CRISPR/Cas9 Disruption of *TRP1* in yeast

Laboratory Manual

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# Design of CRISPR/Cas9 Gene Editing in Yeast

## Background

In the following weeks, we will design and implement experiments in *Saccharomyces cerevisiae* (budding yeast) using CRISPR/Cas9 gene editing system. Please review your lecture notes over CRISPR.

### Why yeast?

*S. cerevisiae* is a robust eukaryotic model system, with ample and sophisticated tools for genetic manipulation. Moreover, previous studies (Laughery *et al.,* 2015) have shown that the DNA repair mechanisms in yeast are robust and quickly repair double stranded breaks generated by Cas9 endonuclease. The ability to repair these breaks is essential, as organisms that are unable to repair CRISPR/Cas9 mediated genome breaks will not be able to further replicate and thus will undergo apoptosis. Furthermore, yeast have a proficient homologous repair mechanism that allows efficient, error-free repair of double stranded breaks.

### A little background on yeast

The existence of natural yeast extrachromosomal circular plasmid (circumference of 2 m) paves the way for many genetic tools in the organism. This plasmid has a single origin of replication, referred to as “2” origin. Plasmids capable of autonomous replication in yeast must contain either the high copy number origin (2 or a *CEN* origin of replication, which allows only one copy of the plasmid per cell.

Selection of yeast that contain plasmid DNA requires a selectable marker (Griffiths *et al.,* 2000). Unlike bacterial selectable markers, that usually confer antibiotic resistance (*e.g.,* ampicillin), yeast plasmid markers allow for production of a nutrient that is lacking in the media. For example, the *URA* marker is a *URA3* gene that allows production of uracil. Most laboratory strains of yeast lack the endogenous *URA3* gene (*ura3* mutant). As long as uracil is provided in the culture medium, these yeast will survive. If uracil is lacking in the media, the yeast will not be able to survive, therefore no growth will be observed. However, yeast transformed with a plasmid that contains the *URA3* gene will be able to survive on media lacking uracil. Similarly, *HIS3, LEU2*, and *TRP1* are marker genes that encode enzymes for synthesis of the amino acids histidine, leucine and tryptophan, respectively. Yeast that are mutant in these genes (auxotrophic for that nutrient) must either have these nutrients provided in the media or contain a plasmid DNA containing the wild-type gene (Pronk, 2002).

Research goal: Use CRISPR/Cas9 to mutate the *TRP1* gene in the yeast genome, generating tryptophan auxotrophs.

### Experimental design for Disruption of the TRP1 gene

We have talked about CRISPR and designed theoretical experiments in the lecture. CRISPR utilizes Cas9, a double stranded endonuclease. Cas9 relies on two short RNA guide sequences to find foreign DNA, then cleaves the target sequences, thereby eliminating the DNA of invaders. The system is specific and efficient enough to stave off viral infections in bacteria. Researchers have re-programmed the process so that the site of Cas9 cleavage can be directed by a single short RNA molecule (sgRNA). Upon cleavage of the genomic DNA, survival requires cells to repair this break by one of two DNA repair pathways: Non-homologous end joining (NHEJ) or Homologous Repair (HR). The DNA repair pathways can restore the site of the break to the pre-break sequence, generate random short insertions or deletions (indels), or introduce a specific mutation directed by HR and a template piece of DNA (Sander *et al.,* 2014).

In this lab, you are going to design an experiment to disrupt expression of the *TRP1* gene in *Saccharomyces cerevisiae* laboratory strain using CRISPR/Cas9 gene editing technology. The yeast strain that we will use is BY4742. (What is the genotype of this yeast strain?) You will find the sequence of the *TRP1* gene and design CRISPR guide RNAs to target the *TRP1* locus. In the future, we will clone the gene for these guides into the yeast vector pML104 that also expresses Cas9 endonuclease. Find a map for this plasmid (include the proper reference). What markers does this plasmid have for selection in yeast and *E. coli*? How would you select for the presence of pML104 in yeast and *E. coli*? pML104 has restriction enzyme sites (*Swa*I and *Bcl*I) for cloning your guide gene sequence (Laughery *et al.,* 2015). Based on your design, short synthetic oligonucleotide sequences will be ordered that will make up the guide RNA gene for cloning into pML104.

Designing guide RNA is relatively simple, but there are considerations to be made: First, you will need to optimize your guide RNA, minimizing the chance of off-target cleavage events by making sure your guide is unique and does not target other parts of the yeast genome. (How will you assure your guide RNA is unique?) Second, these experiments will attempt to introduce a specific mutation, harnessing the power of HR to do so. You will need to decide what specific mutation to introduce and then design a single stranded DNA template that will direct the integration of the desired mutation. (What types of mutations will knock out gene expression from *TRP1* gene?) Finally, as part of your discussion, you will need to consider how yeast will be screened for the *TRP1* disruption, both phenotypically and genotypically. It is likely that your genetic screen will include PCR, so while you have the genomic sequence of the *TRP1* gene available, you should also design oligonucleotide DNA primers flanking the area of genetic disruption in the *TRP1* gene.

Lab today will be devoted exclusively to experimental design. In future labs, these experiments will be implemented. It is important that today you understand the overall goals and process so that later we can focus on the fine details required to complete the experiments.

## References

Griffiths, Anthony J. F., Jeffrey H. Miller, David T. Suzuki, Richard C. Lewontin, and William M. Gelbart. 2000. *An Introduction to Genetic Analysis*. W. H. Freeman.

Laughery, Marian F., Tierra Hunter, Alexander Brown, James Hoopes, Travis Ostbye, Taven Shumaker, and John J. Wyrick. 2015. “New Vectors for Simple and Streamlined CRISPR-Cas9 Genome Editing in Saccharomyces Cerevisiae.” *Yeast* 32 (12): 711–20.

Lin, Steven, Brett T. Staahl, Ravi K. Alla, and Jennifer A. Doudna. 2014. “Enhanced Homology-Directed Human Genome Engineering by Controlled Timing of CRISPR/Cas9 Delivery.” *eLife* 3 (December): e04766.

Pronk, Jack T. 2002. “Auxotrophic Yeast Strains in Fundamental and Applied Research.” *Applied and Environmental Microbiology* 68 (5): 2095–2100.

Richardson, Christopher D., Graham J. Ray, Mark A. DeWitt, Gemma L. Curie, and Jacob E. Corn. 2016. “Enhancing Homology-Directed Genome Editing by Catalytically Active and Inactive CRISPR-Cas9 Using Asymmetric Donor DNA.” *Nature Biotechnology* 34 (3): 339–44.

Sander, Jeffry D., and J. Keith Joung. 2014. “CRISPR-Cas Systems for Editing, Regulating and Targeting Genomes.” *Nature Biotechnology* 32 (4): 347–55.

