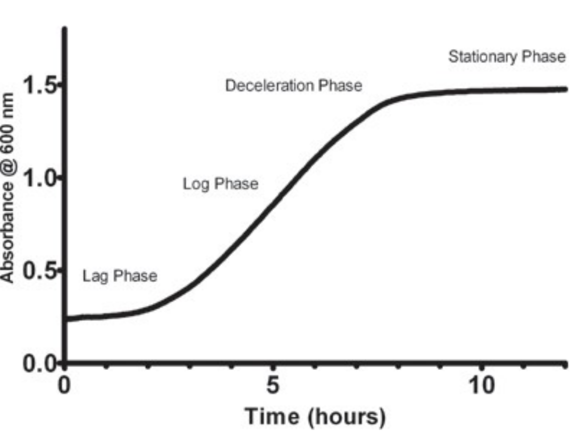


Figure 6. There is a positive, linear relationship between yeast cell density and absorbance @ 600nm while yeast have abundant nutrients to continue budding.

a.



b.

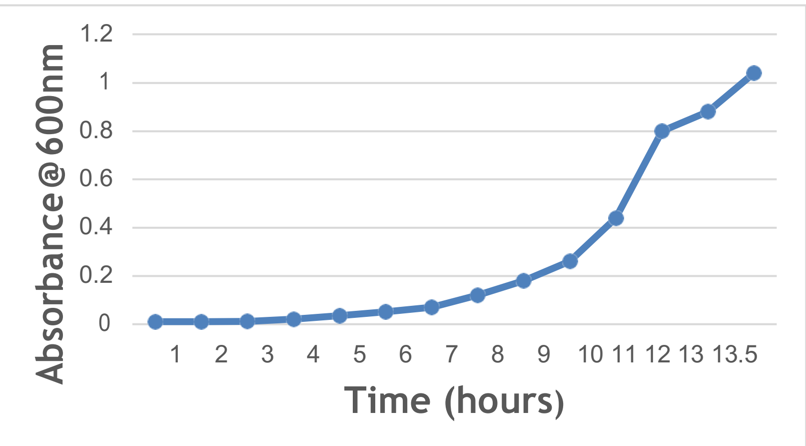


Figure 7. (a) Idealized growth curve with cells at low density exposed to optimal environmental conditions at time = 0. Cell division resumes at the end of lag phase and occurs at the most rapid rate during log phase. Cells stop dividing during stationary phase. (b) Yeast growth curve estimated from optical density (absorbance @ 600mn) over time. Yeast growing under our Biocore 384 lab conditions are metabolically most responsive to alpha factor during the log phase of growth (OD 600nm readings of ~**0.3- 0.85**). Stationary phase is not shown.

Because yeast are metabolically most responsive to α-factor pheromone during the log phase of growth, it's crucial to monitor your cell culture densities during your experiment. Yeast growing under our lab conditions are metabolically most responsive to alpha factor during the **log phase** of growth (OD 600nm readings of ~**0.3- 0.85;** see Fig. 7b).

β-galactosidase Reporter Assay

A beta-galactosidase reporter assay is a good measure of transcriptional activity of mating genes in yeast. You will use a colorimetric reporter assay for β-galactosidase. The substrate in this case is o-nitrophenylgalactopyranoside (ONPG), a colorless compound that is cleaved by β-galactosidase to produce galactose and o-nitrophenol. o-nitrophenol is yellow and can be measured with a spectrophotometer. The amount of yellow color produced is proportional to the β-galactosidase activity/per unit yeast present and is thus a good indicator of the transcription of the mating genes (Fig. 8).

Figure 8. Conversion of ONPG substrate to yellow o-nitrophenol product by β-galactosidase enzyme.

Western Blot Assay

A Western blot, also known as an immunoblot, is a molecular method used to detect individual proteins by their structure and sequence. In essence, the method is based on a protein-protein interaction between a protein of interest in a sample of lysed cells and an antibody that binds the protein of interest. You will use Western blotting to determine the relative presence of β-galactosidase protein in samples of yeast in the presence and absence of α-factor and an additional factor of your choice (i.e. environment, or chemical). One benefit of using a Western Blot to detect β-galactosidase protein is that you will be able to confirm your evidence of relative transcriptional activity of the *fus1* promoter, via the B-galactosidase enzymatic assay.

Western Blot Overview

1. Lysing cells, etc.
2. Separating denatured proteins through a polyacrylamide gel via electrophoresis
3. Transferring proteins from gel to membrane (i.e. nitrocellulose)
4. "Blocking" the remainder of the membrane that has not yet bound any protein with a non-specific protein
5. Binding of primary antibodies to a specific protein of interest
6. Binding of secondary (2°) antibodies to the primary antibodies
7. Detecting the protein bands via an enzyme-substrate reaction that is conjugated to the 2° antibody

Isolation of Proteins

To measure -galactosidase protein, you will first harvest yeast protein for Western blotting at the same time you harvest yeast for the β-galactosidase assay. The yeast cells must be broken in order to access proteins in the cytoplasm. You will shear the yeast cells with glass beads and a lysis solution containing Tween-20, a detergent that destabilizes the cellular membrane. Afterward, the protein will be denatured with sodium dodecyl sulfate (SDS), β-mercaptoethanol, and heat.
Adding SDS and β-mercaptoethanol to a solution of proteins will disrupt quaternary, tertiary, and secondary structures. The SDS is an amphipathic detergent, meaning it has a hydrophobic tail and negatively charged head. The hydrophobic tail disrupts hydrophobic interactions within a protein, thereby causing its unfolding. The charged head coats the protein with a net-negative charge. β-mercaptoethanol is a reducing agent and breaks disulfide bridges in proteins, thus disrupting tertiary structures.

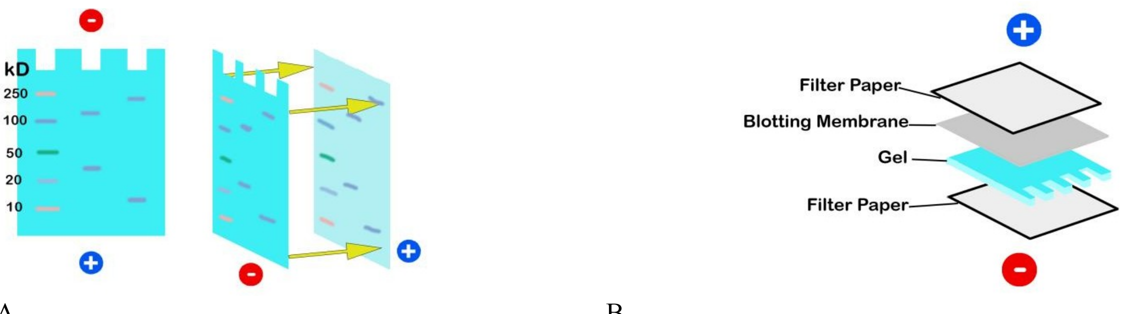
Separation of Proteins by Electrophoresis

Why would we want to go through all the effort of unfolding proteins? Doing so allows us to separate proteins by size (or number of amino acids that make up each protein) rather than by 3-dimensional structure or charge/isoelectric point (pI) (Table 1).

Table 1. Effect of SDS and β-mercaptoethanol detergents on protein structure and charge.

protein characteristic	detergent	purpose of detergent
Structure	β-mercaptoethanol	Reduces disulfide bridges; disrupts tertiary structure
Structure	SDS	Hydrophobic tail disrupts hydrophobic interactions of protein, causing unfolding
Charge	SDS	Anionic detergent coats all amino acids equally, thus giving the amino acid sequence a net-negative charge

Gel electrophoresis is a technique commonly used for the separation of macromolecules like DNA, RNA, or protein. Denatured proteins are separated from one another by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS- PAGE). When a current is applied to the polyacrylamide gel the SDS-coated, negatively charged protein migrates in an electrical field through the gel toward the positive charge. The polyacrylamide gel acts as a molecular sieve, or a lattice, and the movement of proteins through the gel is dependent on their size. Smaller proteins migrate through the gel faster than larger proteins (Fig. 9A).



A. B.

Figure 9. (a) Proteins denatured with SDS migrate toward a positive charge in a polyacrylamide gel. Lane 1 is a protein ladder. (b) Schematic of how to layer the components of the gel transfer apparatus.

B.

Transfer of Proteins to Nitrocellulose Membrane

After separation, proteins are transferred from the gel to a nitrocellulose membrane that binds proteins very well.
This transfer is done because in a Western blot, proteins are eventually detected with antibodies, and the antibodies will bind more quickly to a protein on nitrocellulose compared to a protein embedded within a gel. The electric field is placed perpendicular to the acrylamide gel, with the blotting membrane located in between the gel and the positive pole of the electrical field (Fig. 9B). When current is applied the proteins migrate out of the gel, bind to the membrane's surface, and are thus immobilized. Note that the membrane is actually a mirror image of the gel. Also, protein on the membrane will be found only in locations where protein has migrated from the gel to the membrane. The membrane must be "blocked" with a cheap protein, such as the milk □-protein, casein. "Blocking" the membrane prevents antibodies (which you will use to detect galactosidase protein) from binding to the membrane non-specifically.

Detection of Β-Galactosidase with Antibodies

The detection of proteins with antibodies can be done directly (with a single antibody) or indirectly (with two antibodies).
Indirect detection is usually performed because it is more sensitive and can detect smaller amounts of protein compared to direct detection. For an indirect detection, the primary antibody binds your protein of interest and the secondary antibody binds the primary antibody (Fig. 10). In addition, the secondary antibody is conjugated to an enzyme.

Our target, β- galactosidase, can be detected via an enzymatic reaction of a substrate with the enzyme conjugated to the 2° antibody. The 2° antibody you will use is conjugated to an enzyme, alkaline phosphatase (AP). AP has a high binding affinity for terminal phosphate groups and allows water to hydrolyze them. BCIP and NBT will be used as substrates for AP, and when acted on by AP they leave a purple precipitate on the nitrocellulose thus indicating where β-galactosidase is located.

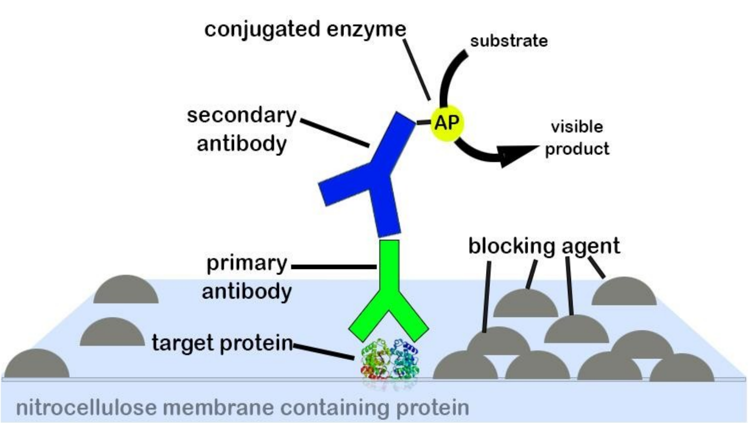


Figure 10. The steps of an indirect Western blot from blocking the nitrocellulose membrane to the detection of protein. Target proteins are concentrated on the nitrocellulose membrane and the primary antibody recognizes a short (20 amino acid) sequence of the protein.

Quantifying the Amount of Β-Galactosidase in Each Sample

Western blots can be quantified; one cannot tell the absolute amount of a protein, but can say with confidence the amount relative to another sample (reported as "relative amount"). Before comparing the relative amount of β-galactosidase present under each treatment condition, the values must be normalized to the amount of protein that is present in each gel lane. This is because it is common to load different amounts of total protein in each lane due to treatment effects and/or experimental variability. You will normalize the relative quantities of β-galactosidase by comparing the relative quantities of total protein. Total protein is estimated by putting our gels on a UV light. Amino acids on the proteins that we've loaded undergo a light-induced reaction with a compound in the SDS-PAGE gel. The reaction results in the visible fluorescence of all (most) proteins. If one lane has more total protein than another, we can see it on a gel and normalize when analyzing our protein of interest. Once a ratio of β-galactosidase : total protein has been determined for each sample, then samples can be compared for their relative amount of β-galactosidase.

Your Project

Each team will design an experiment to investigate some aspect of the signal transduction pathway involved in the yeast response to mating pheromone. Specifically, your team will select a treatment that you predict will influence *S. cerevisiae* yeast haploid "a" cell type response to α factor mating pheromone.

Your signal transduction hypothesis should thus address whether your experimental treatment in combination with alpha factor pheromone affects yeast "a" cell phenotype as well as mating gene transcription (as detected by the β-galactosidase reporter assay and Western Blot). This is how we investigate whether there is an *interaction* between 2 independent variables (treatment and α factor).

