**BIOLOGY 510 RECOMBINANT DNA TECHNIQUES LABORATORY**

 Lab: MW 2:00 – 4:50 p.m., 224 T.H. Morgan

 Lecture: F 2:00 – 2:50 p.m., 109 T.H. Morgan

**Instructors:**  Dr. Brian Rymond, 335A T.H. Morgan Building, 257‑5530, rymond@uky.edu

Office hours Tuesday and Thursday 9:15 ‑ 10:15 AM (or by appointment)

**Assistants:** Min Chen, 335 THM (chen.min@uky.edu)

**Course Content:** This four-credit course will familiarize the advanced undergraduate and the beginning graduate student with the theory and practice of recombinant DNA technology and molecular genetic applications. Emphasis is placed on learning though direct experimentation. The techniques and skills acquired (e.g., prokaryotic/eukaryotic cell transformation, DNA isolation and subcloning, RNAi knockdown, in vitro mutagenesis, protein purification, DNA sequencing, RNA analysis, PCR, phenotypic selection, *in vitro* transcription ‑ among others) are broadly applicable in modern medical, industrial and basic biological research. The Friday lectures will supplement the laboratory assignments with background information and describe alternative or additional methodologies to what is done in the laboratory. In general, the Friday lectures will not involve discussion of the laboratory assignments or results.

**Prerequisites:**This advanced course has requirements for previous coursework in GENETICS [Bio 304/404G or equivalent] and CELL BIOLOGY (BIO 315 or equivalent; Biochemistry BCH 401G can substitute). Students may bypass these prerequisites upon consent of the instructor. However, students are expected to apply genetic and biochemical principles in their understanding of recombinant DNA technology. Time does not permit the review of basic genetic or biochemical principles for BIO 510. Students that require additional background preparation should consult the resources listed below.

**Required Textbook & Background resources:** *Principles of Gene Manipulation and Genomics,7th Edition* S.B. Primrose, R.M. Twyman (Blackwell Science Press, 2006). Laboratory exercises will be provided by the instructor in class as a protocol set. The Cold Spring Harbor Press web site (<http://www.cshlpress.com/>) is a particularly rich source of excellent reference manuals on PCR techniques, molecular genetics, bioinformatics, and other topics related to molecular biology. Good books for background reading include *GENES* *VII* (B. Lewin, Oxford Press, 2000), *Molecular Biology of the Cell* (B. Alberts et al., Garland Press, 2002); *Genomes* (T.A. Brown, Garland Science, 2002), Biochemistry (Berg et al., [W. H. Freeman & Co.](http://www.whfreeman.com/college/book.asp?disc=BIO&disc_name=Biology&@id_course=1058000060&id_product=1124001045), 2002 and [*Modern Genetic Analysis*](http://www.ncbi.nlm.nih.gov/books/bv.fcgi?call=bv.View..ShowTOC&rid=mga.TOC)(Griffiths et al., 1999). These (and related books) are **available free online** at: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=books> . Many other relevant books can be found at the UK library.

**Additional Materials:** Each student will receive a copy of ***New England BioLabs*** (NEB) product and resource catalog. The NEB catalog contains a wealth of information on many of the enzymes and reagents commonly used in a molecular biology laboratory. In addition, this book contains multiple appendices with practical information (genotypes of common bacterial strains, restriction maps, genetic code, etc.) and is well referenced throughout with primary literature citations.

**PLEASE NOTE** that BIO 510 quizzes and exams will cover information presented in lectures, lab handouts, homework assignments, and other assigned readings (textbook, NEB catalog, special in-class assignments).

**A lab coat must be worn at all times in the laboratory.** Please have it on *before* the start of the lab. Also, safety regulations state that ***you cannot wear open-toed shoes such as sandals in the BIO 510 laboratory***.

**Web site link:** [**https://bio.as.uky.edu/rymond/bio-510**](https://bio.as.uky.edu/rymond/bio-510)

**Grading Policy:** Your grade will be assigned based on your performance in:

**Exam 1 25%**

**Exam 2 25**

**Quizzes or homework 30**

**Notebook 10**

**Class participation 10**

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 **100%**

The first half of BIO 510 emphasizes techniques and approaches whereas the second half of the course focuses more on application of techniques and experimental design to address questions in biology. Questions on quizzes and examinations may include essays, short‑answer and multiple choice questions. One or more quizzes may be “open book” and require you to use your New England Biolabs catalog in class.

Homework exercises will be announced in class or listed in the lab manual or on the course web site; unless otherwise noted the ***homework is due one week from the assignment date*** (for instance, a homework assignment appearing in a Monday lab assignment is due the following Monday).

Students are expected to accurately record and analyze every laboratory exercise in a laboratory notebook. The lab manual may be used instead of a separate lab notebook; please use blank sheets (or the back of protocol pages) to record you data. The notebook will be graded twice, once on the day of the mid-term exam and again at the end of the. In order to maximize the likelihood of getting full credit*,* ***students are encouraged to ask the instructor for feedback*** on notebook quality prior to submission for grading.

Graduate students are required to complete two additional homework assignments; one in the first half of the semester and one in the second half. The undergraduates have the option of completing these assignments and dropping two other homework assignment (but not quiz grades) from the average quiz + homework grade calculation. Note that only one homework assignment can be dropped per half semester (that is, one before the midterm and one after the midterm – you CANNOT drop two assignments from either the first half or second half).

**Numerical Grade Letter Grade** (The +/- grading system will not be used for BIO 510**)**

100‑90 A

89‑80 B

79‑70 C

69‑60 D (not available for graduate students)

<60 E

**Attendance Policy:** Attendance is mandatory. At a minimum, multiple (2 or more) unexcused absences (as defined by the UK University Bulletin) will lower your final grade by one letter.

**Plagiarism and Cheating Policy:** The University of Kentucky recently revised its rules on plagiarism and other forms of academic cheating. Infractions of these rules may result in serious consequences, including but not limited to receiving a failing grade of “E” for this course. A full description of this policy, implementation procedures, and outcomes can be obtained at: <http://www.uky.edu/Ombud/Plagiarism.pdf> . Students are encouraged to read this policy.

**The textbook – what it does and does not do.** Our textbook provides a general overview of virtually all the techniques and approaches we use in the laboratory. It includes many scientific citations of historical interest and references relevant to technical innovations and other topical, directed reading. The text helps bring our exceptionally diverse enrollment up to a common level of understanding and is a useful starting point for our lectures and discussions. ***The textbook is not a lab manual and is not meant to explain the lab exercises****.* In addition, this textbook is not meant to replace the primary literature in your scientific education.

**Lab order and design.** Many protocols in molecular biology might be classified as “hurry up and wait” experiments where one step in a protocol (e.g., cell transformation) is initiated then requires hours (or days) to “finish”. A major goal of the lab is to have students learn and apply as many molecular biological approaches as possible within the time constraints of the lab, consequently, ***we usually work on several different lab exercises in parallel.***That is, we carry our “parts” of several experiments in each lab meeting. The flow chart shown below provides an overview of what we hope to accomplish during the first half of the semester. A central “theme” of this work might be termed “characterization of a eukaryotic gene”. **The most important goal,** however, is for you to learn as much recombinant DNA technology as possible. With this in mind, you will note that we also include number of experiments that teach important concepts/techniques but do not directly relate to the characterization of the DNA provided in our first working lab.

**Student participation.** The success of this class depends upon your active participation. Your opinions are valued, please be an active participant - ask questions, present your data to your lab partners/the TAs/instructors, discuss your own lab experiences & professional goals, suggest lab protocol modifications, consider alternative approaches. We use three approaches to enhance student interaction during the semester. These are the Q&A strips (see below), a one-on-one instructor conference, and a homework assignment to “design a lab” for next year’s class. Other ideas to encourage participation are welcome.

**Question and answer (Q & A) strips** are to be submitted the day of participation (explained in class); please print your name clearly. No “first half” Q&A strips will be accepted after October 13th. Question and answers must be directed to Dr. Rymond verbally in class and must be 1) clearly formulated to raise or answer a substantive point of discussion, and 2) spoken loudly enough to be heard by the entire class. No simple (yes, no, why, I don’t know) type responses/questions will be accepted. Each question and answer strip will count equally and **will be summed to form the class participation grade** in the first half of the semester. Undergraduates cannot use this as one of their “dropped” homework assignments.

**Meet with the instructor:** We will schedule one office visit for each student during the semester. The purpose of this visit is to gauge student learning and to provide feedback opportunities. Attendance at this meeting will be scored as one Q & A strip.

**FIRST Half of the Semester**

**Thematic Goal: Characterization of a Eukaryotic Gene**

For the sake of organization (& fun), many of the exercises in the first ½ semester will simulate an actual research experience in which you will test the hypothesis that an unknown segment of DNA provided by the instructor encodes an essential eukaryotic gene.

**Flow Chart**

Isolate a "foreign" DNA molecule (gel purification of the "insert" DNA)

↓

Create a recombinant DNA molecule (ligate the insert DNA to a plasmid vector; prepare competent cells and transform *E. coli*)

↓

Purify the recombinant DNA (plasmid DNA miniprep; restriction digests)

↓

Determine whether the cloned DNA contains a transcribed gene (RNA extraction from yeast; 32P labeled probe preparation by vitro transcription; northern hybridization with +/‑ sense probes)

↓

Establish the DNA sequence of the cloned gene (single stranded DNA preparation from virally infected cells; DNA sequence analysis)

↓

Demonstrate that the cloned DNA actually defines the gene detected by hybridization

(in vivo complementation; yeast transformation)

↓

**Other experiments included in this half of the semester:** Identify a genomic DNA polymorphism & inverse PCR as a mutagenesis tool; RNAi knockdown and phenotypic characterizations; purification of a recombinant protein (a previous year’s “design a lab” winner)

**Along the way,** you will also learn other valuable techniques: RNAi disruption of gene expression; preparation of yeast genomic DNA; organic extraction of proteins; nucleic acid concentration by ethanol precipitation; gene cloning by insertional inactivation and selection; native agarose gel electrophoresis; denaturing agarose and denaturing polyacrylamide gel electrophoresis; probe fractionation & quantification; autoradiography; isolation of a recombinant protein by affinity selection; safe use of radionucleotides and lab equipment.

**Second Half of Semester**

**Thematic Goal: Defining and testing a novel hypothesis in Biology**

**Preparation: Read the article “Next-Generation Sequencing Techniques for Eukaryotic Microorganisms: Sequencing-Based Solutions to Biological Problems” on the lab web site.**

The results of a recent “deep sequencing” study will be presented and used to develop a hypothesis concerning the intracellular function of the Prp43 RNA-dependent ATPase. Students will work in teams to test aspects of this hypothesis.

In addition, we will conduct a yeast two-hybrid (Y2H) experiment to map a protein-protein interaction domain in vivo, a chemical random mutagenesis experiment to generate loss-of-function mutations in an antibody resistance gene, use RNase protection to compare gene expression between two strains, affinity purify a recombinant protein and do a western blot to visualize an epitope-tagged protein.

**Tentative Class Schedule**

**Reading assignments**. The reading assignments are offered **roughly** in the order of presentation; completion in the timeframe presented below will enhance understanding in the lab and lecture assignments (ideally, the reading is completed by the date of assignment). However, please know the precise topic of discussion on a given day may not match the description below as student interests & questions can influence the presentations. You do not have to memorize the tables & graphs assigned in the NEB catalog but you will need to know how to use these as tools for homework or in open book exam segments. **Homework is due** one week after the assignment is made. **GRADED HOMEWORK ASSIGMENTS are in bold.** ***Note*** *that the quizzes and exam will cover the lab assignments & discussions, required readings & homework, and the Friday lectures.* Reading assignments in addition to those listed below are found in the lab manual and others may be assigned during the semester.

**Tentative Class Schedule**

**Aug. 22**: Course overview; lab group assignments, online safety training; basic lab technique.

1. [**Chemical Hygiene Plan/Laboratory Safety**](http://ehs.uky.edu/classes/chemhyg/train.html)
2. [**Hazardous Waste**](http://ehs.uky.edu/classes/hmm/hwaste/training.html)
3. [**Fire Extinguisher**](http://ehs.uky.edu/classes/fire/firetrain.html) **Use**
4. **Biological Safety**

**These online training sessions can be accessed at:** [**http://ehs.uky.edu/classes/**](http://ehs.uky.edu/classes/)

**Note: after logging in, select “8E300 Department of Biology” as the Department listing.** You are required to provide a certificate for completion for each of these by the end of the second week of classes.

*Friday, August 24:* Historical perspectives on recombinant DNA technology & toolbox of enzymes

**Aug 27: LAB 1.** Agarose gel fractionation & purification of DNA; double restriction endonuclease digestion of vector DNA. Reading assignments: PDF files on Qiagen agarose gel recovery; New England Biolabs (NEB) catalog pages 16, 48 (HindIII), 44 (EcoRI); 111; 158; 272-277 **homework assignment**

**Aug 29: LAB 2.** Influence of DNA conformation on gel mobility; preparation of transformation competent *E. coli.* EtBr vs SYBR safe; PDF files on pTZ19u map (print out for your notebook); 1 kbp DNA ladder (print out for your notebook) map, SYBR safe; NEB 108-109; 175-177; 168; 318-319 Principles of Gene Manipulation & Genomics (PGMG) chapters 1-3 and Box 15.1 on pages 310-311 **homework assignment**

*Friday August 31***:**  Restriction endonucleases and associated methylation enzymes; specificity, function, and conditions of use

**Sept 3:** **Labor Day – academic holiday**

**Sept 5: LAB 3.** Joining DNA ends with T4 DNA ligase; isolation of total RNA from yeast; impact of increased salt concentration on DNA migration; NEB 129-130 (CIP)

190-191 (Impact system); 278-321 (know how to read charts); 322; 329 **homework assignment**

*Friday September 7:* RNA- and DNA-dependent polymerases

**Sept 10: LAB 4.** *E. coli* transformation; determination of RNA yield and purity. NEB 200-202; 323; 352-355 **short quiz and** **homework assignment**

**Sept 12:LAB 5**. Phenotypic characterization of recombinant DNA clones; fractionation of RNA by denaturing gel electrophoresis; northern transfer, Riboprobe manual PDF; PGMGpage 408, **homework assignment**

*Friday September 14:* Radiation Safety 2 lecture; (Radiation Safety Officer, Fred Rawlings)

**Sept 17: LAB 6.** Isolation of recombinant plasmid DNA from *E. coli*. Qiagen Miniprep PDF; Epicentre Yeast DNA isolation PDF; NEB 84-88; 97; 166-171; 325; PGMG chapter 4 (pp55-66)

**Sept 19: LAB 7.** Analysis of recombinant clones; preparation of in vitro transcription template; northern blot pre-hybridization; isolation of genomic DNA from *Saccharomyces cerevisiae.* Seraphin group TAP web page (see course web site); PGMG Chapter 6 (pp 96-116)

*Friday September 21*: Common RNase and non-restriction enzyme DNase activities

**Sept 24: LAB 8.** *In vitro* transcription; chromatographic separation of RNA and unincorporated; ribonucleotide triphosphates; NEB 137 (M13K07) **homework assignment**

**Sept 26: LAB 9.** Hybridization of northern blot with strand-specific probes. preparation of single‑stranded DNA template for DNA sequencing. RNAi knockdown, *Caenorhabditis elegans* (part 1**)** PDF files on Realtime PCR and I-cycler; PGMG chapter 7, chapter 15 (pp 315-319)

*Friday Sept 28:* DNA & RNA polymerases

**Oct 1: LAB 10.** . Wash blots; autoradiography; Analysis of northern blot; quality check on single stranded DNA sequencing template; RNAi-knockdown in *C. elegans* (part 2); PGMG chapter 9; PDF file on Typhoon; **short quiz**

**Oct 3**: **LAB 11**. Analysis of northern blot; PCR amplification of genomic DNA; inverse PCR plasmid mutagenesis; assembly of template/primer hybrid; gene identification by in vivo complementation. RNAi-knockdown in *C. elegans* (part 3). Reading, Affy hybridization and analysis PDF; PGMG chapter 23 (protein-interactions),chapter 8 (pp141-146) **homework assignment**

*Friday October 5:* RNAi as an experimental tool*,* Ligase, kinase, phosphatase, proteases, recombinases,

**Oct 8**: **LAB 12.** Analysis of PCR reactions; Yeast two-hybrid (Y2H) experiment, part I; “plasmid shuffle” to score for protein function, part I; completion of *C. elegans* experiment & any make-up experiments and review. PGMG Chapter 4

**Oct 10: Exam 1 & Notebooks due**

*Friday October 12* Bacterial plasmids, promote and protein fusions

**Oct 15: Lab 13** Yeast two-hybrid (Y2H) experiment, part I; “plasmid shuffle” to score for protein function, part II

**Oct 17: Lab 14** Real-time PCR; Isolation of a recombinant protein, part I; Y2H, part II; PGMG Chapter 11 (pp 202-212)

*Friday Oct 19*: Gene expression studies, DNA microarray & nanodrop technology

**Oct 22: Lab 15** Y2H, part III; Isolation of a recombinant protein, part II, PGMG chapter 20

**Oct 24: Lab 16** Isolation of a recombinant protein, part III; Validation of deep sequencing predictions, part I, cDNA synthesis, part I

*Friday Oct 26*: Visit to the UK Microarray Core Facility

**Oct 29: Lab 17**; Validation of deep sequencing predictions, part II, rtPCR and northern blot;

RNase A mapping, part I; Short quiz

**Oct 31: Lab 18** Validation of deep sequencing predictions, northern pre-hybridization & probe preparation by random prime labeling, RNase A mapping, part II

*Friday Nov 2:* DNA and RNA sequencing strategies

**Nov 5: Lab 19** Validation of deep sequencing predictions, hybridization**;** RNase A mapping, part III; rtPCR validation of new introns

**Nov 7: Lab 20** Validation of deep sequencing predictions, washing blots; Random mutagenesis of pBR322, part I (EMS mutagenesis) PGMG page 404 (chemical mutagenesis)

*Friday Nov 9*: *Visit to the Advanced Genetic Technology Center*

**Nov 12: Lab 21** Analysis of northern blots; Random mutagenesis of pBR322, part II (DNA isolation); short Quiz

**Nov 14: Lab 22** Random chemical mutagenesis of pBR322, part III (E. coli transformation); Detection of epitope-tagged proteins by Western blot, part I (protein preparation) PGMG chapter 21

*Friday Nov 16* Interactome – genome-wide studies on gene & protein interactions

**Nov 19: Lab 23** Random chemical mutagenesis of pBR322, part IV (replica plate); Detection of epitope-tagged proteins by Western blot transfer & PAP antibody presentation, part II

**Nov 21: Thanksgiving Break, academic holiday**

*Friday Nov 23:**Thanksgiving Break, academic holiday*

**Nov 26: Lab 24** Random chemical mutagenesis of pBR322, part IV (replica plate results); Detection of epitope-tagged proteins by Western blot, part III (HRP & AP detection) PGMG 26

**Nov 28: Lab 25** Detection of epitope-tagged proteins by Western blot, part IV (AP detection) short Quiz

*Friday Nov 30 Visit to the Proteomics Core Facility*

**Dec 3: Lab 26** Complete ongoing experiments, final lecture topics

**Dec 5: Lab 27** Lab cleanup, exam review

*Fri Dec 7 TBA*

**Dec 12** – Final exam, 1:00 PM, THM 109

**Homework Assignments & Plagiarism**

**CUT and PASTING text from the internet or other direct quotation from web sites or other sources, including you lab partner, is not allowed even if attributed.**

**When is it allowed and appropriate?** When reporting the output of a Web-based computer program you ran such as BLAST, or the RNA folding programs, or when providing a data in the form of a figure or table from the primary literature (that is, a journal article). In last case, the full literature citation must also be provided.

**Otherwise, when is it allowed?** Almost never – the one exception that comes to mind is copying the literature citation itself, e.g., *Wang, Q., He, J., Lynn, B. and Rymond, B.C. 2005. Interactions of the yeast SF3b splicing factor. Mol. Cell Biol, 25: 10745-10754.* Unless otherwise directed, use scientific journal citations only (**not** textbook, Wikipedia, or sales materials).

In all other cases, your response to a question should be ***your own intellectual output.***  Using search engines to find relevant information is fine, but simply transferring such output from machine to paper is not the goal. You are being asked to read, integrate, and respond in your own words – showing that you understand the issue under question, not simply that you are adept in keystrokes and database mining.

**Direct, unattributed quotations represent plagiarism, are unethical and may be illegal. The first instance of plagiarism will result in a grade of “0” on the assignment. Additional instances of plagiarism will be treated much more seriously and could even result in dismissal from the University, see:** [**http://www.uky.edu/StudentAffairs/Code/part1.html**](http://www.uky.edu/StudentAffairs/Code/part1.html)

 and also:

<http://www.uky.edu/Ombud/Plagiarism.pdf>

**Direct, attributed quotations are appropriate *only if I ask for direct, attributed quotation*s – otherwise, everything should be in your own words.**

**LAB 1**

 This section of Bio 510 will focus on the structural characterization of a yeast transcription unit. We will first recover a possible gene‑containing DNA fragment from an agarose gel and insert this DNA into two convenient plas­mid vectors, the pTZ18u and pTZ19u purchased from USB/Amersham Corporation. As will be discussed today, these vectors allow high level DNA amplification in *E. coli,* and permit the in vitro synthesis of gene‑specif­ic single stranded RNA and the recovery of either double stranded or single stranded DNA from bacterial cultures. We will use the RNA synthesis capacity of these vectors to prepare RNA probes that will allow us to whether this DNA contains a gene that is expressed in yeast and, if so, to deduce the direction of transcription with respect to physical markers (e.g., restriction endonuclease recognition sites). Single‑stranded DNA will be prepared for DNA sequencing in order to learn the primary structure and, ultimately, the identity of the cloned gene.

