M2D5: Confirm gRNA sequence

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

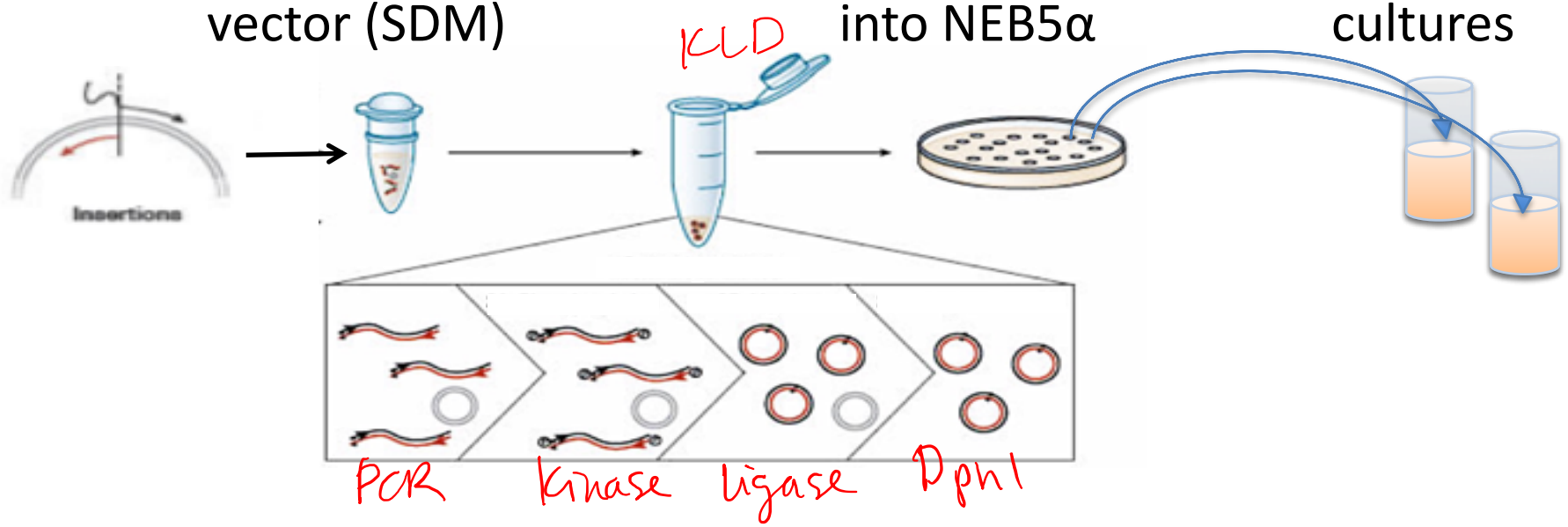
Date	Upcoming Event
Wed (10/31)	Journal Club 2
Friday (11/2)	Comm Lab Workshop
Friday (11/2)	Blogpost due
Mon (11/12)	M2 Research Article Due

Last time (& while you were away):

Insert gRNA sequence into vector (SDM)

