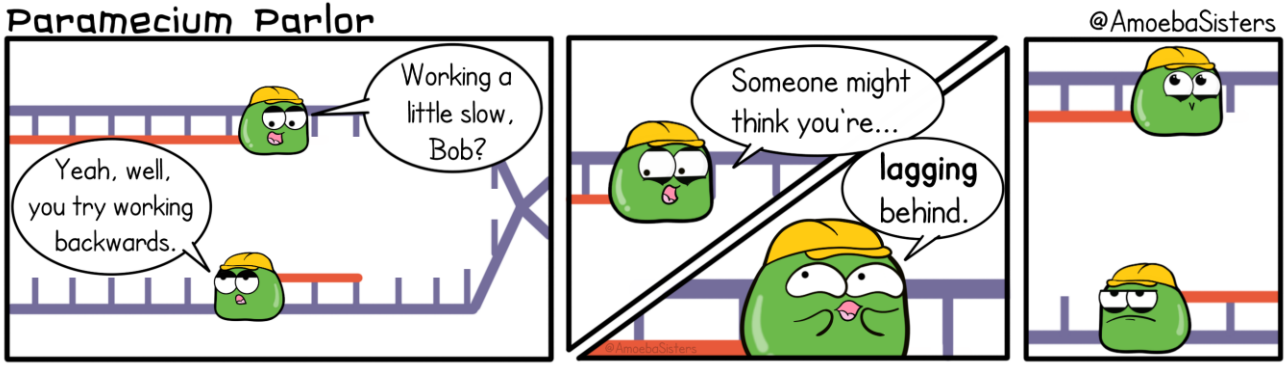
Finish this (start PCR) before prelab

1. Make 100 uM gRNA stock (e.g. 20 nmol → 200 uL nuclease-free water)
2. Make primer mix (10 uM of each primer in 20 uL volume)
   - 2 uL gRNA (forward primer)
   - 2 uL RevP (reverse primer)
   - 16 uL nuclease-free water
3. Take PCR tube and label with your team
   - 10.25 uL nuclease-free water
   - 1.25 uL primer mix (from #2) (OK to use P20)
   - 1 uL pgRNA plasmid (Pipette up at the front)
   - 12.5 uL Q5 Hot Start 2x Master Mix

M2D3: Clone psgRNA expression plasmid

1. Set up reaction to generate gRNA_target plasmid
2. Pre-lab discussion
3. Continue generation of psgRNA
4. Transform pgRNA_target into bacteria



Mod2 Overview

Research goal: Increase the yield of commercially valuable byproducts in *E.coli* using CRIPSRi technology to target genes involved in mixed-acid fermentation pathway.

Last Lab:

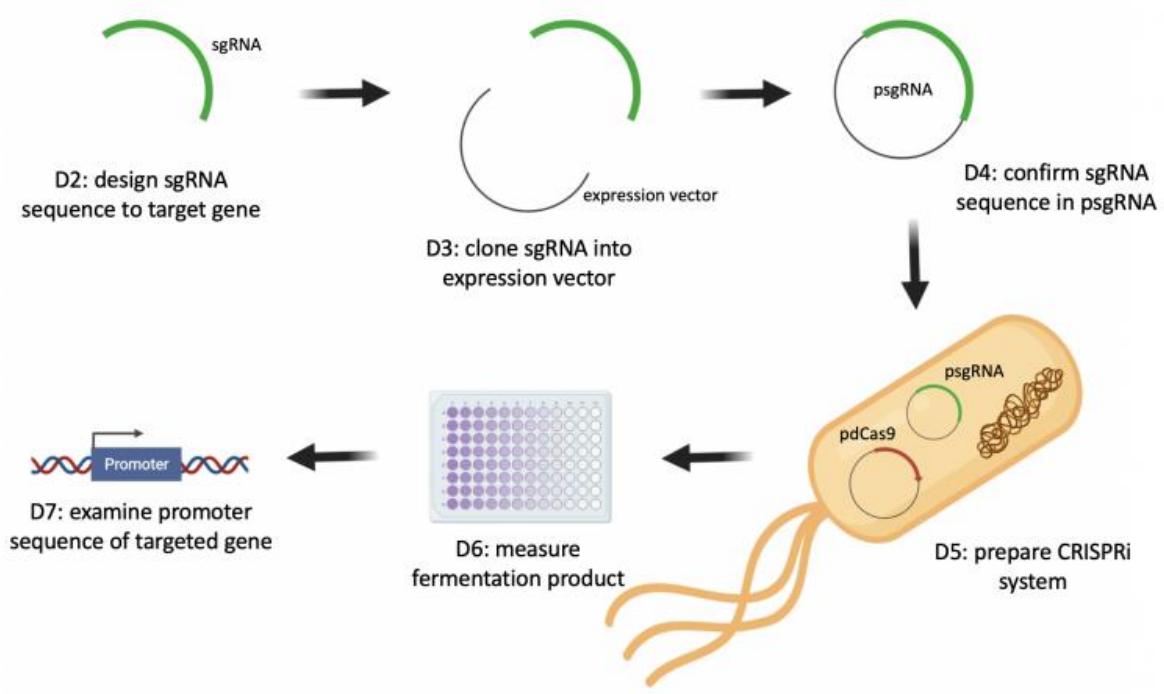
Chose metabolic gene of interest and designed sgRNA to target it

