M2D5: Confirm gRNA sequence

1. Pre-lab discussion
2. Lab
    - Purify gRNA plasmid (mini-prep)
    - Transform CRISPRi system into MG1655
    - Send pgRNA_target plasmids to be sequenced

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:

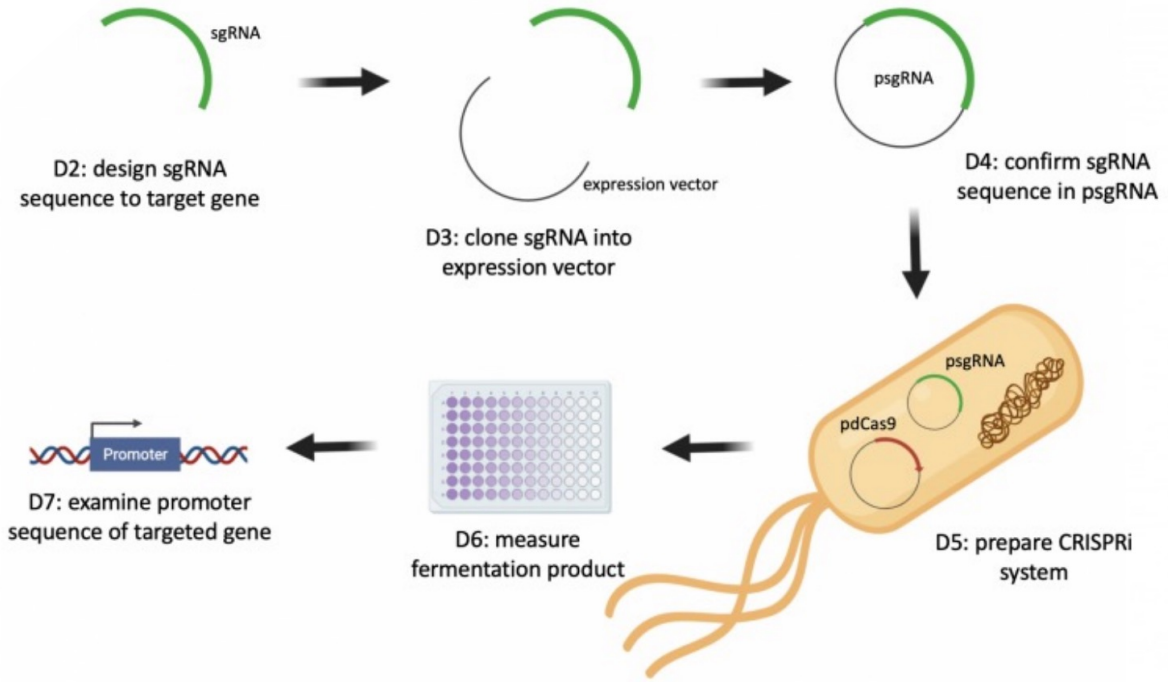
Cloned sgRNA into vector to create psgRNA plasmid

This Lab:

Confirm sgRNA sequence, Prepare CRISPRi system

Next Lab:

Measure fermentation product!



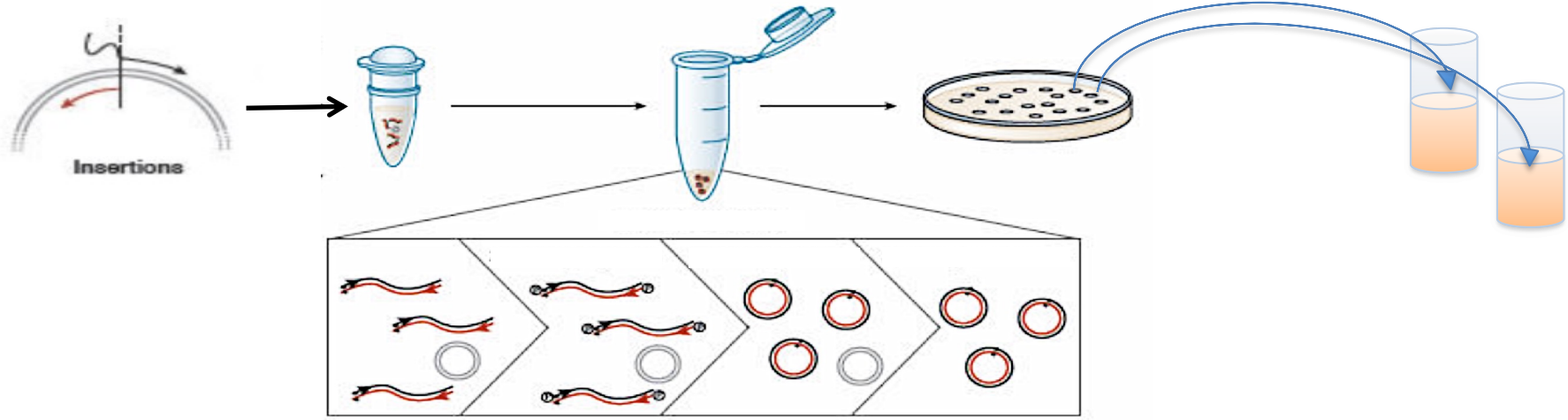
From Becky

Last time (& while you were away):