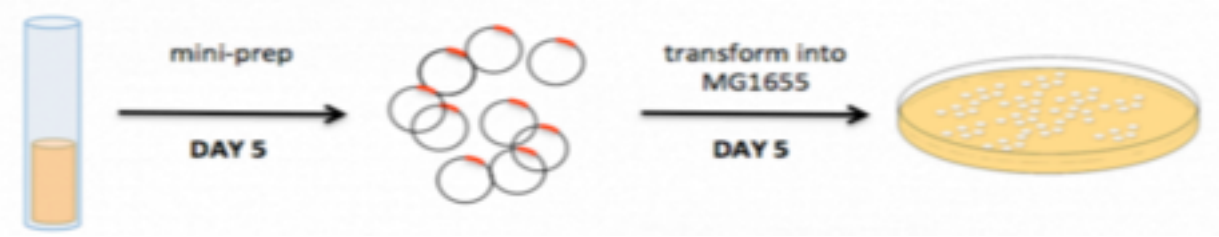
Transform into NEB5α

Inoculate liquid cultures



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

Today: confirm psgRNA_target



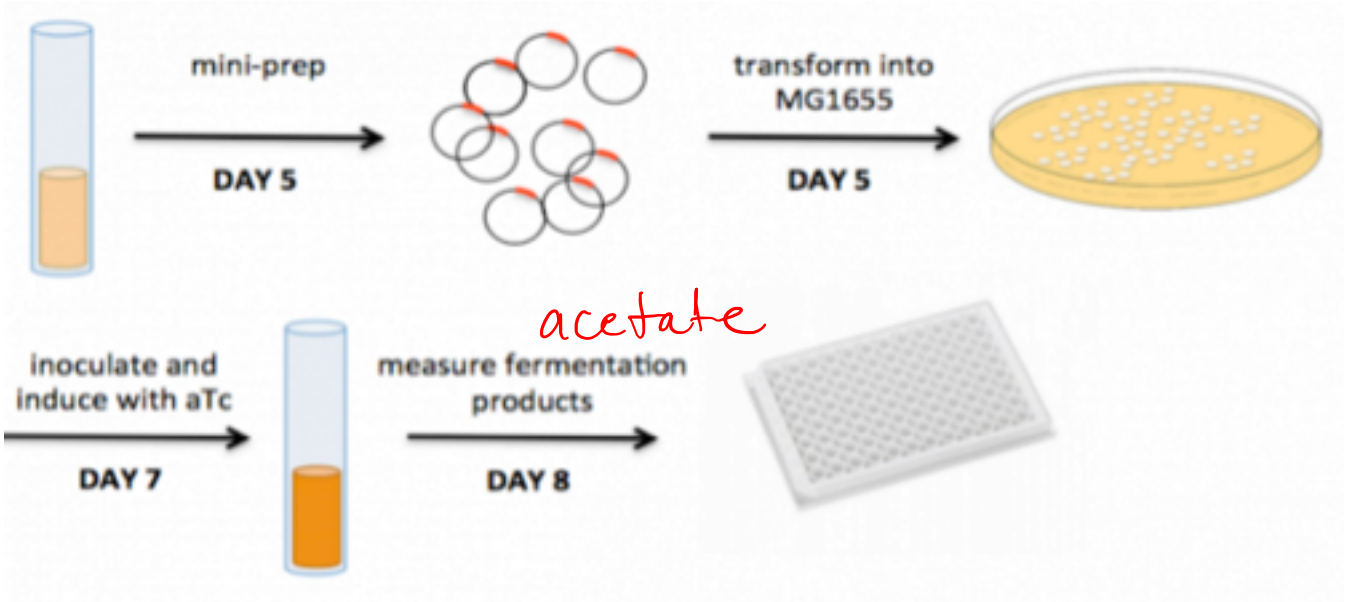
1.) Isolate pgRNA_target from *E. coli* cultures (mini-prep)

2.) Co-transform pCas9 & pgRNA_adhE into MG1655 cells

3.) Submit pgRNA_target for sequencing to confirm product

gRNA = sgRNA

Today: confirm psgRNA_target



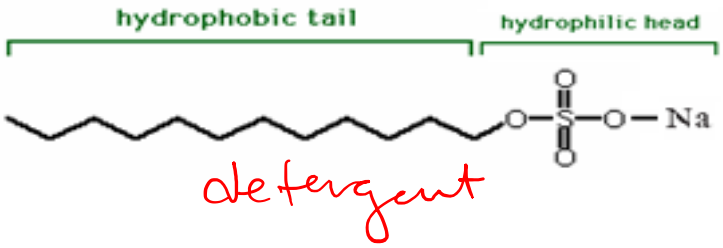
Mini-prep pgRNA_target clones

- Goal of mini-prep: purify plasmid
- Strategy:
  - lyse cells to extract DNA
  - Separate DNA from proteins + lipids
  - Separate plasmid DNA from genomic or chromosmal DNA
  - Purify and collect plasmid from other soluble factors

http://parts.igem.org/Help:3A_Assembly_Kit/Miniprepd

Prepare and lyse cells

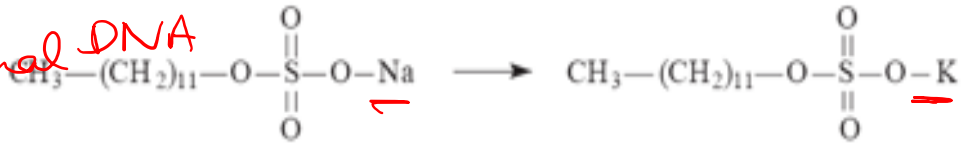
- Prepare cells (P1)
  - Tris/EDTA buffer weaken outer membrane + block DNase
  - Rnase degrades RNA
- Lyse cells (P2)
  - Sodium dodecyl sulfate (SDS)
    - dissolve lipid membrane
    - denature protein
  - Sodium hydroxide (NaOH)
    - alkaline lysis
    - denatures DNA