**NOTEBOOK REQUIREMENT (grading policy):** Note any changes (errors or alternative conditions) in protocol that you make during the experiment in the margins of your lab manual. **Observations must be recorded for all experiment results (color, size of nucleic acid pellets; relative DNA band stain intensities; number of yeast colonies, etc.).**  Document your observations on the back side of your lab manual pages and/or pages added to the end of each experiment. In addition, include in your notebook all photographs, graphs, etc. and the answers to all questions asked in your manual. **EVERY experimental result should be analyzed and discussed – even if the manual does not ask a specific question.**  Were the results obtained as expected based on the experimental design? If so, provide details, if not, offer possible explanations for the discrepancy.

**NOTE: LabLife is a free web-based program useful for keeping and storing information about plasmids, creating restriction maps, inventories of bacterial and yeast strains, protocols, etc (see:** [**https://www.lablife.org/ll?section=mission&page=first**](https://www.lablife.org/ll?section=mission&page=first) **). While not required for use in class, students may find this program helpful this class and (more importantly) for use in their research laboratories.**

**Agarose Gel Fractionation & Purification of a DNA Fragment**

Here you will electrophoretically separate and then isolate a yeast EcoRI-HindIII DNA restriction fragment from second piece of DNA present in the preparation. The recovered DNA fragment contains a segment of the yeast (*Saccharomyces cerevisiae)* genome that we will analyze for the presence of a transcription unit. This gel isolation procedure can also be applied to recover size fractionated molecules from complex mixture of DNA (e.g., cleaved genomic DNA).

**NOTES**: The second piece of DNA (which we will not purify) is a linear form of the plasmid pUC19 (NE BioLabs) which contains some, but not all of the features that we want in our subcloning vector. ALWAYS wear gloves and a lab coat when working with any DNA or RNA sample.

**Each Group of Two (read the pdf file on the Qiagen QIAquick spin handbook – gel recovery section)**

‑Mix 4 μl of dye (50% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol, 5X TAE) with 15 μl of your DNA digest in a clear microfuge tube. Pipet 9.5 µl of this DNA/dye mixture carefully into each of two wells of your 1 % agarose gel. Run at 70 volts until the fast dye (bromophenol blue) migrates ½ to 3/4 the gel length.

‑With gloved hands remove the gel by lifting the glass slide (not the gel) on which it rests. Place the gel in the 2.0 μg/ml ethidium bromide solution. Ethidium bromide is mutagenic and a suspected carcinogen, take care not to get it on your skin.  *Go to our class web* site to find information the structure of ethidium bromide and safe handling procedures. Also, see PGMG pages 16-17, 56.

‑After 15 minutes in the EtBr stain remove the gel and rinse it briefly with water.

‑Place the gel on the UV transilluminator to visualize the DNA bands. Two sharp bans should be visible (and perhaps a third, fuzzy one near the bottom of the gel). NOTE: Wear UV-protective goggles when using the transilluminator.

‑Excise the second band from the top (i.e., well side) of the gel with the plastic knives. **Work quickly** since the UV light damages the DNA in your sample (and in your skin). Try to limit the amount of excess gel (that is gel that borders but does not contain DNA) as excel gel reduces you DNA yield. Place equal amounts of this gel slice in each of two microfuge tubes ‑ **one for each lab member.**

‑Estimate the volume of your gel slice by spinning the sample briefly (~ 1 minute) in the microfuge to compress the gel. Alternatively, weigh the gel slice on pan balance after using an empty tube to TARE the scale – as this is mostly water, assume 100 mg is roughly 100 µl of gel volume. Add three volumes volume of QG buffer (proprietary mix containing guanidine hydrochloride or guanidine isothiocyanate) to the gel slice.

* Incubate for 15 minutes at 55C. (Vortex briefly at 10 minutes, then replace at 55C)
* **Binding is to the matrix is strongly pH dependent**; pH must be less than 7.5. To be sure your pH is appropriate, add 5 μl 3M NaOAc (pH 5.0) and mix on the vortex.
* Add 1 **gel volume** amount (NOT gel volume plus QG) of isopropanol. Vortex.
* Put a QIAquick (Qiagen) spin column (containing a silica-based gel resin) in a 2 ml collection tube. Add DNA to the column, spin 1 minute in a microfuge (full speed). DISCARD the flow-through.
* Add 0.5 ml of QG to column, spin 1 minute as before. Discard the flow-through.
* Add 0.75 ml of PE wash buffer (proprietary composition), allow to sit at room temperature for 1 minute then spin for 1 minute as before, and discard the flow through.
* Spin again for an additional 1 minute (to remove residual liquid).
* Place the column in a clean 1.5 ml microfuge tune.
* Add 50 μl of elution buffer (EB 10 mM Tris, pH 8.5) pre-warmed to 55C, INCUBATE at room temperature for 1 minute. Substitution of water for EB is NOT recommended as the pH difference may result in lower recover. Spin 1 minute.
* Your DNA is now ready for ligation; store at -20C until use. **Please remember to label your tube with the date, your initials, and a description of its contents (e.g., 8/29/11, BR, purified “insert” DNA).**

The web site for Qiagen gel kit (QIAEX II) and related products is: <http://www.qiagen.com/literature/default.aspx?Term=&Language=EN&LiteratureType=1&ProductCategory=230>

 *You will also find the Qiagen kit information in a PDF file on our class web site.* **NOTE:** The Qiagen kits are very convenient and work well, but are expensive to use. If high-grade low melt agarose is used, ligations can generally be done directly in the melted gel slice (provided that magnesium and ATP are added) without further purification.

**Double Restriction Enzyme Digestion of Plasmid DNA – Preparing the Vector for Directional Cloning.**

This protocol allows you to prepare a plasmid vector for asymmetric insertion (i.e., directional cloning) of the DNA recovered from the agarose gel (i.e., your “insert” DNA). The DNA maps/sequences for the pTZ19u vector can be obtained at: <http://fermentas.com/en/products/all/molecular-cloning/sd014> **Go to this site, print out the information, and include it in your lab notebook**. The pTZ18U vector is identical except that the orientation of the multiple cloning site (also called a polylinker) is reversed relative to the T7 promoter (in 19u the orientation is: T7 HindIII->EcoRI; in 18u the orientation is: T7 EcoRI-HindIII).

**Each Student**

Digest your pTZ vector (18u or 19u) with EcoRI in a final volume of 25 μl:

10.5 μl water

2.5 μl 10X HindIII salts (1X = 10 mM Tris‑HCl, 10 mM MgCl2, 50 mM NaCl, 1 mM dithiothreitol, pH 7.9; final concentration)

11 μl DNA (2 μ g, ~ 1 picomole of 18u or 19u but not both)

1 μl EcoRI (or 10 units; excess units because our lab time is short & you want to ensure a complete digestion)

Vortex the sample very briefly (~2 seconds) and then spin briefly (~5 seconds). Note that the air/liquid interface (foaming) can denature proteins, so limit the amount of vortexing time and avoid conditions that cause foaming.

‑Incubate for 15 min at 37oC.

‑Next, add 1 μl (or 10 units) of Hind III followed by 1 μl (0.3 unit) of shrimp alkaline phosphatase(SAP). Mix well then spin as before and then incubate at 37oC for 30 min. For description of SAP see <http://www.promega.com/tbs/9pim820/9pim820.html> (note: you might need to select the “United States” country of origin before being routed to the SAP page)

‑Add 150 μl of TE, extract 1X with 150 μl of phenol/CHCl3/isoamyl alcohol (50:49:1) by repeating the vortexing over a 5 minute period. Spin the sample in the microfuge for 5 minutes at full speed. ***Be careful***, this organic solvent mix can burn your skin.

‑Remove upper phase (aqueous, includes DNA) place into fresh microfuge tube.

‑Add 100 μl of 3M sodium acetate and 1 ml of 100% ethanol, vortex. Label your tube with the date, your initials, and the experiment (i.e., pTZ18u E/H). Place in the -20 freezer until the next lab period.

**NOTE**: In order for DNA or RNA precipitation to work, *the sodium acetate and ethanol must be* ***mixed well*** with your sample.

**EcoRI recognition site = 5'---G/AATTC---3'**

 **3'---CTTAA/G---5’**

**HindIII recognition site = 5'---A/AGCTT---3'**

 **3--- TTCGA/A---5'**

The slash (/) indicates the position of endonucleolytic cleavage, the dash (-) indicates an indeterminate length of DNA flanking the cleavage sites.

**HOMEWORK QUESTION**

**Question 1**. Go to the literature (journal articles only, no Web sites, **no vendor/supplier catalogs**, or books, please) and identify one transformation/transfection vector for each of the following. Use PubMed to find the information (<http://www.ncbi.nlm.nih.gov/sites/pubmed> ) although the index in PGMG may provide some leads).

1. Tobacco plant *(Nicotiana tabacum)* vector (1 point)
2. Fission yeast (*Schizosaccharomyces pombe*) (1 point)
3. a vector that can be used in human tissue culture (1 point)

For each, provide the following information:

1. the vector type (plasmid, virus, etc) (2 points)
2. a literature citation (provide all authors, article title, journal, volume, pages) that describes use of the vector (1 points)
3. the selectable marker or screening marker. State whether this is a selectable marker or a screening marker, say what the gene is and how it is used (for instance, if an antibiotic resistance gene, state which drug is used) (2 points)
4. Answer the following question: What is the basic difference between a gene that can be used for genetic selection vs a gene that can be used in a genetic screen? (2 points)

**Here are recipes for two common buffer systems** used for nucleic acid gel electrophoresis.  In BIO 510 we use TAE for non-denaturing agarose gels and, later, TBE for denaturing polyacrylamide gels.

**TAE Gel Buffer** =  40 mM Tris-acetate, 1 mM EDTA (final concentration of 1X working solution). To prepare 1L 50X stock mix 242 g Tris base with 57.1 ml glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0).  Final pH is ~8.1.

**TBE Gel Buffer** = 0.089 M Tris-borate, 25 mM EDTA (final concentration of 1X working solution). To prepare 1 liter of 10X stock mix 108 g Tris base with 55 g boric acid and 9.3 g  disodium EDTA (dihydrate) (final pH 8.3).

**Check out the Human Genome Project Web site at:**

<http://www.ornl.gov/sci/techresources/Human_Genome/project/timeline.shtml>

Ethidium bromidesilica

**Chaotropic agents:** Materials (e.g., high KCL or guanidinium isothiocyanate) which interfere with the structure of water and can increase the solubility of nonpolar compounds in aqueous environments. Here guanidinium isothiocyanate promotes the binding of DNA to silica resins in the Qiagen column (which does not occur in low salt). This is the basis for many DNA recovery systems. See: Chen, C.W. and Thomas, C.A. Jr. (1980) Recovery of DNA segments from agarose gels. Anal. Biochem.**101**, 339–41. Marko, M.A. et al. (1982) A procedure for the large-scale isolation of highly purified plasmid DNA using alkaline extraction and binding to glass powder. Anal. Biochem.**121**, 382–7. Boom, R. et al. (1990) Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol.**28**, 495–503.

**LAB 2**

Today we will use agarose gel electrophoresis to compare the mobilities of the linear and circular forms of the pTZ18u/19u vector DNA. We will also resolve the purified linear DNA fragment (henceforth called the "insert" DNA) isolated previously and determine its apparent size based on its mobility relative to a set of DNA size standards. In addition, we will make *E. coli* cells competent (receptive) for DNA mediated transformation.

**Influence of DNA Conformation on Gel Migration**

The mobility of DNA in an electric field depends upon numerous factors, including the type and concentration of the separation media, the buffer’s salt concentration and pH, the conformation of the DNA molecules, the strength and direction of the applied electric field. Today you will observe the electrophoretic migration of the various conformational forms present in a standard plasmid preparation from *E. coli*. Also see page 56 in PGMG.

**Each Lab Member**

‑Spin your microfuge tubes containing the vector DNA in the microfuge for **5** minutes on the highest speed setting.

‑Remove the supernatant with a sterile 1 ml pipet tip. It is important to remove the ethanol *without* disturbing your pellet. Leave no more than 30 μl behind (to see what 30 μl "looks like" pipet this volume of liquid into a fresh tube (a tube with nothing in it). Mark the top of the meniscus with your magic marker for future reference).

‑Add 1 ml of fresh 80% ethanol to the DNA pellets. Mix briefly and then spin for 2 min. in the microfuge. Note: the precipitated DNA will not re-dissolve in the 80% ethanol – so don’t worry about using the vortex to mix.

‑ Remove the supernatant. As before, add 1 ml of fresh 80% ethanol to the pellet, and spin again for 2 minutes. This second rinse makes sure that you carry over no salt which might inhibit the subsequent enzymatic steps.

‑Remove the ethanol with the P1000 then use the P20 to remove as much additional ethanol as possible. Place your tubes into the Speed-vac vacuum dryer (this really three machines, a centrifuge, a vacuum pump, and a refrigerated vapor trap).

‑When the samples have dried (~10 minutes), add 20 μl of sterile water to the vector.

- Thaw the “insert” DNA from the last lab.

- Vortex both the insert and vector tubes and then spin briefly (5 seconds) to collect the DNA into the bottom of the tube. **Note**: you should always mix a frozen sample after thawing to assure a homogeneous solution.

‑ Mix 2 µl of dye with 2 µl of your cut vector and, in another tube, 2 µl dye with 4 μl of your purified insert DNA. Load the 4 μl of each sample on the pre‑poured 1% agarose gel. **Mix the DNA with the dye in a clean microfuge tube, do not add dye to your stock tube of purified DNA**. In addition to your insert & cut vector, each gel should include 1 µl of uncut plasmid DNA (add 1µl of dye) and 3 µl of the DNA molecular weight markers (dye already added, don’t add more).

**NOTE:** **Be sure** **that you write down the loading order of your samples**. **For 3-member lab groups load the uncut vector only once per gel (either 18U or 19U). The 2- and 3-member groups should load the 1 kbp ladder only once per gel.**

‑Run the samples at ~70 volts until the bromophenol blue runs 3/4 the length of the gel bed length.

‑Stain the gel and photograph alongside a fluorescent ruler (the T.A.s will help).

**‑Create a standard curve by graphing the migration (in cm) of the DNA size standards against the log of their lengths.** Use semi‑log paper, the X-axis (non‑log) is for the distance value, the Y-axis (log) is for the DNA lengths in kilo base pairs. Note that since the Y-axis is graphed on log paper you simply graph the numerical value of the DNA lengths. Include this graph in your notebook. A blank copy of semi-log paper is on the class web site.

**NOTE: Finger tips (even gloved finger tips) are a major laboratory source of DNase** and **RNase contamination**. It is almost impossible to open a microfuge tube with your hand without touching the inside surface of the cap (and thus potentially contaminating your sample). To avoid this error - *always use the plastic tube opener*!

**HOMEWORK QUESTIONS**

**Question 1 (3 points): What is the length in base pairs of your insert DNA based on the graph you created? What is the mass in Daltons of this DNA? How many double stranded DNA molecules are present in 3 micrograms of this DNA (hint, look for related information in the NEB catalog)? How many 5’ ends are present in the same sample? Be sure to show the calculation (conversion of micrograms to molecules) and include all units.**

**Question 2: What is an isoschizomer? (2 points)**

**Question 3: The genetic code is NOT actually universal. Provide two different examples where a specific codon (group of 3 nucleotides) defines different genetic information than what is represented in human cytoplasmic mRNA. List the codon, state what is encoded, and state the organism involved (5 points)**

**Competent Cell Preparation.** This procedure makes *E. coli* receptive to DNA mediated transformation by the calcium chloride method. CaCl2 treatment and alternative approach to competent cell preparation and transformation (electroporation) is discussed in PGMG pages 24-25.

**For Each Group**

‑A fresh M9 (minimal medium plus biotin) plate of *E. coli* strain TG1 was used to inoculate an overnight liquid culture in rich broth (2XYT). This morning, the saturated culture was diluted into 1/150 in fresh **2XYT** broth and incubated at 37C for two hours before class. For maximal transformation efficiency, the culture density at the time of cell harvest should be between an OD 600 nm of 0.2 to 0.6. ***A dramatic decrease in cell competence occurs when cultures are grown to higher densities (or is cultured at lower pH).***

‑Spin the 30 ml of culture for 10min. at 5,000 rpm.

‑Pour off the broth and **gently** resuspend (no vortexing) the pellet in 15 ml of ice buffered cold calcium chloride solution (75 mM CaCl2, 200 mM NaCl, 10 mM PIPES (pH 7.0)).

Incu­bate on ice for 1 to 2 hours (in the research lab this step would be extended to 8-12 hours). Invert the tube every 20 minutes to keep the cells from settling.

‑Spin out the culture at 5,000 for 5 min.

‑Resuspend the pellet in 1.5 ml of ice cold calcium chloride/NaCl/Pipes solution. Mix gently but completely by inversion & shaking (no clumps should be visible). Add 1.5 ml of chloride/Pipes/ solution made 30% glycerol and 5% with dimethysulfoxide (DMSO; NOTE, DMSO is added to cool solution after autoclave sterilization). Gently but completely mix again (the glycerol takes a bit of effort to mix in). **NOTE**: The glycerol is cryo-protective (that is, keeps the cells alive when placed in the freezer) and DMSO is both a cryo-protective and enhances transformation efficiency by facilitating DNA uptake.

‑Place the cells in the dry ice container at the front of the room. The cells will be kept in a freezer at ‑85oC until the next lab meeting.

**NOTE: Use sterile technique when handling microbial cultures and always wear gloves when handling DNA or RNA samples.**

**Ethidium bromide vs SYBR safe.** Ethidium bromide (EtBr) is a very sensitive compound for the detection of DNA. Unfortunately, EtBr is also a strong mutagen and a potential carcinogen. Invitrogen sells a safer alternative dye, called SYBER safe (product description on the class web site), which we will evaluate this year. One student will do a series of 5-fold dilutions of the 1Kb+ DNA preparation, load adjacent lanes of a gel as:

2 µl (undiluted),

2 µl (1:5 dilution),

2 µl (1:25 dilution),

2 µl (1:125 dilution)

followed by 2 empty lanes then

2 µl (undiluted),

2 µl (1:5 dilution),

2 µl (1:25 dilution),

2 µl (1:125 dilution)

After the run is complete, the gels will be cut in half and stained either with EtBr at 1 µg/ ml or the recommended dilution of SYBR safe. We will photograph each gel half for precisely the same length of time with filters compatible for the two dyes. The signal intensities will then be compared to test the vendor’s claim that SYBR safe is as sensitive as EtBr. **(ANSWER these questions in your notebook).**Include a copy of the gel image in your notebook, record you observations - is it as sensitive? Do you recommend using SYBR safe instead of EtBr? In responding to the last question, what features of SYBR safe other than its sensitivity should be considered when considering routinely using this dye in BIO 510 lab?

**LAB 3**

Today you will covalently join (i.e., ligate) your doubly cleaved plasmid vector (pTZ18/19u) with the gel purified insert DNA. This is an enzymatic reaction catalyzed by the enzyme T4 DNA ligase in the presence of the essential co-factor, ATP. T4 ligase requires only that the 5' and 3' ends of the DNA substrate molecules be directly juxtaposed. As a consequence, this enzyme will join any blunt ended, double stranded DNA or DNA with cohesive (compatible) single-stranded ends. DNA ligase can even be used to join RNA ends when held together by a bridging oligonucleotide (e.g., 5’ UCUU GGUCCA3’

 3’ AGAA-CCAGGT5’ this is meant to represent a discontinuous (nicked) top (RNA) stand and a continuous DNA bottom strand).

**Joining DNA Ends with DNA Ligase**

**Each Lab Member.** NOTE: *The volumes of vector and insert DNA may need to be altered depend­ing on your recovery so be sure to show the photograph of your gel to the instructor or T.A. before continu­ing.* To increase the frequency of the bi-molecular ligation, we will use approximately 4X the molar amount of insert to vector. The insert is roughly 1/4 the length of the vector, so equal mass amounts of DNA (that is EtBr intensity bands) will give ~4X the molar amount of insert to vector. See pages 44-48 in PGMG.

Assemble the reaction in the following order:

10 μl of sterile water (or sufficient water have a final reaction volume of 20 μl

2 μl of 10X ligase buffer\*

2 μl (or as directed by the T.A.) of the pTZ18 or 19u Hind/Eco cut vector

5 μl (or as directed by the T.A.) of isolated gel fragment (i.e., insert DNA)

Mix briefly, then add:

 1 μl (1 to 2.5 units) of T4 DNA ligase. Mix briefly.

**‑*Each student* should also set up a second, control ligation reaction as above but with additional water instead of insert DNA.** This control will score the amount of vector self-ligation.