Insert gRNA sequence into vector

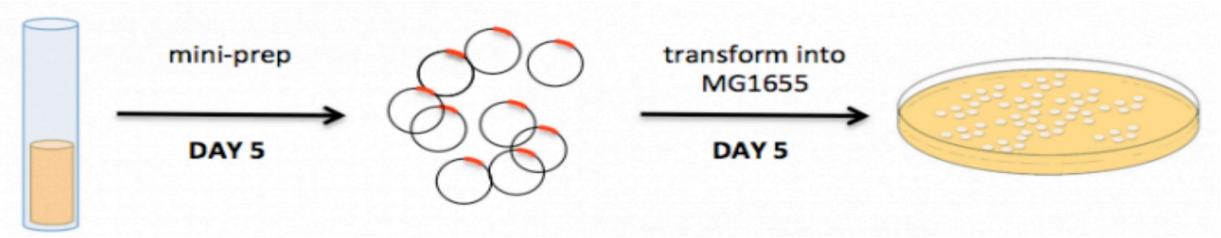
Transform into NEB5α *E. coli*

Inoculate liquid cultures



https://www.neb.com/products/e0554-q5-site-directed-mutagenesis-kit#Product%20Information

Today: confirm psgRNA_target

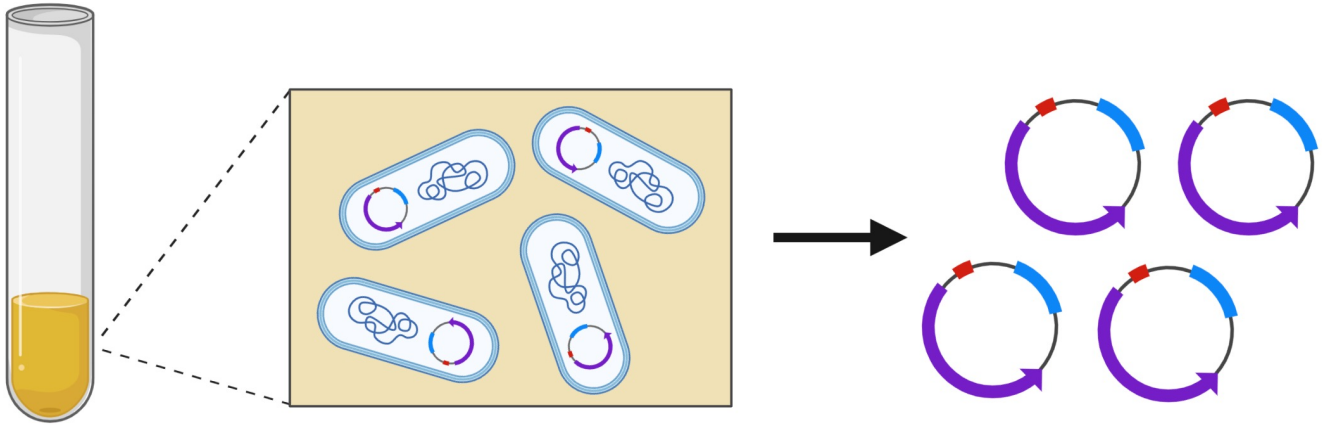


1.) “Miniprep”: Isolate pgRNA_target from *E. coli* cultures

2.) Co-transform _____ & _____ into MG1655 cells

3.) Submit pgRNA_target for sequencing to confirm product

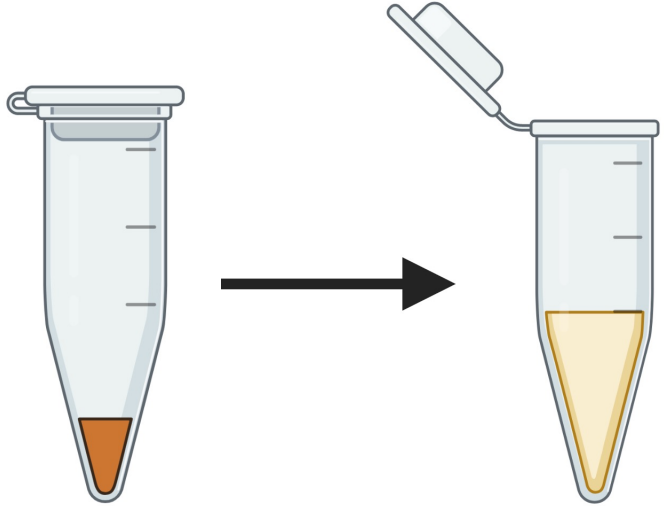
Mini-prep pgRNA_target clones



- Goal of miniprep: purify plasmid DNA from bacterial culture

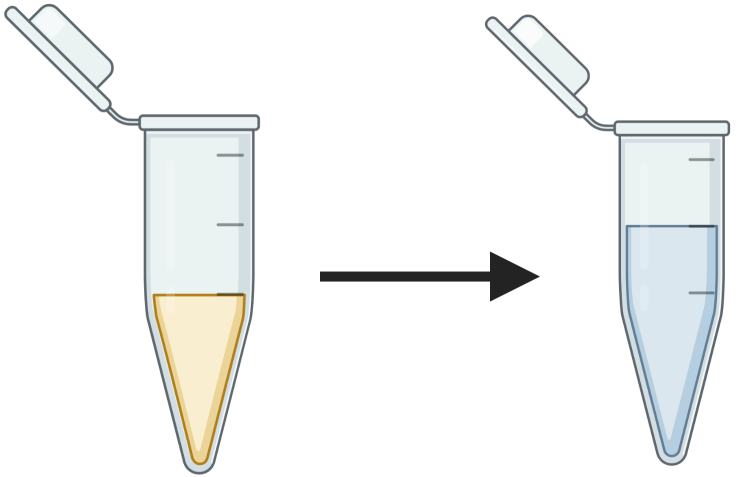