To address your hypothesis, consider an experimental design with two independent variables (treatment and α factor) and three dependent variables or lines of evidence (cell cycle arrest, mating morphogenesis (shmooing) and mating gene transcription). Thinking in terms of a 2x2 factorial experimental design may help you in your planning, as shown below:

	No Treatment	**Treatment**
No α factor	Control #1 • Predictions for cell cycle arrest? • Predictions for mating morphogenesis? • Predictions for mating gene transcription?	Control #2* • Predictions for cell cycle arrest? • Predictions for mating morphogenesis? • Predictions for mating gene transcription?
α factor	Control #3 • Predictions for cell cycle arrest? • Predictions for mating morphogenesis? • Predictions for mating gene transcription?	**Experimental Treatment** • Predictions for cell cycle arrest? • Predictions for mating morphogenesis? • Predictions for mating gene transcription?

* If the treatment in the absence of alpha factor treatments might alter the cell cycle or cell growth more generally, this could be considered as a second experimental treatment group instead of as a second control group.

If you suspect your treatment could stimulate the yeast mating response *without* alpha factor, should you propose a second hypothesis?

- This 2x2 factorial experiment includes three control groups. What will each control group tell you?
- Which data should be treated with non-parametric tests such as the Chi square test of independence?
- Which data should be treated as parametric data?
- After your initial overall test (2-way ANOVA, Chi square test), which pairwise tests would be most appropriate? (*e.g.*, independent t-test, Chi-square comparing just two groups)?

Schedule

After practicing assays in week 1, we expect you to do a literature search that will contribute to the feedback presentation your team will give in week 2. During weeks 3 & 4, you will write an individual research proposal, receive feedback, set up your experiment, do pilot studies, and collect data. Your team will present your research findings in a PowerPoint presentation in week 5.

Refer to Appendix A in Yeast Lab Manual Part B when designing your experiment, especially when establishing replicates. Think about the importance of maintaining equivalent cell density in your cultures, and what you will do if densities differ widely between treatments and/or replicates. These steps are critical for development of your rationale and experimental design carried out in lab.

Individual Research Proposal

Your individual research proposal paper should follow the rubric and guidelines in the *Biocore Writing Manual*. Use week 1 data to help you estimate β-galactosidase activity, cell shape (budding, unbudded, and shmooing proportions), and Western Blot expected results. Use the list of references below to begin your literature search (we strongly recommend the Citation Search in the Web of Knowledge database!). Proposals will be peer reviewed in week 3 discussion.

Group PowerPoint Presentation

The final products of your independent study on signal transduction will be in the form of an oral team PowerPoint presentation during the last lab (week 5). Consult the *Biocore Writing Manual* for details on the oral presentation rubric. *All* team members should contribute equitably to both the preparation and presentation. After the team presentations, each student will be asked to answer a few questions specific to their experiment. Please hand in a copy of your abstract, methods and results sections for our legacy folder.

Remember, we are more concerned with your rationale, hypothesis, experimental design and interpretation of your results than the results themselves. *Good luck, and enjoy this capstone Biocore 384 lab project!*

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Lodish H, Berk A, Kaisar CA, Krieger M, Scott MP, Bretscher A, Ploegh H, Matsudaira P. 2008. Molecular Cell Biology, 6th ed. New York (NY): W.H. Freeman and Co. 973 p. http://bcs.whfreeman.com/lodish6e/default.asp?s=&n=&i=&v=&o=&ns=0&uid=0&rau=0 *Click the, "Chapter 3: Protein Structure and Function" link. Under, "Animations," view the, "SDS Gel Electrophoresis," and "Immunoblotting," videos.*

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Helpful Websites

- Basic yeast molecular biology. Horst Feldman, Adolf Butenandt-Institute, University of Munich (2005) http://biochemie.web.med.uni-muenchen.de/Yeast_Biol/
- General information about the yeast life cycle was put together by The GENE project at Kansas State University http://www.phys.ksu.edu/gene/a1.html
- Yeast genome database https://www.yeastgenome.org/
- Western Blot Visual Protocol Series. (2012) Novus Biologicals. http://www.youtube.com/watch?v=uFu8aie4QFI&playnext=1&list=PL79DF2FB08E95D63D&feature=results_main

Yeast Lab Manual Part B
Protocols

This section of the lab manual contains the protocol for growing yeast cultures and instructions for performing three assays: cell shape assay, β-galactosidase reporter assay, and western blot assay. The experiment begins with starting an overnight culture, diluting the culture the following day, dividing the culture and adding pheromone and treatment to some of the groups. After the treatment period, the cultures can be subdivided to perform the various assays.

Growth of Cells for Assay

(This has been done for you the first week; you do it for your independent investigation.) Liquid cultures from your transformed colonies must be started **15-30hrs** before lab. Remember that you must use sterile technique for this.

Note about treatments: Application of your treatment will be specific to your own experiment. The generic protocol below calls for the treatment to be applied at the time when the α-factor is added. Given your rationale, it might make sense to develop your own protocol and administer your treatment at a different time point.

Note about volumes: Volumes of SD(-)ura broth might vary based on your specific protocol. If your experiment requires volumes other than those listed in these instructions, refer to the proportions outlined in Appendix A to determine the volumes you will need.

1. Begin your experiment by starting an overnight. Pour ~20mls of SD(-)ura broth into a 50ml sterile flask. An exact volume isn't necessary so use the 20ml graduation line on the flask for this measurement.
2. Use a flamed loop to remove 1/8 to ¼ of a loop of yeast from the transformation culture plate and add it to the flask containing 20ml broth. Twizzle (rolling back-and-forth between fingers) the loop to get the yeast into the broth. Allow the yeast to grow for 15-30hrs in the 30°C rotating water bath. During this time, the cells will divide until they generate a culture that has as many cells as the media can support (*i.e*. the carrying capacity for that environment).
3. 3.5-4hrs before you plan to perform the assays, create dilution replicates. Using a P5000, add 3.3ml of overnight culture into a new 50ml flask. Repeat to create 5 flasks with 3.3ml of overnight in them. Pour SD(-)ura broth into the flasks to reach a total volume of 30ml. Place the flasks in a shaker at 30°C and allow to grow for 2hr. This step ensures that the yeast population in each flask will be in an exponential growth phase when you add pheromone.
4. 2 hours after the dilution step, divide each of the flasks into 4 separate culture tubes. Refer to Appendix A for treatment guidelines. **Label with tape near the top of each tube.** To the "+ α" tubes, add 20µL of 100 µM α-factor. α -factor is located in the refrigerator. Place the tubes at a slant in a rack in the 30° shaker to incubate for 90-120 min.

β-galactosidase Reporter Assay

After your cultures have incubated for **at least 90 minutes** with α-factor you are ready to begin the β-galactosidase reporter assay. Note: You will need a hot water batch at a temperature of 30°C.

A. Estimating Cell Density

Cell density can be estimated by measuring the amount of light scattering that occurs in the culture using a spectrophotometer. Note: you do not need to measure the cell density of the nontransformed control culture.

1. **Briefly vortex each culture right before** taking samples from the tube. This is extremely important for estimating cell density as well as when you are taking samples from the tubes for use in an assay. Always mix your cultures. Yeast cells settle out fast!
2. Zero the spec at 600 nm with water.
3. Pour enough culture into a cuvette to reach the white line. Be sure the level as is at or just above the line.
4. Record the absorbance at 600 nm in a table in your lab notebook. Note: cell density should be at least OD 0.3 to measure β-galactosidase activity reliably. If less, let your cultures continue to

grow.

5. Rinse the cuvette with water between measurements. If the absorbance is over 1, use a portion of the culture to make a 10-fold dilution of the cells with water and repeat the measurement. This absorbance measurement will be used in the calculation of β-galactosidase activity. (Remember to multiply the absorbance by 10 if you made a 10-fold dilution.) Record your absorbance measurements and assay data in a table **in your lab notebook** modeled after the one shown here.

	Culture ______________		Culture___________________	
	Rep 1	Rep 2	Rep 1	Rep 2
Exposure time to A-factor (min)				
A600				
Assay time (min)				
A420				
Miller Units				
% shmooing				
% budding				
% unbudded				

B. Performing the β-galactosidase reporter assay – read completely BEFORE starting

1. Briefly vortex your tubes and place 1.5ml (pipette 750µl twice) of each culture into separate **labeled** microcentrifuge tubes. Also place 1.5ml of "nontransformed control" yeast (located in the shaker) into a microcentrifuge tube. This control is simply used to gauge color change during the reaction. You do not ever need to measure the absorbance of this culture. The control pellet tube should be set up exactly like the other tubes. Do you think you should add ONPG to the control pellet? What about Na_2CO_3?
2. Pellet the yeast cells bycentrifuging them in the microcentrifuge for 2 minutes (remember to counterbalance your tubes). Pour off and discard the supernatant. (The control tube will have a yellow supernatant because it is suspended in LB medium.)
3. Note how long each of the + α tubes has been exposed to alpha factor in your data table.
4. To each tube add:
   - 150 µL Z buffer
   - 50 µL chloroform (**Chloroform is toxic and tends to drip from pipette tips.**

   Use care not to spill any on yourself. If you do, wash it off immediately.)
   - 20 µL 0.1% SDS
5. Vortex the tubes vigorously for 30 seconds making sure the pellets have dissolved.
6. Place tubes and the ONPG* bottle in 30°C water bath to warm for 3 minutes.

****Do not use your ONPG if it is yellow, as this indicates contamination****

Note: Z buffer (see Appendix C) is a solution of buffered salts favorable for assaying β-galactosidase activity. It also contains β-mercaptoethanol, a sulfhydryl reducing agent--that is the reason for the bad odor. The chloroform (organic solvent) and SDS (detergent) act to permeabilize the yeast cells so that the substrate can get to the β-galactosidase. **These compounds are toxic!**

6. This is a timed reaction that may go very quickly once you add the substrate (ONPG). Before you start, be sure you are very clear about the following steps in the procedure and who will be stopping the reaction.
7. Stop watches ready! Follow these steps and be very consistent in your technique:
   a) Add 0.7 mL of ONPG solution to one of your experimental tubes. **Start your stopwatch at the exact time you add the substrate.**
   b) Quickly cap the tube, invert once, then open the cap.
   c) Hold the tube next to the control tube to assess color change

   d) When a difference in color is observed, add 0.5ml of 1M Na_2CO_3, then cap the tube and invert once. **Record the exact time** at which you added the Na_2CO_3.

Note: If there is a lot of β-galactosidase in the cells, the reaction will turn yellow within seconds. If the reactions do not turn yellow after 5 minutes, add the Na_2CO_3 at the 5 minute mark.

8. Repeat the assay for each of the remaining samples.
9. Pellet the cell debris by spinning the tubes in the microcentrifuge for 60 sec (you do not need to pellet the untransformed control tube).
10. Pipette 2ml of water into a spectrophotometer cuvette using a P1000 twice. Using the same pipette and tip, add the top 1 mL of your supernatant to the 2 mL of water in a cuvette. You just did a 1:3 dilution of your sample.
11. Vortex briefly to mix. Measure the absorbance of the reaction at 420 nm (use water to zero).

C. Calculating the Units of β-galactosidase Activity

The amount of β-galactosidase activity is generally expressed in Miller units.

$$\text{Miller units} = \frac{A_{420} \text{ of assay} \times 1000 \times 3}{A_{600} \text{ of cells} \times \text{ml of culture used} \times \text{time of reaction } \textbf{in minutes}}$$

The factor of 3 in the numerator of this equation is to correct for the dilution made for measuring the absorbance at 420 nm. The absorbance at 600 nm and the volume of culture (1.5 mL for our reactions) in the denominator allow different numbers of cells to be directly compared. (Remember to multiply the A_{600} by 10 if you used a dilution to make the reading.)

Cell Shape Observations *(Shmooing, Budding, or Unbudded)*

Wait at least 90 minutes after adding α-factor before making observations.

Observations of budding and shmooing cells allow you to determine whether **α-factor** is capable of arresting the cell cycle in G_1 for a particular strain of yeast. We can tell something about where yeast cells are in the cell cycle by observing the size of the bud. Bud formation begins in the S phase, and the bud enlarges as the cell cycle progresses. After mitosis, the daughter cells separate. In a rapidly growing yeast culture, cells will be found in all stages of the cell cycle. If the cells have been depleted of nutrients or are existing under conditions that arrest their cell cycle in the G_0 or G_1 phase, most cells will be unbudded (round). In contrast, cells in G_2 will appear to have a smaller cell attached to them. These are budding cells. Shmooing cell shapes indicate that cells have arrested in G_1 phase and then undergone mating morphogenesis.

Before you begin, ask your teammates performing the β-galactosidase reporter assay for the OD readings at 600nm.

Record this data in you notebook in a table modeled after the one under Part A above. How similar are the cell densities?