Incubate the ligation reactions at room temperature (23oC – room temperature) overnight. The T.A.s will collect the tubes for you in the morning & store the ligation reactions in the freezer until the next lab meeting.

* 10X ligase buffer =

500 mM Tris‑HCl, pH 7.8

100 mM MgCl2

100 mM DTT

10 mM ATP

250 μg/ml BSA

Here are several **outstanding WEB sites** for Molecular Biology protocols and tools:

<http://expasy.org/tools/>

<http://www.ncbi.nlm.nih.gov/Tools/index.html>

<http://searchlauncher.bcm.tmc.edu/>

***DICTIONARY OF MANY COMMON GENETIC AND MOLECULAR BIOLOGY TERMS:***

[**http://www.genome.gov/glossary/index.cfm**](http://www.genome.gov/glossary/index.cfm)**?**

**HOMEWORK**

**Question. Conduct a BLAST search at:**

[**http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi?PROGRAM=blastp&BLAST\_PROGRAMS=blastp&PAGE\_TYPE=BlastSearch&SHOW\_DEFAULTS=on&LINK\_LOC=blasthome**](http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi?PROGRAM=blastp&BLAST_PROGRAMS=blastp&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on&LINK_LOC=blasthome)

**to identify the following protein sequence. Note, just copy & past the sequence and hit the BLAST button (do not fill in any other fields).** Also, you might want to “read ahead” in chapter 9 of PGMG for help with this question.

 1 mlwnllalhq igqrtistas hrhfknkvpe kqklfqeddg iplylkggia dallhratmi

 61 ltvggtayai yqlavasfpn kgvtsiipai twftfiqlsm dqksdk

What is the name of the encoded protein (1 point)?

What organism is this particular DNA sequence from (1 point)?

What is the *biological process* with which it is associated (here your answer should include several sentences – not simply a word or two - make it clear that you investigated the topic by reading the literature) (3 points)?

Show sequence **alignments** (not simply the list of sequences) between this protein and the homologous protein ***from two other species at least one of which is NOT mammalian* (3 points)*.*** The sequence alignments should be printed from your computer as returned from the database (not hand drawn). You can “copy & paste from the sequence alignments (only) but **change to the Courier 8 point font so the alignments stay in register.**

Use Entrez-Pubmed: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?DB=pubmed>

to find two papers published after 2007 on this protein (or its homolog from any organism) – provide the full literature citations (authors/titles/journal/date/volume/pages) (2 points).

**Small‑scale Preparation of RNA from Yeast Cultures (Each Student).** DNA is a double stranded molecule and *both* strands can be transcribed. RNA synthesis is always 5’->3’, with new nucleotides being added to the reducing end (i.e., 3’ hydroxyl) of the growing RNA chain; the DNA template is present in an anti-parallel orientation. Any given gene may be transcribed from either the "top" (or Watson) strand or the "bottom" (or Crick) strand but not both. So, a given mRNA is complementary in sequence to one strand of the double-stranded gene and identical in sequence to the other DNA strand. We will extract RNA from the simple eukaryote, *Saccharomyces cerevisiae* (baker's yeast), to learn the direction of transcription of our cloned gene (in essence, to learn which strand is transcribed). For an intronless, protein coding gene of known sequence this would immediately reveal the encoded amino acid sequence. The stock name of the yeast strain we will use is MGD353-46D. Its genotype is: *MAT α (alpha) leu2-3,112 trp 1-289, ura3-52 his CyhR* (the nomenclature lists the sex of the strain (MAT alpha) and mutations in genes required for the biosynthesis of leucine, tryptophan, uracil, histidine and cycloheximide drug resistance, respectively)

**NOTE:** Ribonuclease is everywhere (e.g., saliva)! Wear gloves throughout this procedure.

1. Grow yeast to saturation in 5 ml of YPD broth @ 30oC (12‑24 hr., depending on the inocu­lation and how "healthy" the strain is).

2. Dilute the culture to an O.D. 600 of ~ 0.10 in fresh YPD. For analytical purposes 1.5 ml of cells should be sufficient.

3. Incubate at 30C until the culture reaches ~O.D. 600 of 0.4- 0.6 (3‑5 hours).

**BIO 510 Students Start Here - BE CERTAIN YOU HAVE GLOVES ON; BE SURE TO USE THE PLASTIC TUBE OPENER. Also, once you start breaking the cells, speed is of the essence for getting a high quality RNA preparation.**

4. Spin out cells (1 min in the microfuge) and wash once with 1-ml ice cold RE buffer (100 mM LiCl, 100 mM Tris‑HCl pH 7.5, 1 mM EDTA). To “wash”, simply add the RE, vortex to mix well, and spin again (as above) to re-pellet the cells. Resuspend the cell pellet in 0.4 ml of cold RE buffer and 150 μl of Tris‑buffered phenol/CHCl3/isoamyl alcohol (PCI, 50:49:1).

5. Yeast have a very strong cell wall, you will beak the cells by mechanical grinding with glass beads. Add approximately ~2/3 of the cell suspension volume of sterile glass beads (acid washed and siliconized). Break cells using the vortex mixer, 8 X 30 seconds on high speed (place on ice for 30 seconds between vortexing to keep the solution cold). To reduce cleavage by endogenous nucleases (that is, to improve the quality of the RNA sample). **WORK QUICKLY** though the cell breakage step.

6. Spin the sample 3 minutes at full speed in the microfuge and transfer the upper layer to a fresh tube with 300 μl of PCI. Vortex over a 3 min.period. Spin for 2 min. in the microfuge. Remove the upper (aqueous) layer and extract once more with phenol/CHCl3/isoamyl alcohol. Finally, extract the aqueous layer one last time with chloroform (CHCl3) only. Like the PCI, the chloroform layer sinks to the bottom of the tube.

7. Transfer the upper (i.e., aqueous) phase to a fresh tube and add 100 μl of 3 M sodium acetate (pH ~ 5.5) and 1 ml of 100% ethanol. Vortex well and then place on dry ice for 10 minutes.

8. Thaw, vortex the sample again and then spin in a microfuge at 4oC for 8 min., decant (discard) all the supernatant, and wash the pellet with 1.5 ml 80% ethanol. Spin 2 min., decant the wash twice with 80% ethanol and dry the pellet under vacuum.

9. Resuspend the pellet in 25 μl of sterile H2O.

10. Store the RNA sample at ‑85oC until used. The typical yield is 20‑40 μg of total RNA (of which ~ 95% is rRNA and tRNA).

**Impact of increased salt on DNA migration.**  Three gels will be set up and run with equivalent amounts of DNA at the same voltage and for the same length of time. Gel 1 will be run as a standard 1X TAE gel, gel 2 with 1/3X TAE and gel 3 with 3X TAE. Assume that no change occurs in the pH (which it won’t under the conditions of use), the difference can be thought of as decreased or increased tris-acetate salt. **Answer these questions in your notebook.** How does this impact the running of the dye? The resolution of the DNA bands?

**LAB 4 (Short quiz today)**

***E. coli* Transformation** **(each student)**

This protocol allows the DNA present in your ligation reaction to move into the *E. coli* cells where the plasmid vector will replicate to amplify your recombinant DNA clone to a copy number of 50 to several hundred molecules per cell. An underlying assumption of this technique is that any given transformed cell propagates one (and only one) plasmid molecule from the ligation mixture.

‑Mix 500 μl of competent *E. coli* cells with 5 μl of your experimental ligation (i.e., vector + insert) in the capped plastic tubes provided (label this tube **EXPERIMEN­TAL LIGATION**, include your initials). In a second tube do the same for your **CONTROL LIGATION** (i.e., vector only).

‑Incubate the cell/DNA mixture on ice for 30 min.

‑Transfer the tube to the 42oC water bath for 1 minute (timing is important during this step). Be sure that the water bath is correctly set at 42oC before incubating your cultures since 42C is close to the upper limit for *E. coli* cell viability.

‑Add 2 ml of 2XYT broth. Incubate at 37oC with vigorous shaking for 45 min. **Why do you want to be sure that no antibiotics are present at this step?** Answer this in your notebook but do not turn in for grading.

-During this 45-min incubation spread 20 μl of 100 mM IPTG and 50 μl of 2% X‑gal solution on 6 LB‑amp (containing 125 μg/ml ampicillin) plates. ***Spread the chemicals evenly***, don't pool the solution in one posi­tion on the plate.

‑After the 45 min incubation pipette 20 μl of the experimental ligation culture onto an LB‑amp/IPTG/X‑gal plate labeled **EXPERIMENTAL LIGATION, 20 μl, today’s date, student initials**. Plate 250 μl of the experimental ligation culture on a second plate labeled the same (except substituting 250 μl for 20 μl). Finally, spin the remaining cells for 10 minutes in the centrifuge, pour off the culture medium, and plate the pellet on a third plate. Do the same for the control ligation culture on three fresh plates (6 plates total per student).

**NOTE** : Be sure to sterilize your spreader before **each** plating.

‑Immediately invert the plates (agar side up) and place them in the 37oC dry incubator at the front of the lab. The T.A.s will refrigerate the plates after 18 hours of incuba­tion. Longer periods of incubation at 37oC can cause the appearance of satellite colo­nies in the vicinity of the true transformants. **Why should you always label Petri plates on the bottom (i.e., agar side)?**

‑Only *E. coli* transformants (i.e., cells containing the β‑lactamase‑containing plasmid) will grow on these antibiotic plates. **The TA.s will** determine the total number of cells/ml in your culture by plating the cells on agar without antibiotic and determine the background level of ampicillin resistant bacteria in the culture by plating an equivalent amount of untransformed cells.

2XYT = 16g Bacto‑tryptone, 10g bacto‑yeast extract, 5g NaCl, water to 1 L (pH 7.5).

**Quantifying RNA – don’t touch the tubes unless you have gloves on.**

The spectrophotometer is used in this experiment to acquire an estimate of your RNA yield and quality. You will read your sample both at the wavelength for which RNA absorbs maximally (260 nm) and a peak of protein absorbance (280 nm) to determine these values.

1. Thaw your RNA samples and vortex vigorously.

2. Spin 1 min. in the microfuge.

3. Transfer 5 μl of the supernatant to 1 ml of sterile water. Immediately place the remaining RNA sample on ice.

4. Read the A260 and A280 on your sample in a spectrophotometer.

5. A 1 mg/ml solution of RNA in a 1 cm path will read 25 @ A260. Multiply your A260 by 200 (dilution factor) then divide by 25 (the extinction coefficient) to get the concentration of your RNA in units of mg/ml. A typical yield is 1-2 µg/µl.

6. The A260/A280 ratio for RNA is ~1.9‑2.0. Values lower than this suggests protein contamina­tion; greater values likely are due to phenol contamination.

7. Add 15 µg of RNA to a clean microfuge tube. Label the tube and bring it to the front of the room. This sample will be dried and returned to you NEXT LAB PERIOD for resuspension in the denaturing loading buffer.

**NOTE:** A scientific report (Nucleic Acids Research 27:910-911, 1999) suggests that a minor modification of the electroporation protocol that includes a tRNA assisted ethanol precipitation of the ligated DNA can increase plasmid transformation efficiencies by 400-fold)

**Here is a link to descriptions of RNA structure prediction, modification, function, and related topics.**

<http://www.imb-jena.de/RNA.html>

**HOMEWORK**

**Question. Find the sequence of the yeast proline tRNA on the Stanford *Saccharomyces Genome Database* <http://www.yeastgenome.org/> (HINT: this is encoded by a gene called** tP(UGG)L**). Now use the mFOLD RNA folding program on this web site:** [**http://mfold.rna.albany.edu/?q=mfold**](http://mfold.rna.albany.edu/?q=mfold)

**to predict a secondary structure. Be absolutely sure that you fold the processed RNA (listed as “non-coding exon”) and not the entire gene with the intron. What is the calculated stability of the “best” structure suggested (1 point)? Next, introduce mutations by changing every third nucleotide to a uracil and recalculate the structure. Present the image and compare and discuss this structure and calculated stability with the first trial. Explain fully what you observe in the two structures and the predicted changes in thermostability. Which structure will more likely form spontaneously?**

**LAB 5**

**Analysis of *E. coli* transformants**

Count the number of blue and white colonies on each plate. Assume that approximately 0.1 μg of ligated vector DNA was used for entire transformation (that is, 0.1 μg of DNA was mixed with the *500* μl of competent *E. coli -* ***remember, you increased this volume by 2 ml prior to plating (total volume = 500 + 2000 + 5 = 2.505 ml)and each plate only received a fraction of the total transformation mixture).* NOTE: If you had no transformants on your plates, use another group’s data to answer the following questions.**

**HOMEWORK QUESTIONS (1-5 based on the CaCl2 transformation).**

1. (3 points) What was your transformation efficiency (the number of transformants per microgram of vector DNA)? State the dilution factor and numbers of colonies on the plate that you use for this calculation.
2. (2 points) Using the T.A.'s viable cell count, determine the percentage of cells transformed and ampicillin resistant i.e., (**number of AmpR colonies present/ total number of cells plated) X 100**).
3. (2 points) How could you determine experimentally whether DNA or competent *E. coli* was limiting for the recovery of transformants?
4. (1 point) Based on the blue/white screen, what percentage of the experimental ligation's transformants may contain recombinant DNA plasmids?
5. (1 point) How would you expect the blue/white ratio to change if uncut pTZ plasmid (rather than HindIII/EcoRI cut) was used in the ligation?
6. (1 point) How do you account for any blue transformants on the experimental transformation plate? How do you account for any white or blue transformants on the control transformation plate?

**Preparation of *E. coli* Plate Cultures**

‑**Each student** should patch two colonies on an ampicillin plate. Note no IPTG/X‑gal need be added. Use one plate per group and divide into four sections by marking the back with a Sharpie marker. Be sure to label your quadrants with your initials and each plate with your group number (e.g., group 1, GR, TY, EE). Each should patch a white colony from the experimental plate and a blue colony (from either the experimental or control ligation plates). **NOTE:** Leave 1/4 inch open on each side of each patch to avoid cross-contamination between the cultures. *Each student should label a sterile glass test tube similarly to the plate designation*. *The TAs will use your plates/tubes to start cultures before next lab period.*

**Formaldehyde Northern Blot (Each Student) NOTE: Wear gloves throughout this proce­dure.** Here you will resolve the RNAs present in your yeast nucleic acid sample based on relative lengths. All RNAs have equivalent charge/mass ratios yet larger RNAs move more slowly due to the diffusion (frictional) barrier presented by the agarose matrix (assuming secondary structures are melted). See PGMG 18-24.

1. Add 10 μl of the RNA denaturation solution to each dried RNA sample. The solution was prepared by mixing 5X MOPS buffer [0.1 M 3‑[N‑morpholino]propanesulfonic acid); 40 mM sodium acetate; 5 mM EDTA, pH 7.0], formaldehyde, formamide in a 1:1.75:5 ratio, and 400 ng of ethidium bromide.

3. Vortex the RNA sample vigorously several times over a 5 min. period. Spin the sample in the microfuge briefly to collect the liquid at the bottom of the tube.

1. Incubate the sample at 65oC for 15 min. and then place on ice for 2 min. Be sure that you use the correct (i.e., 65oC) incubator.

5. Add 2 μl of tracking dye (50% glycerol, 1 mM EDTA, 0.1% BPB/XC) to your sample. Mix and immediately load the sample into a gel well.

6. Load 4 μl of your sample to your 1% agarose/formaldehyde gel well. Load your group's samples in wells 1, 2, and 3, leave wells 4 and 5 empty, load your samples a second time in wells 6, 7, and 8. Record the order of student loading.

7. Run the gel @ 55 volts (HIGHER voltages will greatly distort the sample migration) in 1X MOPS buffer until the tracking dye is near the bottom of the gel (at least 3/4th the length).

8. Soak the gel 1 X 5 min. in 50 ml of water.

9. Soak the gel 1 X 10 min. in 50 ml of 10X SSC (1.5M NaCl, 0.15M Na citrate, pH 7.0).

10. Set up the gel transfer by stacking in the following order (from bottom to top): Plastic wrap sheet, blotting paper saturated with 10X SSC, gel (**well side facing down, why? – answer in your notebook**), positively charged nylon membrane­, blotting paper soaked in 10X SSC, dry blot paper, 2 inch stack of paper towels. **NOTE:** Use a syringe needle to **stab a hole in the position of each well**. In order for the transfer to work, the SSC in the gel must be drawn upward by capil­lary action through the charged nylon membrane. Air bubbles trapped between the gel and the filter membrane inhibit this process. To **remove any bubbles**, roll a Pasteur pipette over the gel surface and over the membrane and the next two layers of filter paper. Also, be sure that the paper towels do not "hang over" the gel and touch its sides or the towel/plastic wrap beneath. This would cause the system to short circuit (that is draw the liquid from a direction other that bottom to top). **Notch the membrane** on the top and bottom to distinguish the two halves of the blot. Blot overnight on the bench top. **The T.A.s will photograph the membrane along side of a florescent ruler** and then store the blots for you until the next lab period. The bottom of the well will correspond to “0” on the ruler.

11. An alternative (& faster) blotting system, the vacuum blot, will be set up for demonstration.

**NOTE:** The yeast 25S rRNA band is 3700 nucleotides (nts) in length, the 18S rRNA is 1700 nts.

Why is it important to heat your sample before loading the RNA on the gel? Why do you “quick cool” your sample immediately after heating it? **(Answer in your notebook, do not turn in for grading).**

**1% Formaldehyde Gel Preparation (made for you)**

- Mix 1 gram of agarose with 62.1 ml of sterile water. Microwave to dissolve the agarose. Bring it (just to) boil 3 times, then place on the gyratory shaker for 10 minutes. Cool to ~ 60C (at this temperature the flask bottom is hot but can be held without burning your hand).

- Add 20 ml\* of 5X MOPS buffer (0.1 M MOPS, 40 mM sodium acetate, 5 mM EDTA, pH 7.0). Dissolve 20.6 gm of 3-(N-morpholino)propanesulfonic acid (MOPS) in 800 ml of water, add 10 ml of 0.5M EDTA (pH 8.0) and sodium acetate to 40 mM. Adjust pH to 7.0, increase water to 1L, filter sterilize and store in the dark at 4C.

- Add 17.9 ml of high quality (Fluka Biochem.) formaldehyde, swirl to mix and pour into the gel mold in the hood. It helps to have the MOPS and Formaldehyde warmed to 42C before adding. Be sure that the gel platform is level before pouring the gel (***use the bullet level to check***). Solidify the gel for > 2 hours.

- Pre-run the gel for 5 minutes at5V/cm before loading (~70 volts for a 14 cm long gel). Run the gel submerged in 1X MOPS buffer.

**Lab 6**

**Isolation of Recombinant DNA from *E. coli* the Alkaline Lysis Method**

This protocol allows provides a quick way to isolate plasmid DNA from *E. coli* in sufficient quantity and quality to screen for the presence of recombinants using restriction endonuclease and agarose gel electrophoresis. It is worth noting that this preparation is a “partial purification” of the plasmid, other compounds (RNA, *E. coli* DNA, limited carbohydrate) co-purify and may inhibit certain laboratory procedures (e.g., DNA sequencing reactions, mammalian cell transformation).

1. Collect cells by brief (1 minute) centrifugation of 1.5 ml of a saturated liquid culture (in 2X YT/amp) of bacteria. **Note:** use the setting of “12" on the microfuge. Longer spin times or greater speeds will make the pellet difficult to resuspend. Mix the pellet by vigorous vortexing in 100 µl of solution one (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0).The pellets must be dissolved completely. Hold the tube up to the light and roll the liquid around to determine if any “clumps” of cells are present. If need be, use a P-200 tip to disrupt the clumps.

2. Add 200 µl of solution two (200 mM NaOH, 1% SDS). Mix by rapid inversion 5 times; ***do not vortex.*** Incubate at room temperature for 3 minutes.

3. Add 150 µl of solution three (made by mixing 300 ml of 5M potassium acetate with 57.5 ml of glacial acetic acid and 142.5 ml of sterile water). Mix by rapid inversion 5 times; ***do not vortex.***

Incubate on ice for 5 minutes.

4. Spin the tube for 5 minutes in the microfuge.

5. Pour the supernatant (upper layer) to a fresh tube containing 300 μl of phenol/chloroform/isoamyl alcohol (PCI). Vortex vigorously (periodically) over a 5 min. period.

6. Spin for 3 min. in the microfuge. Transfer the upper phase (the DNA‑containing aqueous phase) to a fresh tube (discard the waste phenol).

7. Add 200 μl of chloroform to your sample. Vortex vigorously for 2 minutes. This additional organic extraction is very helpful in removing trace phenol and SDS that might inhibit subsequent nucleic acid manipulations. Spin in the micro­fuge for 2 min. Transfer the upper phase to a fresh tube.

8. Add 1.0 ml of 100% ethanol to your DNA. Vortex well. Allow the sample to remain at room temperature for 5 min.

9. Spin the sample for 10 min in the microfuge (at room temperature or at 4C). Carefully discard the sup. by aspirating off the ethanol with a sterile Pasteur pipette.

10. Add 1 ml of 80% ethanol, mix and spin as before for 3 minutes.

11. Discard the ethanol, repeat the wash with 80% ethanol. Spin in the microfuge for 3 minutes. Remove the 80% wash and dry the samples under vacuum ‑ be sure that your tubes are well labeled.