## Protocol

1. Find the sequence of *TRP1* gene in yeast
2. Go to *Saccharomyces* Genome Database (Google “SGD”. It’s the first thing that comes up.)
3. Type “TRP1” into the search bar. This should bring you to a page with a full description of the *TRP1* gene, its products, location in the genome, etc. Feel free to look around here and get to know the *TRP1* gene**.** 
   1. What is the name of the enzyme that *TRP1* encodes? On which chromosome is the *TRP1*?
   2. Scroll down and click on the genomic sequence for the *TRP1* gene
   3. Use this sequence to design a CRISPR sgRNA. (Keep this screen up, or cut and paste the sequence into Word for reference later.)
4. Design three guide RNAs for targeting Cas9 to the *TRP1* gene
5. You can design guide RNAs by hand or use an available web tool. CRISPR RNA recognition can be unpredictable, so you will need to design and test three RNAs to trial in your experiments.
   1. Web tool: There are many web tools available now to help you design your guide RNA. I’ve played around with a few and think these are user friendly. You can use either of these, or another tool you have found on your own. (Cite any tool you decide to use!)
      1. <http://wyrickbioinfo2.smb.wsu.edu/crispr.html>.
      2. Chopchop: <http://chopchop.cbu.uib.no/index.php>
   2. By hand:
      1. Find a PAM sequence (NGG), remembering that either strand is eligible for sgRNA recognition.
      2. Highlight 20 nt upstream (toward the 5’ end of that strand)
6. You also need to be sure that your RNA is as unique as possible to minimize off-target affects. You can either BLAST RNA sequences to the yeast genome (SGD has a BLAST function you may use), or some of the web tools listed above or that you find on your own may include this information. In this case, you may simply state that the RNA is unique and cite the web tool.
7. Design a template for Homologous Repair

For these experiments in a haploid yeast strain, we will be utilizing HR and a template to repair the double stranded break in a way that introduces a specific mutation that will disrupt *TRP1* expression. Several papers have shown that ~100 bp piece of single stranded DNA, from the same strand targeted by the RNA, is an acceptable template for this repair (Lin *et al.,* 2014). A template should have arms of homology (regions of sequence identity) on either side of the Cas9 cut site and include a mutation that will disrupt *TRP1* gene expression. Some suggest that the 5’ arm of homology should be longer (60-70bp) than the 3’ arm (30-40bp) (Richardson *et al.*, 2016). Design one template that suits at least one of your guides.

1. What is the sequence of your template?
2. Underline the arms of homology.
3. Circle and describe the mutation in your template.
4. What is the reasoning behind choosing this mutation?
5. Will this template work for each of your guide RNAs?
6. Data Analysis (Include this information in the data analysis portion of your notebook)

*Genotypic screening*: Once you have introduced Cas9 with your guide RNA into *yeast*, genotyping will be used to determine if Cas9 cleaved at the desired site (guided by your RNA) and introduced the intended mutation (as directed by your template for HR repair). Describe a potential genotyping protocol. Include expected outcomes.

*Phenotypic screening:* Once you have a line of *yeast* with the *TRP1* gene disrupted, describe a phenotypic screen for disruption of the *TRP1* gene activity. Include expected results.

# Cloning sgRNA for CRISPR/Cas9 gene editing, Part I

## Background

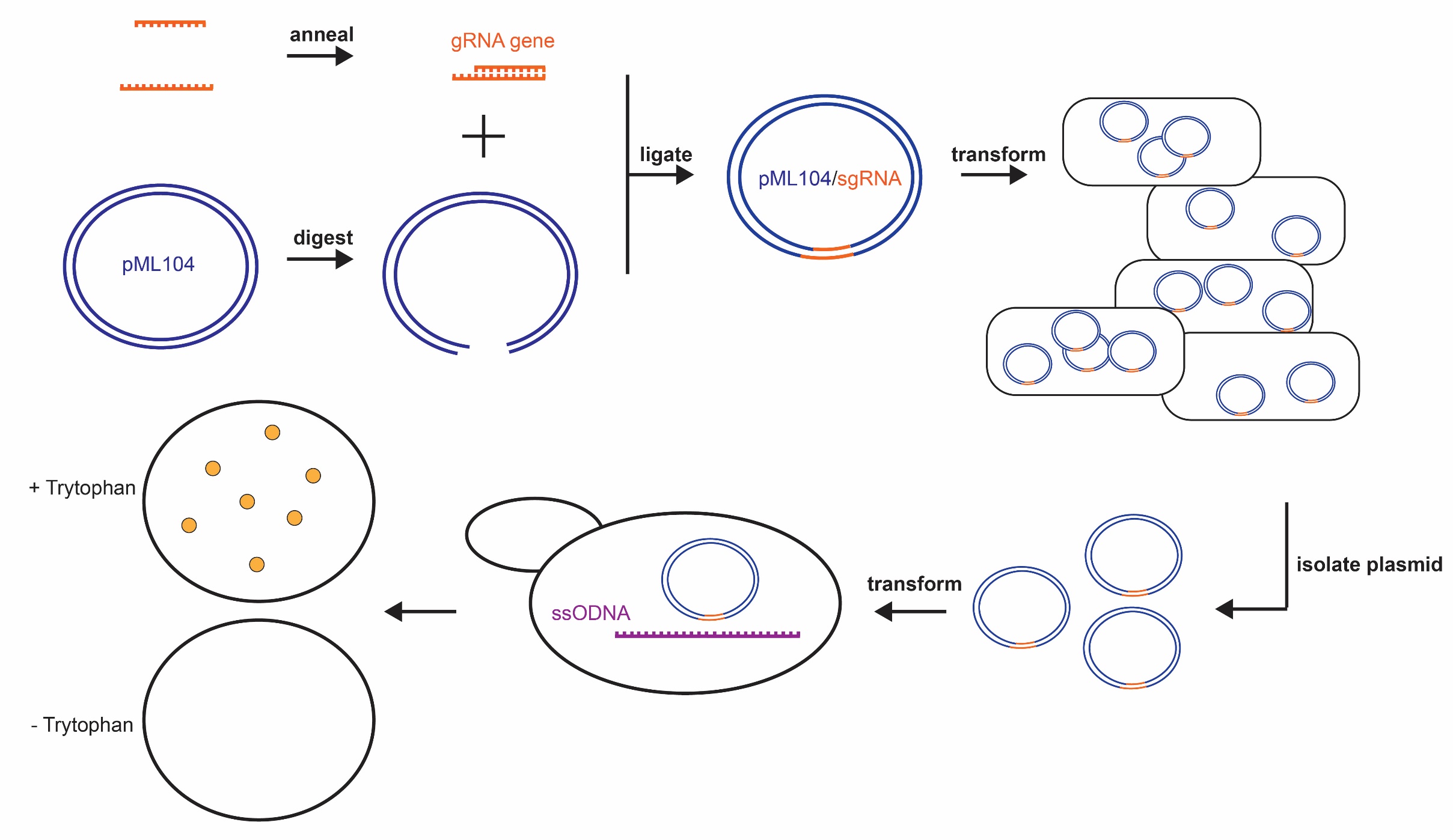
pML104 is a plasmid vector designed for cloning and expression of the essential components for CRISPR/Cas9 gene editing: Cas9 endonuclease, tracrRNA and CRISPR RNAs (Laughery *et al.*, 2015). pML104 has a region for cloning in a targeting sequence to produce an sgRNA that will target your gene of interest. Scientists have conveniently combined the separate small RNA segments of tracrRNA and CRISPR RNA into one single guide RNA (sgRNA). This sgRNA will be transcribed from a RNA Polymerase III promoter, while Cas9 gene is transcribed from an RNA Polymerase II promoter. Cleavage of the plasmid with the restriction enzymes *Bcl*I and *Swa*I will allow cloning of the sgRNA gene cassette from synthetic DNA oligonucleotides that contain the targeting sequence (20 nt) flanked by a region compatible for annealing with the *Bcl*I cut site (GATC) and a structural portion of the sgRNA (Figure 1). (What sequences do these enzymes recognize? Are they influenced by Dam methylation? If they are, will this be a problem? Why or why not?)