It is essential that your view your cells using an aligned microscope. Refer to pages 1-5 of the Tools and Techniques manual for instructions on how to align your microscope. **The alignment steps must be performed each time you sit down to use a microscope.**

1. Shake cultures well and then pipette 200 μL of each culture into an appropriately labeled microcentrifuge tube. The person identifying cell shapes should be blinded to the identity of the

culture. (Why is this important?)

2. **Just before observation** (do not leave culture in EDTA for an extended period of time!) add 200 µL of 0.5 M EDTA to a selected culture. Also, if it is a + α tube, note how long it has been exposed to alpha factor.
3. For each culture, vortex the tube for 30sec to break up clusters of cells. Place 20 µL on a slide and add a cover slip. Observe the yeast cells using first the 10X and then the 40X objective and phase contrast microscopy. Designate all the cells in the field of view as shmooing, budded, or unbudded. (If you are unsure of the cell shape, use the fine focus knob to focus up and down through the depths of the cells. Also refer to the time course images below.) To avoid recounting the same cells start in the upper right corner of the slide and pan to the left as you count. One (blinded) team member should tally each type of cell while another team member records the data. **Count at least 100 cells per culture and record numbers in your data table.**
4. Repeat the previous steps for the remaining yeast cultures. Switch roles and have your partner determine thenumber of shmooing, budded, and unbudded cells for each culture.

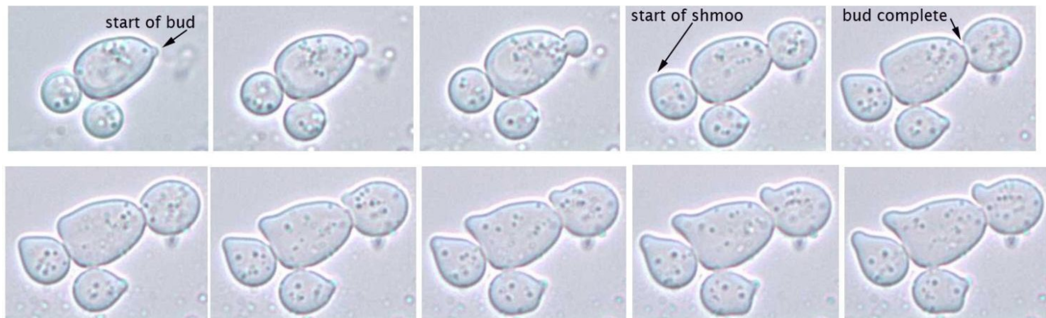


Figure 11. Time series of yeast cells under phase-contrast microscopy (1000x) with images taken at ~15 min intervals (left to right) before and after the addition of α factor. Bud initiation (image 1-3) looks as if the cell is blowing a bubble. A small sphere forms and enlarges over time while constantly maintaining its **spherical shape**. An early shmoo (image 4) is detectible by the morphing parent cell into a **raindrop shape**. When a bud is at least 50% the size of the parent cell, it is considered an independent cell – aka **unbudded**.

Western Blot Assay

The western blot protocol begins after the 90min α-factor incubation period.

Timing of each step	
Pelleting the yeast	20min
Possible stop point	
Creating lysate	30min
Possible stop point	
Setting up and loading the gel	45min
Run gel	30min
Photographing gel	15min
Set up gel transfer	1hr
Transfer to membrane	45min
Set up blocking	15min
Block membrane	1hr *or* over night
Possible stop point	
Antibody incubation	3hr (including two 1hr wait periods)
Visualizing the protein	30min

Pelleting the Yeast
Purpose: to measure the density of the culture and suspend further development of the cells
Time: 1 hour

Note: you will be performing these steps for all 4 treatment groups.

1. Zero the at OD_{600} spectrophotometer with dH_2O.
2. Pour enough of your culture into a cuvette to just reach the white line (not below the line) and read OD_{600}.
3. Fill in the values below.

OD_{600} Values	Without Treatment	With Treatment
Without α Factor		
With α Factor		

The OD_{600} must be greater than 0.3. If it is lower, let the culture continue to grow.

4. Of the remaining culture in your test tube, pipette 1.5ml into a labeled microcentrifuge tube.
5. Centrifuge the tubes at maximum speed for 2 minute.
6. Carefully pour supernatant out of the tube into a sink or liquid waste container leaving only a pellet of cells atthe bottom of the tube.
7. Add 1mL of chilled, sterile dH_2O. Pipette up and down 5 times to suspend the cells in the dH_2O.
8. Centrifuge the tubes again (be sure to counterbalance tubes) at maximum speed for 2 minutes.
9. Carefully remove as much supernatant as possible with a P1000 without disturbing the cell pellet.

You can freeze the pellet of cells at -20°C (small freezer near the door) and continue the protocol at a later time or continue to next step immediately.

Creating a lysate
Purpose: To rupture the cells and denature the proteins
Time: 30 minutes

10. Start to boil water in a beaker containing marbles on a hotplate.
11. Suspend the cell pellets in a calculated volume of chilled lysis buffer (which contains DTT). To equilibrate for differences in cell densities among the four cultures, multiply the OD_{600} reading by 150µL.

Sample Name	OD600	Volume Lysis Buffer (µL)
Example Sample	0.65	0.65 x 150 = 97.5µL

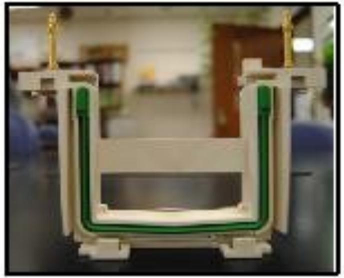
12. Locate a small tube containing 50 µl glass beads and pour the entire contents of the tube into your yeast andlysis buffer tube. The beads help shear the cell walls during vortexing.
13. Vortex vigorously by holding the microcentrifugation tubes on the vortex for 5 minutes.
14. Centrifuge the microcentrifuge tube for 2 minutes at maximum speed to pellet cellular debris and glass beads.

15. Transfer 50µl of the supernatant, which contains the protein, to a new labeled centrifugation tube.
16. Add 50µl of Laemmli buffer (which contains β-mercaptoethanol) to each tube.
17. Place the tubes in a float rack and incubate the samples in boiling water for 5 minutes.
18. Centrifuge the tubes for 10 seconds

The combination of SDS and β-mercaptoethanol in the Laemmli buffer along with boiling will denature the protein.

You can freeze the lysates at -20°C and continue the protocol at a later time or continue to next step.

Preparation of Protein SDS-PAGE Gel Electrophoresis
Purpose: To separate proteins via SDS-PAGE
Time: 45 minutes



Warning: Wear gloves while working with polyacrylamide gels; unpolymerized acrylamide is toxic.

19. Obtain a PAGE gel package from the fridge and open the plastic packaging containing the gel between two plastic plates. The buffer in the package can be poured down the drain and the wrapper can be thrown away.
20. Position your thumb on the indentation (middle of the comb) and remove the comb by pulling upward in one smooth careful motion.
21. Remove the green tape from the bottom of the cassette.
22. Using a sharpie, draw a small symbol at the top of the plastic cassette so you can distinguish which gel is yours later.
23. Load the gel cassette into the cassette holder with the shorter plate facing the center of the cassette holder.
24. Load the buffer dam (it looks like a gel cassette) on the other side of the cassette holder. Look for a label on the dam and be sure to put the proper side towards the green gasket.
25. Slide the cassette holder into the clamping frame and close the hinges to create a tight fit between the gel, buffer dam (or a second gel if you are running gels with another group), and gaskets on the cassette holder.
26. Load the clamping frame into the buffer tank.

27. Fill the area between the plates in the gel cassette holder to the top (it should spill over a bit) with chilled Running Buffer.

If the level of the buffer above the wells on your gel is decreasing, a part of your gel setup is either faulty or not tightly enough put together.

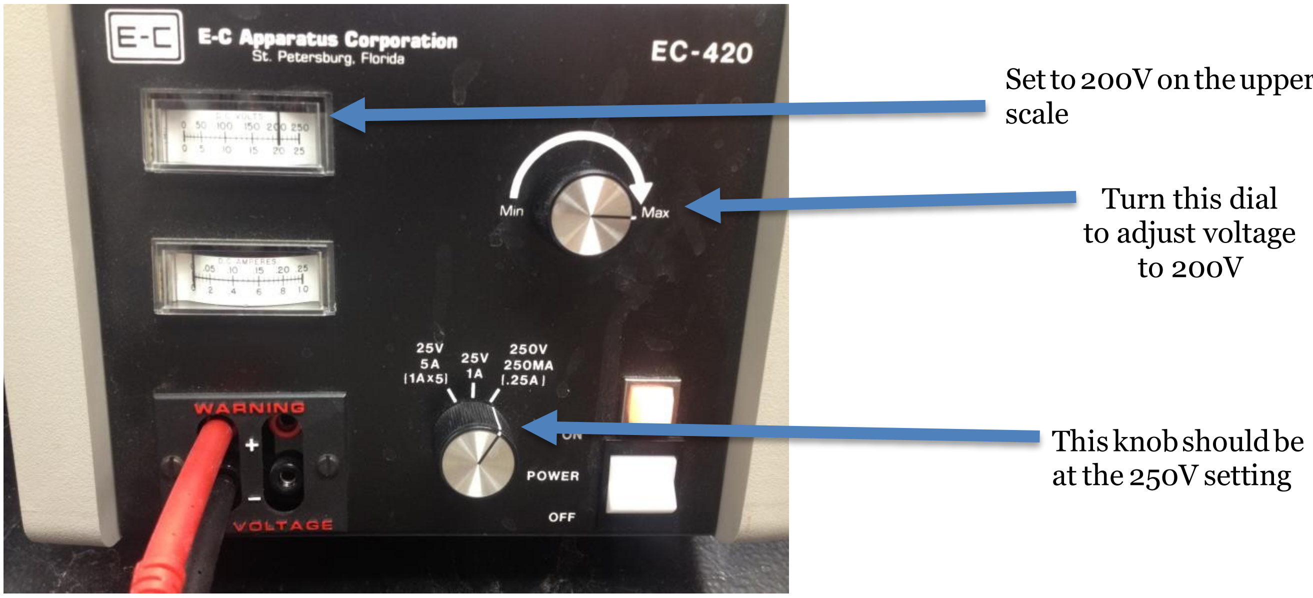
28. Add running buffer to the gel box up to the “Running Buffer line”.
29. Take note of the table below. You will be loading your samples into 5 lanes of the gel (including the ladder). Note: two groups can use one gel, just fill in the other boxes accordingly. If you’re the only group using the gel, you can leave open lanes between samples to avoid cross contamination.

Lane Number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Sample name	Ladder	+ alpha control	- alpha control	+ alpha experimental	- alpha experimental					
Volume added (μl)	**7**	**20**	**20**	**20**	**20**					
Experimental notes										

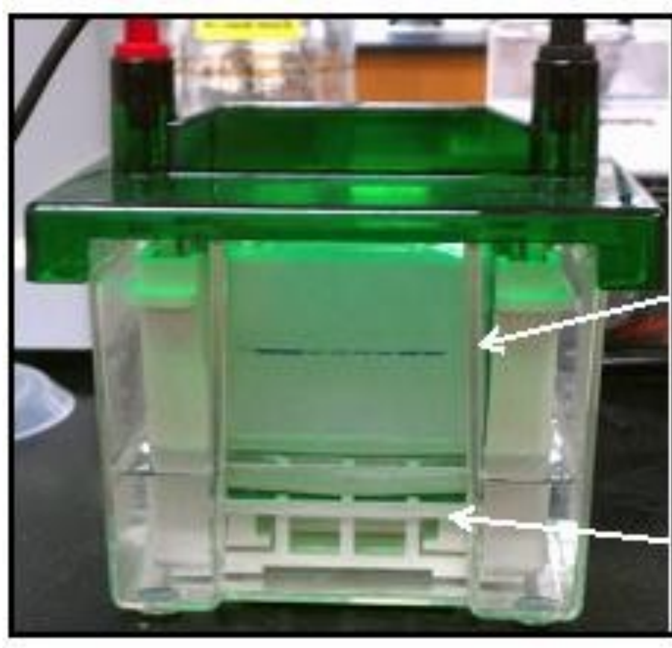
30. Add 7 μl ladder to the appropriate lane of the gel. **Use a p20 with a gel loading tip and load the well very slowly.**
    Ask for advice if you’ve never loaded a well before.
31. Add 20 μl of each sample to the appropriate lanes of the gel. Use a p20 with a gel loading tip attached.
32. Place the tank lid with power cables onto the top of the buffer tank making sure the red cable is on the positive terminal.

Tank lid with power cables

33. Plug the power cables into the power box making sure the black terminal is in the negative receptacle. Activate the power box to start the electrophoresis.
    Volts: 200
    Total Time: Around 30 minutes



34. After 5 minutes have elapsed, check the gel to make sure the samples have migrated out of the lanes and down the gel. You should see tiny bubbles coming off the wire at the bottom of the gel. This ensures a solid electrical current.
35. Every 10 minutes or so, check to make sure the blue dye is migrating down the gel. Keep an eye on the buffer level. If it drops down to the top of your wells that means you have a slow leak. If that's the case, you'll need to turn the power off and fill it back up as needed. Do not let the buffer level drop below the top of the wells.
36. Turn the power off when the leading blue dye has migrated to the green gasket (this takes around 35min). If the dye goes off the gel that's ok because your protein of interest is large (120kD) and should be towards top of the gel at that point.