12. Resuspend your DNA pellet in 30 μl of TE made 20 μg/ml with RNase A (be sure that the RNase A you use was previously boiled to inactivate DNase). Vortex well to mix, spin briefly to collect your pellets. Incubate for 5 min. at room temperature (23C).

1. Run the 2 μl of DNA from each sample on a gel along with an original pTZ18u or 19u empty (uncut) plasmid control. Freeze the remainder of your samples at ‑20oC until needed. Be sure to label your tubes well (contents, today’s date, and your initials).

**Northern Blot Prehybridization (Each Group).** The positively charged nylon membrane has a very high affinity for single stranded nucleic acids. If your labeled hybridization probe was added immediately, it would randomly stick to the membrane surface. **The prehybridization step “blocks” the exposed portions of the membrane filter** with a non-specific nucleic acid. Thus, when your labeled hybridization probe is added it will associate by base pairing with your size fractionated RNA and not directly adhere to the membrane surface.

1. Your blots were crosslinked with a Strategene Stratalinker (measure dose of 120,000 micro joules at 254 nm –see pdf on the class web site) have been baked in a vacuum oven at 80C for 2 hours. Photographs were taken prior to baking. Label the bottom left hand corner of your blot with your initials.
2. **Wet your blots** by soaking for 10 minutes in 250 ml of 6X SSC.
3. Roll your blot in a mesh strip and place both inside a glass roller bottle. **NOTE: Be sure to remove all air bubbles** from between the mesh and the membrane – simply roll a freshly gloved finger over the membrane to squeeze out the air bubbles. Holding the glass bottle in your right hand with the open end pointing to your left, the mesh should be inserted such that the fold is going **over** the top of the roll.
4. **Add 10 ml 6X SSC** and roll at room temperature for 5 minutes (this will spread out the mesh in the tube if you inserted the roll correctly; the mesh will remain in a tight roll if inserted incorrectly).
5. **Heat 2.5 mg (0.25 ml of 10 mg/ml) of salmon sperm DNA at 100oC for 15 min**. Rapidly transfer the denatured DNA to ice for 5 min.
6. Add your denatured salmon sperm DNA to 10 ml of: 6X SSC, 5X Denhardts (0.1g Ficoll, 0.1g poly­vinylpyrrolidone, 0.1g bovine serum albumin per 100 ml), 50mM sodium phosphate, 1% SDS. Mix by shaking the Falcon tube.

Ficoll is a highly branched sucrose polymer.



Polyvinlypyrrolidone is a second synthetic polymer – both added as stabilizing agents that inhibit background binding of probe to the membrane and promote nucleic acid hybridization.



1. Pour out the 6X SSC from the roller bottle and replace with the 10 ml prehybridization plus salmon sperm DNA.
2. Tightly cap the roller bottle and incubate with rolling at 60oC for 24 hours. Tomorrow, the TAs will place the roller bottles into the refrigerator until used.

**Answer the following in your notebook – do not turn in for grading.**

**Question:** Describe how does the mobility of the DNA from the "blue" colony compares with that from the "white colony".

**HOMEWORK:**  Data mining, or the extraction of useful information from complex experimental results files, is a critical function for the modern biologist. Here I want you to answer the following question: how many proteins encoded by the yeast genome contain the four amino acid carboxyl-terminal motif associated with prenylation? To do this use SGD <http://www.yeastgenome.org/>

* (5 points) First, define what prenylation means, then
* Select the “PatMatch” option in the brown menu bar near the top of the page
* Select the “translations of all S.c. ORFs” as the sequence database
* **Use the consensus sequence**: cysteine, aliphatic amino acid, aliphatic amino acid, any amino acid as the consensus sequence. The aliphatic amino acids should include: alanine, valine, isoleucine, leucine, glycine.
* **Restrict the peptide as being present at the carboxyl terminus** of the protein
* Download and submit to me the results that INCLUDE the corresponding gene or protein names.
* (5 points) Most of these proteins share a common biological function. Discuss what common theme (types of proteins, the purposes these proteins serve in the cell & how this is functionally related among most members of this protein set) unites most of these proteins. **A thoughtful response will require at least two paragraphs of discussion**. One or two sentence replies or copy and paste responses are not acceptable.

**LAB 7.** Two sets of hybridization probes will be needed to determine the in vivo direction of transcription of your putative yeast gene. The two probes, though made of RNA, will correspond to the two complementary strands of the double stranded DNA of the insert. One of these in vitro transcribed probes will be complementary to the yeast produced transcript believed present in your cellular RNA preparation(and therefore will hybridize) while the other will have the identical polarity (and won’t hybridize). In order to produce these two probes, your recombinant clones, pTZ18u+insert (one orientation) and pTZ19u+insert (other orientation) will first be cleaved directly 3' of the insert DNA. Next, the T7 RNA polymerase promoter 5' of the insert will be used to produce an in vitro (i.e., synthetic) RNA corresponding to one strand of the yeast DNA insert.

**Restriction Endonuclease Digestion for *In Vitro* Transcription**

In this step you will cleave the recombinant vector just 3' (downstream) of the insert DNA as a preamble to *in vitro* transcription.

1. Cleave 2 μg of your recovered recombinant plasmid DNA (~ 7 μl – **show the gel image of your DNA to the instructor or TA to confirm the amount prior to setting up the reaction)** with HindIII (those with vector plasmid pTZ18u) **or** with EcoRI (those with vector plasmid pTZ19u). Add 3 μl of the appropriate 10X buffer (recipes are in the NEB catalog), 17 μl (or as needed to increase the volume to 27 μl) of sterile water, ***mix*** then add 3 μl (~30 units) of the appropriate of restriction enzyme (always add enzyme last to a mixed solution). Mix briefly, spin briefly, then incubate for 45 min. at 37C (be sure that the water bath is set to the proper temperature).

2. Load 2 µl of uncut recombinant plasmid and 4 µl of the cut DNA alongside of 2 µl of the linear (before ligation) non-recombinant pTZ18u or 19u, and 3µl of the 1 kb DNA marker.Run the gel until the leading dye is ¾ the gel length. Photograph the gel and based on this image determine whether the plasmid was digested to completion.

**Answer the following in your notebook – do not turn in for grading.**

**Question:** Is the size shift of the recombinant DNA consistent with the addition of one insert segment (tell how you evaluated this)?

**Question:** How does the mobility of the DNA from the "blue" colony compare with that from the "white colony"?

While the gel is running:

3. Add 150 μl of PCI and 120 μl of TE to your digestion. Vortex intermittently over 3 min. Spin to separate the phases and transfer the aqueous layer to a fresh tube (discard the phenol in the waste container located in the hood).

3. Extract the sample (the upper phase from the PCI extraction) with 100 μl of chloroform. Mix as before, spin and transfer the sample to a fresh tube.

4. Add 20 μl of 3M sodium acetate and 450 μl of 100% ethanol to your sample. Vortex, place on dry ice for 5 min.

5. Be sure that the sample is not frozen (hold in hand to thaw if needed; then vortex briefly). Spin for 10 min. in the microfuge. Discard the ethanol and wash the pellet with 1 ml of ice cold 80% ethanol. Vortex and spin the sample for 3 min. in the microfuge (no dry ice step this time).

1. **Repeat the 80% ethanol** wash, and then dry the pellet under vacuum.
2. Resuspend the pellet in 10 μl of sterile water. Freeze @ ‑20oC until needed.

**NOTE:** The next lab uses radioactivity, be sure to bring your lab coat and also read the in **Riboprobe** vitro transcription manual **(on the web site**). A second good overview of in vitro transcription is presented in the Ambion web site:

 <http://www.ambion.com/techlib/basics/transcription/>

**Isolation of Genomic DNA from *Saccharomyces cerevisiae* (each student)*.***  The purpose of this lab is to obtain eukaryotic DNA in a form suitable for restriction digestion and PCR analysis. Two cultures will be used (coded simply **A, B, and C**) that differ in specific gene polymorphism (in this case, an experimentally introduced gene disruption). **One is a haploid wildtype *UPF1* strain, another is a haploid that bears the gene disruption (*upf1::KAN*), and one is a heterozygote diploid with both a wildtype and gene disruption allele.** PCR will be used to distinguish the wildtype culture from that bearing the targeted gene disruption. The predicted sizes of the wildtype *UPF1* and mutated *upf1::KAN* PCR products will be provided in class. The details on the chromosomal deletion strategy can be found at: <http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html>

The reagents in today's experiment are supplied by Epicentre Technologies <http://www.epibio.com/category.asp?CatID=9> as part of their MasterPure Yeast DNA Purification kit (pdf on our class web site). This protocol works very well. Unfortunately, the reagent compositions are proprietary information and not disclosed. Other protocols for yeast DNA isolation are available in the public domain (for instance, see Methods in Enzymology, volume 194, 1991).

**(Each Person)**

1. Grow a 1.5 ml yeast culture A, B, or C to saturation (typically overnight) in rich broth (e.g., YPD = 1% Bacto‑yeast extract, 2% Bacto‑peptone, 2% glucose).
2. Spin out cells (2 min. in the microfuge).
3. Remove ***all*** of the supernatant and wash the pellet with 1 ml of sterile water and spin out as before.
4. Remove all the supernatant (use a P-200 tip to get the last bit). Add 300 μl of lysis solution and resuspend pellet very well (use your Pipetman tip to dislodge the pellet then vortex vigorously until ***all*** the clumps are ***fully*** dispersed (1-2 minutes).
5. Incubate at 65C for 20 minutes.
6. Place on ice for 5 minutes.
7. Add 150 μl of protein precipitation reagent and vortex for 10 seconds.
8. Pellet the debris in the 4C microfuge at full speed for 10 minutes.
9. Transfer the supernatant to a fresh tube and add 700 μl of isopropanol. Mix by rapid inversion 10 times.
10. Spin in the 4C microfuge for 10 minutes. Remove the supernatant and wash the pellet in 1.5 ml of 80% ethanol.
11. Spin for 3 minutes in any microfuge, remove all the supernatant, dry the pellet briefly in the Speedvac (5 minutes should be fine, it’s ok if a small amount [<15 μl] of ethanol remains).
12. Resuspend the DNA in 50 μl of sterile water. Freeze at -80C until use.

**Lab 8**

**In Vitro Transcription**

You will now synthesize an RNA equivalent of one of the insert’s DNA strands using your cleaved template, the bacteriophage T7 RNA polymerase and required nucleotide triphosphates and co-factors. The synthesis - like all template-directed nucleic acid syntheses - progresses 5'-3' on the growing RNA strand. While this RNA will be used as a hybridization probe, in other applications such RNAs could be used to program an in vitro translation mix to make protein, function a substrates for RNA processing reactions (e.g., splicing, polyadenylation, capping), or used to study RNA-directed catalysis. PGMG 82-84

**PROBE PREPARATION (Each Student)**

**NOTE: Use only DNA from the "white" colonies. Students that prepared DNA from the "blue" colonies should borrow some DNA from their lab partner.** Remember, the 18u and 19u probes are prepared from the same DNA fragment and are equivalent to the top and bottom strands of the DNA duplex. That is, the 18u and 19u probes are complementary to one another.

1. Add 3.0 µl of cut recombinant DNA (18u or 19u plus yeast DNA insert) to 7 µl of reaction mix contain­ing:

Final concentrations = 0.5 mM ATP,CTP,GTP; 0.05 mM UTP; 50 mM Tris‑HCl, pH 7.5; 6 mM MgCl2; 2 mM spermidine; 10 mM NaCl; 10 units of RNasin; 10 mM dithiothreitol

(DTT), 5 units of T7 RNA polymerase, 2.5 µl of 32P‑UTP (25 µCi). **Label the tube** with your initials and “ 32P”.

Cap the tube well and mix very briefly (1 second) on the vortex. Spin very briefly (3 seconds) and then incubate at 37oC for 45 min.

[NOTE: Save the remainder of your cut plasmid DNA in the freezer ‑ we will use it again later.]

2. After the 45 min. incubation, spin again briefly and then carefully open the tube with a plastic tube opener. Add 1 µl (1 unit) of DNase I to the reaction and cap the tube well. Vortex briefly, spin briefly, and then incubate for 5 min at 37oC.

3. After the 5 min. incubation, spin again briefly and then carefully open the tube with a plastic tube opener. Add 120 µl of TE to the probe solution.

4. Separate the unincorporated nucleotides by pipetting the 130 µl of probe **(NOTE: to avoid contamination of your Pipetman, pipette ~70 µl twice and move the Pipetman plunger very slowly)** into the pre‑packed Bio-Spin 6 column (BioRad; see “documents” at: <http://www.bio-rad.com/prd/en/US/adirect/biorad?cmd=BRCatgProductDetail&productID=211001> also on our class web site) with a collection tune inserted into a glass test tube. Spin for 5 min at ~1,500 rpm.

6. The solution that flows into the collection tube contains your RNA probe; the column retains the unincorporated nucleotides. DISCARD THE **column** SAVE THE **flow through**.

7. Place 10 µl of your probe into a scintillation vial, determine the cpm (counts per minute) of the sample. Assume a 90% counting efficiency (e.g., 90 cpm = 100 dpm (decays per min.)) and that 100 ng of RNA is synthesized during in vitro transcription (that is 0.1 µg of RNA in your 200 µl of stopped reaction), **determine the specific activity (in dpm/µg) of your probe. Determine the amount (volume and µg) of probe to add to provide 1X106 cpm per ml of hybridization**. The TAs will provide you with the number of cpm in the probe mixture before column chromatography. **Determine the % of the radioactive UTP incorporated into your probe.**

**Include the following in your notebook, do not hand in for grading.**

1. Describe the process which achieves DNA separation in your column. That is, what is the basis of the separation? What factors influence whether a piece of DNA exits the column early or late?

**NOTE:** Prepackaged sepharose or agarose columns which greater exclusion limits can be used to purify PCR products from free nucleotides and primers (e.g., the online catalog can be found at: <http://www.bio-rad.com> ).

**HOMEWORK**

**Question.**

1. (4 pts) Describe in detail a non-radioactive detection methodology for the detection of RNA or DNA that has been resolved by gel electrophoresis and transferred to a membrane.
2. (3 pts) Describe a method to determine if a protein is phosphorylated
3. (3 pts) Describe a method to determine if a specific mRNA (for instance, the yeast *ACT1* mRNA) is polyadenylated and, if so, to estimate the length of the poly A “tail”.

**LAB 9**

**Northern Hybridization (Each Group)**

Now you will incubate your probe with your blot in a solution that will foster hybridization. We discard the prehybridization solution in case any RNA was released from the membrane during the incubation - such released RNA will compete with the filter bound RNA for hybridization to your probe. In most protocols (including this one), the prehybridization and hybridization solutions differ only in the whether or not the labeled probe is present.

1. Open your prehybridization roller bottle and remove the membrane. Place the membrane on a clean piece of plastic wrap. Rapidly cut your membrane (**BUT NOT THE MESH**) in ½ using the notches on the top and bottom as guides. Be sure to wear gloves and do not place the blot down on any surface other than on the plastic wrap.

2. Wrap one half of your membrane and one half of the membrane from another student in mesh. Obtain a membrane from a student that is using the other recombinant clone (if you are using pTZ18u, exchange with a group that is using pTZ19u and vice versa) and give this group your other membrane half. Be sure to label the bottom of your membrane with your initials. Add 10 ml of hybridization solution ‑ salmon sperm DNA was already added to this by the T.A.s.

3. Place approximately 10 million cpm of probe into the roller bottle and seal. NOTE: We will choose which probes to use in class. You do not have to heat denature this single stranded RNA probe.

4. Incubate with rolling at 60oC until Wednesday.

1. **Discard the remainder of your probe in the radiation waste container.**

**Isolation of Single Stranded DNA from M13KO7 Infected Cultures**

Today you will isolate mock viral particles that contain the ssDNA form of your PTZ18u **(and 19u)** + yeast DNA recombinant. This DNA will be used as a template for DNA sequenc­ing. Prior to today's lab, the T.A.s did the following. A culture of pTZ19u containing your yeast DNA insert was grown overnight in LB‑amp. 1.0 ml of culture was added to 50 ml of 2 X YT broth with 100 µg/ml ampicillin and incubated with shaking for 30 min. @ 37oC. After 30 min. of growth 10 µl of M13KO7\* helper phage was added. A multiplicity of infection of 5 (that is 5 viruses for every bacterial cell) was used. The culture was incubated @ 37oC for 30 min. with shaking. Finally. 70 µl of a 50 mg/ml kanamycin sulfate solution was added and the culture grown overnight at 37oC.

\* The M13K07 phage provides the DNA polymerase necessary to activate F1 ori, the single‑stranded DNA origin of replication on the pTZ19u vector. The single stranded DNA produced is predominately from your pTZ construct (a small amount of M13K07 ssDNA is also generated). This DNA is covered with viral coat protein and secreted into the culture media (i.e., the single‑stranded DNA is packaged into mock virus particles and transported out of the cell). The M13K07 phage contains a kanamycin resistance gene. Addition of kanamycin will prevent the growth on competing non-infected bacteria, which cannot produce the desired single-stranded DNA. See pages 72 & 81 in PGMG.

**Each Student**

1. Spin 1.5 ml of cells at full speed for 5 minutes in the microfuge (be sure to keep track of whether you are using the 18U or 19U.

2. Transfer 1 ml of the sup. to the tube containing 328 µl of 20% PEG (polyethylene glycol) 8000, 3.5 M ammoni­um acetate. Mix well.

3. Incubate on ice for 30 min.

4. Spin in the microfuge for 10 min. Carefully remove and discard the entire sup. Place the tube upright for 2 min. then remove (using your P200) all liquid collected in the bottom of the tube.

5. Resuspend the pellet in 400 µl of TE.

6. Add 350 µl of phenol/CHCl3/isoamyl alcohol. Vortex 30 sec., allow to settle 30 sec. Repeat this mixing process 5 times. Spin 3 min in the microfuge.

7. Transfer the sup. to a fresh tube and repeat the extraction with P/C/I as above. Spin 3 min. Repeat the P/C/I extraction a third time.

8. Transfer the sup. to a fresh tube and extract with 350 µl of chloroform. Vortex well, spin 3 min.

9. Remove the upper phase and transfer to a fresh tube. Add 100 µl of 3M sodium acetate and 1 ml of 100% ethanol. **MIX WELL**. Incubate on dry ice for 5 min., thaw then spin 10 min in the microfuge.

10. Carefully remove all the sup, wash the pellet twice with 1 ml of 80% ethanol. Spin 3 min., remove sup. dry. Resuspend the pellet in 8 µl of sterile water. Freeze at ‑80C until used.

***Caenorhabditis elegans* RNAi knockdown lab (part 1)**

In today’s lab we are going to learn about knockdown of gene function in the nematodes *C. elegans* using RNAi technique. ***This is another student-requested lab exercise***. PGMG 315-317; 405.

Strain: NL2099 rrf-3(pk1426) (this mutation makes the strain very sensitive to RNAi

|  |  |
| --- | --- |
| Strain:  | KH1125 |
| Description:  | ybIs733[myo-3::EGL-15BGAR::GFP + lin-15(+)]. GFP/RFP chimeric expression of EGL-15BGAR reporter in body wall muscles. |

Details about knockdown of gene function in worms can be found in the website <http://wormbook.org/chapters/www_introreversegenetics/introreversegenetics.html#d0e60>. The genes selected for knockdown are *C. elegans* homologs of yeast splicing factor *CLF1* (M03F8.3), Asd-1 (alternative splicing factor), Unc-22, and Dpy-1 (details provided in class). To monitor the effect of knockdown of the gene function we observe the morphology and activity of the animals after exposure to each RNAi construct or a control. In addition, for Asd-1 construct, we will use a transgenic reporter worm strain (Kuroyanagi *et al*, Nature Methods, 2006) that allows visualization of changes in tissue-specific splicing patterns. Information about the specific genes, expression patterns and knockdown phenotypes can be found at <http://www.wormbase.org/>.

**Vector Design & Insert Information:** Genomic fragments obtained by PCR were cloned into the Timmons and Fire feeding vector (L4440), which is a modified version of Bluescript with a T7 promoter on each side of the MCS driving transcription of each DNA strand (Nature, **395**, 854). Information about the L4440 vector (including sequence information & map) can be found at <http://www.addgene.org/pgvec1?f=c&identifier=1654&atqx=L4440&cmd=findpl>

PCR fragments information on the clones that are expressed were obtained from <http://elegans.uky.edu/lab/c_elegans_chrom_all.txt>.

**Bacteria Information:** Genomic fragments cloned into L4440 were transformed into **HT115** (DE3), an **RNase III-deficient** *E. coli* strain with IPTG-inducible T7 polymerase activity (Gene, 263, 103-112). The strain is available from the *Caenorhabditis* Genetics Center (http://www.cbs.umn.edu/CGC/CGChomepage.htm). The HT115 genotype is as follows: F-, mcrA, mcrB, IN(rrnD-rrnE)1, lambda -, rnc14::Tn10 (RNaseIII mutation; Tn10 confers tetracycline resistance), DE3 lysogen: lac UV5 promoter –T7 polymerase) (IPTG-inducible T7 polymerase). This strain grows on LB or 2xYT plates (and is resistant to **tetracycline)**, and competent cells can be made using standard techniques. The parent for this work, the **lac UV5 promoter**, differs from lac wild type in that the latter contains nonconsensus nucleotides at positions −8 and −9 (TATGTT), WT is TATAAT. The consensus UV5 substitutions increase transcription from 5- to 50-fold.