This Lab:

Clone sgRNA into vector to create plasmid that targets gene of interest

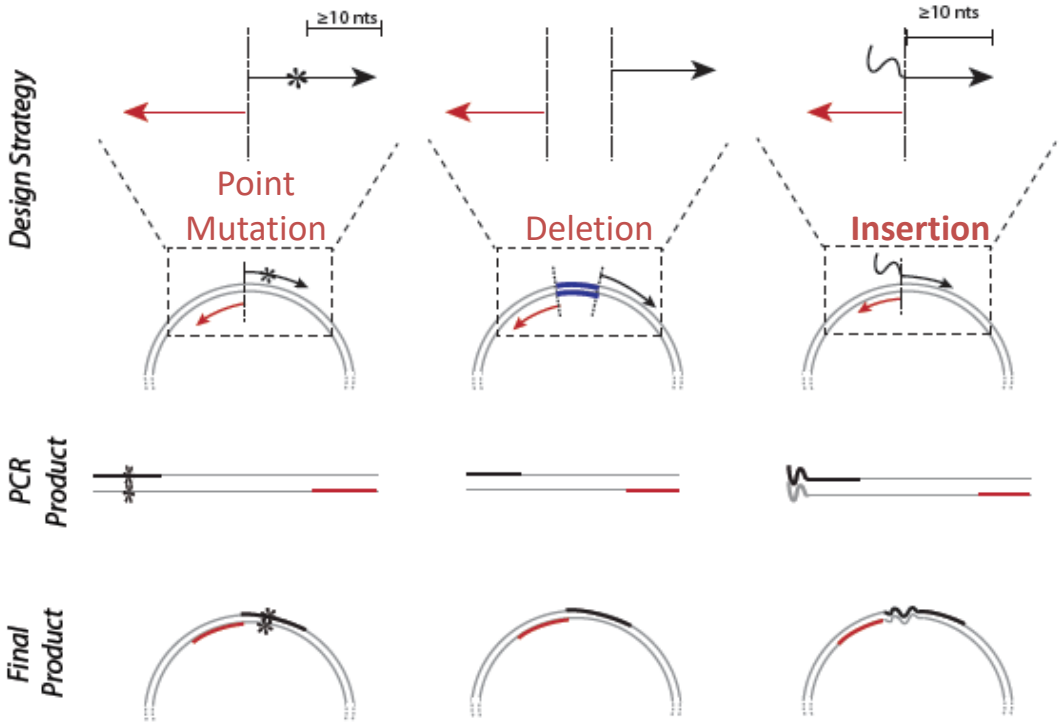
Next Lab:

Confirm correct sgRNA cloning and do preliminary CRISPRi system preparations



Use site-directed mutagenesis (SDM) to engineer plasmid DNA

- NEB Q5 SDM kit
- Create specific, targeted changes in double-stranded plasmid DNA