Step 1: Resuspend bacteria

- P1 Buffer
  - Tris/EDTA buffer
  - RNAse



Step 2: Lyse bacteria

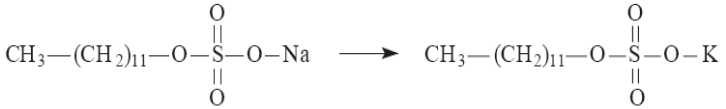
- P2 Buffer
  - Sodium dodecyl sulfate (SDS)
  - Sodium hydroxide (NaOH)



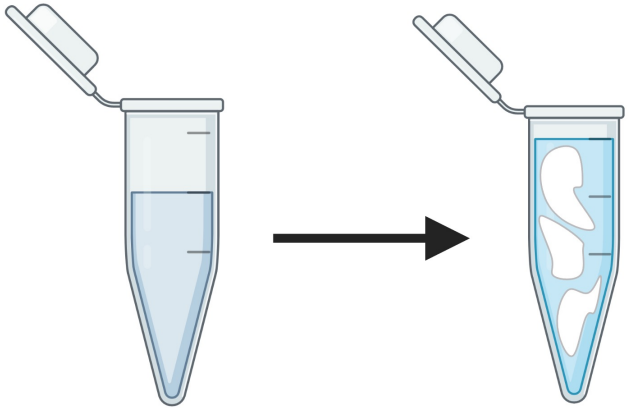
Lysate is clear(er)!

Step 3: Neutralization

- N3 Buffer
  - Acetic acid / potassium acetate solution



  - Guanidinium hydrochloride (GuHCl): a chaotropic salt



Cloudy white precipitate forms

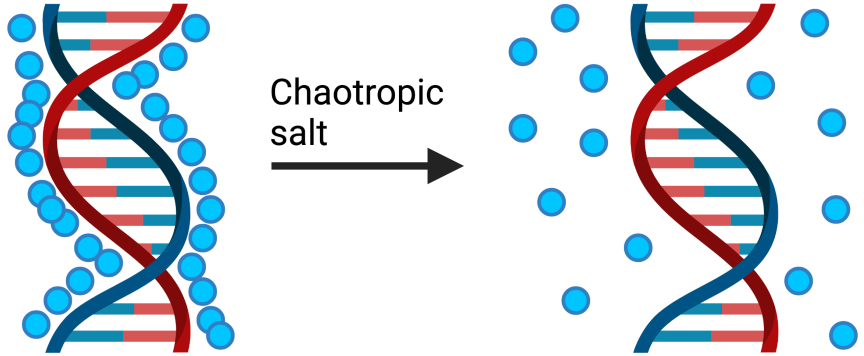
After centrifugation:

  - supernatant:
  - pellet:

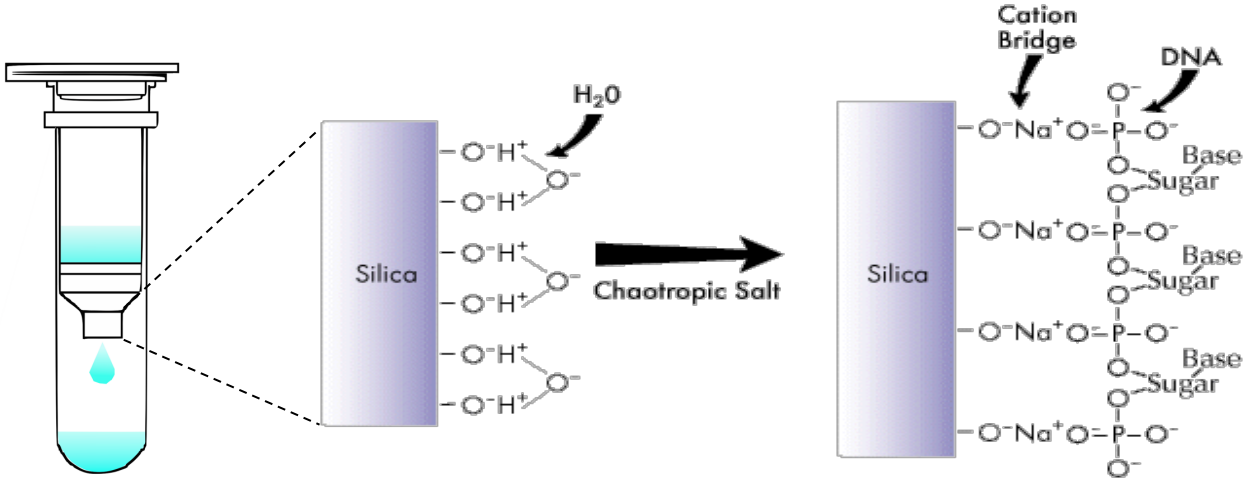
Step 4: Bind DNA to column

Chaotropic salts:

- disrupt hydrate shell surrounding DNA

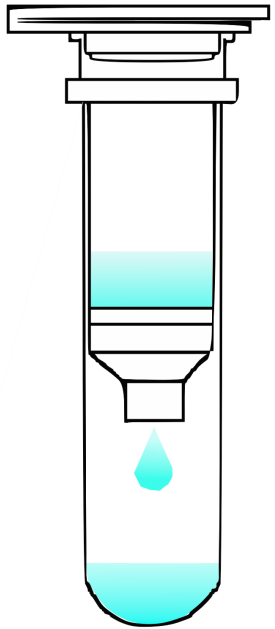


- facilitate DNA binding to silica column



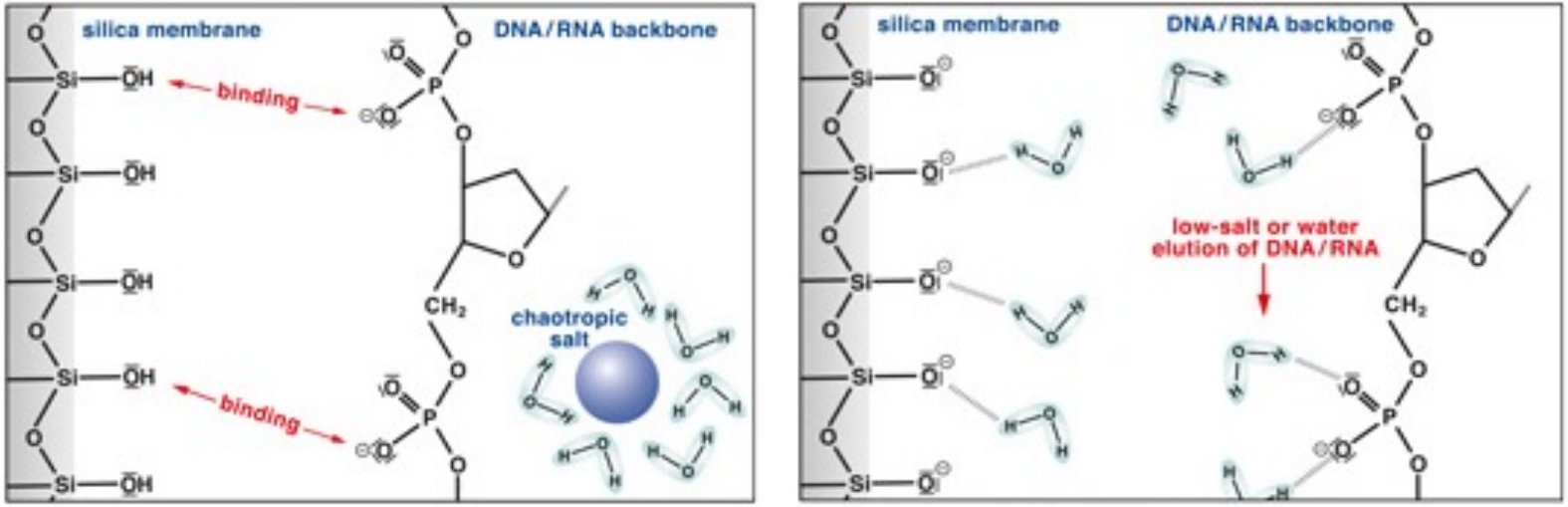
Step 5: Wash column

- PB Buffer
  - Isopropanol
  - Guanidinium hydrochloride
- PE Buffer
  - Ethanol
- Dry spin

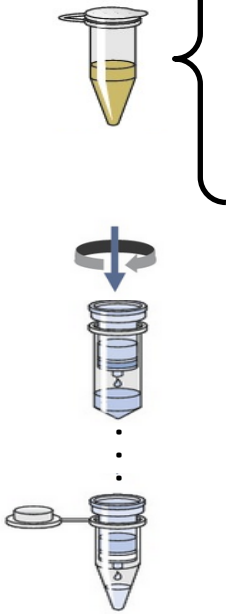


Elute DNA with water (pH 8)