**Today**, we will simply spread the various *E. coli* strains expressing each of the RNAi constructs on NGM agar medium. The *C. elegans* eat the bacteria and when ingested, the RNAi genes are expressed in the host worms. The plates are incubated overnight at 37C and then stored at 4C until used.

Spin out 1.5 ml of the four *E. coli* cultures in the microfuge. Resuspend the cell pellet in 300 μl of fresh broth containing 1 mM IPTG Incubate at room temperature for 20 minutes then pipet the cells onto plates NGM with 100 μg/ml ampicillin that were previously spread with 50 ul of fresh 100 mM IPTG. Swirl to roughly spread the culture and incubate at 37C.

**HO-CH2-(CH2-O-CH2-)n-CH2-OH** polyethylene glycol – molecular “crowding agent” acts like a sponge to absorb water and increase a solutes effective concentration. The phase transition prompted by PEG addition promotes the aggregation of phage particles to facilitate recovery by centrifugation.

**1X NEB buffer 3 = 100 mM NaCl, 10 mM MgCl2, 1 mM DTT, 50 mM Tris-HCl, pH 7.9**

**LAB 10 (short quiz today)**

Today we will resolve our single stranded DNA on an agarose gel. For comparison purposes, we will run the double stranded forms in the adjacent lanes. Also, we will combine single-stranded versions of 18U/19U into the same tube to see if we get a partial hybrid that migrates differently.

**Washing Northern Blots (each group)**

In this step, you will remove the probe adsorbed (i.e., wetting) directly on the transfer membrane with a low temperature, low salt wash and the probe associated by weak non-specific nucleic acid contacts through a high temperature wash, low salt. What probe remains bound is associated through extensive base pairing with RNA fixed to the membrane.

 1. Carefully pour the waste probe in the large waste beaker. Rinse the roller bottle with 1/2 volume of **0.2X** SSC, 0.1% SDS (recall that your hybridization conditions contained **6X** SSC). Simply add the wash, cap the bottle, invert the bottle 5 times, the pour out the wash into the large waste container.

2. Wash the filters 3 X 15 min. at 60oC. The TAs will pre-warm the wash solution to 50-60C before you use it. Fill the roller bottle 3/4ths full with wash solution for each wash. Be careful not to spill the radioactive wash solutions.

3. Remove the membrane and blot off the excess liquid with a Kim wipe. Place the membrane between two pieces of plastic wrap. The TAs will assist you in exposing the membranes to X-ray film. The correct order of placement is: intensifying screen/film/blot. Be sure to mark the position of the wells (or outline your blot) on the film (not on the intensifying screen). Expose your 18u and 19u hybridizations side by side. Recall, one of the in vitro synthesized riboprobes will be complementary to the cellular RNA (and therefore hybridize) while the other will be equivalent to the cellular RNA (and therefore not hybridize).

**Single-Stranded DNA Gel Mobility (as a group)**

1. Each student should run 3 µl of their **uncut plasmid miniprep** and **1 µl** of their single stranded DNA. Add each sample to 2 µl of sample dye (do not add the sample dye to your stock tube of DNA). Load in the following order:

**lane 1-** 2µl (miniprep, double stranded)

**lane 2 -** 3µl of the 1 kb molecular weight markers

**lane 3 -** 1µl of the ssDNA, group member 1

**lane 4 -** 1µl of the ssDNA, group member 2

**lane 5 -** 1 µl single-stranded DNA from of other type (19U if you had 18U; 18U if you had 19U

**lane 6 –** 1.5 µl of a 1:1:1 mixture of single stranded 18U + 19U+ 3X NEB buffer 3; **note: pre-heat the 3µl mix at 65C for 10 minutes then cool to room temperature. Spin briefly before adding dye and loading**

‑Be sure to record the order of student loading. Run @ ~60 volts until the dye is ~3/4 the way through the gel. Photograph (you don't need the ruler). **NOTE: Please place your single stranded DNA into the dry ice when finished. Be sure that each tube is labeled with your group number and your initials. This will be sent to the DNA sequencing facility for analysis.**

**ANSWER THIS IN YOUR NOTEBOOK**: **The M13KO7 helper phage DNA runs near the 5 or 6 kbp ladder band. A linear plasmid with insert would run at ~3.4 kbp, how does this compare with what you observed? Why can't you use the 1 kbp DNA ladder to obtain an accurate measurement of the lengths of your (uncut or single stranded) plasmids?**

***Caenorhabditis elegans* (part 2)** Today we will pipette 100 µl of the juvenile worms onto the bacterial surface. Transfer will be done with the pipetman (details provided in class). The worms tend to settle in the tube, so gently invert the tube a few times before each worm transfer. C. elegans eat the bacteria expressing the RNAi construct and develop in the presence of this “knockdown” reagent which is complementary to an endogenous (that is, natural worm) mRNA. The RNAi/mRNA duplex targets the mRNA for destruction. The result is to remove from the cell the protein encoded by this mRNA.

**Here is a list of the genes for which we will target the corresponding mRNA for destruction by RNAi using plasmid constructs expressed in HT115. The HT115 bacteria are then fed to the C. elegans nematodes and the double stranded siRNAs enter the cells of the worms and destroy the corresponding mRNA in the animal. Once the siRNA is inside the worm it is amplified (more is made) through a worm encoded RNA-dependent RNA polymerase.**

***Asd-1*:** *Alternative splice defective-1.* A largely uncharacterized gene that encodes a 404 amino acid protein which, together with the FOX-1 protein acts to promote **alternative pre-mRNA splicing**. This gene is expressed in the larval and adult pharynx. The knockdown does not noticeably inhibit animal development but does change the splicing pattern of the egl1-15 mRNA (we will look at this in Bio 510 using a dual reporter gene in which the alternatively spliced mRNAs make either GFP (which fluoresces green) or RFP (which fluoresces red).

***Unc-22*:***Uncordinated 22* encodes twitchin, a giant (6049 amino acid) intracellular protein with multiple fibronectin- and immunoglobulin-like domains and a single protein kinase domain that is homologous to titin. UNC-22 is required in muscle for **regulation of the actomyosin contraction-relaxation cycle**. RNAi knockdown animals move slowly and show constant trembling when compared to wild type worms..

***Dpy-1*:** *DumPY* -1 encodes a 1286 amino acid protein important for normal **fat metabolism**. The dpy-1 knockdowns are shorter and stouter than wildtype and also show lower levels of lipid.

***CLF1*:** *Crooked neck-like factor 1* encodes an essential 744 amino acid protein that is a required **subunit of the spliceosome** (the mRNA splicing enzyme). The RNAi animals die during embryonic development.

***Dyn-1*** encodes the C. elegans ortholog of the *dynamin GTPase*; dyn-1 activity is required for endocytosis, synaptic vesicle recycling, cytokinesis, and the CED-1 pathway that regulates engulfment and degradation of apoptotic cells; mutations in dyn-1 affect locomotion, egg-laying, defecation, and embryonic development, indicating that dyn-1's endocytic function is required for a number of diverse processes; dyn-1 reporter fusion constructs are expressed in motor neurons, intestinal cells, and pharyngeal muscle. The RNAi reports embryonic and larval lethality and maternal sterility.

|  |
| --- |
| ***Erm-1*** The erm-1 gene encodes an ortholog of the **ERM family of cytoskeletal linkers** with approximately equal similarity to ezrin, radixin and moesin. The RNAi reports slow growth, uncoordinated movement with some larval lethality and reduced fertility. |
|  |  |

**HT115-L4440:** This is the **bacterial host** strain that is transformed with the empty vector plasmid used to produce in E. coli double stranded RNA for the genes listed above. F-, mcrA, mcrB, IN(rrnD-rrnE)1, lambda -, rnc14::Tn10 (RNaseIII mutant; Tn10 confers tetracycline resistance), lambda DE3 lysogen: lac UV5 promoter –T7 polymerase; IPTG-inducible T7 polymerase)

**LAB 11**

**Analysis of the Northern Blot**

1. Identify each of the bands present on the northern blot. **Are any bands unique to the 18U or** 19U probes? What is the *in vivo* direction of transcription (relative to the EcoRI and HindIII restriction sites) of the gene encoded by your yeast DNA? What is the length in nucleotides of this RNA transcript?  **Use the 25S and 18S rRNA to create a standard curve on semi-log paper and use this to determine the length of the unique RNA band. Include this graph in your notebook.**

**Polymerase Chain Reaction (PCR)**

Today we will perform the polymerase chain reaction (PCR) on 1) the yeast genomic DNA iso­lated last week and 2) a plasmid DNA (details discussed in class**).** The PCR reaction simply consists of multiple rounds of DNA synthesis on a predetermined region of your target DNA. The target DNA is located between a pair of synthetic DNA oligonucleotides (oligos) that are: 1) complementary to opposite strands of the target and, 2) oriented with their respective 3' ends towards each other across the target. Taq DNA polymerase is a heat stable enzyme that uses the oligos as primers for DNA synthesis. Each round of DNA synthesis is followed by a high temperature dissociation of the double stranded reaction products. The released single‑stranded reaction products can then bind a new oligo to initiate another round of amplification. For the yeast DNA, the wildtype *UPF1* gene amplification should be ~3000 bp while the deletion mutant (*upf1::KAN*) is 1700. PGMG 26-28.

**Polymerase Chain Reaction (PCR)**

**(Each Student)**

Add: 2.0 µl of yeast genomic DNA template (either strain "A" “B” or C strain; DNA concentra­tion ~0.1 mg/ml) to18.5µl H2O in a 0.2 ml PCR tube

**Add 3.5 µl of a mix containing:**

 [0.25 µl of upstream (or 5') primer\* (0.5 µg of 20‑mer)

 0.25 µl of downstream (or 3') primer\* (0.5 µg of 20 mer)

 2.5 µl of 10X buffer

 0.5 µl of 10 mM dNTP]

**Add**

 1.0 µl of NEB Taq polymerase (~5 units)

\* final primer concentration ~1 µM

Mix briefly and spin briefly. Be sure to label the **cap** of your tube.

**3’ at 94C followed by 30** cycles: [45” @ 94C (denaturation), 45” @ 58C (annealing), 3.5 min @ 72C (extension)]

10X Buffer = 500 mM KCl

 100 mM Tris‑HCl, pH 8.3 (at room temperature)

 15 mM MgCl2

 0.1% gelatin

**Inverse PCR to create an in-frame deletion within a protein coding sequence.** Here the PCR reaction will be repeated as above but substituting the SP6-HZ18 plasmid (details provided in class) for the yeast genomic DNA (plasmid details provided in class). The primers are designed to delete out a 400 bp intron sequence from the yeast *RPS17A* gene cloned into this plasmid. After PCR, you will compare the length of the full-length linear plasmid with the amplified DNA (which should be shortened by 400 bp)

1 Students add 1.0 µl of the SP6 HZ18 DNA template to19.5 µl of H2O in a 0.2 ml PCR tube

Add 3.5 ul of the following pre-mix

 0.25 µl of upstream (or 5') primer\* (0.5 µg of 20‑mer)

 0.25 µl of downstream (or 3') primer\* (0.5 µg of 20 mer)

 2.5 µl of 10X NEB LongAmo buffer

 0.5 µl of 10 mM dNTP

Add:

1.0 µl of Roche expand long polymerase (~5 units)

**2’ at 94C followed by 30** cycles: [45” @ 94C (denaturation), 30” @ 45C (annealing), 6 min @ 65C (extension)]

1X Buffer = 60 mM Tris-SO4 (pH 9.0 at 25C), 20 mM (NH4)SO4, 2 mM MgSO4, 3% glycerol, 0.06% IGEPAL CA-630, 0.05% Tween 20 (Note: both IGEPAL and Tween are non-ionic detergents)

**PCR (Polymerase Chain Reaction) amplifies specific segments of DNA from complex mixtures.**

**The cycling reactions :** There are three major steps in a basic PCR reaction , which are repeated for 15-40 cycles. This is done on an automated thermo cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

**Denaturation** at 94°C (or as specified by the vendor of the specific polymerase used). During the denaturation, the double stranded template melts open to provide access for the single stranded DNA oligonucleotide primers.

**Annealing** typically between 37-60°C (parameters determined by the hypothetical annealing temperature of the primer/template pair. A number of web sites are available to assist with this, e.g., <http://frodo.wi.mit.edu/primer3/>

During the annealing phase, the oligonucleotide primers basepair with the template DNA.

**Extension** at 72°C (or as specified by the vendor of the specific polymerase used). A good rule of thumb is 1 minute per 1,000 basepairs. Repeat as needed to generate suitable quantities of DNA (often 20-30 cycles although fewer or greater numbers may be required). **Note that, starting with a single DNA molecule, the number of DNA products with primer-defined PCR sequences at both ends of the molecule is 2n –2n (where N is the number of cycles).** The first fully primer-defined double stranded DNA molecule is not made until the 3rd PCR cycle

.

**Note that, starting with a single DNA molecule, the number of DNA products with primer-defined PCR sequences at both ends of the molecule is 2n –2n (where N is the number of cycles).** The first fully primer-defined double stranded DNA molecule is not made until the **3rd PCR cycle. QUESTION: There is an error in the diagram above, what is it?**



**Gene identification by in vivo complementation: yeast transformation (each group does a set of three transformants)**

A critical test of whether a cloned DNA encodes a gene of interest is to determine whether that DNA genetically complements a strain deficient in the activity under study. Today, we will transform a conditional‑lethal strain of yeast with a set of yeast/E. coli shuttle plasmids. This yeast strain is deficient in the *URA3*‑encoded enzyme (orotidine‑5'‑phosphate decarboxylase) required for the biosynthesis of uracil. Hence the untransformed strain will not grow unless uracil to be added to the growth medium; transformants will grow in the absence of uracil. This yeast also has a temperature sensitive mutation (ts) mutation in the U1 snRNA gene, *SNR19*. At room temperature, this ts mutation is silent (the organism lives), at 37oC this mutation is lethal. One of the plasmids is pTZ18u and this cannot transform yeast. Two of the pasmids have 2 origins of replication (one for *E. coli*, one for Saccha­romyces cerevisiae), selectable marker genes for bacteria (amp resistance) and yeast (*URA3*) and one of three possible yeast DNA segments (A, B, or C). Only one of these plasmids (i.e., segments A, B, or C) encodes the wild‑type (normal, not temperature sensitive) allele of *SNR19*, the other two contain unrelated DNA sequences. You will select yeast transformants on uracil deficient agar media and determine which transformants possess the *SNR19*‑contain­ing DNA by screening for cell growth at 37oC.

**NOTE: Use sterile technique throughout this protocol.** Before each use of the Pipetman, wipe its shaft with ethanol. Use the fresh, sterile pipette tips provided and do not leave the top of the box open between pipetting.

**Each Group 510 protocol**

**Rapid Yeast Transformation (see PGMG 202-206)**

**Each Group**

1. Start off with ~30 ml of a mid to late log culture of yeast (OD 600 between 2 and 4) grown in YPD medium.

2. Wash the culture twice with 5 ml of Li buffer (100 mM LiOAc, 10 mM Tris pH 7.5, 1 mM EDTA).

3.Spin as before and then resuspend in 2 ml of Li buffer.

**Each Student**

4. Add 500 µl of culture to each 13X100 mm (small) capped glass culture tube. Prepare and label **one** tube for each DNA to be tested. **Be sure to note the specific construct used**.

5. Add plasmid DNA (1-3 µg in 10µl), incubate for 5 minutes at room temperature.

6. Add 25 µl of 10 mg/ml salmon sperm DNA (heat denatured for 10 min. @100C prior to use)

7. Add 25 µl of DMSO incubate 10 minutes at room temperature.

8. Add 2.5 ml of 40% PEG 3000 in Li buffer. Incubate for 30 minutes at 30C.

9. Heat shock at 42C for 15 minutes.

10. Spin out the culture, wash once in 1 ml of sterile (i.e., freshly autoclaved) water. You can vortex to mix the cells. Spin the cells for 5 minutes at full speed in the clinical (tabletop) centrifuge. Pour off the liquid. Vortex what is left in the tube (usually ~ 150 µl of liquid remains) and **plate the yeast evenly on *two* selective plates (estimate volumes)**. You can expect 200-1000 transformants per microgram of plasmid DNA. Incubate one plate at 23C and one plate at 37C.

**NOTES:**

1. Use sterile technique throughout the experiment but do not hold the pipettes over a flame or you will kill the cells.

2. Check to be sure that the water bath is at the right temperature before starting the experiment.

3. Use the clinical centrifuge for all spins. A 5 minute spin at room temperature should be sufficient to pellet the yeast.

4. Be sure that you use water that was recently autoclaved for your washes.

***Caenorhabditis elegans* (part 3).** Under the dissecting microscope, compare the sizes, shapes, numbers movement of the RNAi control worms with that of those containing an RNAi selected to destroy a cellular mRNA. Record these characteristics in your notebook – clearly highlighting the RNAi defects. We will also be using a florescence microscope on the 3rd floor to monitor changes in alternative pre-mRNA splicing (discussed in class).

**Homework (10 points):** Chromatin immune precipitation (CHIP) is a common technique designed to identify DNA cellular targets for transcription factors or to determine the histone modification state of DNA. Design a CHIP experiment using Saccharomyces cerevisiae that can be used in the BIO 510 laboratory.

**In order to receive full credit you need to include each of the following:**

1. Your statement of the experimental goal.
2. A full reference for the paper from which you have taken this protocol. A photocopy of the methods pages from which you adapted the protocol. You MUST use approaches that can be done “in class” and reagents (i.e., antibodies) proven to work with S. cerevisiae.
3. A picture of what the “results” might look like (taken from the same or another paper).
4. A step-by-step student-adapted protocol written in your own words (not photocopied from the paper) similar to what is presented to you for other BIO 510 lab exercises. Be sure to include a timeline for completion, that is, what steps are done in each lab meeting. Also describe the necessary control experiments needed to interpret your results. This should include all steps (e.g., chromatin preparation/crosslinking, IP recovery, PCR analysis)
5. Complete vendor, catalog number and price listing for all the enzymes and any “specialty” reagents that might be needed (assume that if we haven’t already used it in class, then it is a “specialty” reagent, these might include antibodies, oligonucleotides etc).

**Lab 12**

**Analysis of the genomic PCR reactions (each student).** Your PCR reactions were placed in a –20C freezer after completion of the 30 programmed cycles Resolve **4** µl of your PCR reaction alongside 3 µl the 1 kbp marker. Use 1 or 2 µl of the blue loading dye. Recall that there is oil on top of the PCR reaction – so, put the pipet tip to the bottom of the tube to remove the sample and not the oil layer on top. Run the 1% agarose gel at 60 volts until the fast dye (bpb) is ¾ the gel bed length.

**Question.** Which genotype was stain A? Stain B? C?

**Inverse PCR results.** Run the inverse PCR results on the same gel as the genomic DNA PCR reactions. If the experiment worked, you should see that the deletion primer set generates a band shortened by the length of the predicted deletion. The predicted size of the PCR amplification if correct, is ~4,800 bp. The linearized plasmid DNA is 400 bp longer (or ~5200 bp). Recall that this is a full plasmid amplification – so the bands are both large, you need to run the gel a long time in order to resolve the mutant and wildtype DNAs. Run 2 µl of your inverse PCR product and 4 µl of linear plasmid DNA (the same DNA used as a template for the inverse PCR but cut with EcoRI by the TA).

If you wanted to clone this deletion mutant derivative (the PCR removes the intron from the ***RPS17A* gene**), all you would need to do is to blunt the ends (since Taq leaves non-encoded 3’ adenosines), phosphorylate the (since oligonucleotide primers typically do not 5’ phosphates when purchased), ligate & transform *E. coli.* Since PCR is error-prone, it is important to sequence the recovered DNA to confirm the change and rule out the introduction of unexpected mutations.

**NOTE:**  This same approach can be used to introduce more limited mutations (e.g., single base substitutions, alanine scans, etc) or to modify the DNA to introduce a new restriction site for subsequent use. Since a wildtype plasmid was used for amplification, it is possible that full length (i.e., non-mutated) plasmid also will be recovered. Should this be a problem, simply cut the ligated DNA with the Dpn I 4-base cutter prior to *E. coli* transformation. DpnI specifically cleaves methylated and hemi-methylated DNAs – only the original plasmid DNA (which comes for *E. coli* & is methylate) will be cut & destroyed. The PCR –generated plasmid is not methylated and hence is protected.

**Plasmid Shuffle**

The plasmid shuffle is a common way to **test whether particular mutation on a cloned gene inactivates (or other ways alters) the function of that gene**. To do this, we need a strain of yeast with the following characteristics: a **chromosomal mutation** that fully inactivates an essential “gene of interest” (here we will use a *prp43* null mutant), and mutations in at least two genes that can be used for plasmid selection (here we will use *ura3* and *trp1* mutations). Figure 7 in the Vincent et al paper on the class web site shows how we used this technique to score the function of a newly identified component of the yeast splicing apparatus.

**NOTE**: *PRP43* encodes a nuclear enzyme essential for two biological processes, 1) the splicing of pre-mRNAs into mRNA that is exported to the cytoplasm for protein production and 2) the endonucleolytic processing of ribosomal RNA (rRNA) into the 25S and 18S rRNA core components of the ribosome.