Protein location at around 10 minutes, denoted by the blue banding.

When the leading blue bands reaches the green gasket, stop the gel. That should be around 30-35 minutes.

Photographing the gel
Purpose: To capture an image that will be used to determine total protein
Time: 15 minutes

37. Place the buffer tank in the plastic bin on the bench so you don't make a mess during disassembly.
38. Remove the cassette holder from the buffer tank.
39. Pour the running buffer from the buffer tank into the "used running buffer" bottle. Rinse the tank with tap water
40. Remove the clamping frame from the cassette holder.
41. Unclamp the hinges and remove your gel cassette.
42. Remove the polyacrylamide gel from the gel cassette by prying the plastic gel plate off the cassette using the metal cassette prying tool at the arrow points on the cassette (it will sound like it's breaking).
43. The gel should be stuck to one of the cassette plates at this point. Cut the wells (the very top of the gel) away with a spatula.
44. Notch the top corner of the gel above the protein ladder in lane #1 to serve as a marker for the orientation ofyour gel.
45. Gently transfer the gel to the UV box using a spatula or square piece of Parafilm. This can be a bit tricky so take your time. Be very careful not to rip the gel. It might help to put a small amount of running buffer on the spatula or Parafilm so it is less sticky.
46. Expose the gel to UV light for 3 minutes to visualize all proteins that migrated into the gel.
47. After 3 minutes, turn the camera on and zoom to the appropriate level to see your entire gel.
48. Take a picture of the gel with the following settings on the camera:
    - Exposure time: 2.5 seconds
    - Aperture (F): 3.5
    - No flash
    - ISO speed: 400
    - Up close image (flower icon is on)

    Be sure not to move the camera in the least bit while the photograph is being taken. If you do, you'll have a blurry image.
49. Transfer the image from the camera to a computer via the memory card.
50. Rinse the clamping frame and gel cassette holder **(careful not to get the electrodes wet)** with dH_20 and set out to dry.

TRANSFER OF PROTEINS TO A NITROCELLULOSE MEMBRANE
Purpose: To transfer the proteins from the gel to the membrane
Time: 20 minutes

51. Remove a cassette from the transfer machine, turn the dial to unlock and remove the lid.
52. Obtain a transfer pack from the refrigerator, place it flat on the lab bench and carefully peel away the packaging.

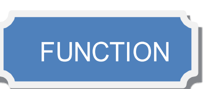
53. Remove the bottom stack from a transfer pack and place it onto the center of the cassette. Be sure the stack is not touching the green gasket. Lay the stack down just as it was laying in the transfer pack (don't flip it over). The stack consists of a piece of filter paper containing transfer buffer and a nitrocellulose membrane.
54. Carefully transfer the gel onto the bottom stack. Try to align it exactly with the membrane. It is often easiest to first put the gel on a piece of Parafilm and then slowly flip it/lay it down onto the membrane. It does not matter what side of the gel faces the membrane.
55. Gently place the top stack on the gel and use the blot roller to remove any air bubbles in the assembled sandwich.

56. Once the sandwich is complete, place the cassette lid on the base. The lid is reversible so the orientation doesn't matter.
57. Press the lid down firmly and turn the dial clockwise to engage the lid pins into the locking slots.
58. Slide the cassette (with the dial facing up) into one of the instrument bays until it clicks into position. The bays are labeled A (top) and B (bottom) and it doesn't matter which bay you're in or if there are one or two cassettes inthe

    machine during your transfer. A second cassette can even be inserted and started independently while the other cassette is running.

Transfer Using the Turbo Protocol

59. If not already on, turn the machine on using the switch located on the right side of the instrument.
60. On the home screen press the Turbo button.
61. Choose "1 Mini gel"
62. Press the Run button that corresponds to the bay your cassette is in.
63. The protocol will run automatically and the screen will display the conditions and progress of the run.
64. When the transfer is complete the screen will display RUN COMPLETE and a beep will sound.
65. Remove the cassette from the bay by pulling it straight out of the instrument (be careful it may be warm).
66. If no other groups will be using the machine, turn it off using the power switch.
67. Label a petri dish with your name (use a square dish if you have a full membrane or a round dish for ½ membrane). Pour ~10ml of TBST + 5% milk into the dish. The volume need not be exact but should be sufficient to just cover a membrane.
68. Unlock the cassette and remove the lid. If the blot sandwich sticks to the lid, use forceps to place it back in thetray in the same orientation that it ran (do not flip it over).
69. Disassemble the blotting sandwich. Throw away the top filter paper and the gel. Using a scalpel or scissors, notch the upper left corner of your membrane just like you did to your gel. Using forceps, carefully pick up the membrane and place it with the side that faced the gel facing UP in the dish with TBST + 5% milk.



You place the gel face up because the proteins are most abundant on the side facing the gel. This step is called "block" as the milk helps to inhibit non-specific antibody binding.

    Note: if you shared a gel/membrane with another group, you can divide the membrane now using a scalpel and proceed in your individual groups if you choose.

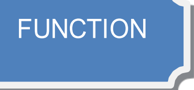
70. Place the lid on the petri plate.
71. At this point you can either:
    a. Put the dish on the rocker for at least an hour
       OR
    b. incubate the dish overnight **in the fridge**
72. No matter your choice, make sure to clean up right now. The old blot sandwich can go into the trash. Rinse out the cassette tray by spraying it with diH20. Leave the tray upside down on a paper towel next to the machine to air dry.

Antibody incubation
Purpose: To bind antibody to β-gal protein immobilized on the nitrocellulose membrane
Time: 3hr

73. Pour the TBST + 5% milk into the sink. Pour slowly so that the membrane doesn't fall out of the dish (don't worry, it sticks well).
74. Using a rinse bottle, cover the membrane in deionized water, swirl to wash, and then pour the water out of the dish.
75. Pour 10mls of diluted **primary antibody** solution (which is anti β-gal) into your dish containing the membrane.
76. Incubate the membrane on the rocking platform at room temperature for 1hr.

(OR, incubate the dish overnight in the fridge)

77. After 1hr, pour the primary antibody solution into the sink.
78. Pour enough TBST+5% milk into the dish to just cover the blot (about 5-10 ml). Put the dish on the rocker for 5 minutes.
79. Pour the TBST+5% milk into the sink and repeat the TBST+5% milk wash two more times. Be sure to pour out all the wash each time to ensure that no residual antibody is in the dish.



The washing step removes the unbound primary antibody and blocks the nitrocellulose again to limit nonspecific binding of the secondary antibody.

80. Pour 10mls of diluted **secondary antibody** solution (which is anti-primary) into your dish containing the membrane.
81. Incubate the membrane on the rocking platform at room temperature for 1hr.

WAIT 60 MINUTES

82. After 1hr, pour the secondary antibody solution into the sink.
83. Pour enough TBST+5% milk into the dish to just cover the blot (about 5ml). Put the dish on the rocker for 5 minutes.
84. Pour the TBST+5% milk into the sink, cover the membrane with diH2O and swirl to wash.
85. Pour the water out and rinse again with water two more times.

Visualizing the Protein
Purpose: To visually detect β-gal protein on the membrane via Alkaline Phosphatase reaction
Time: 30min

The next steps are timing sensitive so read through them fully before proceeding.

86. Add 10 ml of Western Blue detection reagent (it contains 333 µg per mL NBT and 167 µg/mL BCIP) to your dish.
87. Watch as the protein bands develop. Focus on the area of the membrane where your protein of interest would be (~120kd). It is possible to overexpose the membrane, so be ready to stop the reaction once the bands start getting dark. The trick here is to get the bands dark enough to see well, but not so dark that any differences between lanes are not detectable (oversaturated bands). This can take anywhere from 1 to 20 minutes depending on how much protein is on your membrane.
88. To stop the reaction, pour the liquid into the sink and wash the membrane in diH2O in your dish.
89. Put the membrane on a paper towel and fold it over to gently blot it dry.
90. Scan the membrane on a scanner.

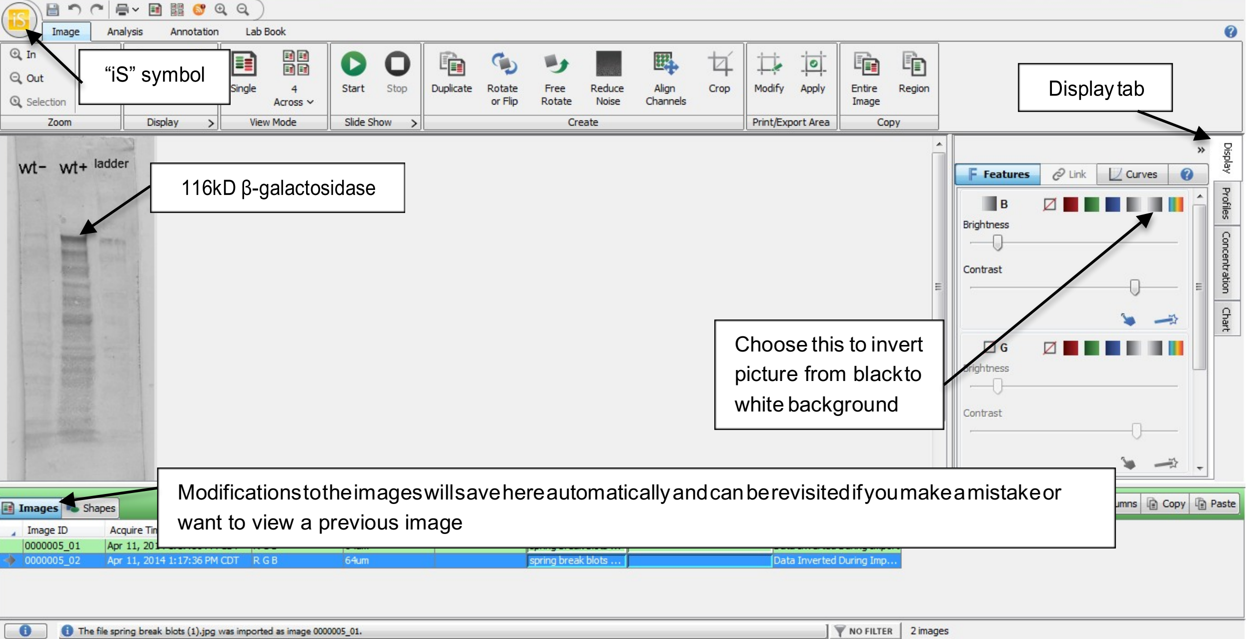
On a PC, open the program Windows Fax and Scan Choose “New Scan”
Scan as: color DPI: 600

91. Write your name on the blot and place it in the page protector.

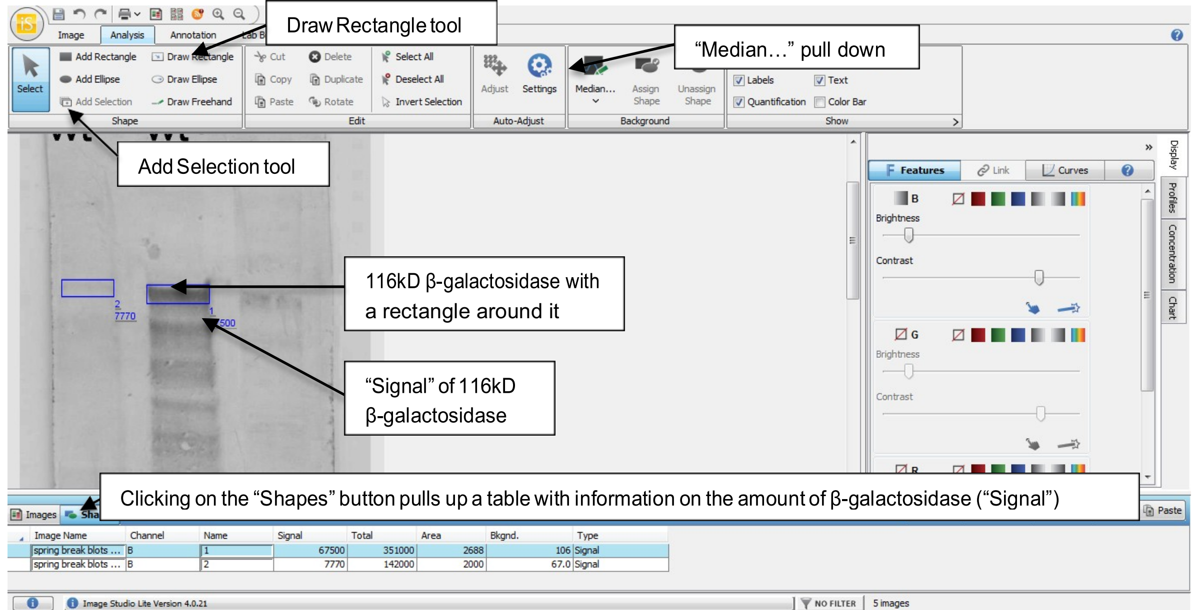
Analysis of Protein Bands using quantification software
Purpose: To analyze and quantify the amount of β-galactosidase in each sample relative to either the positive or negative control
Time: 30-60 min

Download the Image Studio Lite Software from the following website: http://tinyurl.com/BiocoreWBsoftware The program is free, but you will need to supply your email to download the software.

