CRISPR/Cas9 Disruption of *TRP1* in yeast

Instructors’ Notes

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# Week1: CRISPR design

## Objectives:

1. Students will be able to describe how to screen for auxotrophic phenotypes in *Saccharomyces cerevisiae*, and describe at least one example of when auxotrophic yeast strains are useful to researchers.
2. Students will be able to locate the coding sequence, flanking sequence, protein product and characteristics of a given gene from the *Saccharomyces* Genome Database.
3. Students will be able to design and defend the design of guide RNA in CRISPR/Cas9 gene editing studies to eliminate the expression of a gene.
4. Students will be able to describe the qualities of a single stranded DNA template for homologous repair of double stranded breaks in haploid eukaryotic organism.

## Materials:

* Computer with internet access for every student or every group of students

## Mini-lecture:

1. Overall goal of today’s lab:
   1. Design experiments to disrupt the *TRP1* gene in *Saccharomyces cerevisiae.*
   2. This design will contribute to experiments conducted in laboratory for the duration of the semester
2. Background
   1. Yeast:
      1. What are they?
      2. How do you grow them?
         1. Dishes with media
         2. Need nutrients: Sugar (or carbon source), essential amino acids and bases
      3. They make their own amino acids, such as tryptophan through biochemical process that requires several enzymes.
      4. Mutating the gene for one of the enzymes that help synthesize the amino acid will eliminate the ability to make that amino acid.
         1. Mutants can grow if they are supplied that amino acid
         2. Called auxotrophy
         3. Commonly used as a genetic marker
            1. i.e. *URA3* gene in plasmid DNA
            2. if yeast *ura3* gene is mutant, transfect yeast with plasmid allows them to grow on media lacking uracil because *URA3* gene is provided on plasmid
            3. Mechanism of selection for yeast that contain plasmid
   2. We will use CRIPSR/Cas9 to generate Tryptophan auxotrophic yeast
      1. Knockout the *TRP1* gene
3. Activities
   1. Find the sequence of the gene on the *Saccharomyces* Genome database.
      1. By default, SGD will give you the coding sequence.
      2. May also need some sequence upstream of the start codon… custom sequence retrieval
      3. Put this sequence in a Word or Google Doc
      4. Get to know SGD a little. It is a useful website for yeast biologists.
   2. Based on this sequence, design THREE guide RNAs. How?
      1. Find PAM sequence (REMEMBER DNA IS DOUBLE STRANDED).
      2. 20 nt upstream of PAM
      3. Make sure it is unique. (Might need to BLAST it against the Yeast genome)
      4. Can use web-based tools for design. Find your own or there are some tools listed in handout. Hint: ChopChop is really easy to use and gives a score for uniqueness of the RNA.
      5. Highlight the binding sites for these RNA in the *TRP1* genomic sequence on your word doc.
   3. Template for homologous repair
      1. Yeast almost exclusively perform HR for double stranded breaks
      2. But these yeast are haploid
      3. Must provide a homologous strand of DNA for repair, otherwise death
      4. This template should be similar to the genome so that it can be recognized by DNA repair proteins, but also have desired mutations so that product of repair is a mutant genome.
      5. We will use asymmetric single strand synthetic DNA
         1. Arms of homology – sequences on either side of the mutation that match the genomic sequence
         2. Best if mutation is closest to the Cas9 cut site as possible
            1. Cas9 cuts 3 bp upstream of PAM
         3. Also need to eliminate PAM sequence so that Cas9 cannot cleave once this DNA is repaired
         4. Think about the types of mutations, and locations of these mutations that are most likely to eliminate expression of a gene. (i.e. inside the coding region, near the 5’ end, indel not a multiple of 3).
      6. Only need to design one template
      7. Underline the arms of homology in the *TRP1* genomic sequence on your word doc. Write the mutation above the region you will mutate
   4. Thinking ahead: If your CRIPSR/Cas9 gene editing is successful, describe how you would genotype and phenotype and what you would expect those results to look like.

# Week 2: Cloning

## Objectives:

1. Students will be able to perform a restriction digest of plasmid DNA
2. Students will be able to describe how DNA strands are denatured and annealed
3. Students will be able to explain how phosphates on 5’ carbon of deoxyribose affect joining of DNA molecules by ligation
4. Students will understand the basic structure of a gene, and the essential elements for expression of small non-coding RNA and protein-coding genes.

