**2016 BIO 510 Final Exam Guide**

The final exam will focus mostly on the second half labs and lectures. This will **lab 13 forward** plus any experiments started in the first half but completed in the second half (e.g. the yeast two hybrid assay). For each lab experiment, ask yourself – *what did we do and why did we do it*? Understand the goals and experimental steps involved – including the reagents used. Be able to *“design an experiment”* using these same techniques to answer a novel question. Review the posted readings associated with the experimental work.

**Friday lecture PowerPoint slides 119 to the end** will be included. The **in lab discussion PowerPoint slides 151to the end** will be included. At least two questions will be derived from our mid-term first exam, two from last year’s final exam, one question from a homework assignment and at least one question from the trip to the DNA microarray core, the DNA sequencing or Proteomics core facilities. One question will be on the papers we discussed at the end of the semester showing how **molecular genetic approaches** can be combined with recombinant DNA techniques to define sets of genes involved in a biological process (in class PowerPoint slides and associated papers on the class web site).

Your **notebook** must be turned in for grading before the start of the final exam on **Wednesday , Dec 14 at 1:00 PM in THM 109**. During the exam please ask for clarification when needed – the more questions, the better.

**Lab overview**

**Fundamental Principle:** Use of recombinant DNA techniques for the cloning and characterization of a eukaryotic gene.

**Experimental Goal:** To test the hypothesis that a segment of *Saccharomyces cerevisiae* DNA encodes a yeast gene.

**Experimental Design***: Clone a segment of yeast DNA*

1. Gel fractionate and purify a piece of yeast DNA (“insert DNA”)
2. Cut pTZ18u/19u cloning vectors with EcoRI and HindIII
3. Prepare competent TG1 *E. coli*
4. Ligate PTZ vector with insert DNA and transform into the competent *E. coli*
5. Select transformants on ampicillin plates and score for insertional inactivation of the lacZ gene using X-gal/IPTG for blue/white screen
6. Isolate DNA from the presumed recombinant and non-recombinant transformants and determine whether your cloning experiment was successful (i.e., that you generated the desired vector plus insert construct)
7. *Determine whether the cloned yeast DNA contains a gene naturally expressed in yeast*
8. Isolate total RNA from yeast. Quantify and fractionate this RNA by denaturing gel electrophoresis
9. Transfer the gel-fractionated RNA to a charged nylon membrane and pre-hybridize the membrane.
10. Prepare + and – strand “riboprobes” by in vitro transcription of your recombinant DNA with T7 RNA polymerase and NTPs (including 32-P UTP). Quantify radiolabel incorporation and add probe to membrane transfer of yeast RNA in the hybridization step
11. Wash off excess probe and expose the membrane to a phosphorimager screen. Use the Typhoon phosphoimager to detect the radioactivity signatures stored on the phosphoimager screen. Based on the signals obtained with the two hybridization probes determine whether the cloned DNA segment contains at gene and if so, determine the direction of transcription in yeast of the natural transcript with respect to the EcoRI and HindIII restriction sites (assume that these same sites are present in the genome of yeast at this locus).
12. *Identify the specific gene encoded by your “insert DNA”*
13. Infect your TG1 cells containing the recombinant pTZ18u(+insert) and pTZ19u(+insert) with M13K07 in order to produce single-stranded DNA for subsequent mutagenesis
14. “Qiagen” purify dsDNA plasmid for sequencing.
15. “Blast” search to identify the gene encoded on your recombinant DNA

**Class Outcome:** While the ssDNA yield was low,we had great results on each step of this experiment and identified the cloned gene as *SNR19* in which the U1 snRNA transcript is made in yeast in the EcoRI->HindIII direction.

**Fundamental Principle:** Test of gene function by the selective removal of cellular mRNAs using the natural RNA interference (RNAi) pathway in *C. elegans*

**Experimental Design**: Prepare agar medium seeded with **HT115** *E. coli* that express dsRNA from the L4440 vector corresponding to the *C. elegans* *CLF1, sup-12, Unc-22*, and *Dpy-1* genes plus an empty vector control which does not make a targeting ds RNA. IPTG is added to induce expression of the dsRNA gene. Plate L1 larvae and allow the nematodes to grow and develop. Newly hatched worms mature in ~4 days and lay eggs to start another generation. We compared number of live animals, body size & shape, worm movement and, in the case of *sup-12*, alternative splicing in the control and RNAi expressed cultures.

**Class Outcome:** Fantastic results on the phenotypic consequences of the four ablated gene products. **NL2099 (rrf-3)** showed embryonic lethality noted for *CLF1* & much reduced viability for sup-12, impaired mobility for Unc-22, short fat worms with Dpy-1 and greatly altered muscle-specific pre-mRNA splicing patterns in the **KH1125** worms after knockdown with sup-12.