Neutralization (N3)

- Acetic acid / potassium acetate solution
  - Neutralizes pH
  - Converts soluble SDS into insoluble PDS (white fluff)

precipitates w/ proteins, lipids, chromosomal DNA

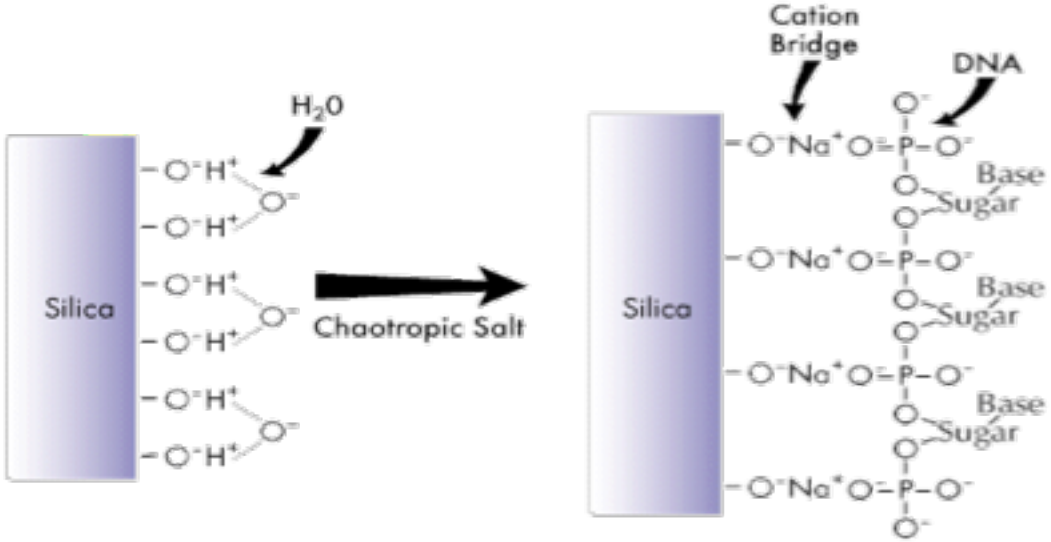


- Chaotropic salt
  - facilitates DNA binding to silica

disrupting hydrogen bonding ($DNA + H_2O$)

➢ After centrifugation
  - supernatant: plasmid (and soluble cellular constituents)
  - pellet: PDS, lipids, proteins, chromosomal DNA