In addition, this strain will be is simultaneously transformed with **two different plasmids** (that is, two plasmids in the same cell). **One plasmid** will have a functional copy of the *URA3* selectable marker (which complements the *ura3* chromosomal mutation) plus a fully functional copy of the gene of interest (here, *PRP43*). **The second plasmid** will have the *TRP1* selectable marker (to complement the chromosomal *trp1* mutation) plus (IMPORTANTLY) **the mutant version of the gene of interest that one wants to score** (here a point mutation called *prp43-H218A*).

As described, this strain survives on –ura, -trp medium since it has plasmids that complement both nutritional markers (i.e., *trp1* and *ura3*) and at least one copy of functional *PRP43* (which is required for life).

The question you want to answer is whether the *prp43-1* point mutation is capable of supporting life. To do this, you need to determine whether or not yeast can live without the *URA3-PRP43*(WT) plasmid. This can be done by streaking the culture on medium containing 5-fluoro-orotic acid which kills cells that have the *URA3* gene (since the enzyme encoded by *URA3* (orotidine-5'-phosphate decarboxylase) converts the 5’FOA compound into the toxic anti-metabolite 5-fluorouracil, Yeast cells normally have a low level of plasmid loss during mitosis. So, if the yeast culture is able to survive with the TRP1-*prp43-1* allele as its sole source of the essential Prp43 protein, then the yeast will grow on the 5’FOA medium after spontaneous *URA3-PRP43* plasmid loss. If the *prp43-H218A* mutation is lethal, the yeast will not be able to grow on the 5’FOA medium after spontaneous *URA3-PRP43* plasmid loss. **Streak cultures containing the following plasmids on the FOA medium and, in parallel, on YPD medium.**

1. *URA3-PRP43, TRP1- prp43-H218A*
2. *URA3-PRP43, TRP1-PRP43 (positive control)*
3. *URA3-PRP43, TRP1-empty vector (negative control)*
4. *URA3-SNR19-YCpLac33 from our earlier transformation)*
5. *URA3-empty-YCpLac33 from our earlier transformation)*

***5’ FOA URA3-> ***5-fluorouracil

**Lab 13**

**Plasmid Shuttle (part 2). Today we will score the *prp43-H218A* as biologically functional or not based on its ability to serve as the sole source of the essential Prp43 protein.**

Record your observations. Which of the following formed colonies on the FOA plate?

1. *URA3-PRP43, TRP1- prp43-H218A*
2. *URA3-PRP43, TRP1-PRP43 (positive control)*
3. *URA3-PRP43, TRP1-empty vector (negative control)*
4. *URA3-SNR19-YCpLac33 from our earlier transformation)*
5. *URA3-empty-YCpLac33 from our earlier transformation)*

**Answer these questions in your notebook:** What do you conclude about the *prp43-H218A* mutations – does it inactivate the gene or not? Defend your answer (tell why you believe this to be so).

The FOA selection enriches to cells that have spontaneously lost the *URA3* plasmid. A prediction of this observation is that FOA+ cells should not grown on uracil. A further prediction of this observation is that any other genes present on the originally transformed URA3-marked plasmid will be lost. To test these observations,

**Patch** two colonies from each of the strains supporting growth the 5 FOA plates onto one plate that lacks uracil (-ura plate). Include as positive controls the original (that is, before FOA selection) *URA3-SNR19-YCpLac33* and *URA3-empty-YCpLac33* transformants. For this to work well, you want to patch the colonies sparingly (that is, transfer a small amount of the colony, not a big “gob”). Incubate the plates at 30C.

We saw that *URA3-SNR19-*YCpLac33 complements the temperature sensitive (ts) growth defect of our *snr19* mutant (Lab 11). We predict that the loss of *URA3-SNR19-*YCpLac33 after 5 FOA selection should result in this strain re-acquiring the ts growth defect.

**Streak for single colonies** on two YPD plates (which has uracil) the original *URA3-SNR19-YCpLac33* and *URA3-empty-YCpLac33* transformants and the FOA+ versions of these same constructs. Place one YPD plate at 37C and one at 30C. For this to work well, you will need to carefully follow the in-class instructions for streaking for single colonies.

**Defining a Protein-Protein Interaction Domain by the Yeast Two-Hybrid (Y2H) Assay.** *The basis of this assay and its applications is described in detail on pages 458-464 in your textbook. This is* ***required reading*** *for a full understanding of this technique. In addition, I added a paper on the yeast host strain system used here on the web site –* ***this is also required reading.*** Note that in this latter paper, the plasmids used for two-hybrid construction are not pACT and pAS2 but the principles remain the same.

**The Pxr1 protein** physically binds to the Prp43 enzyme to promote Prp43 activity in ribosomal RNA processing. The site of Pxr1 interaction with Prp43 is unknown. Here we will determine the site of interaction by testing for the ability of different regions of Pxr1 to bind Prp43 in the yeast two hybrid system. In essence, what occurs is that we bisect the Gal4 transcription activator into two parts. To begin this study, we created a protein fusion between the Gal4 DNA binding (AS2) domain with the full length Prp43 sequence. We then created a set of overlapping Pxr1 domain fusions with the Gal4 transcriptional activation (ACT) domain. We then test each Gal4-Pxr1 protein fusion for its ability to interact with the Gal4-Prp43 domain fusion in yeast. Where the interaction occurs, the Gal4 DNA binding domain and transcription activation domain are brought together to reconstitute the Gal4 protein – this reconstituted Gal4 protein is then able to stimulate transcription of three specific reporter genes in the yeast host. The bottom line is that when the fusion proteins interact, the yeast is able to grown on medium lacking histidine or adenine – this “positive” interaction defines the site(s) on Pxr1 that bind Prp43. When the fusion proteins do not interact, the yeast is not able to grown on medium lacking histidine or adenine – this “negative” interaction suggests that the Pxr1 segment being tested does not have a high affinity site for Prp43.

 <http://cmbi.bjmu.edu.cn/cmbidata/proteome/method/research05.htm>

(this site has some nice references for the use of two-hybrid approaches)

**Host Strain**

PJ69-4A ***MATa*** *trpl-901 leu2-3,112 ura3-52 his3-200 ga14 Δ ga18OΔ* ***LYS2::GALl-HIS3*** *GAL2-ADE2 met2::GAL7-lacZ*

**Yeast Transformation Plasmids:** pACT (contains the *LEU2* reporter gene plus the GAL4-activation domain; pAS2 (contains the *TRP1* reporter plus the *GAL4*-DNA binding domain). In both cases, the Gal4 segment is at the amino terminus of the fusion protein. Please see the lab web site for a pdf that contains additional descriptions of the pACT and pAS2 two plasmids.

**Observation:** Pxr1 stimulates Prp43 enzyme activity. **Question:** What region(s) of the Sqs1 protein bind Prp43? **Approach:** Score a set of Pxr1 deletion derivatives for Prp43 interaction by the yeast two-hybrid method. **Rationale:** Pxr1 fragments that give a positive response interact and must retain the Prp43 binding surface. Pxr1 fragments that give a negative response do not interact and therefore do not possess a functional binding surface.

**Here is a map of the Pxr1 surfaces to be scored against the full-length Prp43 protein**. Note, other than the full-length construct labeled “A”, all other images show the region of the protein that is deleted in the construct to be tested. For instance, construct “B” lacks amino acids 25-70 of the native protein but has everything else; construct “C” has a larger deletion that extends from amino acid 1 through position 101. The “G-patch” and KKE/D labels refer to specific sequence features of the Pxr1 protein.



Today, we will start with the PJ69-4A strain that we already transformed with pAS2-Prp43 (full-length Prp43, Gal4-DNA binding domain) construct and introduce by transformation the various Pxr1 peptide- Gal4 activation domain constructs. Each group will transform a different Pxr1, Gal4-activation domain plasmid and together, the lab will score the surface of Pxr1 for Prp43 binding sites.

**Rapid Yeast Transformation (see PGMG 202-206)**

**Each Group**

1. Start off with ~30 ml of a mid to late log culture of PJ69-4A yeast already transformed with pAS2-Prp43 (OD 600 between 2 and 4) grown in YPD medium.

2. Wash the culture twice with 5 ml of Li buffer (100 mM LiOAc, 10 mM Tris pH 7.5, 1 mM EDTA).

3.Spin as before and then resuspend in 2 ml of Li buffer.

**Each Student**

4. Add 500 µl of culture to each 13X100 mm (small) capped glass culture tube. Prepare and label **one** tube for each DNA to be tested. **Be sure to note the specific construct used**.

5. Add plasmid DNA (1-3 µg in 10µl), incubate for 5 minutes at room temperature.

6. Add 25 µl of 10 mg/ml salmon sperm DNA (heat denatured for 10 min. @100C prior to use)

7. Add 25 µl of DMSO incubate 10 minutes at room temperature.

8. Add 2.5 ml of 40% PEG 3000 in Li buffer. Incubate for 30 minutes at 30C.

9. Heat shock at 42C for 15 minutes.

10. Spin out the culture, wash once in 1 ml of sterile (i.e., freshly autoclaved) water. You can vortex to mix the cells. Spin the cells for 5 minutes at full speed in the clinical (tabletop) centrifuge. Pour off the liquid. Vortex what is left in the tube (usually ~ 150 µl of liquid remains) and **plate the yeast evenly on selective medium lacking both leucine and tryptophan**. You can expect 200-1000 transformants per microgram of plasmid DNA. Incubate one plate at 30C. **NOTE, the TAs will do the transformation for the full-length (positive control) Gal4-Pxr1 construct. In addition, the TAs will do a class negative control** (no plasmid DNA) to estimate the frequency of Leu+, Trp+ revertants (these will be rare).

**NOTES:**

1. Use sterile technique throughout the experiment but do not hold the pipettes over a flame or you will kill the cells.

2. Check to be sure that the water bath is at the right temperature before starting the experiment.

3. Use the clinical centrifuge for all spins. A 5 minute spin at room temperature should be sufficient to pellet the yeast.

4. Be sure that you use water that was recently autoclaved for your washes.

**Lab 14**

**Plasmid Shuttle (part III).** Score the growth at 30C and 37C of the *URA3-SNR19-YCpLac33, URA3-empty-YCpLac33*  transformants and the same cultures after selecting for cells that have spontaneously lost the Ycplac33 plasmid on FOA medium. ***Record your observations in your notebook and provide a detailed discussion/explanation of what you see.***

**Yeast Two-Hybrid (Y2H) Assay, part II.**

Patch two transformants from each plate onto –leucine, -tryptophan medium. These purified double-transformant colonies will be incubated until Wednesday and then scored for trans-activation (that is, a positive two-hybrid signal) on medium that lacks histidine.

**Modified Realtime PCR Experiment**

**Real Time PCR.** Real time PCR is the current gold standard to measuring the change in abundance of RNA or DNA in a sample. We will use the iCycler iQ from BioRad, see the lab web site. Today we will set up a class standard curve using a known amount of target DNA (yeast gene ***SQS1***) and individual student reactions containing an unknown quantity of DNA. The primers amplification flank a sequence that is **126** bp in length. We will use SYBR green dye that fluoresces in the presence of double stranded DNA several folds higher than in the presence of single stranded DNA (similar to EtBr). The Real Time PCR uses a CCD camera to detect the fluorescence and the fluorescence increases with the production of double stranded DNA product – this is observed during the experiment (i.e., in real time). The real time PRC system is linear over 8 orders of magnitude in DNA content. PGMG 30-31

We will also use a melt curve to detect the products obtained at the end of the reaction instead of running a gel (conventional PCR). At the melting point of the PCR product (which depends on the GC content and size of the product), there is a rapid change in the fluorescence. A melt curve is plot of the change in fluorescence over temperature. An ideal PCR run would yield just one peak.

Record your data and estimate (using a graph of the control data) the amount of DNA in your sample. Similar experiments (but with controls) can be used to measure viral loads (e.g., during HIV infection) or the presence/abundance of pathogens (anthrax spores).

**2X plus Master Mix (purchased from BIO-Rad):**

**Student 1 in group**

Set up four 10-fold dilutions of your starting control DNA. Do this by pipetting 9 µl of sterile water into each of 5 microfuge tubes. Your stock DNA is at a concentration of 100 ng/ µl. Transfer 1 µl of the stock DNA to the first dilution tube mix and label this tube 10 ng/ µl. Next transfer 1 µl of the 10 ng/ µl DNA to the next dilution tube, mix and label this 1 ng/µl. Continue the 10-fold dilutions to prepare 0.1 ng/µl and 0.01 ng/µl dilutions

**Student 2 in each group. Set up7X Master Mix:** Add the following in order:

45.5 µl sterile water

87.5 µl 2X iQ Sybr green supermix (100 mM KCl, 40 mM Tris-HCI, pH 8.4, 0.4 mM of each dNTP (dATP, dCTP, dGTP, and dTTP, 50 units/ml iTaq DNA polymerase, 6 mM MgCl2, SYBR Green I, 20 nM fluoresein, and stabilizers).

17.5 µl 5 uM Primer 1(SQS1 G patch F – 20 mer)

17.5 µl 5 uM Primer 2 (SQS1 G patch R – 20 mer)

Mix briefly.

**Student 1)**

- Pipet 24 µl of the 7X Master Mix into 5 fresh microfuge tubes labeled 100, 10, 1, 0.1 and 0.01.

-Transfer 1µl of your stock DNA or 1µl of your diluted DNA to each of the labeled tubes (for example, the tube labeled “100” gets one microliter of the stock = 100 ng; the tube labeled “10” gets one microliter of 10 ng/µl dilution) BE SURE TO LABEL EACH OF YOUR FIVE TUBES SO THAT YOU KNOW WHAT DILUTION IS IN EACH SAMPLE.

**Student 2 in group (DNA of unknown concentration)** Pipet 24 of the 7X Master Mix into 1 fresh microfuge tube. Add 1 µl of your DNA of known concentration, mix and label “unknown”

Mix briefly and transfer your samples into the microtiter dish using the coordinates Min provides.

****Collect and analyze the data with the yellow lens. Choose filter set 4 and select Sybr 490 as the fluorophore for all wells/tubes.  *Your “unknown” samples will fit somewhere on that curve – your job (using the computer software) is to use the standard curve to determine the DNA concentration in your sample.* The threshold cycle is the PCR cycle where the fluorescence is considered to be significantly above the background fluorescence measured at the beginning of the experiment. Significant is defined here as 10 times the mean of the standard deviation of the fluorescence during the first 10 cycles.

**Purification of a Recombinant Protein – use of a protein-fusion approach.**

**NOTE: *This is a previous year’s “winner” of a student suggested laboratory exercise.***

It is often the case where purified protein is desired (e.g., for use as an antigen for the production of antibodies, to characterize enzymatic activity or to study protein-protein or protein-nucleic acid interactions, to create a commercial product such as a bioactive hormone). While purification from the original source organism (e.g., human, chicken, yeast) may be impractical if the protein is in very low abundance. A variety of vectors have been created that allow the high-level expression of recombinant proteins in *E. coli*. The use of a heterologous system assures that the protein will be purified without the co-selection of interacting partners that may alter activity. The inclusion of affinity selection tool in the protein coding sequence facilitates protein recovery. Here we will use the pTXB1 vector from NEB to purify a 121 amino acid peptide cloned from the N-terminus of the yeast Spp382 protein. This vector creates a T7 RNA polymerase transcribed gene in which the Spp382 protein coding sequence is fused to the chitin binding domain (CBD) of *Bacillus circulans.*

Chitin is composed of ß(1-4) linked units of the amino sugar N-acetyl-glucosamine that is found throughout nature (in the exoskeletons of lobsters, insects, cell walls of fungi, etc).



The Spp382-CBD protein fusion can be selected on a chitin-agarose matrix. After selection, the matrix can be washed to remove non-specifically bound proteins and then the peptide eluted. Elution of the protein free of the CBD motif is made easy with pTXB1 as a self-cleaving intein peptide present in the vector between the CBD sequence and the cloning site for your gene (here, the *SPP382* segment). Cleavage results simply by the addition of the reducing agent dithiothreitol (DTT) which stimulates the intrinsic self-cleaving enzymatic activity of the intein – and thereby releasing the desired recombinant product free of any vector-derived sequence. See the “Impact Expression” PDF file on the class web site for additional details. Also see 90-91 in PGMG.

**Part 1. Purification of a Recombinant Protein: Induction of protein synthesis.** The pTXB1 fusion gene is expressed from a T7 RNA polymerase dependent promoter. We will use the E. coli strain ER2566 as a host since this strain contains the bacteriophage T7 RNA polymerase under the control of an IPTG-inducible promoter (ER2566 Genotype: *fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10*--TetS*)2 [dcm] R(zgb-210::Tn10*--TetS) *endA1* Δ*(mcrC-mrr)114::IS10*). An overnight culture of the pTXB1-SPP382 (1-121) was diluted 1/100 2.5 hours before class. You will induce gene expression by the addition of 100 µl of a 50 mM IPTG solution to the 10 ml culture (final concentration = 0.5 mM IPTG). Likewise add the same amount of IPTG to second culture contains the empty vector pTXB1. Incubate at 30C with vigorous shaking until 4:30 PM. In addition to the induced cultures, two cultures will be grown without IPTG as “uninduced” controls for the pTXB1-SPP382 and empty vector. At that 4:30, concentrate the cells in the centrifuge (10 minutes at 5000 rpm). Pour off the medium and freeze the cell pellets on dry ice (these will be stored at -80C until the next meeting).

**Lab 15**

**Yeast Two-Hybrid (Y2H) Assay, part III.** The yeast double-transformants will be scored for reporter gene trans-activation using medium lacking histidine. We note that this medium also contains 5 mM 3-aminotrizole (3-AT) which is a competitive inhibitor of [imidazoleglycerol-phosphate dehydratase](http://en.wikipedia.org/wiki/Imidazoleglycerol-phosphate_dehydratase) (this His3 enzymatic activity). 3-AT addition “tightens” the selection by making growth dependent on higher levels transactivation. A positive interaction (i.e., growth) is interpreted as indicating that Prp43 interacts with the region of Pxr1 included in that particular pACT plasmid.

Each student will **streak for single colonies** two patches of their double transformant, and one colony each of the same yeast host transformed with either the full-length pACT-*PXR1* (positive control) or the pACT-empty vector (negative control). To confirm that the yeast are viable, we will also streak the same yeast strains on –leucine, -tryptophan agar medium (which selects for the plasmids but does not require reporter gene trans-activation). Incubate all plates at 30C.

**Part 2. Purification of a recombinant protein: protein extraction and affinity selection.** Today we will break the *E. coli* cells by mechanical (freeze/thaw) and chemical means then take cleared supernatant and bind the released proteins to chitin-agarose beads. Non-specifically bound proteins will be dissociated by a high salt wash and then the proteins specifically bound to the chitin-agarose will be released by DTT-stimulated intein cleavage for subsequent analysis by polyacrylamide gel electrophoresis.

Resuspend your cell pellet in 400 µl of the B-PER protein extraction reagent (see lab web page) and vortex vigorously. P-PER contains a proprietary zwitterion (has both positive and negative charges) detergent that facilitates cell lysis. Another example of a common zwitterion detergent used for protein isolation is CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate).

**Structure of CHAPS**



Transfer the cell solution to a microfuge tube. Protein recovery is favored by efficient cell breakage & this is made better by cycles of freezing and thawing. Place on dry ice until the solution freezes solid (5 minutes). Next, thaw by warming the tube between your fingers and shaking. Repeat the freeze/thaw cycle one more time.

Next, add 2 µl (1 unit per microliter) of DNase I and incubate the cell mixture at 25C (room temperature) for 15 minutes. Spin for 5 minutes in the microfuge and carefully transfer 350 µl of the supernatant to a fresh tube. Discard the cell pellet.

Place 20 µl of the supernatant into a fresh microfuge tube with the blue protein loading dye and label **Total-vector or Total-Spp382 (with your initials and the date)** – put these tubes into the dry ice until next lab period.

 Transfer the remaining 330 µl of cell lysate supernatant to the tube containing the 50 µl of a 50% suspension of chitin agarose. Label this tube as Bound Induced-Spp382 or Bound Induced-vector depending on which sample you are working with.

Rotate the tubes for 30 minutes at room temperature.

Spin the tubes at 4,000 RPM for 15 seconds. Carefully remove the supernatant and gently resuspend (do NOT vortex) the agarose beads in 1 ml of the high salt wash buffer (20 mM Hepes pH 8.5; 500 mM NaCl, 1 mM EDTA). Invert the tubes 3X and then spin as before and remove the supernatant. Repeat the wash twice more (for a total of 3 washes) with 1 ml each of the wash solution.

Finally, remove final wash and add 100 of cleavage buffer to each sample (20 mM Hepes pH 8.5; 25 mM NaCl, 50 mM DTT. Mix gently and then incubate the sample overnight at room temperature.

The TAs will then freeze the sample until the next lab period.