**Fundamental Principle:** Use PCR to 1) amplify a genetic locus to detect a genomic polymorphism and 2) to make a site-specific mutation in a plasmid DNA by inverse PCR

**Technique:** Use of the Polymerase Chain Reaction to characterize nucleic acids

**Experimental Design:**  Isolate genomic DNA from yeast by mechanical disruption with glass beads followed by PCI extraction. *SQS1, sqs1::KAN/SQS1 and sqs1::KAN* strains were used. PCR was then performed with oligos that flank the *SQS1* gene to determine the genotype by DNA fragment lengths. For inverse PCR we removed the F1 origin of DNA replication from the pTZ18u-*SNR19* recombinant plasmid.

**Class Outcome:** Some variability but overall the PCR reactions were successful for most students and allowed us to identified the yeast bearing *SQS1* and/or *sqs1::KAN.* The inverse PCR was successful for all in removing the desired target from our recombinant plasmid.

**Fundamental Principle:** Use of the Polymerase Chain Reaction to quantify the abundance of a particular target nucleic acid.

**Technique:** Real time PCR

**Experimental Design:** Conduct real time PCR to set up a standard curve of *SQS1* DNA abundance vs threshold cycle using the double strand specific SYBR green dye. Use this standard curve to determine the DNA concentration of your “unknown” sample of *SQS1* DNA.

**Class Outcome:** *Ghrrrrrrrrrrrrr*, still having problems, may be a bad reagent. If we are unable to resolve, we will use the results from last year (same DNAs, same conditions) which worked pretty well and allowed determination of the target DNA abundance.

**Fundamental Principle:** Determine whether a mutation inactivates or impairs the function of a gene. Negative selection in the plasmid shuffle technique resolves the basic problem of not being able to know whether a gene is essential or not since, under normal circumstances if you inactivate the gene, you kill the cell, and you never “see” the desired clone. That is, you can’t distinguish between a knockout experiment that inactivates a gene and kills a cell from one where the inactivation failed to occur do to a technical difficulty (such as poor transformation efficiency).

**Technique:** Plasmid shuffle, FOA counter-selection

**Experimental Goal:** To determine whether 3 different mutations in the essential yeast *SPP382* gene had functional consequences on yeast growth.

**Experimental Design:** Transform a functional copy of *SPP382* on a *URA3*-based plasmid (*URA3-SPP382(N19))* and different *SPP382* alleles (or an empty vector) on a *LEU2*-based plasmid into yeast where the chromosomal copy of *SPP382* is disrupted (i.e., *spp382::KAN)*. Select the plasmids based on the nutritional markers (e.g., *LEU2* and *URA3*) then score for Spp382 activity on 5’FOA media. 5’FOA is an anti-metabolite toxic in the presence of the *URA3* encoded activity and therefore selects for cells that have spontaneously lost the *URA3*-based plasmid (here containing the functional *SPP382(N19)* gene) during mitotic division.

**Class Outcome:** This experiment worked very well and we were able to demonstrate that the G-patch deletion mutant and the *PXR1* G-patch substitution mutations resulted in a lethal phenotype while yeast transformed with the unaltered allele (i.e., *YCplac111 LEU2, SPP382)* grew well while the YCplac111-*spp382-SQS*1 chimeric showed weak complementation. This shows that the G-patch peptide has limited portability in the context of the Spp382 splicing factor. Subsequent screening on medium lacking uracil confirmed that the *YCplac111 LEU2, SPP382* transformant lost the *URA3-SPP382(N19)* plasmid by FOA selection (and hence was Ura-).

**Fundamental Principle:** Protein-protein interaction can be monitored in living cells based on the bi-molecular reconstitution of the Gal4 transcription factor

**Technique:** Yeast Two Hybrid Assay

**Experimental Goal:** Map the Prp43-binding surface on the Pxr1 protein

**Experimental Design:** Create a full-length Prp43-Gal4 DNA binding domain fusion on the pAS2 plasmid and a series of Pxr1 peptide-Gal4 transactivation domain fusions on pACT2. Introduce into a yeast strain bearing a Gal4-regulated *HIS3* reporter gene plus *trp1* and *leu2* mutations. Select the double transformants based on the *TRP1* and *LEU2* plasmid markers then score for reporter gene transactivation on medium lacking histidine and containing 5 mM 3 aminotriazole 3-AT. 3-AT makes the assay more stringent as this compound is a competitive inhibitor of the *HIS3* enzyme – so, in the presence of 3-AT, greater levels of transcription is required from the *GAL1::HIS3* reporter gene to get growth on minus histidine medium.

**Class Outcome:** This experiment worked flawlessly and we were able to localize the Prp43 binding surface to a 50 amino acid region of Pxr1.

**Fundamental Principle:** Changes in mRNA processing can be inferred by careful measurements of RNA structure.

**Techniques:** reverse-transcription based PCR (rtPCR)

**Experimental Goals:** 1) Determine whether the novel intron and alternate use predicted by our Illumina yeast RNA deep sequencing experiments were valid.