Synthetic oligonucleotides have been purchased that contain the template and non-template strands of the gene that allows targeting of *TRP1*. See the instructor for the sequences of the oligonucleotides. Annealing the antiparallel oligonucleotides will result in a double stranded piece of DNA with a 5’ overhang (non-base paired region) complementary to the overhang on the vector cut with the restriction enzyme *Bcl*I. The other end on the annealed oligonucleotides is blunt (no overhangs), similar to the *Swa*I cut site on the vector. The nature of these ends will allow ligation of digested pML104 with the annealed oligonucleotides, generating a plasmid that will express a *TRP1*-targeting sgRNA to guide Cas9 cleavage of the *TRP1* gene (Table 1).

In order to clone the sgRNA gene cassette into the pML104 vector, the vector must be prepared by restriction digest and then removal of 5’ phosphates. The vector should be digested with two restriction enzymes to facilitate sgRNA gene cloning; *Bcl*I and *Swa*I. Since the optimal conditions for the enzymes differ, the digest cannot be performed simultaneously. What are the optimal conditions for each restriction digest? After digest, the 5’ phosphates on the ends of the cut vector DNA are removed in order to prevent the small fragment cut away from the vector from ligating back to the vector, or allowing partially digested vector to re-circularize. The synthetic oligonucleotides used to construct the sgRNA genes do not have 5’ phosphates, therefore, phosphates will also have to be added to the synthetic DNA oligonucleotide. When the treated sgRNA gene cassette and vector are added to a ligation reaction, the 3’ hydroxyl groups of the vector will only be able to ligate to the 5’ phosphate of the desired insert (annealed oligonucleotides).

Leaving DNA in the presence of active enzymes longer than the suggested incubation times can damage the DNA. In some cases, you can denature enzymes with heat, which inactivates them. Two of the enzymes we will use, *Bcl*I and phosphatase, are resistant to heat inactivation, therefore, will need to be purified away from digested, dephosphorylated pML104 using phenol:chloroform extraction and ethanol precipitation. Phenol and chloroform are organic solvents that will allow separation of water-soluble molecules (like DNA) in an aqueous layer from organic substances like lipids and proteins (Green and Sambrook, 2017).

By the end of this week’s lab, you will have a dephosphorylated, cut vector, and phosphorylated sgRNA gene cassette with complementary ends. Next week we will combine them into a ligation reaction, then transform *E. coli* with the ligated fragments for selection and propagation (Figure 1). Please be mindful of this week’s activities and use your time wisely. There are several incubation periods so you will need assemble reactions quickly. Plan ahead what you will assemble first. If you are confident in your skills, you may assemble your restriction digest prior to the official start of the laboratory class.



**Figure 2. Cloning of 20mer guide sequences.** Unique restriction sites enable efficient cloning of any 20 mer guide RNA targeting sequence into the single guide RNA (sgRNA) expression cassette, pML104. pML104 is linearized by digestion with *Bcl*I and *Swa*I enzymes. Two oligonucleotides (orange) are hybridized to generate the sgRNA gene cassette that will be cloned. The sgRNA contains a 20 mer guide sequence (in this case targeting the yeast TRP1 gene), and will be fused with 5' end of the structural segment of the sgRNA. The sgRNA gene cassette also contains a 5’ overhang and blunt end that are compatible with the *Bcl*I- and *Swa*I-digested pML104. The hybridized oligonucleotides are ligated into the digested plasmid, yielding the final complete sgRNA expression cassette, pML104/sgRNA.

## References

Green, Michael R., and Joseph Sambrook. 2017. “Isolation of High-Molecular-Weight DNA Using Organic Solvents.” *Cold Spring Harbor Protocols* 2017 (4): db.prot093450.

Laughery, Marian F., Tierra Hunter, Alexander Brown, James Hoopes, Travis Ostbye, Taven Shumaker, and John J. Wyrick. 2015. “New Vectors for Simple and Streamlined CRISPR-Cas9 Genome Editing in Saccharomyces Cerevisiae.” *Yeast* 32 (12): 711–20.

## Protocol

1. Digest pML104
   1. Restriction Digest
      1. Fill in the volumes of reagents that you will need in your restriction digest below. The final volume of your complete digest, with all reagents, will be 30l. Use this table as a guide and checklist while you are adding reagents to each reaction. Read down each column, adding the same reagent to all appropriate tubes**.**

|  |  |
| --- | --- |
| \_\_\_\_\_l | pML104 (1-2g) |
| 1 l | *Swa*I enzyme |
| \_\_\_\_\_l | 10X NEB buffer 3.1 |
| \_\_\_\_\_ l | ddH2O |
| 30 l | TOTAL |

1. Add the correct amounts of reagents to each reaction tube. Be sure to watch to make sure that you have delivered the correct amount of each component to the inside of the tube.

*Note: In general, pipette the largest volume of a reaction first, and the restriction enzyme is ALWAYS added last. When you are pipetting small volumes (1-2 l), be sure to touch the pipette tip to liquid already in the tube.*

1. Close the tops of the tubes and mix by gently flicking the bottom of the tube. Centrifuge briefly with just a quick pulse to make sure all reagents of the reaction are in the bottom of the tube.
2. Incubate at 24oC for 12-18 hours.
   1. Assemble reaction mixture for digestion of vector with *Bcl*I.

Remaining volume of *Swa*I digest

1.5 l *Bcl*I enzyme

1l 10X NEB buffer 3.1

\_\_\_\_ l ddH2O

40 l TOTAL

* + 1. Gently mix the reaction and microfuge briefly.
    2. Place reaction at 50°C for 1-2 hours
  1. Dephosphorylation

1. Add 1 l Alkaline phosphatase to the restriction digest
2. Flick to mix and microfuge briefly.
3. Incubate reaction at 37°C for 30 min
   1. Purification of DNA
      1. Add an equal volume of phenol:chloroform to your digested plasmid.

* *Tip 1 - Phenol is an organic solvent and extremely caustic. Wear gloves and long sleeves. If you drip it on you, rinse thoroughly with water immediately.*
* *Tip 2 – The phenol:chloroform is equilibrated with isoamyl alcohol. In the stock container, the phenol is in the lower layer. Both are translucent, but have slightly different appearances. Do not shake this container. You want the phases to be separate. Make sure that your pipette tip is inserted into the lower layer before releasing the plunger.*
* *Tip 3 – Phenol:chloroform has very little surface tension so it will not stay inside your pipette tip. This means you need to be move quickly between pipetting up the phenol:chloroform and releasing it into the tube with DNA. Have your tube open, and near the phenol:chloroform container.* 
  + 1. Vortex.
    2. Centrifuge the mixture at 13000 rpm for 2 minutes. A lower organic layer should separate from an upper aqueous layer. If the organic and aqueous phases are not separated well, centrifuge again for a longer time.
    3. Carefully pipette the aqueous phase (which contains the DNA) to a fresh tube. Discard the interface and organic phase. If you think you may have transferred some of the organic layer with the aqueous solution, repeat the centrifugation and pipette off aqueous solution.
    4. Precipitate the DNA by adding:

1/10 volume of 3 M sodium acetate.