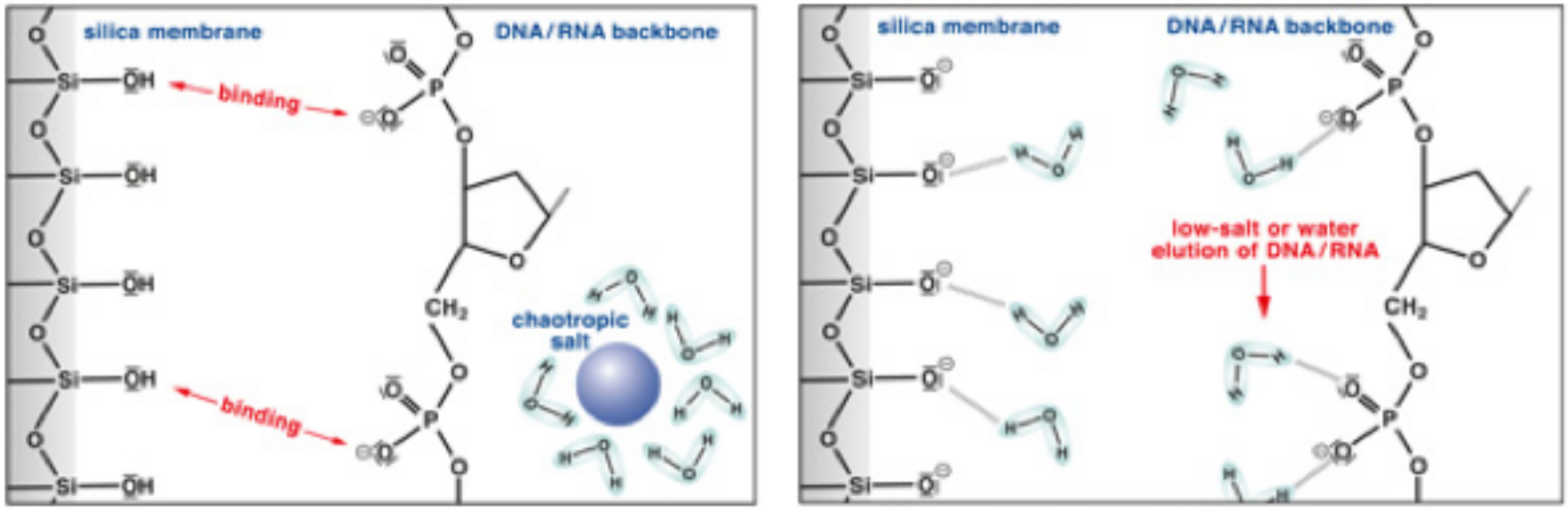
Bind DNA to column



- Washes with PB (isopropanol) and PE (ethanol)
  - remove residual contaminants
  - maintain DNA onto column
- Spin off all ethanol before eluting DNA

Elute DNA with water (pH 8)

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



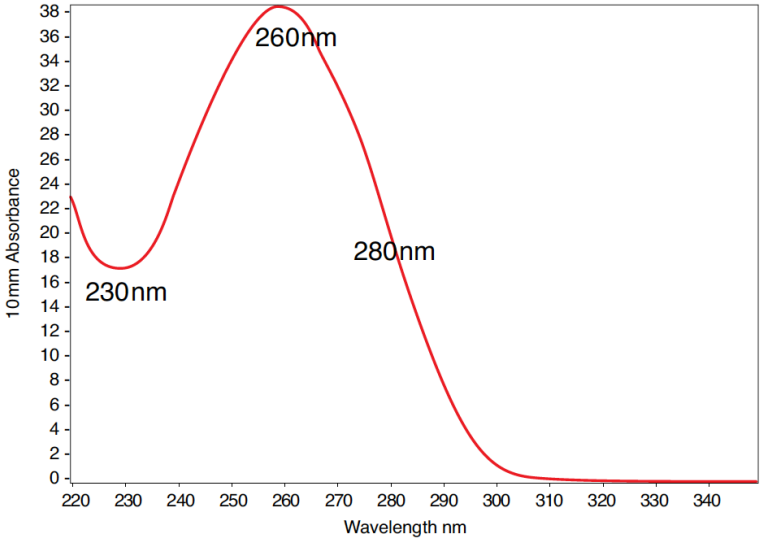
Summary of mini-prep to isolate DNA

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

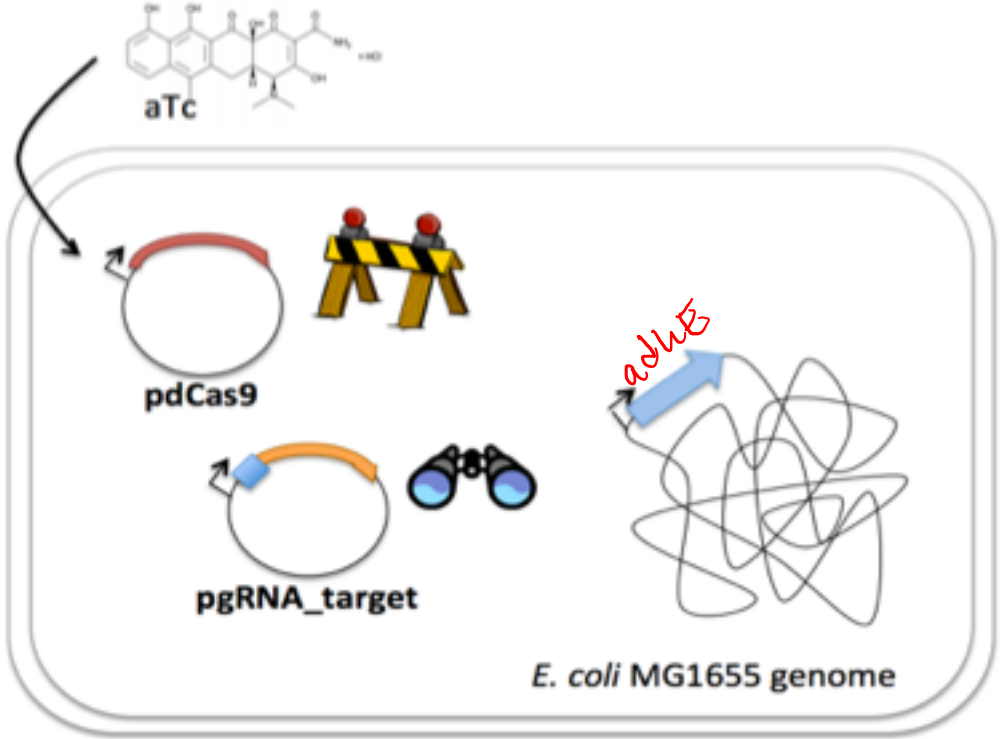
Measure DNA concentration with NanoDrop spectrophotometer