- Water competes DNA off of silica column
- Collect DNA in a new tube



Summary of mini-prep to isolate DNA

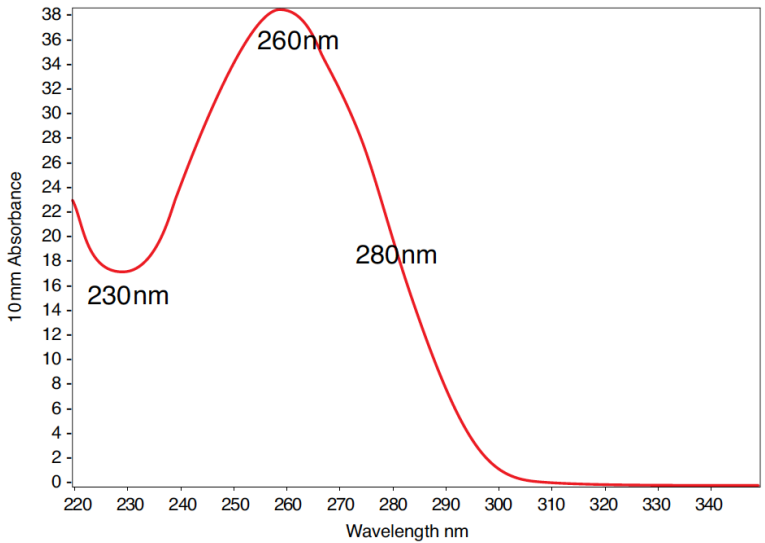


Steps	Contents	Purpose
Prepare (P1)	Tris/EDTA buffer RNase	Resuspend cells, weaken membrane, EDTA blocks DNase, RNase degrades RNA
Lyse (P2)	SDS (detergent) NaOH (alkaline lysis)	solubilize proteins, denature DNA
Neutralize (N3)	Acetic acid, chaotropic salt, potassium acetate	Renature short DNA, precipitate long DNA and protein
Spin		
Bind	Silica column	Concentrate and isolate DNA
Wash (PB, PE)	Isopropanol, ethanol	Remove contaminants
Elute	Water, pH 8.0	Elute all DNA off column

*Note: All liquid waste should be collected in conical tube, never aspirated

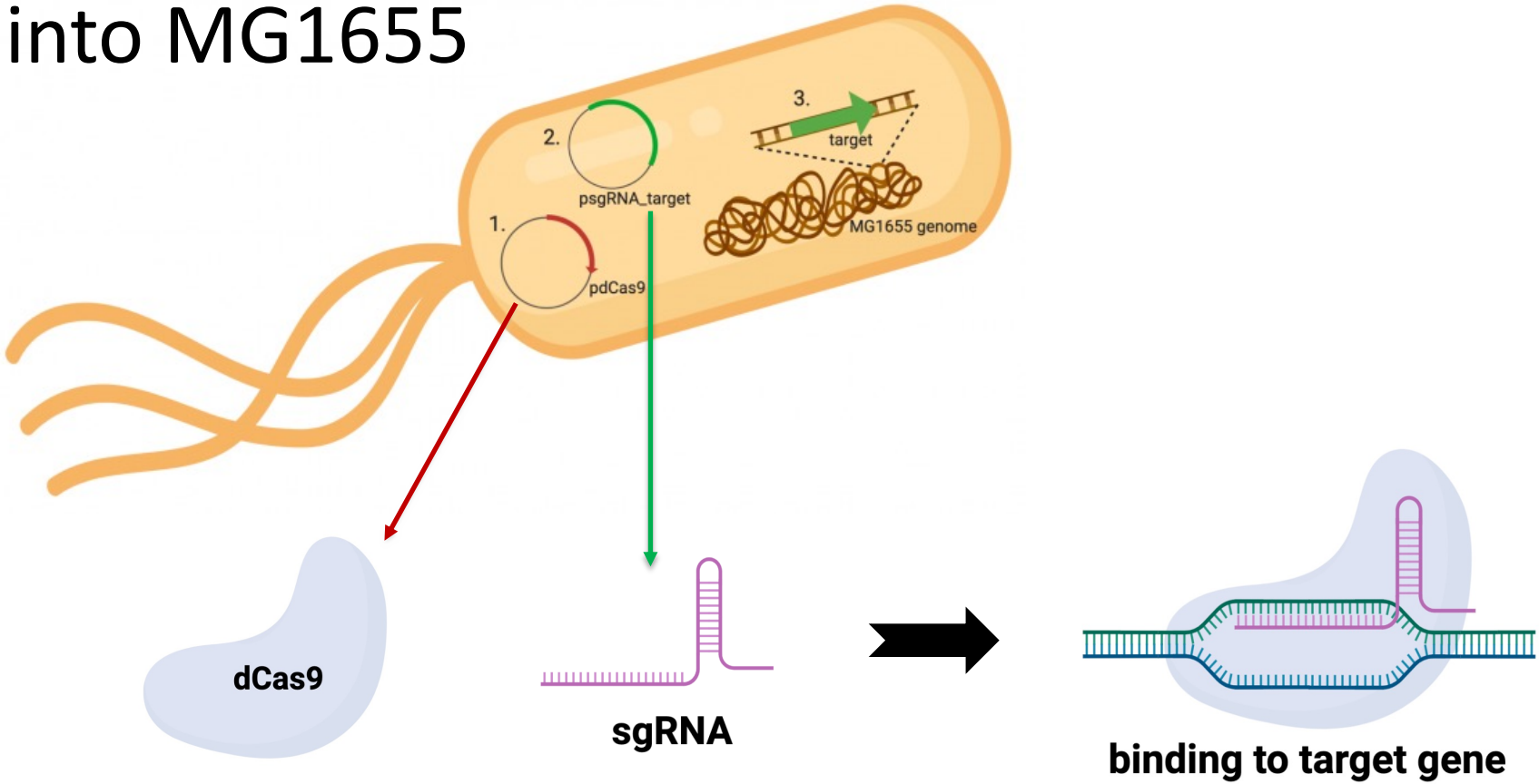
Measure DNA concentration with NanoDrop spectrophotometer