Equal volume of isopropanol or 2.5 volumes 95% ethanol.

*Note: This is a stop point. DNA in ethanol can be stored at -20°C, indefinitely.*

* + 1. Incubate 5 min on ice
    2. Centrifuge 5 min at 13000 rpm
    3. Discard solution and add 200 l 75 % ethanol
    4. Centrifuge 5 min at 13000 rpm
    5. Discard ethanol and dry pellet at room temp
    6. Resuspend in 50 l ddH2O
  1. Store purified DNA at -20 °C until next week

1. Prepare sgRNA gene cassette from oligonucleotides
2. Choose one set of oligonucleotides for cloning.
3. Phosphorylate the oligonucleotides. (Think through this. You CAN fill in the blanks on this reaction).

5 l oligo (200 ng/l)

\_\_\_ l 10 X T4 PNK buffer

5 l 10 mM ATP

1 l T4 phosphonucleotide kinase (PNK)\*

\_\_\_ l ddH2O

50 l TOTAL

* 1. Gently mix the reaction and microfuge briefly.
  2. Incubate 1 hr at 37°C
  3. Heat inactivate the enzyme at 65°C for 20min.
  4. Store at -20°C or proceed to annealing

1. Anneal the oligonucleotides in preparation for cloning

20 l Phosphorylated template strand oligo

20 l Phosphorylated non-template strand oligo

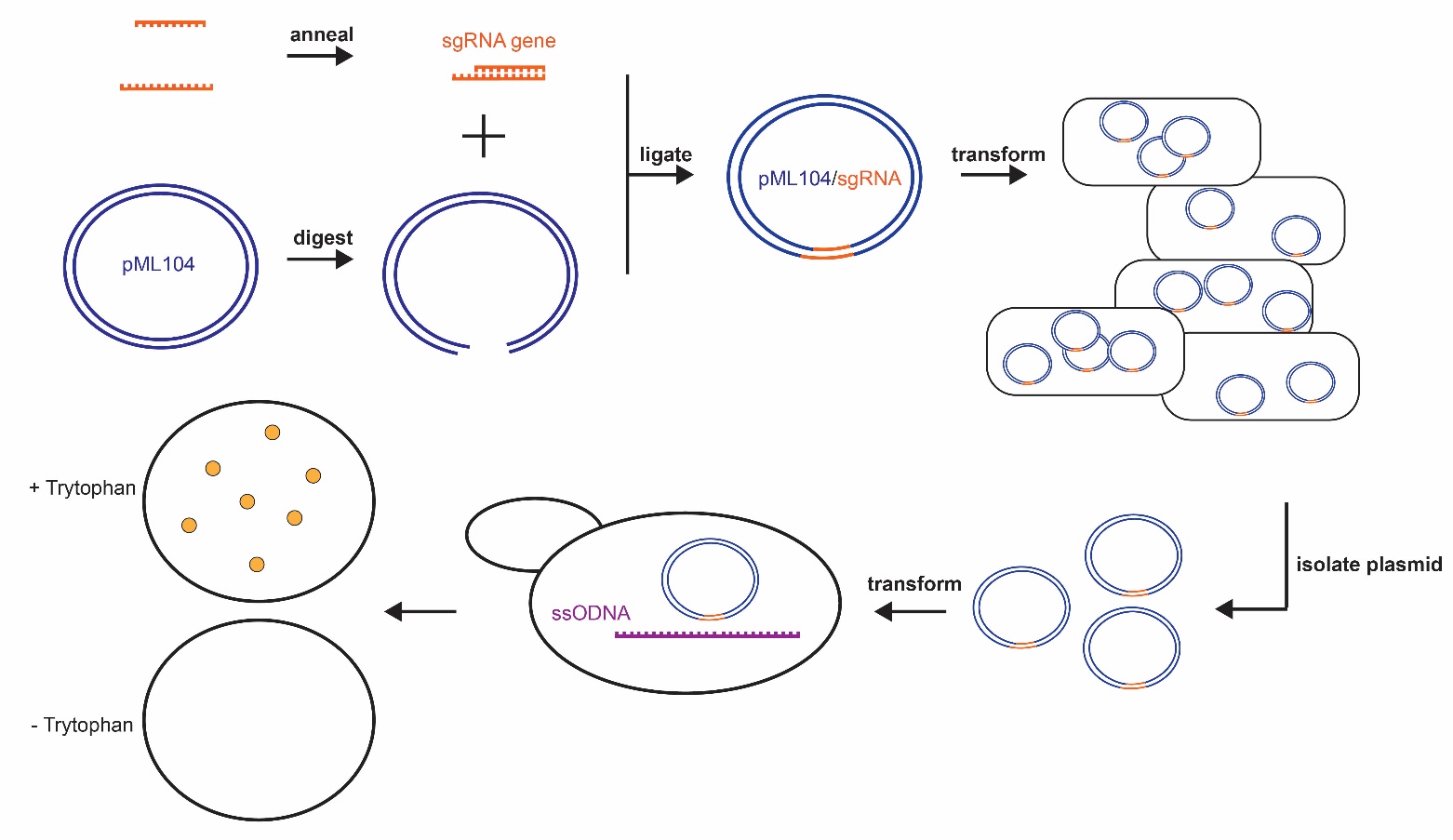
* 1. Gently mix the reaction and microfuge briefly.
  2. Heat annealed reaction to denature any structure in the oligos, then slowly cool to room temperature to allow annealing of complementary DNA sequences.
     1. In thermocycler: 5 min at 95 °C, Ramp to 24 °C at a rate of 1.5 C/min (Slow cooling step necessary for annealing), Hold at 4 °C
     2. In water: Boil water in beaker my heating in the microwave. Add a float with your tube to the hot water. Sit on bench until the water reaches room temp.

*Note: Your reaction can remain at 4 °C indefinitely.*

# Cloning sgRNA for CRISPR/Cas9 gene editing, Part II

## Background

Last week, you digested and dephosphorylated pML104 for cloning in the gene for sgRNA expression, and the sgRNA gene was made by annealing phosphorylated oligonucleotides. Today, you will finish the cloning process by ligating the digested plasmid DNA with the sgRNA gene cassette, then transforming the ligation into competent *E. coli* cells. (What does “competent” mean in this context?)



**Figure 3. Graphical overview of cloning process.** The sgRNA gene targeting *TRP1* was constructed by annealing two oligonucleotides (orange) and ligating into pML104 (blue) that has been digested with two different restriction enzymes. The cloned product, pML104/sgRNA, is transformed into bacteria, which will produce more molecules of the plasmid as the bacteria reproduce.

### Ligation

DNA ligase will fuse the phosphate backbones of the insert (sgRNA gene cassette) with the vector (pML104). DNA ligase requires ATP, therefore, DNA ligase buffers will contain this reagent. In a ligation reaction, the insert is usually added in 3:1 to 10:1 molar excess of the vector. This means for every mole of pML104 you would add between 3 and 10 moles of insert. If the insert and vector were the same size, you would add 100 ng of insert for 10 ng vector in a 10:1 ratio. Ligations of “sticky ends” (DNA that contains complementary overhangs) is often more efficient and can be accomplished with a lower insert:vecter ratio. Ligating DNA with blunt ends is less efficient and therefore should have a higher insert to vector ratio. There are calculators available from many biotechnology resources to help you calculate the moles in a given weight (ng) of DNA, based on length. (Make sure the calculator you use is properly cited.)