- Nucleic acids absorb 260 nm light



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

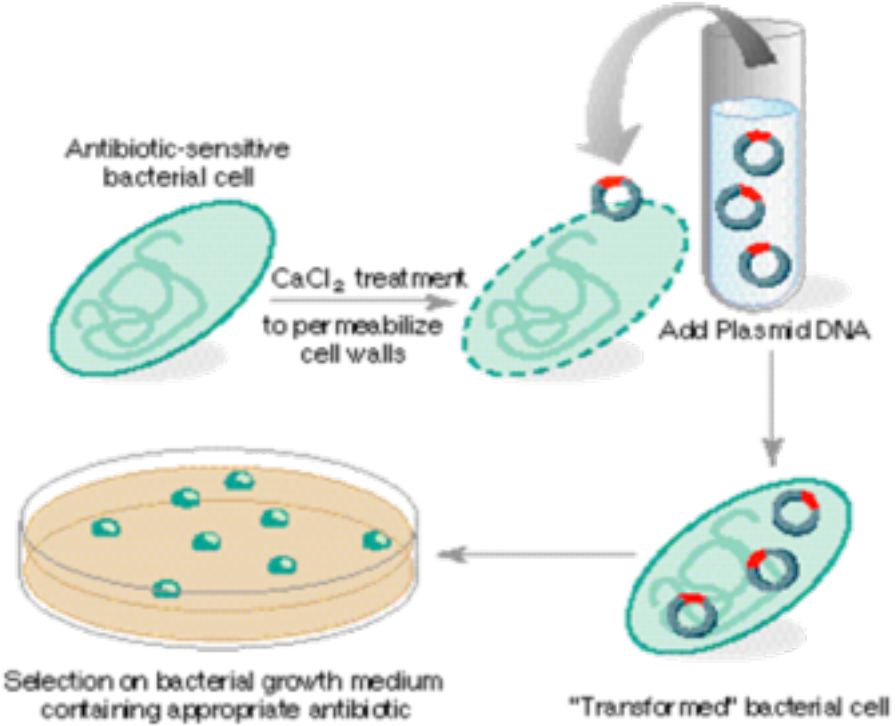
Transform CRISPRi system (two plasmids) into MG1655



- Target gene
- pgRNA_target
- pdCas9

Dr. Lyell's M2 lecture slides

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 both DNA backbone and lipopolysaccharides
  - Handle very gently, or will lyse
    - on ice all the time, and with chilled solutions, not vortexed
- **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