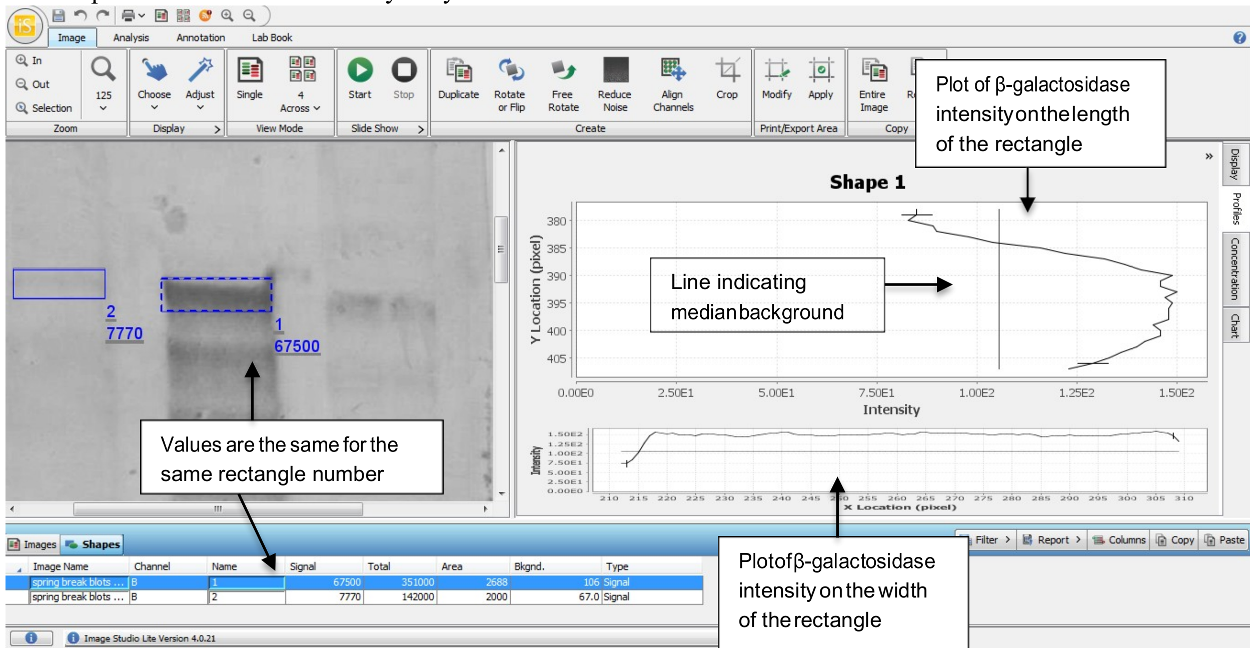
1. Open the program. Open your image by clicking on the “iS” symbol in the top left corner. Go to Import-> Other Image-> Files, to find your scanned blot image. There is a crop tool available under the image tab if needed.



2. Your image will appear inverted from its original color as white bands on a black background. Invert the colors:
   a. Click on the “Display” tab on the right side of the program
   b. You will see 7 color options. Choose the option that is a gradient from white to black ( ).
3. Click on “Analysis” tab at the top of the program. Select the “Draw Rectangle” function and draw a rectangle around the 116 kD band of β-galactosidase in your WT +□ -factor sample. Make the rectangle as small as possible while encapsulating the whole band. To adjust the size of the rectangle, click the “Select” button and then drag the rectangle to the proper size. Use the protein ladder to help you locate the 116 kD β-galactosidase protein.
4. Select, “Add Selection” and then click on the middle of the WT – -factor lane where you expect β-galactosidase to be. The selection box can centered on the band by clicking and dragging it (You may have to press the “Select” button first under the “Analysis” tab). You can be confident in assuming that β-galactosidase migrated the same distance in each lane of the SDS-PAGE.
5. Repeat the previous step for the 116 kD location for each remaining sample.

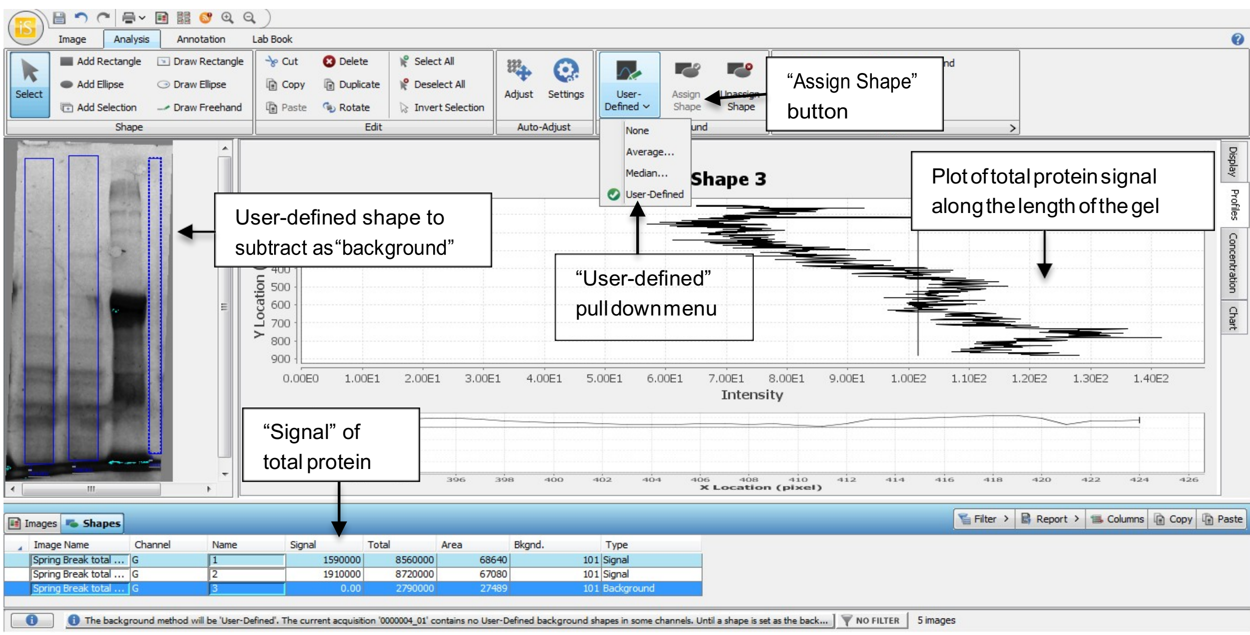


6. To subtract the background "noise" from the β-galactosidase band click on the pull down menu labeled "Median…" in the "Background" box in the top menu and click "Median". A new menu will pop up; set the border width to 1 and the segment to "top and bottom". With these options, the background is set to be the median value of the border one pixel above and one pixel below the rectangle you have drawn.
7. Select the "Profiles" tab on the right side of the program. The images you see after you select a rectangle show the profile of the band within the box. The top image is the intensity of β-galactosidase at each pixel on the length of the nitrocellulose membrane and the bottom image is the intensity of β-galactosidase across the width of the rectangular box. These are simply visual representations of the "Signal" reported in the "Shapes" table at the bottom of the program and also in the corner of each rectangle (see below). The visual images do not need to be reported in your poster presentations. This is merely for your own information.



8. Copy your results for the “Signal” (i.e. β-galactosidase) from the “Shapes” tab at the bottom of the program into an excel spreadsheet. If a signal value is negative, report β-galactosidase as “Not detected” or ND.
9. Next, you must determine the signal of total protein that is in each lane of your gel. These values will be used to
   normalize the amount of β-galactosidase to the total amount of protein in each lane.
10. Repeat steps 1-6 for the image of the **total protein gel** (this will be a separate image- you should have taken it while the protein still resided in the gel and before it was transferred) with the following modifications:
    a. **In step 3**: Set the color of the image; in the “Display” tab, set “B” and “R” to “don’t show this channel ( ), and “G” to a white to black background ( )
    b. **In step 4:** Draw a rectangle the length of the gel in each lane to quantify the total amount of protein. You may need to straighten out your image in order to make an accurate box. To do this, go to the “Image” tab at the top and click “Free Rotate” in the “Create” section.

    c. **Additionally:** You likely have a lane (or some area on the gel) with no protein. Draw a rectangle the length of the gel. Select “User-Defined” in the Background box (found in the same drop down menu as “Median” from **step 7**), and set this rectangle as the background with the “Assign Shape” button (see screenshot below). This will subtract the background noise of the gel from each lane. The signal for this background segment will be zero. The selection should be approximately the same length as the rectangles for the other lanes and preferably the same width (if possible). Record the total protein signal for each treatment on your spreadsheet containing the β-galactosidase signals.



Calculating the relative expression of β-galactosidase in each whole cell lysate

11. It’s possible that the difference in β-galactosidase you observe among the samples is an artifact. For example, maybe there is more β-galactosidase detected in one sample than another because there is more total protein. To control for variability in the amount of protein in each lane, one can determine the ratio (R) of signal (S) of β- galactosidase to that of total protein:

$$R = \frac{S_{\beta-\text{galactosidase}}}{S_{Total\ Protein}}$$

Note that the values do not describe the concentration or mass amount of β-galactosidase.

In the literature these ratios are reported relative to a control sample (i.e. +□ -factor) because it simplifies the data for the reader. To calculate these relative values, set the ratio for either the positive or negative control to 1.00, and use the following equation to solve for each sample (*X*).

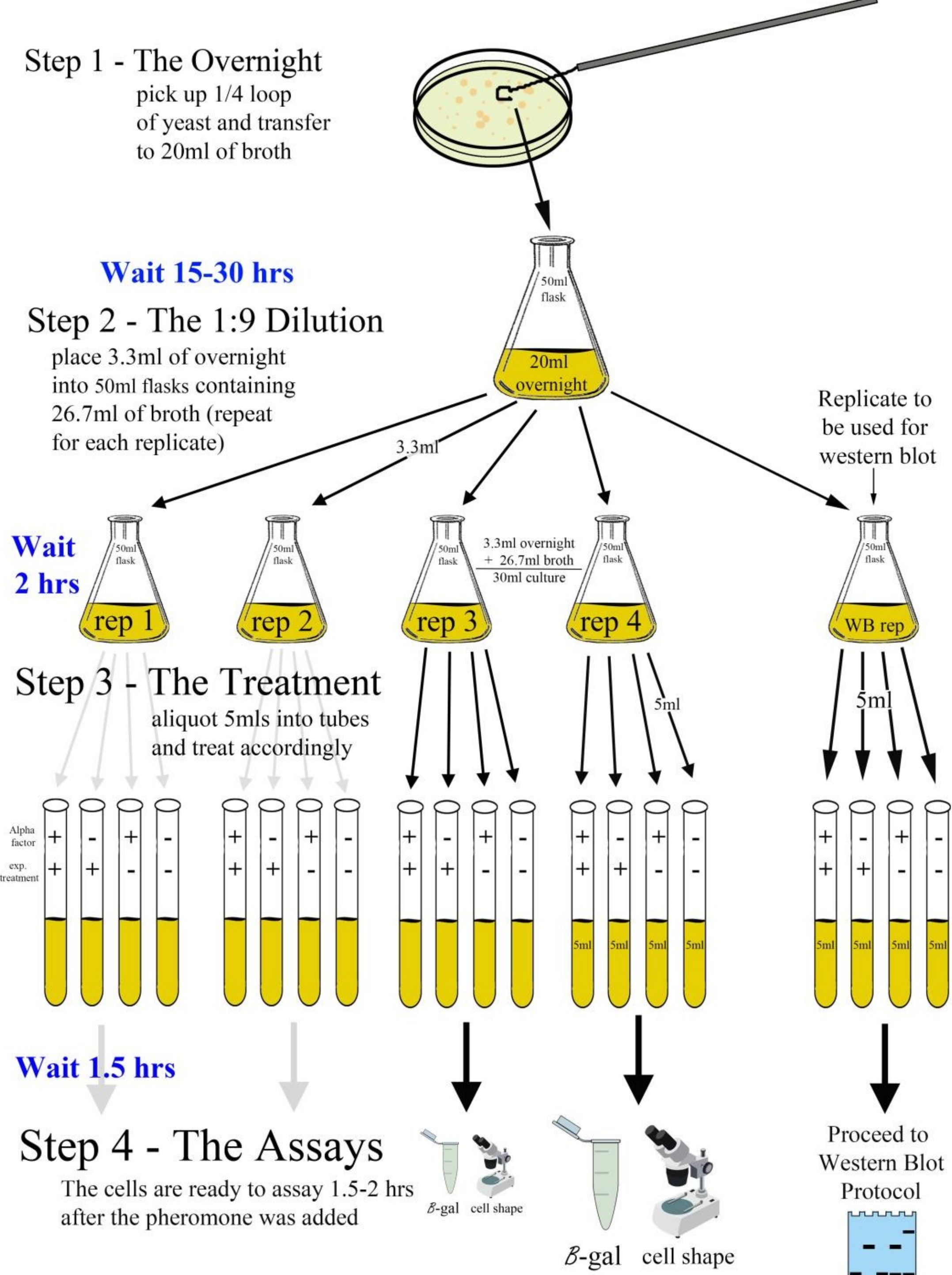
$$\frac{R_{Sample}}{R_{Control}} = \frac{X}{1.00}$$

When presenting your data you can either report R or X. Both are valid, but it is up to you to understand what these values mean and how they would be presented differently.
Take a moment to compare your image with the computational values the software provided. Do your eyes agree with the computer?