- DNA absorbs 260 nm light

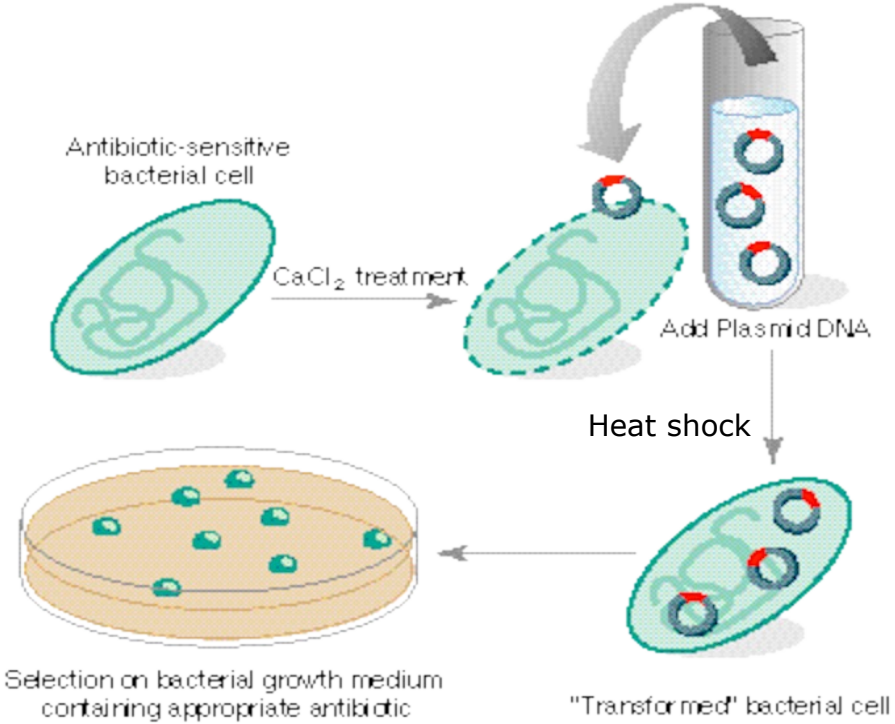


https://tools.thermofisher.com/content/sfs/brochures/Thermo-Scientific-NanoDrop-Products-Nucleic-Acid-Technical-Guide-EN.pdf

Transform CRISPRi system (two plasmids) into MG1655



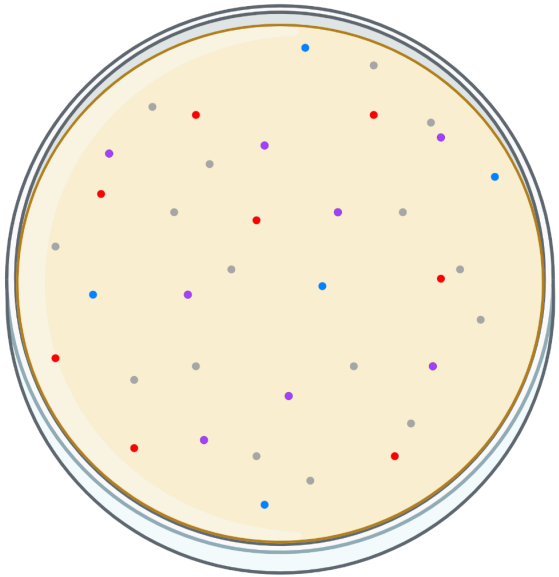
Heat shock competent cells for transformation



- MG1655 made chemically **competent** by $CaCl_2$
  - In exponential growth phase
    - OD_{600} = 0.4-0.8
  - Ca^{2+} ions attract lipopolysaccharide (LPS) cell membrane and _____
- Heat shock competent cells with plasmids
  - 42°C for 45 sec
  - Potentially alters membrane to allow plasmid entry