**Experimental Design:** We used reverse transcriptase to prepare cDNA from a yeast RNA sample and PCR with gene-specific oligonucleotide primers to score for the novel intron and alternate 3’ splice site processing events.

**Class Outcomes:** cDNA synthesis and PCR worked well we were able to validate the predicted presence of an intron in YMR147W and the alterative 3’ splice site for

*IWR1*.

**Fundamental Principles:** Random mutagenesis of naked DNA can be used to score for gene function on a plasmid.

**Techniques:** Nitrous acid (NA) mutagenesis of plasmid DNA; ssDNA->dsDNA

**Experimental Goal:** Determine the effectiveness of NA in generating mutations in the lacZ gene of pTZ18u and identify the mutations.

**Experimental Design:** Treat single stranded (ss) pTZ18u with nitrous acid for 5 or 20 minutes, convert the sspTZ18u to ds pTZ18u by primer extension; transform the treated DNA into TG1 *E. coli* and select on ampicillin plates containing X-Gal/IPTG. Control treatment lacked NA. Next score the number of AmpR, lacZ- and AmpR, LacZ+ transformants in the control and experimental cultures to learn if NA acted as a mutagen.

**Class Outcomes:** Outstanding results ~ 1 % AmpR LacZ- colonies in the NA-treated samples at 5’ and 18% at 20’ while the negative control had fewer than 0.02% AmpR Lac- colonies.

**Fundamental Principle:** Protein fusions to affinity tags offer value in protein isolation.

**Techniques:**  Protein purification by chitin-agarose affinity selection, chemical lysis of *E. coli*, use of a T7 coupled expression system in ER2566 bacteria, intein cleavage.

**Experimental Goals:** 1) One-step purification of a recombinant protein (Spp382 1-121) expressed in *E. coli*.

**Experimental Design:  1)** Use the Impact system from NEB to recover express a recombinant protein in bacteria, lyse with B-Per reagent, select Spp382-121 on chitin agarose then release via intein cleavage with DTT

**Class Outcome: 1)** Excellent results - class recovery of theSpp382 (1-121) peptide was great in terms of yield and purity – with the only contaminant being a small amount of the intein-CBD peptide released from the beads.

**Fundamental Principle:** A common epitope tag can be used to compare relative protein abundance.

**Techniques:** Protein extraction from yeast using chemical treatment approach. Western blot, use of alternative horseradish peroxidase and alkaline phosphatase development schemes

**Experimental Goals:** Use the calmodulin binding domain-protein A TAP fusion to detect three different proteins of yeast.

**Experimental Design:** Extract yeast proteins by LiOAc/NaOH treatment; resolve protein on a denaturing 7.5% SDS polyacrylamide gel; transfer proteins to a polyvinylidene fluoride (PVDF) hydrophobic membrane, block with non-fat dry milk. We first used the PAP (rabbit anti-horseradish peroxidase antibody conjugated to horseradish peroxidase enzyme) to bind to the protein A portion of the TAP epitope. We then added a goat anti-rabbit alkaline phosphatase secondary antibody (which binds PAP) and used a chromogenic substrate mix (NBT/BCIP) to localize the alkaline phosphatase bound TAP band.

**Class Outcome:** Excellent results. The relative sizes and abundances of the TAP-tagged proteins were generally consistent with predictions based on SGD information.

**Fundamental Principles:** Gene editing in cells has become much more efficient with the CRISP-Cas9 system. The Cas9 DNA endonuclease binds a guide RNA (gRNA) that has two purposes: 1) to direct the Cas9 enzyme to the correct DNA target via base pairing with the DNA target and 2) enzymatically activate the Cas9 enzyme. In the absence of a homologous repair template, the resulting dsDNA breaks are generally repaired by non-homologous end joining resulting in small localized deletions and insertions. When a homologous repair target is present (often co-transformed PCR DNA) , recombination between the cleaved chromosomal DNA and the repair target substitutes the repair target for the native chromosomal gene – this can be used to alter the native gene sequence to: 1) correct (or add) a mutation, or 2) create a promoter or peptide fusion allele (e.g., add GFP to the carboxyl terminus of the endogenous gene.

**Technique:** CRISPR-Cas9 site-directed mutagenesis *in vivo*

**Experimental Goals:** Determine the efficiency of CRISPR-Cas9 directed mutagenesis in yeast

**Experimental Design:** We targeted the *CAN1* gene (encodes a cell permease for canavanine) for mutation by transformation with a plasmid-based g-RNA (pRS426- SNR-gRNA.CAN1.Y) that base pairs with *CAN1*. The pRS426 empty vector is used as a control. The yeast genome has an integrated copy of the galactose inducible CAS9 gene (*GAL-Cas9::LEU2).* The transformants are selected on –ura galactose medium and then scored for *CAN1* inactivation on medium containing canavanine (a toxic amino acid). *can1* mutants grow on canavanine while wildtype yeast (*CAN1*) do not.

**Class Outcomes:** Ongoing experiment.