Refer to Appendix D for an example of how to represent western blot data.

Appendix A

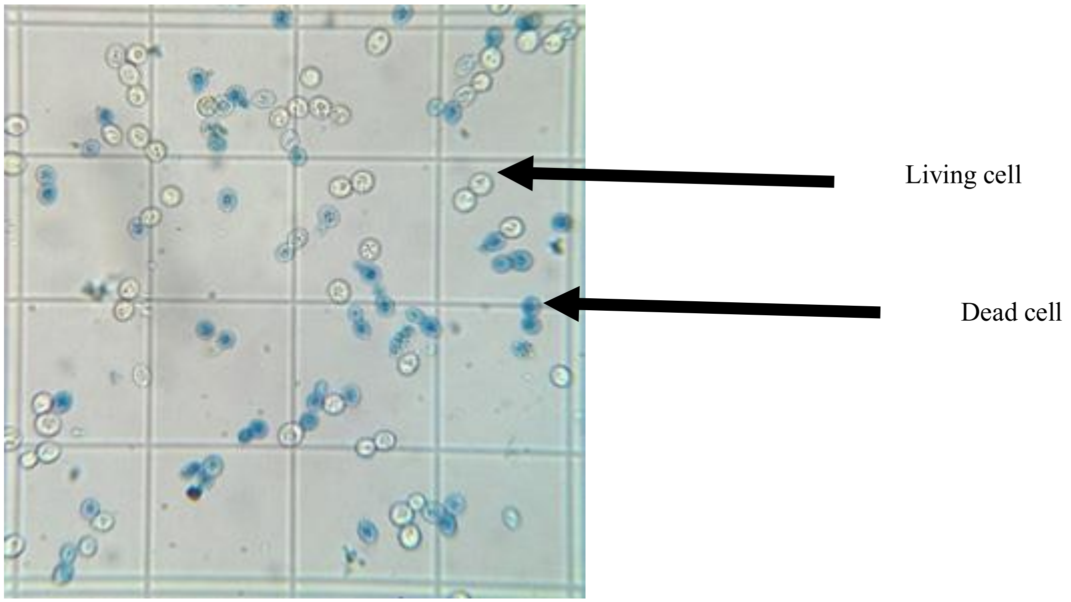
Schematic of an example protocol which examines the effect of treatment “x” on yeast response to mating pheromone. You may use a similar protocol for your independent project. Note the point at which replicates are separated out. This point may change depending on your experimental question; think carefully about an appropriate point to begin your replicates. **Consider staggering** your α-factor introduction among replicates to keep exposure time consistent among your replicates.



Appendix B
Cell Viability Protocol

1. Pipet 1ml cell suspension into a microcentrifuge tube. Spin down suspension for 20 sec and pour supernatant off.
2. Resuspend pellet in 100µl SD-ura media.
3. Add 200µl methylene blue (100ug/mL). Slowly pipette up and down to mix. Immediately, add 100µl 0.5M EDTA to each tube. Mix again.
4. Wait 1 min, prepare slide and score.

Dead cells will be stained blue; living cells will remain unstained. For the most accurate results, be sure to score the cells less than 5 minutes after the addition of methylene blue. Consider which type of microscopy would be best to use for this.



Appendix C Yeast
Reagents and Solutions

SD-ura Broth 1L
- 1.92g SDM (sigma Y1501)
- 6.7g Yeast nitrogen Base (Sigma Y0626)
- 40ml 50% glucose

Z Buffer
8.53 g Na_2HPO_4
5.5 g $NaH_2PO_4 \cdot H2O$
0.75 g KCl
0.25 g Mg $SO_4 \cdot 7H_2O$
H_2O to 1 liter
Just before use, add 2.7 µL of ml β-mercaptoethanol for each mL of the volume to be used.

ONPG
*make just before each lab
120 mL Z-buffer
0.144g ONPG
324µL B-merc
Can crush chunks with glass rod to speed up dissolving

Transfer Buffer
[25 mM Tris, 192 mM glycine, 20% Methanol]
3.03 g Tris Base
14.4 g Glycine
200 mL Methanol
H2O to 1 L
*Dissolve Tris and Glycine in ~700 mL H2O, add methanol, and bring up volume to 1 L with H2O

100µM α factor GeneScript # RP01002
Always store @4°C

1M Na2CO3

50% glucose

Glass beads 50µl/sample (sigma G8722)

0,1% SDS (sodium dodecyl sulfate)

100% chloroform

TBST, pH 7.4
[150 mM NaCl, 2.7 mM KCl, 25 mM Tris, 0.05% Tween-20]
8.8 g NaCl
0.2 g KCl
3.03 g Tris Base
2.5 mL 20% Tween-20
H2O to 1 L
*Dissolve Tris, NaCl, and KCl in 800 mL H20, bring to pH 7.4 with HCl, bring volume to 1 L with H2O and then add Tween-20.

TBST + 5% milk
5% dehydrated (powdered) milk dissolved in TBST; keep refrigerated

1x Lysis Buffer
[150 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 8.0, 0.05% Tween-20, 1mM dithiothreitol]
1.5 mL 5M NaCl
0.5 mL 1 M Tris-HCl, pH 8.0
0.5 mL 500 mM EDTA
0.025 mL 100% Tween-20
H2O to 50 mL
Add 1 □l 1 M DTT for every 1 mL of 1x lysis buffer

The lysis buffer contains Tween-20, a detergent, and DTT, a reducing agent, which disrupts the cellular membrane of the yeast, allowing for the cells to be broken open with the help of the glass beads. The buffer also contains EDTA—a protease inhibitor—that limits the degradation of proteins.

Running Buffer
[25 mM Tris, 192 mM glycine, 0.1% SDS]
3.03 g Tris Base
14.4 g Glycine
5 mL of 20% SDS
H2O to 1 L
Primary Antibody - anti-B-gal (mouse) – Promega z378a
2ul per 10ml of TBST + 5% milk

Secondary Antibody - Anti-Mouse, AP Conjugate – Promega S3721
4ul per 10 ml TBST + 5% milk

Molecular Weight Ladder Biorad 1610374

Trans blot nitrocellulose transfer packs Biorad 1704158

10% TGX gels Biorad 4556-8034

Western Blue Promega S3841

2x Laemmli Buffer
[125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 5% β-mercaptoethanol, Bromophenol blue to desired color]
6.25 mL 1 M Tris-HCl, pH 6.8
2 g SDS
10 mL 100% glycerol
H2O to 45 mL
Add bromophenol blue to desired color
Add 50 µl β-mercaptoethanol fresh for every 950 µl of sample buffer
Laemmli buffer contains glycerol, which has a greater density than water; adding glycerol to the WCL increases the density of the solution, allowing the WCL to fall into the wells of the acrylamide gel. Bromophenol blue is used as a surrogate to visualize the migration of the proteins through the polyacrylamide gel. It forms what is known as the "dye front". SDS is a detergent, and like other detergents it is amphipathic (contains a polar head and hydrophobic tail). The hydrophobic tail of SDS can interact with the hydrophobic regions of proteins and disrupt their tertiary and secondary structure. This leads to the linearization of proteins, contributing to their separation by size rather than their globular structure. The polar head of SDS is anionic, and so when it binds to a protein the SDS coats the protein with a net negative charge. In doing so the SDS negates the charge of the amino acid side chains that make up the protein, and allows for the migration of all proteins in a single direction in the polyacrylamide gel (that being toward the cathode). β-mercaptoethanol (β-ME) is a reducing agent, a name given to a class of chemicals that can reduce covalent sulfide-sulfide (disulfide) bonds by adding hydrogen to each sulfur atom. Disulfide bonds are common in protein and provide tertiary structure to them. The amino acid, cysteine, contains sulfur, and two cysteine side chains can create disulfide bonds. β-ME breaks these bonds, causing the protein to unfold. Thus, β-ME contributes with SDS to linearize proteins

Appendix D
Western Blot Example of Figure & Legend

Figure XX

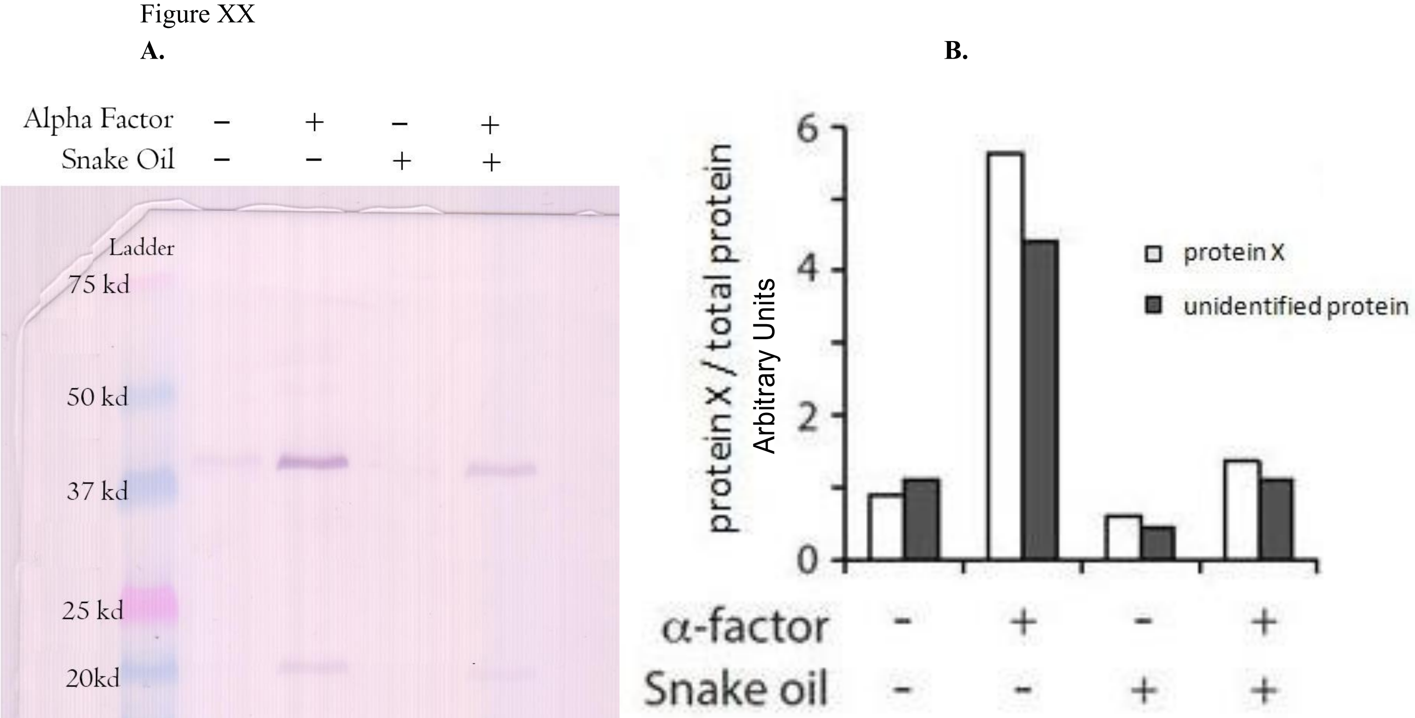
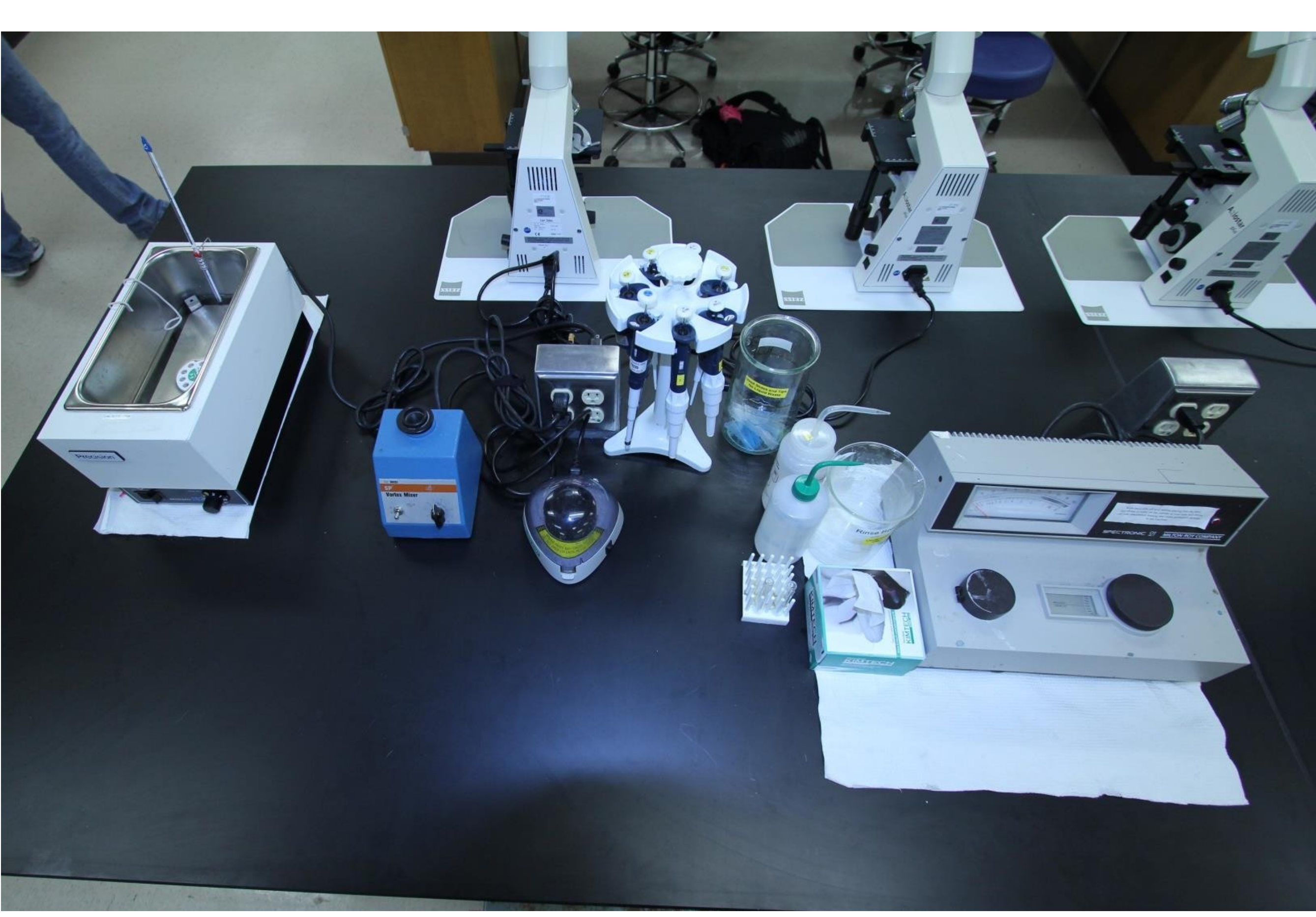