### Transformation

There are a number of ways to prepare competent cells. Each protocol induces small ‘pores’ in the cell membrane and allows extra-chromosomal plasmid DNA into the cell. The cells we will use today are purchased from Invitrogen and have been made chemically competent using CaCl2 (Hanahan *et al*., 1991). The product description and suggested transformation protocol are included in your lab materials. Why is it important to maintain the cells in a cold environment?

In this transformation protocol, we will use heat shock for transformation. What are some other methods of transformation? The order of each step in a heat shock transformation remains consistent between protocols, but the duration of each step varies with cell type, the thickness of the tube, and, sometimes, even the DNA transformed.

Once the host cells are transformed with DNA from your ligations they are then incubated in optimal conditions for growth without selection (this is called the outgrowth step). Outgrowth allows the cells to recover from heat shock and begin to express the necessary genes from the plasmid, before being exposed to the antibiotic on selection plates.

The success of transformation can be measured by calculating transformation efficiency, the number of host cells transformed per microgram of DNA. Transformation efficiency should be included in the results section of your notebook. Calculate as follows (Green and Sambrook, 2017):

# of transformed cells/µg of DNA used x final volume of cell suspension (ml)/volume of cell suspension plated (ml) = number of transformants per µg of DNA

## References

Green, Michael R., and Joseph Sambrook. 2017. “Isolation of High-Molecular-Weight DNA Using Organic Solvents.” *Cold Spring Harbor Protocols* 2017 (4): db.prot093450.

Hanahan, D., J. Jessee, and F. R. Bloom. 1991. “Plasmid Transformation of Escherichia Coli and Other Bacteria.” *Methods in Enzymology* 204: 63–113.

## Protocol

1. Ligate the sgRNA gene cassette into digested, purified vector (pML104). In addition to ligation of the digested vector with the gene insert (annealed oligonucleotides), you will need to decide on and perform a control reaction. The purpose of the control reaction is to assure that the product of the ligation is the gene inserted into the vector, and not undigested vector only re-circularized (or perhaps vector that was never linearized), or that some contaminating plasmid DNA is present in solutions along the way. There are several options for appropriate controls. Reason through this and decide on ONE control ligation reaction. Make sure to set this control reaction up alongside the vector + insert reaction (below) in your notebook.
   1. Assemble ligation reactions

\_\_\_\_\_ l digested vector (200ng, if your DNA is concentrated enough)

\_\_\_\_\_ l annealed oligos (or water, in the vector only control)

\_\_\_\_\_ l 2 X ligation buffer

1 l DNA ligase

\_\_\_\_\_ l ddH2O

10 l TOTAL

* 1. Gently mix the reaction by pipetting up and down and microfuge briefly.
  2. Incubate at room temperature (25 °C) for 15 minutes.
  3. Chill on ice.

1. Transform 5 µl of each reaction (ligation and ligation control) into 50 µl competent cells. You will also need transformation controls (both positive and negative). For the positive control, you will transform the parent plasmid (pML1014, undigested). What do you think would be a good negative control for transformation of *E. coli*? Be sure to include these controls in your procedures. See Invitrogen protocol, with the following exceptions: Plate all of transformation solution on one selective plate. (What selective plate will you use?)

# Screening clones

## Background

You should have individual *E. coli* colonies grown on a selective plate that may (or may not) have your sgRNA gene cassette cloned correctly into pML104 backbone. At the least, these colonies likely contain the pML104 since they grew on media with antibiotic. However, if the restriction digest or dephosphorylation of the vector was incomplete, it is possible that some of the colonies do not contain the sgRNA gene cassette. We will screen the DNA from bacterial colonies to select only colonies with the correct insert. There are several ways to screen plasmid DNA, and the selected screening tool depends on the vector, the insert, time and resources. Often screening involves first isolating plasmid DNA, then digesting with restriction enzyme that will cut a unique pattern when the insert is present and properly oriented. Our insert does not contain a restriction site and is too small to detect by change in size by agarose gel electrophoresis after a digest of the backbone. Therefore, we will use PCR to screen.

## PCR

PCR is a technique by which DNA can be replicated *in vitro*. With this technique, starting DNA material called the target sequence, a gene or a DNA segment can be amplified up to a million-fold. Being able to amplify and produce large quantities of a specific DNA sequence is a very powerful technique in molecular biology. In fact, the PCR technique is a routine technique in any molecular or cell biology laboratory.

The PCR technique relies on several critical reaction components listed below:

**Template DNA** (a double stranded DNA segment)

**DNA primers** (single stranded synthetic DNA complementary to target sequence)

**DNA polymerase** (DNA polymerases responsible for replication of DNA)

**DNA building blocks** (a mixture of dATP, dGTA, dCTP, dTTP, also known as dNTPs)

**Buffering components** (ensure polymerase activity, Mg+2 is critical)

Once the reaction components are well mixed, the reaction needs to be taken through a series of 3 different temperatures representing one PCR cycle. These 3 temperature cycles are repeated up to 30 times to complete the million-fold amplification of the target DNA.

**Denaturing** at ~95oC to make all DNA in the reaction single-stranded

**Annealing** at 45-60oC to allow the DNA primers to complementary base pair

**Extension** ~65-72oC to allow DNA polymerase to replicate DNA

The duration and temperatures of these steps are dictated by the expected product size, primers and exact polymerase. For example, the annealing temperature should be ~2-5 degrees below the estimate Tm of the primers, therefore, each set of primers may have a unique annealing temperature (although some polymerases will recommend slightly higher or lower temperatures). The size of the product will dictate the extension time. Generally, we estimate that it takes about 1 min to extend 1 KB of DNA. Finally, the optimal conditions for the polymerase (*Taq*) may vary. In this lab, we will use GoTaq by Promega. Find the reaction conditions for this polymerase on the company website and record your reaction conditions in your notebook.

The annealing step of PCR allows the primers to base pair with the target DNA sequence by complementarity. Once this takes place, the primers provide a free 3’-OH to which the DNA polymerase can add dNTPs (that are supplied in the reaction). One primer in your reaction, M13 Reverse (M13R), is complementary to the backbone and everyone will use. The second primer in your reaction will depend on the sgRNA you are attempting to clone. This primer should anneal to the sgRNA insert, but to the opposite strand as the M13R primer. (Each primer will anneal to different strand and amplify the region in between them.) Find the sequence of pML104 and find where M13R binds (on Addgene). What oligo will you use as your second primer? (Hint: It will be one of the oligonucleotides you used for your cloning.) How far apart will these primers be if your sgRNA is cloned correctly? (*i.e.,* How long is your expected product?)

**M13R sequence**: AGCGGATAACAATTTCACACAGG

The elongation temperature is the optimum temperature for the DNA polymerase to replicate DNA. The DNA polymerases used in the PCR reaction have been isolated from thermophiles, bacteria that thrive at high temperatures. These bacteria can be found in places such as the hot springs in Yellowstone National Park. The DNA polymerases from the thermophiles are heat-stable and do not denature at 95 oC and will survive in the reaction for the 30 cycles it takes to amplify the DNA.

Within the first 5 cycles of the temperatures, the DNA information defined by the primers begins to be doubled and will be amplified exponentially such that over a million copies of the specific DNA segment are produced. This is so much DNA material that you can electrophorese the PCR reaction on an agarose gel and see the DNA segment that was amplified.