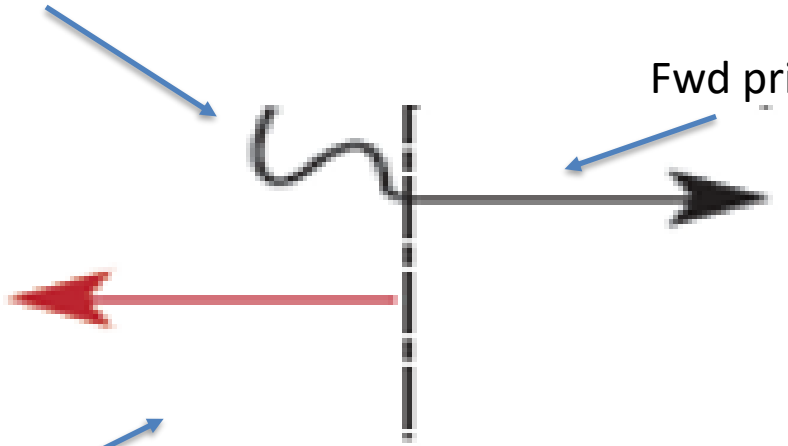
SDM Primers

sgRNA
(made by you!)

Insertion Sequence

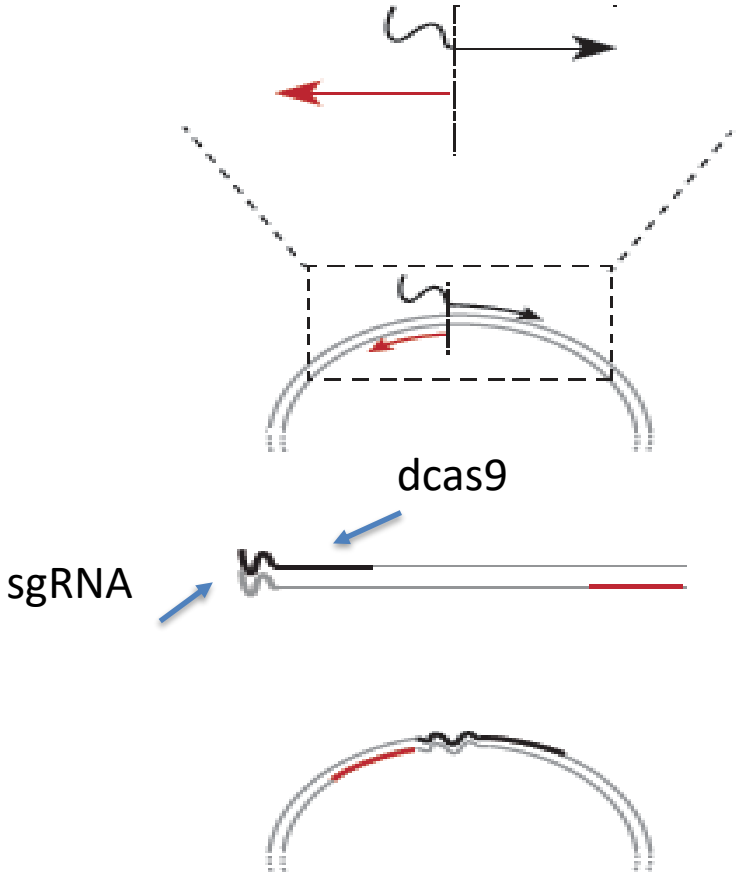
Fwd primer

dcas9 handle



Universal reverse primer

Insertion of DNA via SDM



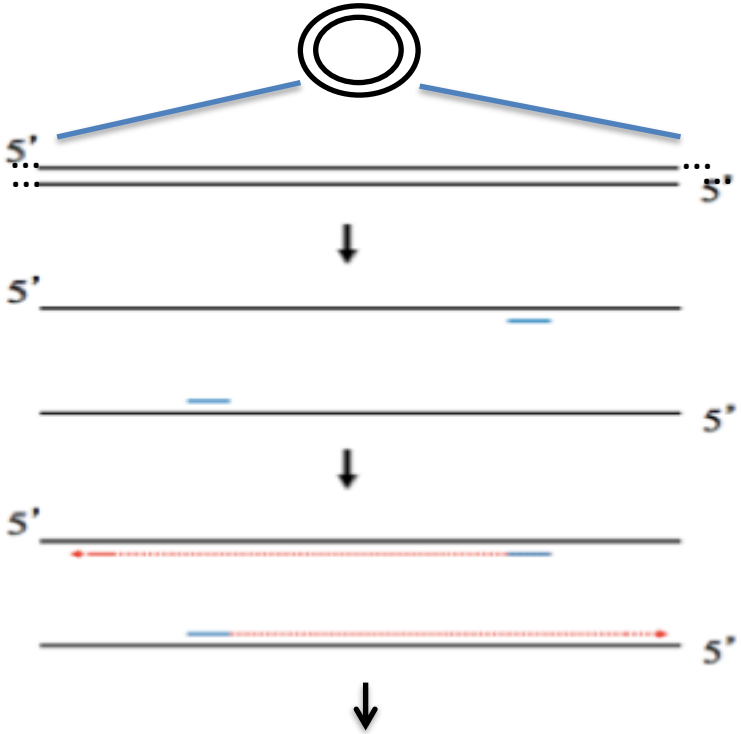
Primers lined up back to back

Linear PCR product

Blunt end ligation

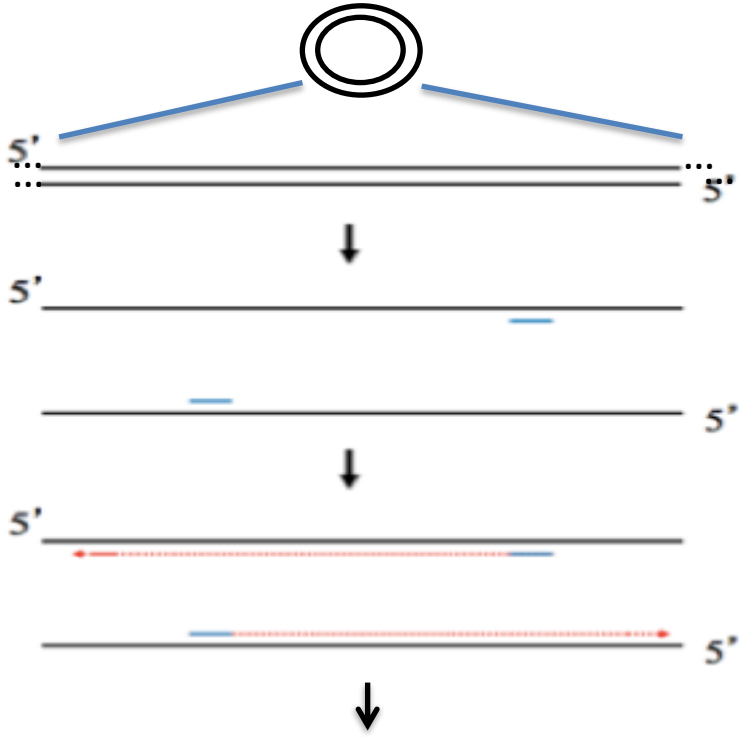
SDM Part 1: PCR amplification of DNA

Ingredients
dNTPs
Hi-Fi Q5 Polymerase
Buffer (pH, cofactors like Mg^{2+})