## Materials:

* Purified pML104 (from Dam- strain of *E. coli*)
* Enzymes (All should be provided on ice)
  + *Swa*I (NEB; should be available the day before the lab)
  + *Bcl*I Enzyme (NEB)
  + PNK (NEB)
  + CIP [phosphatase (NEB)]
* PNK buffer and ATP, or Ligase buffer
* NEB Buffer 3.1
* Diluted oligonucleotides (IDT, 200 ng/ul)
* Phenol:chloroform
* 3M sodium acetate
* Molecular biology grade Isopropanol or 95% Ethanol
* Molecular biology grade 75% ethanol
* Beaker with tube float
* Equipment:
  + Hot plate
  + Microcentrifuges
  + Vortexes
  + 50° C water bath
  + 37° C heat block or water bath
  + 65° C heat black or water bath

## Mini-lecture:

1. Narrated PowerPoint provided on the course website and students are instructed to look at before assembling materials and methods. See Supporting File S1: CRISPR in Yeast - Lectures.
2. Outline cloning scheme:
   1. pML104 –
      1. promoters: PolIII (sgRNA) and PolII (Cas9) promoters
      2. *Bcl*I and *Swa*I sites for cloning your sgRNA gene cassette
   2. Pick at least one guide RNA gene per group to clone. Ideally, it’s one that you’ve already mapped.
   3. Goal is to first clone the genes. Need to have what gene you selected and where it targets the *TRP1* gene in your notebook
3. Week 1:
4. Restriction digests:
   1. Reiterate proper order of reaction assembly (largest volume first, enzyme last)
   2. Not all enzymes use the same conditions (Buffer, temperature, etc.)
   3. Dam methylation will prevent *Bcl*I digestion (but we isolated the plasmid from *E. coli* lacking dam methylase)
5. Dephosphorylation-prevents reannealing of vector pieces
6. Purify DNA - phenol:chloroform extraction\*, ethanol precipitation
7. Phosphorylate oligos – Kinase. Buffer with ATP used 10X ligase buffer instead
8. Annealing oligos:
   1. Heat and slow cool is to denature and allow annealing on complementary oligos.
   2. Purpose of the annealing is to generate the cassette that contains the gene for guide RNA and part of the structural portion of the tracrRNA in CRISPR system.

\*\*The order of these digests can be rearranged. For example, one class digested plasmid with *Bcl*I in class, extracted with phenol:chloroform, and set up *Swa*I digests overnight. We treated these with phosphatase and heat-inactivated the next day.

\*Phenol chloroform extraction and not gel purification is required due to the large size of pML104. Its size is well over that recommended by most kits for gel extraction.

# Week 3: Cloning, Part II

## Objectives:

1. Students will be able to ligate and transform plasmid DNA.
2. Students will be able to describe the concept and reagents needed to make *E. coli* competent.
3. Students will be able to describe the rationale behind selected DNA volumes in ligation reactions.
4. Students will recognize and design appropriate controls for ligation and transformation.

## Materials:

* Digested/dephosphorylated pML104 (from last week)
* Phosphorylated/annealed oligonucleotides (from last week)
* Promega Rapid DNA ligation kit (include ligase, and 2X buffer)
* Invitrogen subcloning efficiency competent cells (store at -80°C. Thaw on ice.)
* 42° C water bath
* 37° C shaker
* 37° C incubator
* LB + amp plates (4 per group)

## Mini-lecture:

1. Narrated PowerPoint provided on the course website and students are instructed to look at before assembling materials and methods. See Supporting File S1: CRISPR in Yeast - Lectures.
2. Ligation: explain molar ratio. Need more molecules of insert relative to vector to drive ligation reaction toward inclusion of insert, but not so much insert that you promote concatemers of insert.