## Protocol

1. Start cultures. (This must be done the night before your lab.)
2. Remove plates from storage at 4 °C.
3. Pick at least two individual colonies from your pML104+gRNA gene plate and start 5 ml cultures in selective media. (Antibiotic is in the freezer in the lab.) If your negative control transformation (vector only) was clean (no colonies), you only need to start these two cultures (plus a non-inoculated control). If you had significant growth on your vector only control transformation (more than 10% of your vector + insert), you should increase the number of colonies you picked to a maximum of six colonies.
4. Return plates to 4 °C for storage.
5. Shake culture at 37 °C for at least 18 h.
6. Screening plasmid DNA
   1. Isolate plasmid DNA from overnight cultures using the protocol from Part 1, exercise 4. (Write out the protocol.)
   2. Assemble the PCR reaction. As a negative control, you should include a reaction without template DNA. (How many reactions will you have in total?)

1l isolated plasmid DNA (or water)

\_\_\_\_l GoTaq 2 X MasterMix (What is in this mix?)

1l M13R primer (200 ng/l)

1l gRNA oligonucleotide (Which did you choose?)

Fill to 20l PCR-grade water

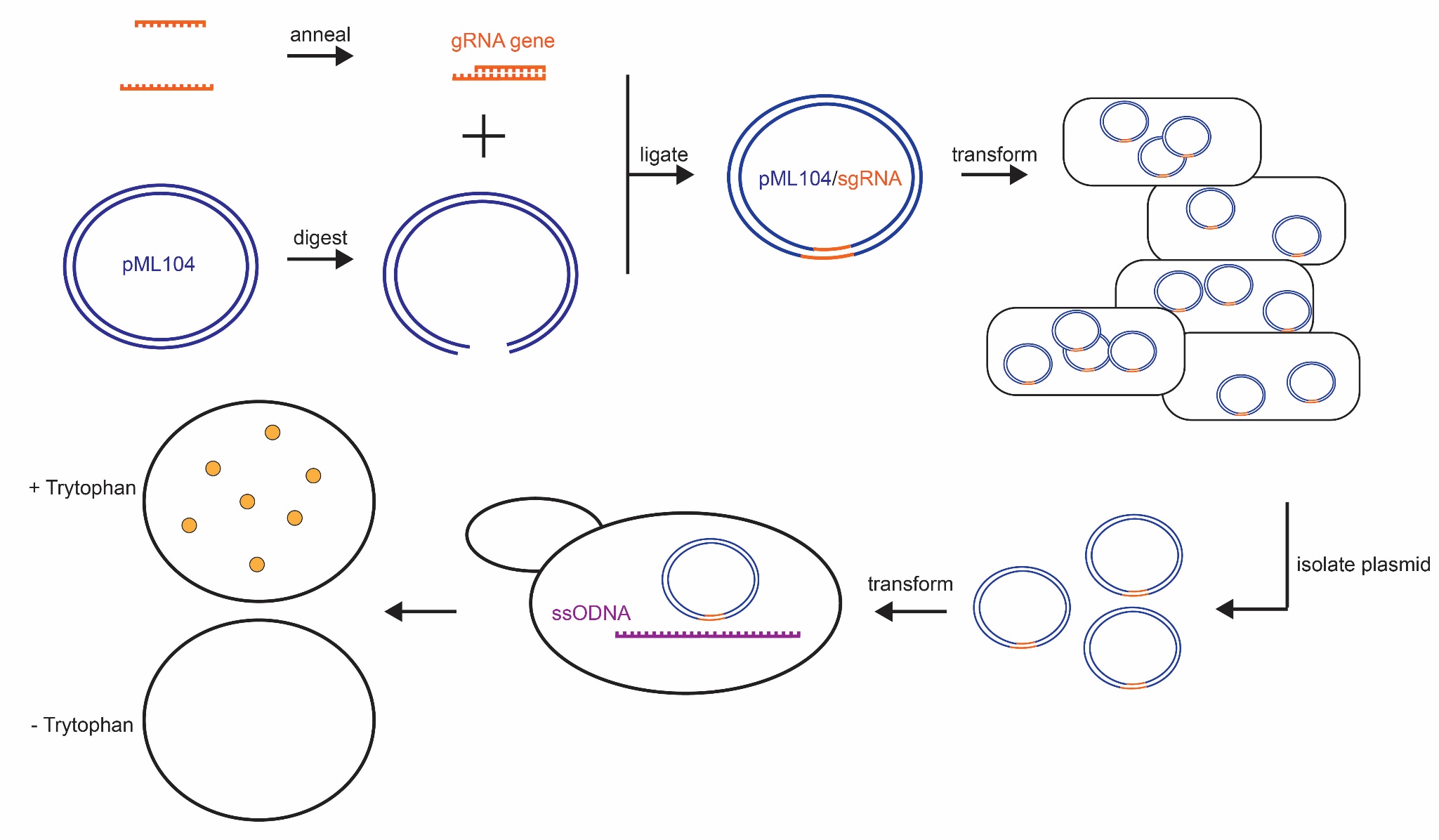
* 1. Securely close the lid on the PCR tube (make sure that the lid is fully closed) and make sure tubes are labeled.
  2. Place in thermocycler and cycle under the following conditions.
     1. 95 °C for 2 min
     2. 95 °C for 30 sec
     3. \_\_\_\_\_\_ for 30 sec (The annealing temp is 5 °C below the Tm of the primer with the lowest Tm)
     4. 72 °C for \_\_\_\_\_ min
     5. Repeat steps ii-iv 30 times
     6. 72 °C for 5 min
     7. Hold at 4 °C indefinitely. (You may leave this at 4 °C until next week.)
  3. Next week: Run 5l of each PCR product on an agarose gel. (Include this information in NEXT WEEK’s pre-lab: What percent gel will you use? How will you make this gel?)

# Selection of clones and Transformation of yeast

## Yeast Transformation

The laboratory *Saccharomyces cerevisiae* (yeast) strain you are using is called BY4742. The genotype is *MATα his3Δ leu2Δ lys2Δ ura3Δ TRP1*. Yeast come in two mating types *MATa* or *MAT* While they often exist as haploid organisms, yeast cells of each mating type can fuse to become a diploid cell. A wild-type gene is indicated by uppercase letters, while a mutant gene is indicated by lowercase letters. Gene and RNA species are indicated in italics. Looking back at the genotype, this strain is lacking a functional the *URA3* gene that allows synthesis of the essential nutrient uracil. However, the *URA3* gene is present on the pML104 backbone so they will be able to survive in the absence of uracil, as long as they contain this plasmid (or any derivative of this plasmid). They also contain a wild-type *TRP1* gene that will allow synthesis of tryptophan. It is the overall project goal to direct Cas9-mediated cleavage of the *TRP1* gene in the genome, and repair the lesion in a way that disrupts the ability of the gene to synthesize functional enzyme. Since our yeast are haploid and rarely undergo non-homologous end joining, but efficiently perform homologous repair, a template for DNA repair is required for lesion repair and thus prevent death of the cell. Therefore, yeast that contain sgRNA (and Cas9) that targets the genome efficiently, but no template for homologous repair, will likely die due to unrepaired breaks in genomic DNA.