Figure XX *S. cerevisiae* strain LM23-3a was cultured for 90 minutes at 30°C in SD -ura media with (+) or without (-) 0.4 µM α-factor and with or without 1 mM snake oil. (A) Proteins in each lysate were separated by SDS-PAGE and protein X (40kd) was detected via Western blot with BCIP/NBT. The total amount of protein in each lysate was determined with NuView gels exposed to UV light for 2 minutes (data not shown). Note the unidentified protein (~20kd) in the + alpha factor treatments. (B) The amount of protein X and the unidentified protein in each lysate is represented relative to the total amount of protein in the lysate. Arbitrary Units were generated by the quantification software LiCore Studio which was used to analyze the gel (not pictured) and the nitrocellulose membrane.

Appendix E
Equipment for Yeast Signal Transduction

Student benches

Two light microscopes
"Used tips" jar
Vortex
Liquid waste beaker
30°C water bath
thermometer
Tube float
ONPG water bath holder
P20, 200x2, 1000x2, P5000
Spec
2 cuvettes
Rinse bottles (water and EtOH)
kimwipes
microcentrifuge



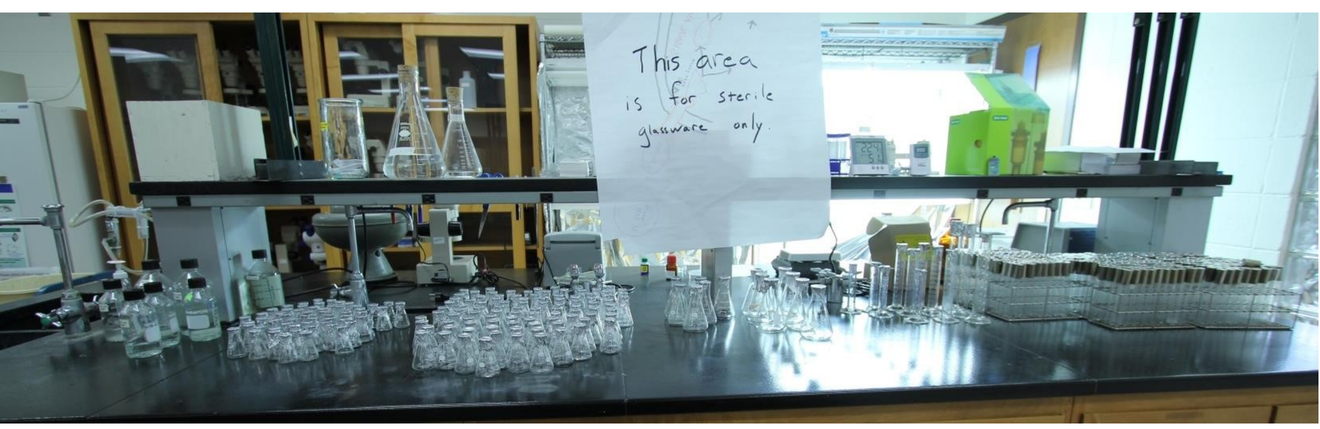
Appendix F
Community Benches

Flask of +α and –α for each strain
Untransformed strain for control
Beakers of EtOH
Inoculating loops
Alcohol lamps
matches
Bottles of water
B-gal kits
- Z buffer
- Chloroform
- SDS (0.1%) (make 100ml)
- 0.5M EDTA
- ONPG (1.2 mg/ml ONPG in Z buffer)
- 1M Na_2CO_3

Extra chloroform, sds, edta and Na2CO3
Sterile microfuge tubes in jars
Extra bag of tubes
Tally counters
Microfuge tube rack
Stopwatch Tube racks Sharpies Tape
Pipette Tips

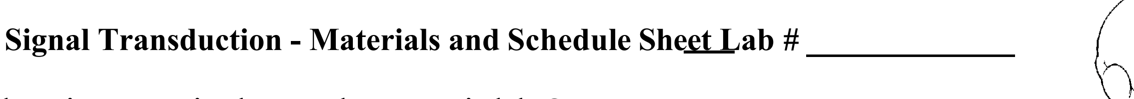
SD-ura broth
Sterile flasks

Sterile grad cyclinders
Sterile tubes



Appendix G

Signal Transduction - Materials and Schedule Sheet Lab # ____________



What is your independent variable? _________________________

Group Members	Email address

Chemical Request - Fill out **every** line.

Chemical name_________________________Molecular Weight ____________

Molarity of Stock Solution_______Volume of Stock Solution________

_______mg needs to be added to_______ml to make this solution

What is the solubility of your compound (look it up)?________
Is this concentration you asked for below the published solubility? _______

Can it be poured down the drain?_____
If you're not sure call Chem Safety to find out disposal procedure

Ordering info

Ask if we have the compound. If we don't, fill this out:

Sigma Cat#________________________Quantity to order_____________

Price____________

Equipment Request

– use this space to request anything outside of the normal experimental setup (i.e. glassware, special media, extra waterbaths, pH meter, pogo stick, rattlesnake, extra thermometers, theremin, etc.)

If you'd like to carry out the β-gal assay outside of normal lab time be sure to **email Seth** to make arrangements.

Appendix H
Examples of Independent Variables chosen by students for Yeast Cell Signaling Research Projects

Actigenin
Adenosylmethionine
Apotinin
Caffeine
Calcineurin
Calcium chloride
Casein
Cholera toxin
Cobalt chloride
Colchicine
Comparing against yeast strains with various mutations
Cyclic AMP
Dansylcadaverine
Dimethyl sulfoxide
Dithiothreitol Doramapimod
Drop-out media (- specific amino acids)
Electrical current
Epinephrine
Ergosterol
Ethanol
Farnesol
Glucose (increased and decreased)
Gonadotropin-releasing hormone
Guanosine-5'-triphosphate
Hydrogen peroxide
Insulin
Latrunculin-A L-captan
L-dopa
Light intensity
Lithium
Mannose
Mutated pheromone (various amino acid deletions/changes on various parts of the ligand)
Myristic acid
Nicotine
Nocodazole
Novobiocin
Ortho-phthalaldehyde
Pepsin
Phenethylamine
Pheromone concentration (higher and lower)
Pheromone exposure time
Pheromone heat exposure
Potassium chloride
Purvalanol
Reduced nitrogen (ammonium sulfate) in media
Renin
Sodium chloride
Sodium pyrophosphate
Sorbitol
Speed of agitation
Tacrolimus
Temperature of culture (higher and lower)
Tosyl-Largininyl-methyl-ester
Tryphostin
Tryptophan (addition)
Ubiquitin
Valproic acid
Zinc sulfate

Appendix I
Western Blot Timeline Form

Lab # ________ *Fill in names and emails on back*

Fill in all the shaded boxes

		Date	Time starting	Group member present
Pelleting the yeast	20min	Do this in lab when starting your other assays		
Flexibility point: cell pellets can be kept frozen for weeks				
Creating lysate	30min			
Flexibility point: lysates can be kept frozen for weeks				
Running and photographing the gel, transferring to the membrane and blocking	2hrs			
WAIT 1 HOUR Flexibility point: You can resume after 1hr or anytime within the next 24hrs.				
Adding primary Antibody	10 min			
WAIT 1 HOUR Flexibility point: You can resume after 1hr or anytime within the next 24hrs.				
Adding secondary Antibody	10 min			
WAIT 1 HOUR Flexibility point: You can resume after 1hr or anytime within the next 24hrs.				
Visualizing the protein	30min			

Appendix J
Signal Transduction Prelab

During your literature search, you read about a compound call Powerpuff X that was shown to specifically disrupt the assembly of actin microfilaments in yeast cells involved in mating morphogenesis. You become curious as to what effect Powerpuff X will have on the yeast mating pathway using type 'a' yeast cells that have been transformed with plasmid pBH315.

1. Being a meticulous scientist, you first set out to generate a testable hypothesis. Choose the most suitable hypothesis for your experiment. (Read carefully)

- **A.** We hypothesize that Powerpuff X will disrupt the yeast cells' ability to undergo mating morphogenesis (less shmoo) but should not interrupt other aspects of the yeast mating pathway
- **B.** We hypothesize that treatment with Powerpuff X in the presence of alpha-factor will lead to a significant increase in cell cycle arrest and decrease in yeast mating morphogenesis as quantified by a lower percentage of shmoos present in the yeast population compared to yeast cells treated with alpha-factor alone.
- **C.** **We hypothesize that treatment of Powerpuff X in the presence of alpha factor will lead to a significant decrease in yeast mating morphogenesis (as quantified by a lower percentage of shmoos) but no change in cell cycle arrest (as indicated by a lower percentage of budding cells) or the transcription of the mating gene *fus1* indicated by B-galactosidase production and activity, as compared to yeast cells treated with alpha-factor alone**
- **D.** We hypothesize that Powerpuff X will disrupt actin microfilaments leading to a lower population of shmoos but no change in cell cycle arrest or Beta-galactosidase transcription compared to yeast cells that were not treated with Powerpuff X

Questions 2- 4: Here is the 2 x 2 table you generate for your experiment.