H_2O



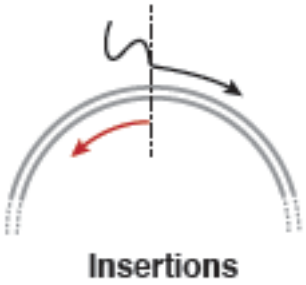
SDM Part 1: PCR amplification of DNA

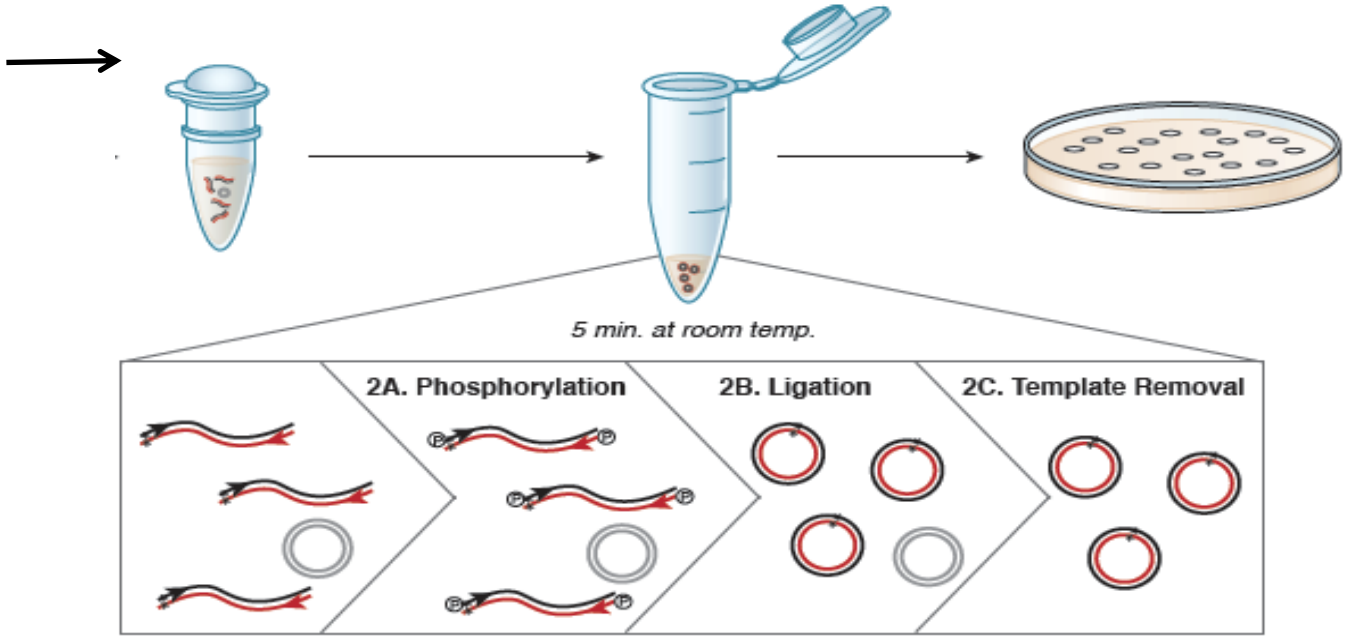
Ingredients
Forward Primer (Insertion)
Reverse Primer (CRISPRi reverse primer)
Template (psgRNA)
dNTPs
Hi-Fi Q5 Polymerase
Buffer (pH, cofactors like Mg^{2+})
H_2O



SDM Part 2: Recover circular plasmid product using Kinase-Ligase-Dpn1 (KLD) enzyme mix

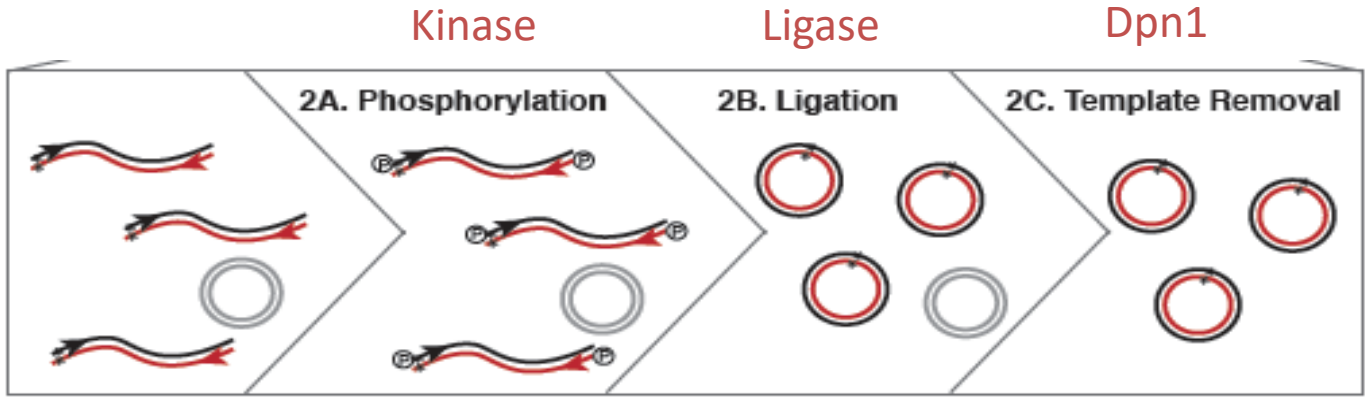
1. Exponential Amplificaion (PCR)
   - Q5 Hot Start 2x Master Mix
2. Treatment & Enrichment: Kinase, Ligase and DpnI
   - 10x KLD enzyme mix
3. High-efficiency transformation
   - NEB 5-alpha competent cells





Phosphorylation video:
https://www.neb.com/tools-and-resources/video-library/the-mechanism-of-dna-phosphorylation

KLD reaction



K: Kinase – Phosphorylation required for phosphodiester bond formation

L: Ligase – Forms phosphodiester bond to circularize plasmid

D: Digests methylated DNA. Why does this target the template?