Select for MG1655 with both plasmids using antibiotics

Plate contains chloramphenicol and ampicillin

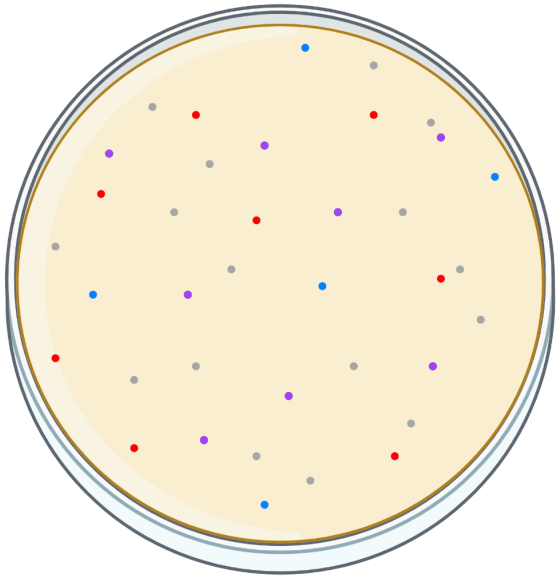


Overnight growth
37°C

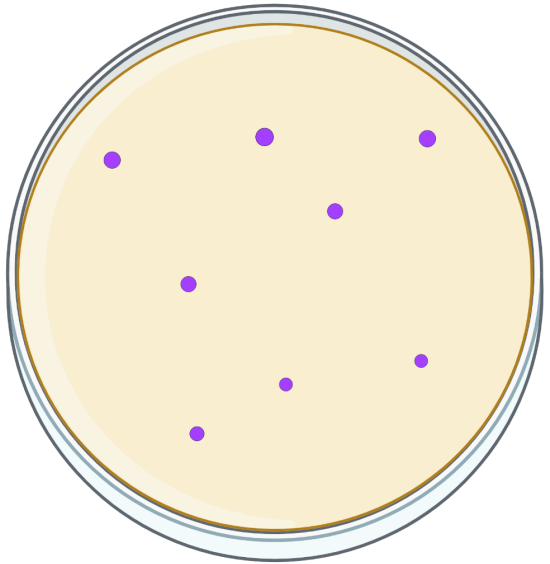
Gray bacteria did not receive plasmid
Red bacteria got pdCas9, CamR
Blue bacteria got psgRNA, AmpR
Purple bacteria got both

Select for MG1655 with both plasmids using antibiotics

Plate contains chloramphenicol and ampicillin



Overnight growth
37°C

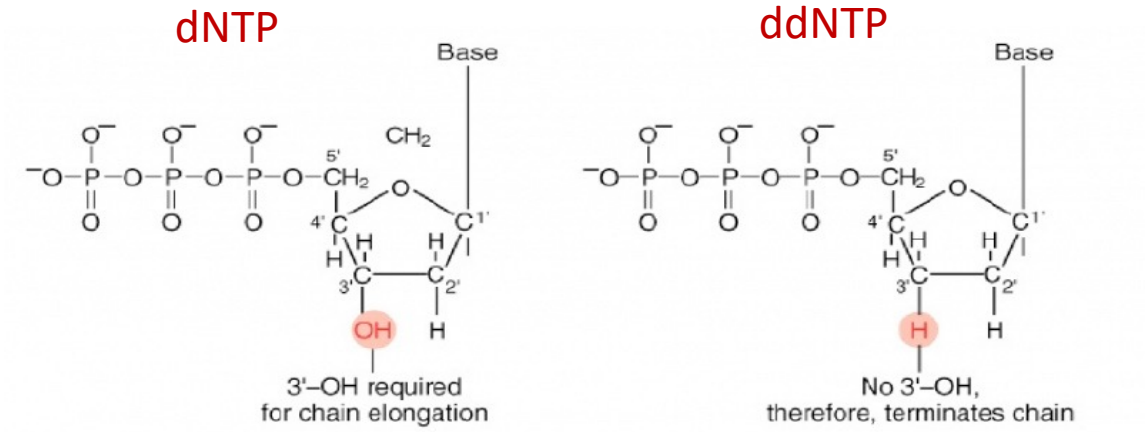


Gray bacteria did not receive plasmid
Red bacteria got pdCas9, CamR
Blue bacteria got psgRNA, AmpR
Purple bacteria got both

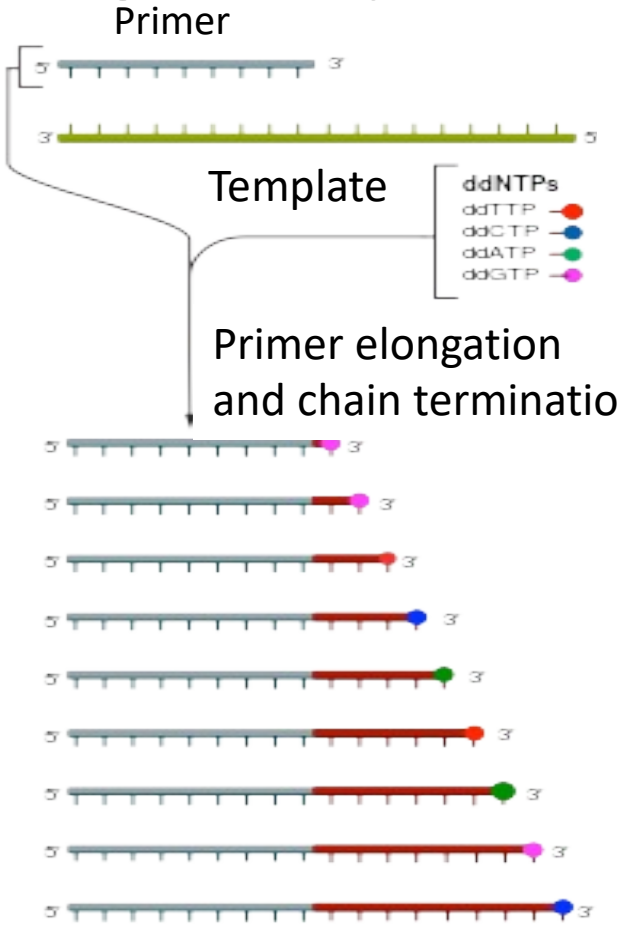
Only bacteria that received both plasmids grow and form colonies