**HOMEWORK:** Go to the class web page and open the file labeled FASTA hexokinase. Copy and paste the files as a group into the “one-click” Phylogeny.fr program <http://www.phylogeny.fr/version2_cgi/simple_phylogeny.cgi>

Note that the last of these sequences is of “unknown” origin. Run the program using the default settings (this may take 5-10 minutes to complete; a return results by email option is also available). Print the phylogenetic tree you complete in the PDF format and include in your homework. Does this tree composed only of one type of protein “make sense” to you in terms of the previously proposed evolutionary relationships of the organisms involved? Why or why not? Scroll through the “**Gblocks 0.91b Results”** window on the“Curation” page – this shows the areas of high & low sequence conservation in color. There are areas of high conservation, low conservation and some alignments are missing roughly half of the protein sequence found in the rest. Given the intracellular role of this protein, what sort of functional domain is likely to be most conserved? Speculate on what the function of “extra” sequences may be that is found in roughly half of the alignments. Is the “unknown” organism likely to be a mammal, fish, plant, fungus, or bacteria – explain the basis for your choice based on the alignment data.

**Lab 16**

**Defining a Protein-Protein Interaction Domain by the Yeast Two-Hybrid (Y2H) Assay.** Based on the results of the Y2H data map the domain within Pxr1 that binds Prp43. Go to NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi> ) and do a Blastp search using the segment of Pxr1 that interacts with Prp43 (not the whole Pxr1 sequence, just the peptide domain that interacts with Prp43. First search all sequences and see what the best 25 hits are that are not from Saccharomyces. Next use only the *Drosophila melanogaster* database and look at the best “hits”. Finally, search only the *Homo sapiens* sequence and find the best hits. **Answer this question in your notebook.** Based on your results, is this domain present in any human proteins or not? Justify your answer using specific examples from your Blastp analysis. Provide a thoughtful response, this is ***not*** a simple yes/no question.

**Purification of a recombinant protein: sample analysis – Part III.** Today we will visualize the proteins recovered by chitin-agarose affinity selection. We will resolve the proteins on a 15% polyacrylamide gel (29:1 acrylamide to bis:acrylamide) resolving gel, soak the gel with Coomassie blue dye (which binds tightly but nonspecifically to proteins), and then remove the excess stain with a methanol-containing solution to reveal the protein pattern.

Spin beads with your pTXB1 empty vector and the pTXB1-Spp382 (1-121) at 4,000 rpm and then transfer the supernatant to a fresh tube containing 100 µl of protein sample buffer. Label this as the ELUTED sample – this will contain the protein released after intein-based cleavage.

**For the Spp382 beads only (the vector beads can be discarded at this time),**

Add 1ml of low-salt bead wash buffer ((20 mM Hepes pH 8.5; 25 mM NaCl) to the invert several times, then spin beads at 4,000 rpm then remove the supernatant with a pipetman. Repeat this wash step two more times to removal any of the intein-released material that no longer is bound via the chitin-agarose link.

Remove the final wash from the beads and resuspend in 50 µl of protein sample buffer. Label this as BOUND sample. What remains on the beads is the chitin-binding domain-intein segment for both the pTXB1 empty vector preparation and the pTXB1-Spp382 (1-121) preparation (the latter assuming complete intein-cleavage).

Heat all samples at 100C for 10 min, remove but do not put on ice. Place a heavy glass plate or beaker over tubes to prevent these from popping open. After 10 minutes remove the whole block (with the glass lid) and allow to stand at room temperature for ~10 minutes before removing the tubes.

Vortex the samples briefly, then spin in the microfuge at full speed for one minute, Load gel (left to right) with 20 microliters of each sample

Lane Sample

1. Uninduced vector only Total
2. Uninduced pTXb1-Spp382 (1-121) Total
3. Protein markers
4. Total induced Spp382 group 1
5. Eluted control group 1
6. Eluted Spp382 group 1
7. Bound sample group 1
8. Total induced Spp382 group 2
9. Eluted control group 2
10. Eluted Spp382 group 2
11. Bound sample group 2
12. Total induced Spp382 group 3
13. Eluted control group 3
14. Eluted Spp382 group 3
15. Bound sample group 3
16. Total induced Spp382 group 4
17. Eluted control group 4
18. Eluted Spp382 group 4
19. Bound sample group 4
20. Total induced Spp382 group 5
21. Eluted control group 5
22. Eluted Spp382 group 5
23. Bound sample group 5
24. Protein markers
25. Previously purified Spp382 (1-121)

When the gel dye reaches the bottom, the TAs will stop the electrophoresis, stain the gel with Coomassie brilliant blue and then take a photograph of the samples. This will be posted to the web site – be sure to put this image into your lab notebook.

**Deep sequencing analysis, part I – Note: *a student-requested lab***

We previously identified four genes with previously unknown splicing patterns using the deep-sequencing approach. While such observations are interesting, one should rule out experimental artifacts by confirming the observation using an independent methodology. We will do this by making a cDNA library form the total yeast RNA (of the wildtype strain) and then using PCR to score for the predicted splice products.

**cDNA synthesis:**

We will use the Protoscript M-MuLV Taq RT-PCR kit provided by New England Biolabs. A PDF file that describes this system is on the lab web site and is required reading. NOTE: all buffers are listed in this PDF you should copy these into your lab notebook. The protocol below includes minor modifications for convenience.

**Each Student (annealing reaction):**

Mix the following in a test tube in the following order:

4 µl of DNase I-treated total yeast RNA (0.5 µg)

2 µl of a 1:1 mix of the random primer mix and sterile water

2 µl of dNTP mix

Mix, vortex briefly (5 seconds) and then put at 70C for 5 minutes

Next, spin briefly (5 seconds) and then transfer to ice.

**Each Student (cDNA synthesis reaction):**

Add each of the following to your annealed RNA/primer mix (from above)

1 µl of the 10X RT buffer

1 µl of a mixture of M-MuLV reverse transcriptase (0.5 µl) RNase inhibitor (0.25 µl) and water (0.25 µl).

Mix briefly, spin briefly, then incubate at room temperature for 5 minutes followed by an incubation at 42C for one hour.

At the end of the hour, inactivate the enzyme at 80C for 5 minutes. Next, spin briefly then add 15 µl of sterile water.

Freeze the cDNA on dry ice and store at -80 until next lab period.

NOTE: The TAs will do the –RT control.

**Mini Protein Gels (scaled down to the Invitrogen mini‑V 8‑10)**

**15% Resolving Gel** This recipe prepares enough acrylamide for one 1.5 mm spacer gel.

40% (29:1) acryl. 3.75 ml

1.0 M Tris (pH 8.8) 3.75 ml

10% SDS 0.10 ml

H2O 2.30 ml

10% APS 0.1 ml

TEMED 0.010 ml

Pour 1% agarose plug to seal the bottom of the gel. Simply pipet ~7 ml of agarose (in running buffer) onto a clear glass plate and then stand the gel upright over agarose. The agarose will move between the plates by capillary action. Note that the hotter the agarose, the further up the plate the plug will go.

**4% Stacking Gel** (NOTE: stack has different % acrylamide & buffer pH)

40% (29:1) acryl. 0.975ml

1.0 M Tris (pH 6.8) 1.25 ml

10% SDS 0.10 ml

H2O 7.58 ml

10% APS 0.1 ml

TEMED 0.010 ml

**Gel Running Buffer:** 3.0 g Tris base, 14.4 g of glycine, 1 gram SDS; adjust pH to 8.3 and the volume to 1000 ml. Run gels at 150 volts for ~ 1 hour.

**Coomassie stain** (per liter) 2.5 grams Coomassie brilliant blue, 90 ml glacial acetic acid, 450 ml methanol, 460 ml H2O

**Coomassie de-stain** (per liter) 50 ml glacial acetic acid, 450 ml methanol, 500 ml H2O

Protein sample dye: 4M urea,

**2X Protein Sample Buffer**

100 mM Tris-HCl, pH 6.8; 20% glycerol; 5% SDS; 5% β-mercaptoethanol 0.02% bromophenol blue

**Lab 17 Short Quiz Today**

**Deep sequencing analysis, part II.** To score for new introns and alterative 3’ splice sites, today we will use PCR to amplify the yeast cDNA prepared during the last lecture. LABEL the tube with your group number and the first three letters of the gene to be amplified (new intron, YMR147W, TAF13; 3' Alt Splice site: IWR1, or TAN1)

Add the following in a 0.2 ml PCR tube in the following order.

9 µl of sterile water

12.5 of Taq2 Master Mix

1 µl of a 1:1 mix of the upstream + downstream your specific primer mix (100 nm)

2 µl of the cDNA

Mix briefly, shake/tap to bring the liquid to the bottom of the tube, then put in the PCR block.

Cycle:

3’ at 94C followed by 35 cycles of:

30” @ 94C

30” @55C

15” @ 68C

Followed by 30” at 72C and a hold cycle at 10C until the sample is removed from the machine.

*Go to the lab web page to find the full composition of the Taq master mix and* ***write this composition in your notebook.***

**Northern blot.** Our comparison of the gene expression changes predicted by deep sequencing the transcriptome of the *PRP43* (wildtype) yeast and *prp43-H218A* mutant suggested that the normally transcriptionally repressed genes found at the telomeres, centromeres and in the area of the replication origins are up-regulated in *prp43-H218A.*

To learn if this is true, we will perform a northern blot to independently score for changes in mRNA abundance. **Each bench (two groups)** will load RNA on a single blot that will be cut in half and probed for one transcript predicted to change (the mannitol dehydrogenase encoded by the YNR073C open reading frame) and a control gene whose RNA is not expected to change (*ADE3*).

**Formaldehyde Northern Blot (each student)**

1. Add 10 μl of the RNA denaturation solution to each dried RNA sample containing 12 micrograms of wildtype PRP43 RNA or *prp43-H218A RNA.* The solution was prepared by mixing 5X MOPS buffer [0.1 M 3‑[N‑morpholino]propanesulfonic acid); 40 mM sodium acetate; 5 mM EDTA, pH 7.0], formaldehyde, formamide in a 1:1.75:5 ratio, then adding 400 ng of ethidium bromide.
2. Vortex the RNA sample vigorously several times over a 2 min. period. Spin the sample in the microfuge briefly to collect the liquid at the bottom of the tube.
3. Incubate the sample at 65oC for 15 min. and then place on ice for 2 min. Be sure that you use the correct (i.e., 65oC) incubator.
4. Add 2 μl of tracking dye (50% glycerol, 1 mM EDTA, 0.1% BPB/XC) to your sample. Mix and immediately load the sample into a gel well.
5. Load 4 μl of your sample to your 1% agarose/formaldehyde gel well. Load your group's samples in wells 1, 2, (and 3 if needed), leave wells 4 and 5 empty, load the second group’s samples a second time in wells 6, 7, (and 8, if needed. Record the order of student loading.
6. 7. Run the gel @ 55 volts (HIGHER voltages will greatly distort the sample migration) in 1X MOPS buffer until the tracking dye is near the bottom of the gel (at least 3/4th the length).
7. 8. Soak the gel 1 X 5 min. in 50 ml of water.
8. 9. Soak the gel 1 X 10 min. in 50 ml of 10X SSC (1.5M NaCl, 0.15M Na citrate, pH 7.0).
9. Set up the gel transfer by stacking in the following order (from bottom to top): Plastic wrap sheet, blotting paper saturated with 10X SSC, gel (**well side facing down**), positively charged nylon membrane­, blotting paper soaked in 10X SSC, dry blot paper, 2 inch stack of paper towels. **NOTE:** Use a syringe needle to **stab a hole in the position of each well**. In order for the transfer to work, the SSC in the gel must be drawn upward by capil­lary action through the charged nylon membrane. Air bubbles trapped between the gel and the filter membrane inhibit this process. To **remove any bubbles**, roll a Pasteur pipette over the gel surface and over the membrane and the next two layers of filter paper. Also, be sure that the paper towels do not "hang over" the gel and touch its sides or the towel/plastic wrap beneath. This would cause the system to short circuit (that is draw the liquid from a direction other that bottom to top). **Notch the membrane** on the top and bottom to distinguish the two halves of the blot. Blot overnight on the bench top. **The T.A.s will photograph the membrane along side of a florescent ruler** and then store the blots for you until the next lab period. The bottom of the well will correspond to “0” on the ruler.

**NOTE:** The yeast 25S rRNA band is 3700 nucleotides (nts) in length, the 18S rRNA is 1700 nts.

**MOVE**

**RNase A mapping of *SNR19* RNA abundance.** Today you will use the anti-sense *SNR19* (U1) snRNA thatyou produced by T7 RNA transcription to distinguish between the RNA recovered from two yeast cells, one a wildtype yeast that has normal levels of U1 snRNA and the second a yeast strain that produces excess U1 snRNA 3 to 5-fold due to the substitution of the *GAL1* promoter for the natural *SNR19* promoter. First, we need to set up the snRNA/anti-sense RNA hybrid.

Each student does one sample:

To the tube containing 10 µg of dried RNA from either strain A or B add:

1. µl of hybridization buffer mix [80% formamide, 400 mM NaCl, 1 mM EDTA, 40 mM PIPES (pH 6.4)]

Mix the sample well over a 5 minute period. Spin, then add

2 µl of antisense RNA probe (~30 ng at 108 CPM per µg)

Close the tube, mix well, spin then heat to 85C for 10 minutes then transfer to a 50C incubator for 12-18 hours (then frozen at -85 until use; this will reheated for 15 minutes just prior to use)

**NOTE**: The TAs will carry forward the same reaction without added cellular RNA to score for probe sensitivity in the absence of other RNA.

**Expanded Homework** – will be scored based on 20 points (twice the value of the standard homework assignment). **Extra time allowed, due November 7th,**

 This assignment requires you to think a bit about the deep sequencing data – there is no simple answer. I expect at least one or two pages of thoughtful double-space response.

The scientific literature describes three specific mRNA sequences that must be present in a yeast intron in order for splicing to occur. What are these (NOTE – give actual sequence strings that are used, not simply descriptions (for examples, /GUAPyGU is a common 5’ splice site where the “/” shows the position of the upstream exon/intron border). As sequences similar to these found in the YMR147W protein coding sequence? If so, print out the sequence and highlight each.

The cryptic introns in YMR147W and *TAF13* and the alternative 3’ splice sites of the *IWR1* and *TAN1* mRNAs produce “minor” or low-abundance mRNAs compared with the major mRNA isoforms predicted by SGD. Speculate on why these RNAs might inefficiently produced. Speculate on why such minor mRNA processing events might be important for this organism – be specific in describing the consequences of producing a mRNA with altered primary sequence.

Your lab head proposes that that the low-level splicing of the YMR147W intron is essential for cell survival. Design an experiment to definitively test this hypothesis.

**Lab 18**

**Deep Sequencing Deep sequencing analysis, part III. Confirmation of Transcriptional Change by Northern Blot. Prehybridization (one per bench).** The positively charged nylon membrane Your blots were crosslinked with a Strategene Stratalinker (measure dose of 120,000 micro joules at 254 nm) and baked in a vacuum oven at 80C for 2 hours. Photographs were taken prior to baking. **Look at the blots – are the lanes for the *PRP43* and *prp43-H218A* loaded equally? How do you know? Record this observation in your notebook.**

Next, label the bottom left hand corner of your blot with your groups’ numbers (i.e., 1+2, 3+4, 5+6 or 7+8)

1. **Wet your blots** by soaking for 10 minutes in 250 ml of 6X SSC.
2. Roll your blot in a mesh strip and place both inside a glass roller bottle. **NOTE: Be sure to remove all air bubbles** from between the mesh and the membrane – simply roll a freshly gloved finger over the membrane to squeeze out the air bubbles. Holding the glass bottle in your right hand with the open end pointing to your left, the mesh should be inserted such that the fold is going **over** the top of the roll.
3. **Add 10 ml 6X SSC** and roll at room temperature for 5 minutes (this will spread out the mesh in the tube if you inserted the roll correctly; the mesh will remain in a tight roll if inserted incorrectly).
4. **Heat 2.5 mg (0.25 ml of 10 mg/ml) of salmon sperm DNA at 100oC for 10 min**. Rapidly transfer the denatured DNA to ice for 5 min.
5. Add your denatured salmon sperm DNA to 10 ml of: 6X SSC, 5X Denhardts (0.1g Ficoll, 0.1g poly­vinylpyrrolidone, 0.1g bovine serum albumin per 100 ml), 50mM sodium phosphate, 1% SDS. Mix by shaking the Falcon tube.
6. Pour out the 6X SSC from the roller bottle and replace with the 10 ml prehybridization plus salmon sperm DNA.
7. Tightly cap the roller bottle and incubate with rolling at 60oC for 24 hours. Tomorrow, the TAs will place the roller bottles into the refrigerator until Wednesday.

**Random Prime Probe Preparation.** Today we will use an alternative approach to in vitro transcription prepare a 32P-labeled nucleic acid probe. This technique is called “random prime labeling”. A full description of the technique is on the web site in a PDF file from NEB. In essence, we heat denature the template DNA and then anneal a population of short oligonucleotides of random sequence composition to the template. Some (but not all) of the oligonucleotides will basepair with the template and serve as primers for DNA synthesis using the Klenow fragment of *E. coli* DNA polymerase I. Unlike the ssRNA proble prepared by T7 transcription, the resulting product of this random prime DNA synthesis will be the synthesis of labeled DNA corresponding to both strands of the original template DNA molecule. Today we will prepare two different probes. Groups 1-4 will prepare probes for the YNR073C ORF (whose mRNA is predicted to increase in the *prp43-H218A* background compare with the WT) and for *ADE3* (whose mRNA is predicted to be the same in the *PRP43* and *prp43-H218A* backgrounds).

1. Heat 12.5 ng of DNA in 10 µl H2O @100C for 7.5 minutes. NOTE: **Label the tune with the probe gene name & your group number**
2. Place on ice quickly for 3 min.
3. Spin briefly to collect liquid.
4. Bring to the back of the room and add 6 µl of the following pre-mix:

2.5 µl 10X buffered random primer solution

3 µl of unlabeled dNTP (0.5 mM each)

2.5 µl of labeled dCTP (specific activity = (provided in lab )

6.5 µl of H2O

0.5 µl of DNA polymerase

Cap the tube well and mix very briefly (1 second) on the vortex. Spin very briefly (3 seconds) and then incubate at 37oC for 60 min.

1. After the 60 min. incubation, spin again briefly and then carefully open the tube with a plastic tube opener. Add 75 µl of EDTA to the stop the reaction. Cap the tube well. Vortex briefly, spin briefly
2. Separate the unincorporated nucleotides by pipetting the probe **(NOTE: to avoid contamination of your Pipetman, pipette ~70 µl twice and move the Pipetman plunger very slowly)** into the pre‑packed Bio-Spin 6 column with a collection tune inserted into a glass test tube. Spin for 5 min at ~1,500 rpm.

7. The solution that flows into the collection tube contains your DNA probe; the column retains the unincorporated nucleotides. DISCARD THE **column** SAVE THE **flow through**.

1. Place **1** µl of your probe into a scintillation vial, determine the cpm (counts per minute) of the sample. D**etermine the specific activity (in dpm/µg) of your probe. Determine the amount (volume and µg) of probe to add to provide 1X106 cpm per ml of hybridization**. **Record this information in your notebook.**
2. Approximately 55,000,000 DPM of total radioactivity was use for probe preparation.
3. The TAs will provide you with the number of cpm in your probe after column chromatography. **Determine the % of the 32P radioactive dCTP incorporated into your probe and record this information in your notebook.**

The probe will be stored in a lead-shielded container @ -80C until use.

**RNase A mapping of *SNR19* RNA abundance, Part II.** Today we will use RNase A to degrade to completion any of the 32P probe (anti-sense) RNA not based paired with the U1 snRNA (i.e., the product of the *SNR19* gene). Any 32P probe RNA based paired with the U1 snRNA will be protected over the length of the RNA-RNA hybrid. The probe was designed to be ~100 nts longer that the U1 snRNA – so, if the RNase A worked efficiently, no full-length 32P labeled probe should remain, and the intensity of the 32P signal will be indicative of the amount of probe “protected” by the U1 snRNA. Recall that one of these yeast RNA preps has only the normal *SNR19* gene and the other has as second copy (*GAL1-SNR19)* and is expected to have 3-5-fold more U1 snRNA. Based on the 32P signals observed, determine which culture (A or B) contained the single copy of *SNR19* and which contained *SNR19 plus GAL1-SNR19.*

**RNase A digestion**

1. Cool the annealed RNA/probe solution to room temperature (5 minutes on the bench top)
2. Add 300 µl of the following mix (300 mM NaCl, 10 mM Tris [pH 7.4], 40 µg/ml RNase A).
3. Incubate 60 minutes at 30C.
4. Add 20 µl 10% SDS and 150 µl of PCI. Mix well and then spin 5 min in the microfuge.
5. Transfer the supernatant to a fresh tube containing 2 µg of unlabeled carrier RNA, add 50 µl 3M NaOAc and 1 ml of 100% ethanol. Mix well then place on dry ice for 5 min.
6. Spin for 10’ at full speed, remove the sup and wash the pellet with 1 ml of 80% ethanol.
7. Spin for 3 minutes then repeat the ethanol wash.
8. Dry the pellet.
9. Resuspend the dry pellet in 20 µl of 7M urea containing 0.1 % bromophenol blue and 0.1% xylene cyanol. Freeze until next lab period.

**Lab 19**

**Deep Sequencing Deep sequencing analysis, part IV. Confirmation of Novel Introns and Alternative Pre-mRNA splice site choice.**

Today you will resolve your PCR products on a 5% polyacrylamide gel made 8% with glycerol. The added glycerol helps the short PCR fragments focus into sharp well-resolved bands.