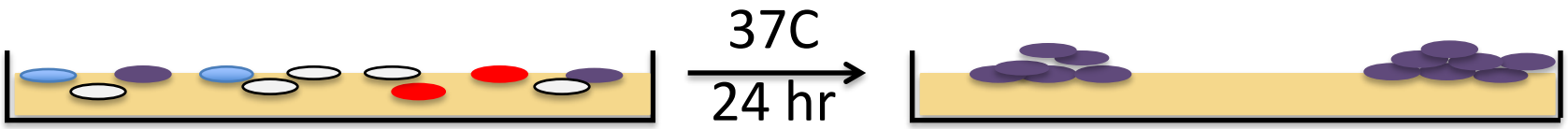


Plate transformation mix on double antibiotic plates

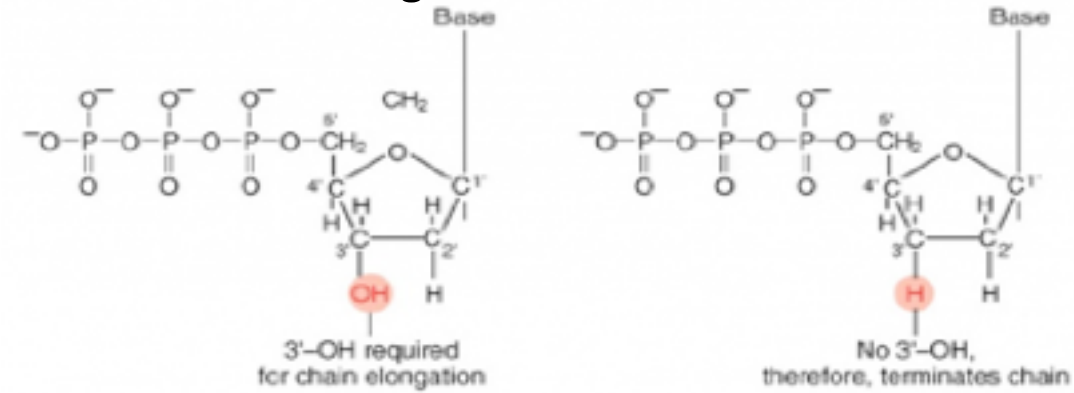
Selecting for transformants that received both plasmids

pdCas9 confers resistance to: chloramphenicol (cam)

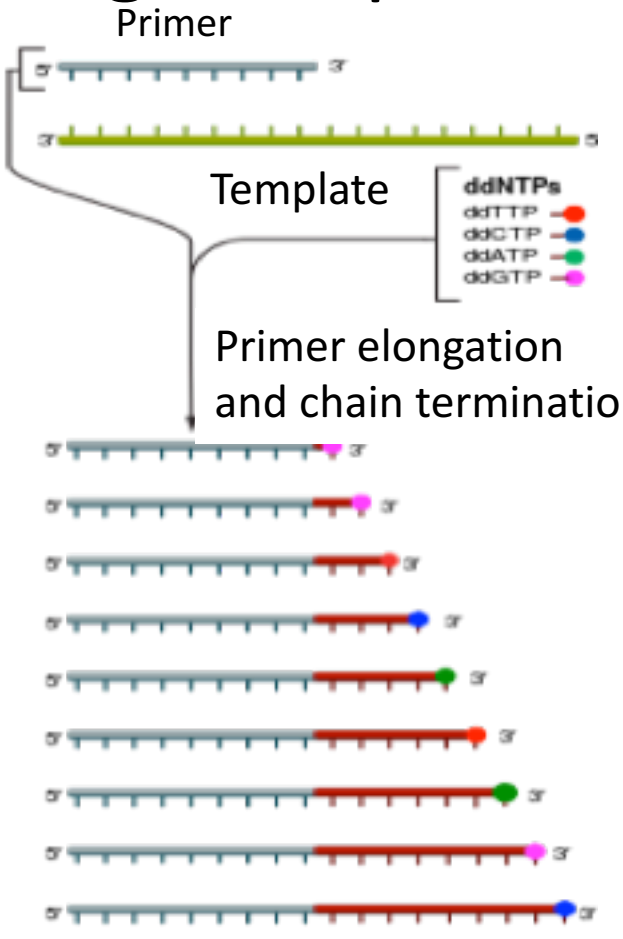
pgRNA confers resistance to: ampicillin (amp)