<https://nebiocalculator.neb.com/#!/ligation>

1. 11.5kb vector
2. 33nt insert:
3. 20 ng vector (= 3 l total of ~8 ng/l): 3ng insert = 7:1 ratio
4. Determining concentration of insert:
   1. 200ng/l x 5ul = 1 g/50 l x 20 l into annealing reaction = 0.4 g/100ul = 0.004 g/l in annealing reaction = 4 ng/l
5. Control – no insert, or no ligase
6. Transformation
   1. Explain competency
   2. Controls FOR TRANSFORMATION They must chose a positive and negative control (no DNA, pML104)
   3. Must keep cells cold
   4. Heat shock
   5. Out growth
   6. Controls
   7. Selection
      1. Amp plates provided
      2. Plate ALL of the transformation on one plate
      3. Dry before inverting for incubation
      4. Must return tomorrow to record results and store plates at 4 oC

# Week 4: Screening clones

## Objectives:

1. Students will be able to describe the method of PCR, including the essential components of a reaction mixture, and thermal-cycling conditions.
2. Students will be able to locate the binding sites of and design primers for PCR and describe the expected size of the amplification product.
3. Students will be able to describe the reasons for each step and each component of a PCR reaction.
4. Students will be able to describe and perform isolation of plasmid DNA from *E. coli.*

## Materials:

1. Available before lab:
2. Micropipetters
3. Sterile glass culture tubes
4. Amp
5. Available day of lab:
6. Transfer pipettes
7. Promega miniprep kit
8. PCR tubes (Strips)
9. Diluted M13R primer (200ng/ul)
10. Diluted oligos used for cloning
11. GO-Taq 2x master mix (Promega)
12. PCR grade water aliquoted into 1.5 ml tubes
13. LB amp plates

## Mini-lecture:

1. PowerPoint provided on the course website and students are instructed to look at before assembling materials and methods. See Supporting File S1: CRISPR in Yeast - Lectures.
2. Isolate plasmid DNA
   1. Students need to find protocol from miniprep kit (spin)
   2. Keep unused culture.
3. Assay concentration of DNA on Nanodrop
4. PCR
   1. Use PCR grade water
   2. Control – no plasmid DNA
   3. Describe how to set up a master mix
   4. Primers?
      1. Extend from 3’ OH so primers must be from opposite strands.
      2. Where do the primers bind? (*M13 binds sense strand*)
      3. Which direction do they extend? (*5’ to 3’*)
      4. How far is cloning site (your primer) from the M13R primer in pML104? (*SwaI site to M13 primer end is Approx 390bp*)
      5. Which oligo (T or NT) should you use to screen?
      6. How will you know you have a positive clone? *(~400bp PCR product in positive, nothing in negative)*
   5. Cycling conditions: We have individual PCR machines for each group (miniPCR), but if you have only one thermocycler, the class must agree on one set of cycling conditions.
      1. Annealing temp (*must look up Tm of primers. Use the lowest Tm of the two*.)
      2. How long do we extend? (*30sec*)
      3. What is extension temp?
   6. Run on gel next week.

# Week 5: Selection of clones and transformation of yeast

## Objectives:

1. Students will be able to describe the how yeast are transformed, including the essential components of a transformation mixture, and conditions necessary for transformation.
2. Students will be able to describe the basic conditions required for cultivating *Saccharomyces*.
3. Students will be able to choose a percentage of agarose in a gel appropriate for separation of a given size of DNA.
4. Students will be able to pour an agarose gel, and perform analyzes of DNA separated by agarose gel electrophoresis (including size estimation).
5. Students will be able to describe and recognize the qualities of a template for DNA repair that allow efficient DNA repair, provide genotyping tools and eliminated repeated CRISPR/Cas9 cleavage.

## Materials:

* Yeast culture, diluted ~4 hrs prior to lab. 50ml per group. (one large culture diluted by instructor)
* Diluted template DNA
* ~200ml flasks (for pouring agarose gels)
* Agarose
* Ethidium bromide
* Gel rigs
* Diluted 100bp ladder
* Sterile conical tubes (1 per group)
* 2 mg/ml salmon sperm DNA
* 1000 ml beaker with water on hot plate (for boiling ssDNA)
* 50% PEG
* LiOAc
* 42° C water bath
* –URA plates (3 per group)
* Votexers
* Microcentrifuges