For today

1. Continue production of sgRNA plasmid
2. Work on journal club in down time

For M2D4…

1. Create a journal club slide (in ppt) and script for 1 figure from your paper
   - Use the homework checklist on the wiki for guidance

For homework, make a Journal Club presentation slide

Craft 1 slide using your JC article to present the data from a figure.

- Your slide should show the data and highlight the key finding(s).
- The information should be clear and large enough to read.
- Keep text to a minimum. (NO figure captions on slide!)
- The title should state the take-home message of the data that are shown.

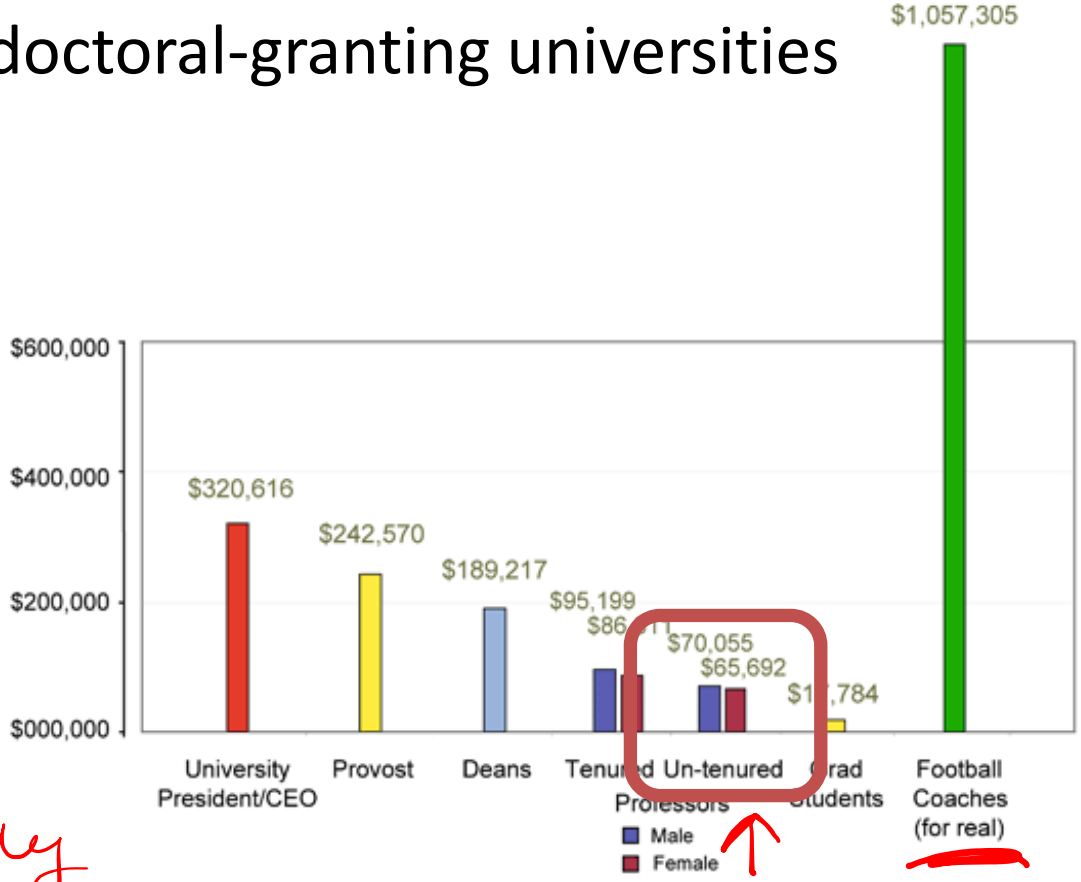
NO A, B, C.

no more than 1/2 of slide is text

EXAMPLE SLIDE: Football coaches are the highest paid academic employees at doctoral-granting universities

- Data represent expression of Y using method A
- Possibly something about the control(s), if applicable
- Perhaps an important note about the data that is not already stated in the title
- Transition to next slide…

in text or verbally



PhD Comics