	No Powerpuff X	+ 1 mM Powerpuff X
No alpha-factor	A	B
+ alpha factor	C	D

2. Which quadrant(s) represent(s) the control(s)?

- **A.** A only
- **B.** B only
- **C.** C only
- **D.** A and B only
- **E.** A and C only
- **F.** **A, B and C**

3. What will the control(s) tell us in your Powerpuff X experiment?

- **A.** The yeast cells are healthy and were not starved for nutrients during the experiment
- **B.** The yeast mating pathway is functioning as expected
- **C.** If the concentration of Powerpuff X we chose was toxic to budding yeast cells.
- **D.** **All of the above**

4. Which quadrant allows us to determine if the concentration of Powerpuff X we chose was toxic to budding yeast cells?

- **A.** A
- **B.** **B**
- **C.** C
- **D.** D

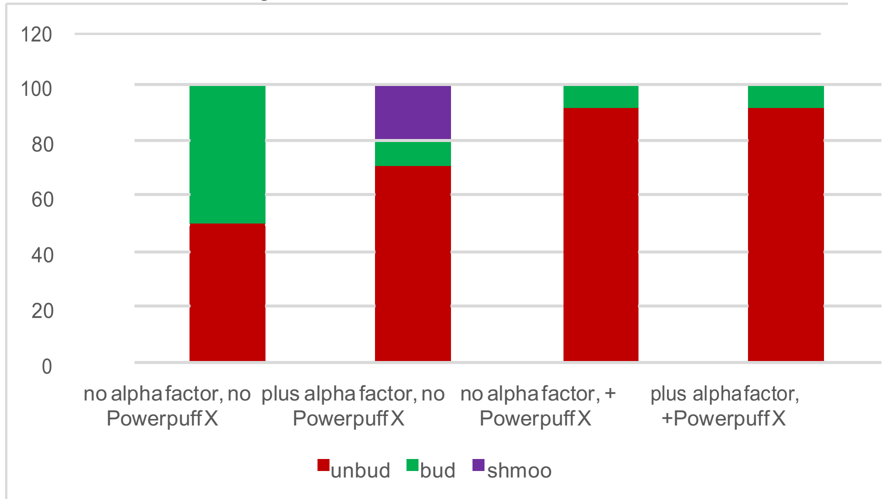
To study whether Powerpuff X has an effect on the yeast mating pathway, you will be performing 3 assays on type 'a' yeast cells transformed with plasmid pBH315: cell shape assay, Beta-galactosidase assay and a western blot.

5. What does the cell shape assay indicate?
   A. Cell cycle arrest
   B. Mating morphogenesis
   C. Mating gene *fus1* transcription
   D. A and B only
   E. A and C only

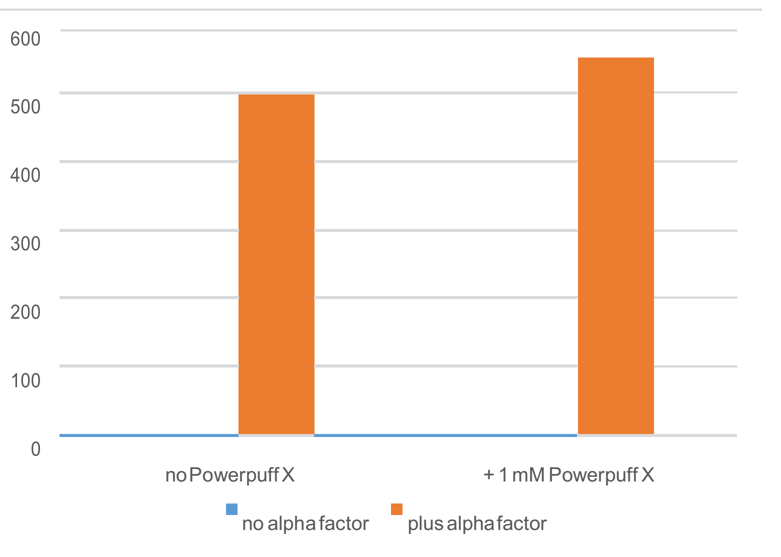
6. Why are we using the Beta-galactosidase (β-gal) reporter assay? Read carefully and pick your best choice.
   A. To assess the expression level of β-gal that is induced when the yeast mating pathway is activated
   B. To assess the expression level of β-gal that occurs when the yeast cells transformed with plasmid pBH315
   C. To assess the expression level of the *fus1* mating gene as indicated by β-gal activity under the control of the
fus1 promoter-_lacZ_ gene construct in plasmid pBH315 of transformed yeast treated with alpha factor
   D. To assess the expression level of the *fus1* mating gene as β-gal activity is required for expression in the plasmid pBH315 of transformed yeast treated with alpha factor

7. You have performed the experiment and obtained the following results:

Cell shape assay



β-gal assay



What are the possible interpretations of your results? Read carefully and select the most reasonable interpretation based on the evidence presented here.

A. **Treatment with 1 mM Powerpuff X impaired the ability of yeast cells to form shmoos and increased cell cycle arrest in the presence of alpha factor while the expression level of *fus1* mating gene as indicated by Beta- galactosidase activity was unaffected.**
B. Treatment with 1 mM Powerpuff X is toxic to yeast because the yeast cells had decreased budding, and were unable to form shmoos or express β-galactosidase
C. Treatment of both Powerpuff X and alpha-factor inhibited the propagation of the yeast mating signaling pathway because they bound to and sequestered the alpha-factor receptor Ste1

8. What is missing from the β-gal assay graph?
A. y-axis label
B. y-axis units
C. error bars
D. all of the above

9. The data that you generate from the cell-shape assay will be______________data that will be analyzed using ______________

A. **qualitative, chi-squared test of independence**
B. quantitative, two-way ANOVA (post-hoc t-tests if appropriate)
C. quantitative, chi-squared test of independence
D. qualitative, two-way ANOVA (post-hoc t-tests if appropriate)

Question 10 – 13: You will be performing western blots on your yeast samples to detect beta-galactosidase protein expression. What is the purpose of the following components/reagents you will be using as you perform your western blots?

10. Beta-mercaptoethanol is used:
A. To bind protein and add an overall net negative charge
B. **As a reducing agent that disrupts disulfide bonds and tertiary structure**
C. To provide a sweet-smelling aroma ☺
D. To degrade protein into single amino acids

11. SDS Poly-acrylamide gel electrophoresis (SDS-PAGE) is used to:
A. To determine the enzymatic activity of beta-galactosidase made in yeast
B. To separate proteins by charge with the more negatively charged proteins migrating faster than uncharged or positively charged proteins
C. **To separate proteins by size with the smaller proteins migrating at a faster rate than larger sized proteins**
D. To separate proteins by size with the larger proteins migrating at a faster rate than smaller proteins

12. Milk protein (casein)
A. Provides proteins to which the primary antibody can specifically bind
B. Provides proteins to which the secondary antibody can specifically bind
C. Binds only to proteins that were transformed to the nitrocellulose membrane from the SDS-PAGE gel
D. **Blocks all parts of the nitrocellulose membrane that are not occupied by the proteins transferred from the SDS-PAGE gel**

13. Alkaline phosphatase enzyme that is conjugated to the secondary antibody:
A. **Reacts with the Western blue reagent substrate to form a colored product that allows us to visualize the Beta- galactosidase protein on the nitrocellulose membrane**
B. Reacts with the Western blue reagent substrate to form a colored product that allows us to visualize the Beta- galactosidase protein on the SDS-PAGE gel

C. Reacts with pNPP substrate to form a colored product that allows us to visualize the Beta-galactosidase protein on the nitrocellulose membrane
D. None of the above

14. Matching:

As you know, experiments don't always go as planned. Match what you might see on a Western blot if you encounter each of the following issues.
A. Protein did not transfer to the nitrocellulous membrane **No bands at all**
B. Either the primary antibody did not detect β-galactosidase protein, the secondary antibody did not detect theprimary antibody, or both **Only ladder**
C. The amount of β-galactosidase protein in the +alpha/+ experimental treatment sample is below the detectable limit
Band in + alpha/ - treatment but no band in + alpha/ + treatment
D. The blot was not blocked with a non-specific milk protein (casein) prior to treatment with antibodies **Darkly stained entire blot**
E. The antibody for β-galactosidase protein cross-reacts with other proteins, which are not β-galactosidase protein **Lots of non-specific binding**

Matching w/
Darkly
stained
entire blot
Only ladder
No bands at all
Band in + alpha/ - treatment but no band in + alpha/
+ treatment Lots of non-specific binding
No band in + alpha/ - treatment but dark band in + alpha/ + treatment

Materials

In Biocore we teach five lab sections of 20-24 students each week (total of 100-120 students/week). Materials supporting our curriculum are contained in these appendices:

- Appendix E - lab bench equipment provided for each team of 4-6.
- Appendix F - pictures of two shared, community equipment and supplies benches that teams use during open lab weeks.
- Appendix G - Materials and Schedule document that each student team fills out after week 2 feedback presentations.
- Appendix H - examples of independent variables that our students have chosen.
- Appendix I - timeline form that each student team fills out before they perform the Western Blot.
- Appendix J - week 2 pre-lab assignment.

Please contact MH (maharris@wisc.edu) for:

- Week 1 Yeast Introductory PowerPoint slideshow with embedded iClicker assessment questions.
- Biocore Tools & Techniques Manual

Notes for the Instructor

Our Challenges

The large variety of novel research questions that our students pursue results in an exciting, ever-changing teaching and learning environment. Instructors must be nimble, creative, open-minded and flexible. While we invest in learning the basic elements of yeast signal transduction and the mating response, we cannot know everything, and we make this clear to our students. We have learned to be comfortable with not knowing; instead we try to model genuine curiosity and logical questioning. We have also found that it is delightful to learn from our students.

Our large teaching teams do present a quality control challenge. We mentor one graduate teaching assistant (TA) + one undergraduate teaching assistant (uTA) per lab section x 5 lab sections. We have also trained several teaching postdocs. Such a large instructional team requires open and consistent communication between all members. Each semester we facilitate two half-day teaching workshops for our TAs. Instructors, TAs and uTAs also meet weekly for 1.5-2 hours to reflect on student progress and teaching approaches during the previous week, to discuss upcoming lesson planning, and to normalize grading expectations. These investments in the training of our teaching team means that our students receive high quality, consistent, and timely feedback.

General Advice

Because each student team poses a novel hypothesis and designs their own experimental protocol, the logistical support of numerous independent projects presents some of our greatest teaching challenges. We list here general advice for instructors:

1. Require students to support their hypothesis with a clearly articulated logical biological rationale ("biorationale") that links independent variable(s) to the dependent variables measured. Logical biorationales are supported by relevant literature, state key assumptions, and point out the knowledge gap to be addressed by the data.
2. Only approve proposed projects that can be carried out safely, are do-able in the 2-week timeframe allowed, and that address a novel/reasonable knowledge gap. We also give each team a spending budget for supplies we do not already have on hand. Students are responsible for researching safe handling and disposal procedures for their chosen variable. Teams must also find out how/where to order any unique consumable supplies, and the cost (see Appendix G for a Materials & Schedule documents that each team must fill out).
3. Support students' data analysis by introducing relevant statistical analyses and graphical options.
4. Data collected by students will vary based on the novel experimental design used by each team. Be flexible, helping students devise "plan B's" to address unexpected protocol challenges.
5. Students often struggle to integrate data from the three lines of evidence for cell signaling (cell cycle arrest, mating morphogenesis, and mating gene transcription) to make a conclusion about their hypothesis. Be prepared to help students with this data integration.
6. It helps if students have open access to classroom lab facilities in order to begin yeast cultures, add alpha factor and conduct experimental treatments outside of class time.
7. Have students present a timeline for their

experiment. This requires them to mentally walk through the necessary steps involved in growing the yeast in preparation for their experiment.

8. Encourage students to do small-scale, relatively quick pilot studies in the first week of open lab before finalizing their data collection protocol. Expect them to repeat their experiment in the second week of open lab, and address if/why they observed variability in their data between the two weeks.

Construct student groups based on knowledge of students' previous performance and group work attributes. Consider making team efforts a part of each students' graded assessment, providing opportunities for students to evaluate the contribution of their teammates as well as each students' graded assessment, providing opportunities for students to evaluate the contributions of their teammates as well as themselves. (See Group Effort Analysis (GEA) rubric in our Biocore Writing Manual; available online www.biocore.wisc.edu/bioresources).

Lab Safety Information

Sodium dodecyl sulfate, chloroform and 2-Mercaptoethanol are hazardous in cases of skin contact, eye contact, ingestion, or inhalation. Gloves, goggles and appropriate clothing should be worn during experimentation. Refer to Material Safety Data Sheet guidelines for each compound and follow disposal procedures in accordance with federal, state and local environmental control regulations.

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About the Authors

Faculty Associate Michelle Harris has been an instructor in the University of Wisconsin-Madison Biocore Program since 1999, is co-chair of the second semester cell biology lab, and chair of the third semester physiology lab course. Seth McGee has served as Biocore's Lab Manager since 2002 and is a co-instructor in the first and second semester Biocore labs. Janet Batzli has been Biocore's Associate Director since 2002, and is the chair for the first semester ecology, evolution and genetics lab and co-chair of the second semester cell biology lab.

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