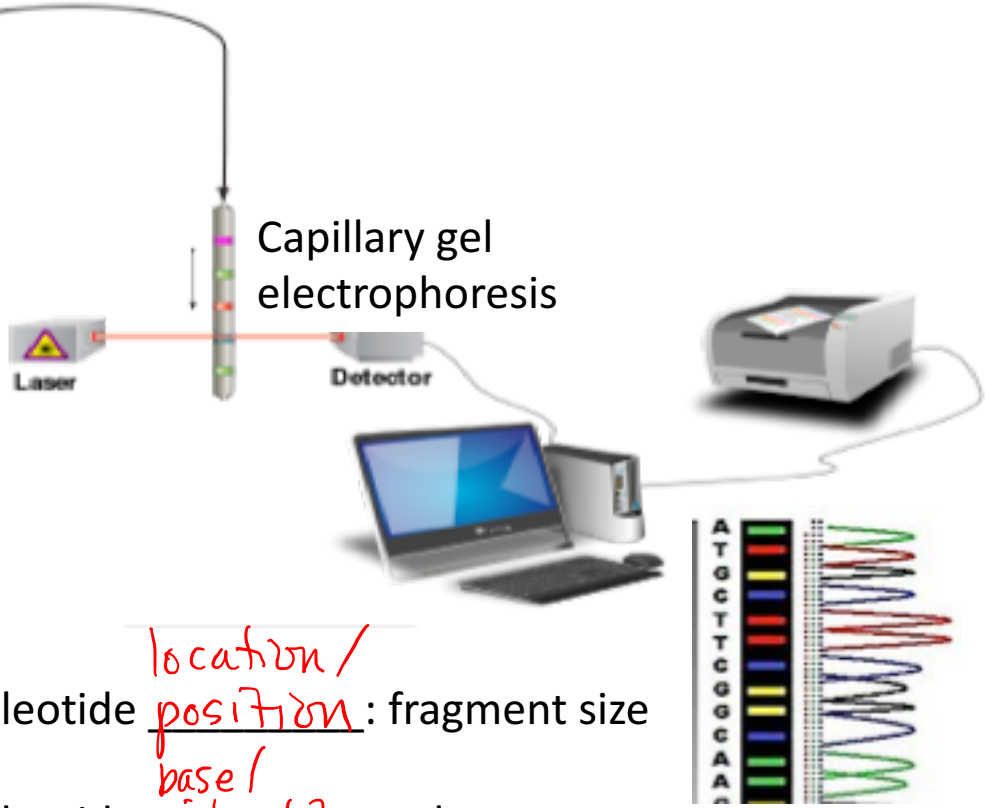
Use sequencing to determine if we have the intended pgRNA_target

- Diagnostic digests check size
- Sequencing
  - good to have both forward and reverse primers
    - More coverage (~1000 bases)
    - good coverage, double check
  - di-deoxynucleotides terminate elongation



Sanger sequencing by Genewiz





Nucleotide location/position: fragment size

Nucleotide base/identity: color

Chromatogram

Tips to write Methods (due M2D7)

- **Methods completed individually and included in your Mod2 Research article**
  - M2D2 through M2D5 (leave out M2D1)
  - Using the phase “per manufacturers protocol” allowed for Qiagen miniprep kit only (not allowed for other protocols)
  - full primer sequences are reported in methods sections
- Include enough information to replicate the experiment
  - list manufacturers name and location (City, ST)
  - Be **concise and clear** in your description
- Use subsections with descriptive titles
  - Put in logical order
  - Begin with topic sentence to introduce purpose
- Use clear and concise full sentences
  - NO tables and lists
  - Passive voice expected
- Use the most flexible units
  - Write concentrations (when known) rather than volumes
- Eliminate 20.109 specific details
  - Example “green team gRNA…”
  - Do not include details about tubes and water!
  - Assume reader has some biology experience

Improving your Methods [1]

pgRNA_template final 1ng/µl AdheF (5'GCTA...) and AdheR(...)

~~Template DNA (5 µL) and primers were mixed with 20 µL of~~

1X Master Mix

~~2.5X Master Mix in a PCR tube. Water was added to 50 µL~~

cycle [98°C for 10sec, 72°C for 30sec ...]

~~and samples put on~~ PCR machine.

Improving your Methods [2]

genotype of bacteria
growth phase

A liquid bacteria culture was pelleted and the DNA was purified using a Qiagen kit.

manufacturers info

* clearly state when step different, elution in H_2O pH8

Improved Methods

[1] ***PCR amplification of inverse pericam (IPC)***

Inverse pericam (IPC) was amplified from a pcDNA3-IPC template (5 ng/uL) with 2 pmol/uL IPC-forward (5’ NNN 3’) and IPC-reverse (5’ NNN 3’) primers, using 1X MasterMix (company, city, state/country) and the following thermocycler conditions: initial denaturation at 98°C for 30 s, 25 cycles of amplification (melt at 98°C for 10 s, anneal at 55°C for 30 s, extend at 72°C for 2 min), final extension at 72°C for 2min.

[2] ***Amplification of the pRSET-IPC plasmid***

The DNA of a 1.5 mL of NEB 5alpha (genotype: *fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*) overnight** culture was collected using a QIAquick mini-prep kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol with a final elution in 30 µL of distilled water pH 8.0.

**grown to saturation (as opposed to exponential growth phase for transformation or induction of expression)

Today in lab…

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_adhE plasmid