## Mini-lecture:

1. Complete screening
   1. Run out 5 l of PCR
   2. Loading dye already in samples if used GoTaq green master mix
   3. 100 bp ladder
   4. Will need standard curve in results, as well as estimation of size of PCR bands
   5. Expected size?
   6. Select at least 1 clone with appropriate product for transformation into yeast
2. Yeast transformation
   1. We will start a culture the night before lab, and dilute it to 0.167 A600 about 4 hours before lab. Usually done by the instructor. This allows yeast to be log phase, which will help our transformation
   2. Must keep sterile technique
   3. Transformation ingredients must be added in this order:
      1. PEG – protective
         1. Very thick. Watch the pipette tip.
         2. Will be hard to resuspend. Pipette up and down
      2. ssDNA – carrier
      3. Diluted plasmid DNA + HR template (See discussion below)
      4. LiOAc
   4. Heat shock – a lot longer heat shock than *E. coli* because yeast have cell wall, whereas *E. coli* do not.
   5. Selection –
      1. What plates should you use to select for the presence of pML104/sgRNA?
      2. We usually have minimal media plates lacking tryptophan and minimal media plates lacking uracil available for students. They must choose.
   6. Template DNA:
      1. ssODNA
      2. Arms of homology
      3. Disrupted PAM sequences
      4. Introduces a restriction site. How will this help with genotyping?

# Week 7: Phenotyping

## Objectives:

1. Students will be able to design experiment to determine auxotrophic phenotypes.
2. Students will be able to predict the outcome of multi-step experiments.

## Materials:

* Transformed yeast plates
* Sterile toothpicks
* Sterile water and 1.5 ml Eppendorf tubes
* –URA OR YEPD plates (1 plate per group)
* –TRP plates (1 plate per group)

## Mini-lecture:

1. Plating on both –TRP (phenotyping, experimental) and –URA (alternatively, YEPD plates can be used as positive control). Allows to know *TRP1* mutants, but keep genetically modified strains for future use.
2. Want to start with approximately the same amount of yeast and make serial dilutions.
   1. Pick a single colony with toothpick and suspend in sterile water.
   2. Read concentration of this solution (A600)
   3. Adjust all resuspended colonies (control yeast and at least 2 colonies of experimental yeast) to similar concentration. C1V1 = C2V2 where A600 is C1 and lowest A600 of all your colonies is C2 and V1 is volume of yeast solution.
   4. At least 2 colonies from pML104/sgRNA + template, one from pML104 control
3. Pipette dilutions onto plates, sitting on the grid.
   1. If you put your initials (in small print) at the top, you know your orientation for later.
   2. Do not move the plate until you’ve spotted all the dilutions of all the strains.
   3. Record exactly what was plated where.
   4. Pics should go in your notebook with labeled strain and dilution
4. One of these dilution spots should have countable colonies.
   1. Count those colonies and calculate the viable cells in this culture by multiplying by the dilution factor.
   2. Compare the viability on -uracil and -trp qualitatively (More or less) and quantitatively (viability on –trp/viability on –ura \* 100)

# Week 8 (Optional): Genotpying

## Objectives:

1. Students will be able to predict the outcome of multi-step experiments.
2. Students will be able to design and perform an experiment to the sequence of a particular genomic locus.

## Materials:

* Transformed yeast plates
* Sterile toothpicks
* Sterile water and 1.5 ml Eppendorf tubes
* Agarose
* Ethidium bromide
* Gel rigs
* Diluted 100bp ladder
* PCR tubes (Strips)
* Diluted M13R primer (200ng/ul)
* Diluted oligos used for cloning
* GO-Taq 2x master mix (Promega)
* PCR grade water aliquoted into 1.5 ml tubes
* PCR purification kit (Promega or Qiagen)

## Notes:

## This protocol is not well practiced. Only a few students have completed genotyping. When they have attempted genotyping, it was out of class, on their own time. I do not have well-designed lectures or specific protocols.

## The premise is that the region surrounding the intended mutation is amplified by PCR, purified and sequenced.

## Sanger sequencing services will provide purification, for a fee, which may save some time. *e.g.* <https://www.genewiz.com/Public/Services/Sanger-Sequencing/PCR-Purification>

## The protocol is actually written to be started the same week as phenotyping (after they plate some of their cell suspension, some of the suspension is then placed in a PCR reaction). The amplified product is then held until the following lab period, where agarose gel electrophoresis and purification are performed.

## Sanger sequencing services have very specific requirements for sample preparation, therefore, instructors should be aware of these specifications. *E.g.* <https://www.genewiz.com/Public/Resources/Sample-Submission-Guidelines/Sanger-Sequencing-Sample-Submission-Guidelines/Sample-Preparation#sanger-sequence>

## It was my intention to prepare the samples for sequencing myself, to minimize error. In this case, students would need to provide me will labeled samples and concentrations.

## For my students, intentional instruction on Sanger sequencing (how it is done and how to analyze the results) would be required.