In order to edit the *TRP*1 gene in the yeast genome, but not kill the cells, you will transform yeast with your isolated plasmid containing the sgRNA and Cas9, as well as a single stranded DNA template to elicit DNA repair. The templates for HR of the CAS9-mediated double-stranded break were ordered as single stranded DNA oligonucleotides (ssODNA). The sequences of the available ssODNA will be provided by the lab instructor. At least one of these templates should work with the sgRNA that you cloned. Choose the most appropriate template for your experiment and describe the qualities of this template that will be useful in your experiment. To choose your template, it might be helpful to think back to the CRISPR design lab and note what qualities make a DNA repair template useful.



**Figure 4. Yeast Transformation.** Isolated pML104/sgRNA (blue/orange) is transformed into yeast along with a single stranded DNA oligonucleotide (ssODNA) that will be the template for homologous repair of the Cas9-mediated double-stranded DNA break.

You will begin by electrophoresing the product from your PCR reaction to screen clones. See last week’s information for the protocol, and your notebook for information on how to pour the gel. Include a detailed protocol for pouring the gel, and any other pertinent information in your materials and methods.

## Protocol

1. Select DNA plasmid for transforming into yeast
   1. Electrophorese 5 l of PCR product from last week alongside 5 l of 100 bp DNA ladder. (See previous protocols. If you are confident in your skills, you may pour and/or run your gel prior to the start of the lab.)
   2. Analyze gel and choose at least one clone for transformation. (Describe results that will indicate a “good” clone.)
   3. Quantify DNA concentration of selected clone.
2. Yeast Transformation
3. Three to five hours before class, dilute an overnight yeast culture in rich medium (YEPD) to make a culture of OD600 0.167 in 50 ml of YEPD. (This step will be done for you.)
4. Incubate the flask on a rotary or reciprocating shaker at 30°C to grow for at least two divisions (it takes about 2 h per division).
5. Harvest and wash cells
   1. Aseptically harvest the cells by centrifugation in sterile conical tube at 3200 *g* for 2 min. Discard supernatant.
   2. Add ~25 ml of sterile water to wash the cells. Spin 3200 *g* for 2 min. Discard supernatant.
   3. Resuspend in 1 ml of sterile water.
6. Boil a 1.0 ml sample of 2mg/ml salmon sperm DNA for 5 min and chill in an ice/water bath while harvesting the cells.
7. Transformation
   1. Transfer the cell suspension to a sterile 1.5 ml microcentrifuge tube, centrifuge for 30 sec at ≥ 3000 rpm and discard the supernatant.
   2. Add sterile water to a final volume of ~1.0 ml (use the graduations on the side of tube to estimate final volume of 1ml) and vortex mix vigorously to resuspend the cells.
   3. Pipette 100 μl into sterile 1.5 ml microfuge tubes, one for each transformation.

*Note: You will need a negative control and a positive control to assure that your yeast transformation results are reliable. What are appropriate controls?*

* 1. Centrifuge at top speed for 30 sec and remove the supernatant.
  2. Each tube of cells receives following reagents/volumes, in this order.

240 μl 50% polyethylene glycol (PEG).

*Note: PEG is very viscous and will move in and out of the pipette slowly. Be patient.*

Resuspend the cell pellet completely in PEG before adding additional reagents. You will have to pipette up and down to disrupt the pellet.

50 μl 2 mg/ml salmon sperm DNA (Denatured)

34 μl sterile water with DNA to be transformed

Use 20 μl of 60 nM ssODNA template

Use 2 μg of DNA plasmid

36 μl 1.0 M LiOAc

* 1. Vortex the mixture well.
  2. Incubate the tubes in a 42 °C water bath for 40 minutes.
  3. Microcentrifuge at top speed for 30 sec and remove the supernatant with a micropipettor.
  4. Pipette 1.0 ml of sterile water into each tube; gently resuspend the pellet by pipetting up and down.

1. Plate 500 μl on one selective media plate. (What type of plate?) Incubate the plates at 30 °C for ~4 days.

# Phenotyping

You should now have yeast that contain pML104/sgRNA and a homologous recombination template (ssODNA). Back in the CRISPR design lab, you outlined a method to genotype and phenotype yeast with the edited *TRP1* gene. What phenotype are we looking for, and why? What was the suggested phenotyping method? What controls do you suggest for this experiment? (Remember that we want to isolate some of the genetically modified yeast strain, so simply NOT growing is not an option. We need to keep some for the future.)

## Protocol

* + - * 1. Pick yeast colonies from selection plate with a sterile toothpick or inoculating loop.

Pick at least 3 colonies from pML104/sgRNA + template DNA plates

Pick at least one colony from the positive transformation control plate.

* + - * 1. Resuspend cells in 100 μl sterile ddH2O in an Eppendorf and mix well.
        2. Assay the density of cells in this tube by measuring the amount of visible light (600 nm) absorbance on the Nanodrop spectrophotometer. Be sure to vortex your cell suspension thoroughly JUST BEFORE you pipette since the yeast cells will settle to the bottom of the tube.
        3. Adjust the densities of your cell suspension to be equal in all tubes. To do this, choose the tube with the lowest density anduse the A600 of this tube as your final concentration. Using 100 l as your initial volume, calculate the final volume and add sterile water to the existing 100 l suspension to equal the final volume. (*i.e.,* amount of water to add = V2 - 100 l)
        4. Make 4 ten-fold dilutions of this suspension (10-1, 10-2, 10-3, 10-4) for each yeast colony isolated.
        5. In small lettering, write your group name and date on the edge of a minus uracil plate. Place the template (Figure 1, below) on the bench top and set the plate on top of it (lid side up) so that you can see the grid through the plate. Align the plate on the template with your group name at the top of the plate on the grid.
        6. Pipette cell suspensions on to the plate, inside one box of the grid on the template. Be sure to vortex your cell suspension thoroughly JUST BEFORE you pipette.

Pipette 10 μl of the highest dilution (10-4) to the appropriate location on the grid. (Start with the box on the top left.)

Pipette 10 μl of the 10-3 dilution in the same row, next to the corresponding 10-4 dilution.

Pipette 10 μl of the 10-2 and 10-1 samples in the same row, so that you have highest dilution to lowest dilution of each same in one row.

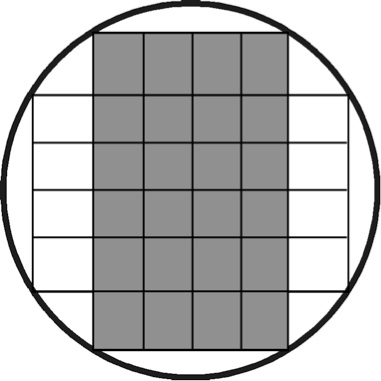
*Notes: If you spot from highest to lowest dilution then you can use the same tip within each sample set.*

*Do not move your plate until you have plated all of your samples. Once the spots dry, you will not be able to see them and therefore will not know exactly where they are.*

*Record in your notebook where you plated each colony and dilution on each plate.*