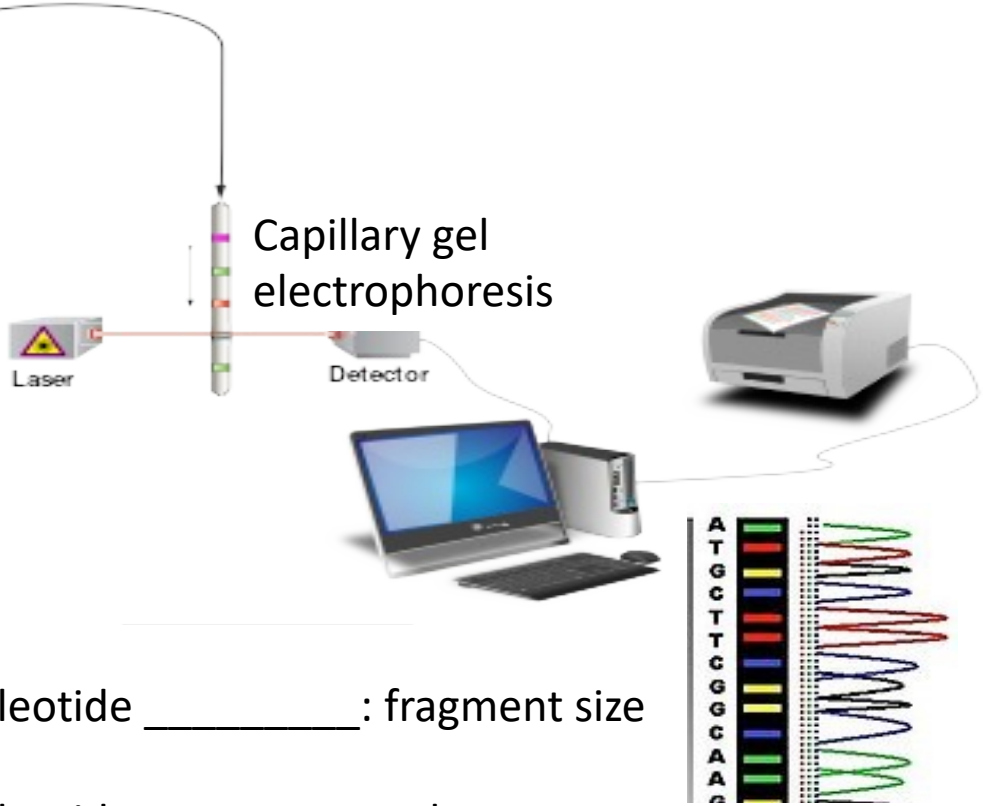
Use sequencing to determine if we have the intended pgRNA_target

- Previously: diagnostic digests to check size and banding pattern
- **Sanger Sequencing** to get exact plasmid sequence
  - fluorescent di-deoxynucleotides terminate elongation



Sanger sequencing by Genewiz





Nucleotide _____: fragment size

Nucleotide _____: color

Chromatogram

M2D5 HW: Creating a figure and text

- Create a figure of the pdCas9 confirmation digest data

- Write associated results and discussion **paragraphs**
  - **Explain ALL results (i.e. all the lanes on the gel)**
  - **Research article separates results and discussion**
  - Results= What did you see? Discussion= What does it mean?

- Review guidelines on the wiki Homework tab and descriptions on the Research Article tab

M2D5 HW: Introduction outline for the Research Article (Noreen's slide from Mod 1)

Include topic sentences for each paragraph in your introduction!

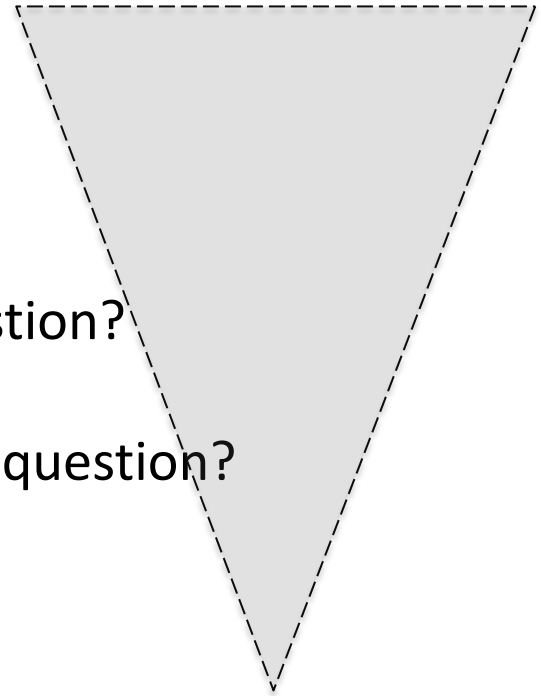
1st paragraph: what is the big picture / problem?

2nd paragraph: what is currently known?

3rd (or 4th) paragraph: what is your research question?

4th (or 3rd) paragraph: how will you address your question?

5th paragraph: here we show…



In lab today…

1. Start your miniprep from liquid culture at front bench
2. Transform miniprep DNA and pdCas9 into MG1655
3. Prepare sequencing reactions for submission and create new sequence file in benchling for your pgRNA_target plasmid