**Gel Order**

**Lane Sample**

1. **TA - YMR147**
2. **TA- TAF13**
3. **TA IWR1**
4. **TA TAN**
5. **DNA marker**
	1. **Student YMR147**
6. **DNA marker**

 **11-14 Student TAF13**

 **15 DNA marker**

**16-19 Student IWR1**

**20-23 Student TAN1**

1. **DNA marker**
2. **TA - YMR147 neg control, no reverse transcriptase**
3. **TA- TAF13 ..**
4. **TA IWR1 ..**
5. **TA TAN ..**

Run gel @ 72 volts overnight, stain with EtBr and image/photogrrah under short wavelength UV light.

**Confirmation of Transcriptional Change by Northern Blot.** Hybridization (one per bench).

Now you will incubate your random-primed DNA probe with your blot in a solution that will foster hybridization. We discard the prehybridization solution in case any RNA was released from the membrane during the incubation - such released RNA will compete with the filter bound RNA for hybridization to your probe. Note that unlike the ssRNA probe, this dsDNA probe must be heat denatured before use.

1. Place 250 µl of the 10 mg/ml ssDNA into a microfuge tube that has a pin hole in the cap. Add the approximately 10 million cpm of probe (either the 32P labeled *ADE3* DNA or the YNR073C DNA) to this same tube. Heat denature at 100C for 10 minutes and then quickly transfer to ice for 5 minutes.
2. Open your prehybridization roller bottle and pour out the pre-hybridization solution.
3. Pipet all of your ssDNA+ probe solution into a capped tube containing 10 ml of the hybridization solution. Invert the tube several times and then transfer the entire contents to your roller bottle. Your bottle should be labeled either *ADE3* or YNR073C.
4. Tightly seal the roller bottle and rotate at 60C until the next meeting.

Hybridization (one per bench; the B partners of each group should participate).

**RNase A mapping of *SNR19* RNA abundance, part III.** Today you will resolve your RNA bands on a 7M urea 5% polyacrylamide gel. The samples are heat-denatured prior to loading and the gel is run under denaturing conditions using the urea and running the gel at 50 to 60C.

Heat the samples at 90C for 3 minutes then load 5 µl each of:

**Gel order:**

**Lane Group Sample**

**1 TA Un-reacted probe (~2000 cpm)**

**2 1 A**

**3 1 B**

**4 2 A**

**5 2 B**

**Etc.**

Run the gel until the fast dye (bromophenol blue) runs off the bottom and the second dye (xylene cyanol) runs 2/3 rd the gel length. Dry the gel on a filter paper then expose to a phosphorimager screen.

**Lab 20**

**Confirmation of Transcriptional Change by Northern Blot.** **Washing Northern Blots**

Here you will remove the probe adsorbed directly on the transfer membrane with a low temperature, low salt wash and the probe associated by weak nucleic acid contacts (that is, incomplete basepairing of sequences not fully complementary) through a high temperature low salt wash. What 32P DNA probe remains bound is associated by extensive base pairing with RNA fixed to the membrane.

 1. Carefully pour the waste probe in the large waste beaker. Rinse the roller bottle with 1/2 volume of **0.2X** SSC, 0.1% SDS (recall that your hybridization conditions contained **6X** SSC). Simply add the wash, cap the bottle, invert the bottle 5 times, the pour out the wash into the large waste container.

2. Wash the filters 3 X 15 min. at 60oC. The TAs will pre-warm the wash solution to 50-60C before you use it. Fill the roller bottle 3/4ths full with wash solution for each wash. Be careful not to spill the radioactive wash solutions.

3. Remove the membrane and blot off the excess liquid with a Kim wipe. Place the membrane between two pieces of plastic wrap. The TAs will assist you in exposing the membranes to X-ray film. The correct order of placement is: intensifying screen/film/blot. Be sure to mark the position of the wells (or outline your blot) on the film (not on the intensifying screen).

**Discussion of DNA Sequencing Technology.** In preparation for next Friday’s (Nov 18th) visit to the *Advanced Genetic Technologies Center* (113 Plant Sciences Building, across Cooper Drive), we will discuss the fundamentals of DNA sequencing technology. We will begin with a discussion of basic enzymatic sequencing and then discuss high throughput and deep sequencing approaches. As preparation for Monday’s discussion, please read the relevant sections in the PGMG textbook 126-134 and view this short video on the Ion Torrent Technology:

[**http://lifetech-it.hosted.jivesoftware.com/videos/1016**](http://lifetech-it.hosted.jivesoftware.com/videos/1016) also, for those seeking additional information, a paper describing the data acquisition and analysis of the Torrent methodology is posted on the class web site.

**Deep Sequencing Deep sequencing analysis, part VI** Confirmation of Transcriptional Change by Northern Blot. Discussion of the phosphorimager principles and the northern blot results.

**Random mutagenesis of DNA. Adopted from** [**Parkhomchuk**](http://www.pnas.org/search?author1=D.+Parkhomchuk&sortspec=date&submit=Submit) **et al.,**

PNAS **December 8, 2009** vol. 106 no. 49 **20830-20835**

It is often valuable to create mutations within a cloned gene. Earlier, we learned how we can use mutagenic oligonucleotides and PCR to introduced precise mutations at pre-defined locations. The value of this approach rests upon knowing where to mutate. Sometimes this is unknown (e.g., where you have an uncharacterized gene or large genetic segment where the gene of interest has not been identified). Here a randomized mutagenesis protocol can be beneficial. The goal is to introduce mutations over a large region then use some biological assay to score for changes that enhance or reduce activity. Today we will use mutagenized the classic plasmid, pBR322, and score for mutations in the tetracycline resistance gene. This protocol was adapted from: **Adopted from** [**Parkhomchuk**](http://www.pnas.org/search?author1=D.+Parkhomchuk&sortspec=date&submit=Submit) **et al.,** PNAS **December 8, 2009** vol. 106 no. 49 **20830-20835**.





**EMS structure; the arrows on the guanine show positions of basepairing in DNA**

Ethyl methanesulfonate (EMS) is an alkylating agent that ethylates O6 of guanine (and other sites but this is the mutagenic hit) to alter the basepairing allowing it to pair with adenosine resulting in G:C to A:T transitions (mutations) in the DNA after replication. EMS is a mutagen and a carcinogen.; It should therefore be handled in a hood with gloves. EMS solutions can be deactivated in a solution of 4g NaOH and 0.5 ml thioglycolic acid in 100 ml.

**Each group:**

**Conduct this work in the hood – two students can work simultaneously. Work quickly but carefully! BE SURE to place the pipet tips and the excess reagent in the specially marked waste containers.**

Hydroxylamine (HA) Mutagenesis of DNA (adopted from *Methods in Yeast Genetics*, Kaiser, Michaelis and Mitchell, 1994 Cold Spring Harbor Laboratory Press). HA causes C to T and G to A transition mutations on double stranded DNA.



Add 1µg of pBR322 DNA(purified by Qiagen column) to 50 µl µl of hydroxylamine solution. (student 1) and to 50 µl of TE (student 2). Incubate both at 37C for 48 to 60 hours.

\*Hydroxylamide solution:

Hydroxylamide HCl 0.35g

5M NaOH 450 µl

H2O 4.55 ml

NOTE: pH should be ~6.7; prepare just before use and store on ice until needed.

pBR322 can be purchased from Promega (D1511) for $61.00/10µg

**Lab 21. Short Quiz Today**

**Random Chemical Mutagenesis of pBR322, part III.**

Stop the mutagenesis reaction by the addition of 10 µl of 5M NaCl, 50 µl of 1mg/ml BSA and 1 ml of 100% ethanol.

Place on dry ice for 10 minutes.

Thaw, mix, and then spin at full speed in the microfuge for 10 minutes. Remove the supernatant and place the supernatant in the chemical waste.

Resuspend the DNA in 100 µl of TE, add 20 µl 3M NaOAc, and 360 µl of ethanol. Place on dry ice for 10 minutes.

Thaw, mix, and then spin at full speed in the microfuge for 10 minutes. Remove all the supernatant and place in chemical waste.

Wash the pellet with 1.5 ml of 80% ethanol

Spin 3 minutes in the microfuge, remove the supernatant, dry the pellet in the speedvac.

Dissolve the DNA 100 µl of TE.

**Deep Sequencing Deep sequencing analysis, part VI** Discussion of northern blot results. **Lab 22. Random chemical mutagenesis of DNA, part III.** The DNA was mutagenized, precipitated, dried and resuspended in 20 µl of sterile water. Today we will begin a test the prediction that of the colonies recovered on ampicillin plates, the number of tetracycline sensitive (amp resistant) clones will increase with plasmid mutagenesis.

***E. coli* Transformation.** Use 10 µl of this DNA for transformation into the E. coli host strain CJ236. This strain has a mutation in the ung1 gene which encodes the uracil DNA glycosylase enzyme that removes uracil from DNA resulting from deamination of cytosine during mutagenesis. The loss of ung activity results in elevated levels of mutagenesis.

In a parallel transformation, use 1 µl of unmutagenized pBR322 for comparison.

Half of your transformation mixture will be plated on plates containing 100 µg/ml ampicillin and half of the transformation will be plated on plates containing 50 µg/ml tetracycline. By using both medium types we will be able to compare the rates of mutagenesis on the two genes.

**Western Blot of an Epitope-tagged Protein (part I)**

1. In order to permit easy tracking (and purification) of the protein, the TAP affinity tag was genetically engineered into the carboxyl terminal coding sequences of almost every yeast open reading frame (see <http://www.openbiosystems.com/GeneExpression/Yeast/TAP/> and references cited). This epitope tag fuses protein A and a calmodulin binding domain to create the tandem affinity purification (TAP) tool. The TAP tag has proved valuable for 1) proteomics, purifying protein complexes from yeast to identify factors involved in specific biochemical pathways, 2) determining the relative cellular abundance of yeast proteins under standard growth conditions and 3) for determining the subcellular location of yeast proteins.
2. There is a commercially available, high sensitivity antibody available to detect the TAP tag. Since the TAP tag is introduced at the natural chromosomal locus, gene expression is assumed to be normal. If we further assume that the TAP epitope does not influence the corresponding RNA or protein stability, then the relative TAP signal observed for different TAP-tagged proteins by western blot reflects the relative abundance of each protein in the cell. Today we will do a western blot of several TAP-tagged yeast proteins to see how this protein-fusion approach can be used to compare protein abundance.
3. **Protein preparation.**
4. - Spin out 7.5 ml of the saturated yeast culture. Resuspend the pellet in 400 μl of cell breakage buffer (50 mM Hepes pH 7.0 (@ 4C), 2 mM MgCl2, 200 mM KCl, 10% glycerol 0.5 mM DTT, 0.5 mM PMSF).
5. - Add ¾ volume of sterile, acid washed glass beads. Vortex the sample vigorously for 4 minutes.
6. - Spin briefly (10 seconds) to pellet the beads. Transfer all of the supernatant to a clean tube and then spin again for 10 minutes at 4C.
7. - Carefully transfer the clear supernatant to a fresh tube and discard the pellet.
8. - Transfer 24 μl of the cell extract into a fresh tube and add 24 μl of 2X protein loading buffer containing 7M urea. Mix briefly. Strore at

**Lab 23 Western Blot of an Epitope-tagged Protein (part II)**

Heat the sample at 100C for 5 minutes and then load the gel with 25 μl of sample as indicated below. **NOTE: Use the tube cap locks to avoid the tops from popping open.**

1. **Two teams (groups 1-4; 5-8)**
2. Load two 7.5% polyacrylamide gels (29:1 acrylamide:bisacrylamide) in the following order:
3. Lane 1 protein molecular weight markers (15 μl)
4. Lane 2. Open Predicted MW in kDa (without TAP)
5. Lane 3 Mud1-TAP (25 μl) \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_
6. Lane 4 Prp28-TAP (25 μl) \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_
7. Lane 5 Prp43-TAP(25 μl) \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_
8. Lane 6 CUS1-TAP(25 μl) \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_
9. Lane 7 Clf1-TAP(25 μl) \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_
10. Lane 8 Sup-35-TAP(25 μl) \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_
11. Lane 9 BY4742 (no TAP) (25 μl) NA
12. Lane 10 SPP381-TAP(25 μl) \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

**IN YOUR NOTEBOOK – add the predicted molecular weight for each protein (use SGD to find this information). The TAP tag will add approximately 20 kDa more to each protein.**

NOTE: The proteins are heated to 100C for 5 minutes just before loading. Run the gel with 650 ml of running buffer at 100V for 10 minutes then at 150 volts for 50 minutes. While the gel is running, soak Immobilon P membrane in 100 ml of: 100% methanol (5 minutes), distilled water (5 minutes), protein transfer buffer (15 minutes). This is a hydrophobic membrane. Be absolutely certain that the membrane stays under the liquid at all times. If it dries out, the proteins will not transfer.

At the end of the 1 hour run, remove the gel to a vat of transfer buffer. Assemble the transfer "sandwich" from top to bottom (scotch brite pad, filter paper, gel, membrane, filter paper, scotch brite pad). Transfer at 150 volts for one hour in 1L of transfer buffer.

Remove the gel, place the filter "protein side up" in a staining tray. Remove the markers by cutting off the strip. Incubate the membrane in the blocking agent (i.e., 5% nonfat dry milk in phosphate buffered saline -PBS-milk) for 15 minutes. Pour off the milk and incubate the membrane overnight (with shaking) in PBS-milk containing a 1:2000 dilution of anti-protein A antibody conjugated to the enzyme horse radish peroxidase (PAP)

NOTE: If your markers were not prestained you would first stain with the light temporary stain Ponceau S to localize the markers. Cut off the markers, rinse the membrane for 5 minutes in water, then stain the markers with 1% amido black for 5 minutes with shaking. Afterwards, you would use the destain solution to remove excess amido black.).

1. **Gel Running Buffer:** (per liter) 3.0 g Tris base, 14.4 g of glycine, 1 gram SDS, pH to 8.3
2. **Transfer buffer:** (per liter) 3.0 g Tris base, 14.4 g glycine, 100 ml methanol, pH 8.3
3. **2X Protein Sample buffer:** 62.5 mM Tris (pH 6.8), 10% glycerol, 0.05% bromophenol blue, 2.3% SDS, 5%(v/v) β mercaptoethanol, 7M urea
4. **Amino Black Stain/destain:** 10% glacial acetic acid, 45% methanol, 45% water (0.1% amido black)
5. **10X Ponceau S stain solution** (2% Ponceau S, 30% trichloroacetic acid (TCA), 30% sulfosalicyclic acid). This is diluted 1/10 in water prior to use.

**Random chemical mutagenesis of DNA, part IV.** Replica plate analysis. Today you will transfer cells from the colony surface on the LB-Amp (100 mg/L) plate to the LB-Tet (50 mg/L) plate using the “replica plate” technique – essentially, using the first plate to make a print of the colony pattern for the second plate. You will also replica plate the colonies that formed on the tetracycline plate onto ampicillin medium

After the overnight incubation, the plates will be refrigerated until the next lab period.

 1) the number of AmpR colonies recovered and 2) the fraction of AmpR  that are tetracycline sensitive (TetS) as well as 3) the number of TetR colonies formed and 4) the fraction of TetR colonies that are ampicillin resistant.

**Each Student:**

1. Choose **one** Amp plate to use as a replica master. Ideally, this plate will have between 50 and 300 well isolated colonies. NOTE: Plates with fewer colonies are of less statistical value and plates with crowded colonies smear together upon replica plating and cannot be easily scored.
2. Place one velvet cloth (velvet side up) over the round replica cylinder (as illustrated in the lab demonstration). Gently slide down the plastic collar to hold the velvet in place.
3. Press the agar surface of the Amp plate onto the velvet. With your fingers, tap the top of the plate all around the surface to get good contact between the velvet and the colonies. You don’t want to “smash” the dish, but do want to use some force in this step.
4. Remove the Amp plate by lifting straight up (do not slide or twist, this will smear the colonies). Cover the Amp plate and save in the refrigerator. NOTE: in removing the plate, you might not notice many colonies outlined on the velvet – this is ok there is likely sufficient transfer for this experiment to work well , avoid the temptation to replace the plate on the agar to “try again” as this will mix colonies and prevent you from interpreting your data.
5. Place the fresh tetracycline plate straight (labeled in advance with your group number, initials, and time of mutagenesis) down over the agar surface and tap the plate as before.
6. Remove the plate by lifting straight up as before. Replace the lid on the tetracycline plate and incubate overnight at 37C.
7. Repeat as above for the colonies formed on the tetracycline plates

**Lab 24**

**Random chemical mutagenesis of DNA, part V.**

**Determine**1) the number of AmpR colonies recovered and 2) the fraction of AmpR  that are tetracycline sensitive (TetS) as well as 3) the number of TetR colonies formed and 4) the fraction of TetR colonies that are ampicillin resistant.

**Homework (answer in your lab notebook, do not turn in)**. The percentage of cells that are ampicillin resistant may not be identical to the percentage of cells that are tetracycline resistant even though both genes were mutagenized at the same time. Discuss at least two different reasons why the apparent rates of mutagenesis may differ.

**Western Blot of an Epitope-tagged Protein (part III)**

The membranes have been incubated in a solution of PBS-5% nonfat dry milk containing the rabbit anti-horse radish peroxidase antibody conjugated to the enzyme horse radish peroxidase (used at a 1:1000 dilution; **NOTE:** the class web site has the PDF describing this antibody and a second PDF describing the Luminol detection system – both are highly recommended reading). This antibody will bind to the protein A sequence of the TAP epitope through the antibody constant region. The fact that the antibody is directed against horse radish peroxidase allows more than one antibody to be recruited to the TAP tagged protein (that is, one against the protein A segment and then others against the horse radish peroxidase covalently joined to the first antibody). This results in considerable enhancement of the signal. Today, we will detect the TAP-tagged protein using the Pierce Supersignal chemiluminescent substrate and image the blot by autoradiography with X-ray film.

Was the membrane 3 times 5 minutes each with 25 ml TBST buffer and a final time with PBS buffer (recipes on board). Be sure to remove all of the liquid during each wash but do not let your membrane dry.

Pour off the final TBST wash and add 2 ml of a 1:1 mixture of the Piece luminol and enhancer solutions (see Pierce PDF file for more information). Be sure that the membranes are completely covered and shake gently for 5 minutes. Briefly dry the membrane on paper towels or blotting paper and expose to X-ray film. NOTE: the chemiluminescent signal decays VERY rapidly so we must coordinate this last step so the membranes can be quickly imaged.

**Imaging with Alkaline Phosphatase.** We have the option to use another system to image the TAP-tagged proteins by chromogenic staining with alkaline phosphatase (AP). While both HRP and AP are widely in use, the AP reaction is preferred by some investigators since you can observe the signal develop in real time and stop the reaction before it reaches saturation.

After imaging the blots, soak for 15 minutes in 5% nonfat dry milk in PBS. Next add containing a 1: 1500 dilution of goat anti-rabbit-alkaline phosphatase conjugate. Incubate at 4C until the next lab.

**Lab 25 (short Quiz today)**

**Imaging with Alkaline Phosphatase.**

Pour off the goat-anti-rabbit-AP antibody and wash 3 times 5 minutes each with 25 ml TBST buffer and 1 time in ml alkaline phosphatase (AP) buffer.

Finally, pour off the 1X AP buffer and add 5 ml of the chromogenic substrate (Gibco/BRL BCIP/NBT - 5-bromo-4-chloro-3-indoyl-phosphate/nitroblue tetrazolium). The dephosphorylation of BCIP catalysed by AP results in the production of an intense blue/ purple precipitate which is deposited on the membrane at the site of antibody association. NBT enhances the purplish-brown color of the precipitate. The precipitate is very stable and resists fading when exposed to light. The alkaline phosphatase reaction creates a blue precipitate on. The time required to develop the image varies (5 minutes to several hours) depending on the amount of protein present.

**Chromogenic substrates for Western blotting with Alkaline Phosphatase.**

**BCIP** 2,2’-Bis(4-nitrophenyl)-5,5’-diphenyl-diphenylene) ditetrazolium chloride: BCIP has a molecular weight of 433.6, and hydrolysis by alkaline phosphatase results in a bluepurple precipitate that can be deposited on nitrocellulose or nylon membranes. BCIP can be used as a chromogenic substrate for both immunoblotting and immunohistochemical studies.

**Nitro blue tetrazolium (NBT)** 3,3’-(3,3’-Dimethoxy-4,4’-biphenylene)phenyl-2H-tetrazolium chloride]: NBT, with a molecular weight of 817.6, is a member of a class of heterocyclic organic compounds known as tetrazolium salts. Upon reduction, the compound yields NBT-formazan, a highly colored, water-insoluble product. The substrate is widely used for immunochemical assays and techniques because the color produced by the formazan is linear and stable over a wide dynamic range. The BCIP chemistry prompted by alkaline phosphatase treatment promotes the reductionof NBT. An ideal system for blotting or staining applications with AP is the combination of NBT and BCIP. Together, they yield an intense, black-purple precipitate that provides much greater sensitivity than either substrate alone. This reaction proceeds at a steady rate, allowing accurate control of its relative sensitivity.