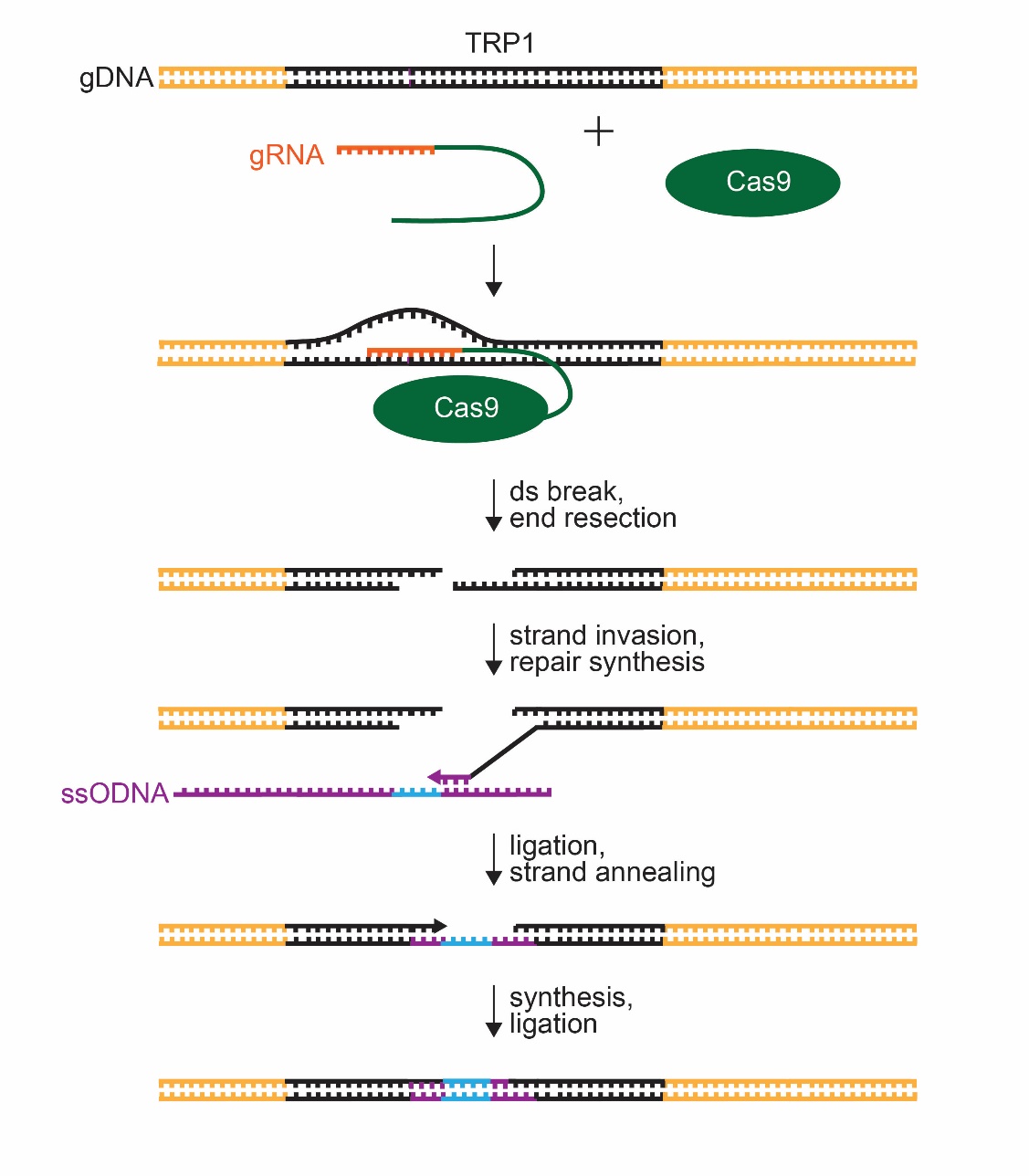
* + - * 1. Repeat the spotting of the suspensions in another row with the dilutions from another yeast colony.
        2. Once each plate is finished, gently replace the lid and move aside until spots have dried.
        3. Repeat this spotting, exactly as was done on the plate lacking uracil (this plate has a blue line on the side), with a plate lacking tryptophan (this plate has a purple line on the side).
        4. Incubate plates inverted at the appropriate temperature for yeast growth. (What temperature is this?).
        5. Examine and photograph after 3-4 days.

**Figure 5. Template for spotting plates.** Be sure to mark the orientation of your plates and to label them on the edge with your initials and the date.



# Genotyping

You should now have yeast that contain pML104/sgRNA and possibly yeast that are tryptophan auxotrophs. You included ssODNA in your yeast transformation that was the template for homologous repair (Figure 6), and introduced a mutation that will disrupt the *TRP1* gene. Predict the sequence of your mutant *trp1* gene based on the sequence of the ssODNA (provided by the instructor).



**Figure 6. Repair of CRISPR/Cas9-Mediated dsDNA Break.** gRNA (orange) recognizes the *TRP1* gene (black) by base pairing to one strand immediately upstream of a PAM sequence. The gRNA is fused to tracrRNA (green) to form a sgRNA. Cas9 endonuclease is guided to cleave the *TRP1* gene by sgRNA recognition. The ends of cleaved DNA are resected. A 3’ overhang will base pair to homologous sequence (purple) within the provided ssODNA template (strand invasion). The ssODNA also contains the desired mutation to disrupt the *TRP1* gene (insertion, deletion, and/or substitution; blue). The 3’ end of the genomic DNA is extended by DNA polymerase (repair synthesis), copying the sequence from the ssODNA. The extended 3’ end ligates with the resected 5’ end in the *TRP1* gene. The gap in the opposite strand is filled by DNA synthesis and ligation.

In genotyping, you will test your prediction and determine the sequence of you affected gene. Genotyping will require isolation and amplification of the region surrounding the edited region of the *TRP1* gene, therefore, you will use PCR to amplify this region. Primers for amplifying this region are provided (sequences shown below). To determine where these primers bind in the yeast genome, paste the primer sequences into BLASTN, back to back with no spaces. Search the *Saccharomyces cerevisae* genome (in the program selection, make sure you select somewhat similar sequences). In your results (scroll down to alignments), you will the genome location of where the primer matches on the subject DNA sequence indicated with numbers beside the subject sequence. Use these genome coordinates to calculate the size of the expected PCR product. Map these primers relative to your sgRNA binding site on the TRP1 gene. (Remember that one of these will be the reverse complement of the *TRP*1 gene.)

|  |  |
| --- | --- |
| Primer #1 | ATGACGCCAGATGGCAGTAG |
| Primer #2 | ACTGGCAAACCGAGGAACTC |
|  |  |

Outline a genotyping method that will allow for selection of yeast that have genomes edited by Cas9 and homologous repair. This method should utilize the PCR product you described above and consider the template used in DNA repair processes. Be sure to include controls and what you expect the results to look like.

**Protocol:**

1. Pick a bit of yeast or an individual colony with a toothpick or sterile pipette tip. Resuspend in 100 l water.
2. Take 5 l of yeast suspension from step 1 above and transfer to PCR tube. (The genomic DNA in the yeast will serve as a template for colony PCR.)
3. Assemble 50 l PCR reactions with each of your yeast colonies (and negative control). Primers (200 ng/l) and 2X GoTaq mixture will be provided. (Write out the protocol for PCR, included all volumes.)
4. Place in thermocycler and cycle. (What cycle conditions will you use?)
5. Electrophorese a small volume (5 l) of the PCR reaction and control on an agarose gel to confirm the size of the PCR product.
6. Purify the PCR product through a PCR purification kit (Qiagen) or Wizard SV PCR and Gel Clean Up Kit (Promega).
7. Analyze the concentration of pure product on the Nanodrop Spectrophotometer.
8. Submit for Sanger sequencing with ONE primer.