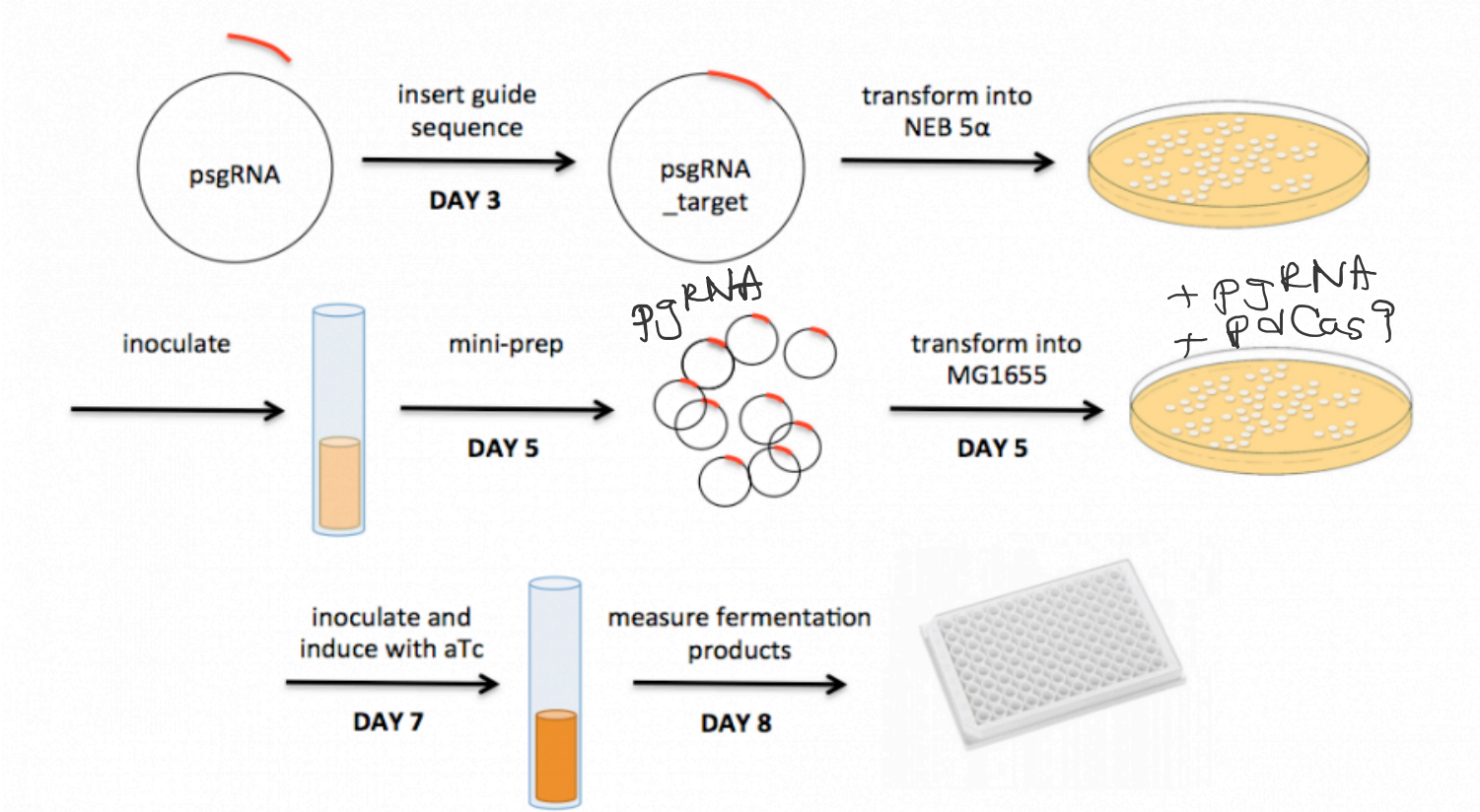
M2D5: Confirm gRNA sequence

10/24/19

1. Quiz
2. Pre-lab discussion
3. Purify gRNA plasmid (mini-prep)
4. Transform CRISPRi system into MG1655
5. Send pgRNA_target plasmids for sequencing

Mod 2 experimental overview



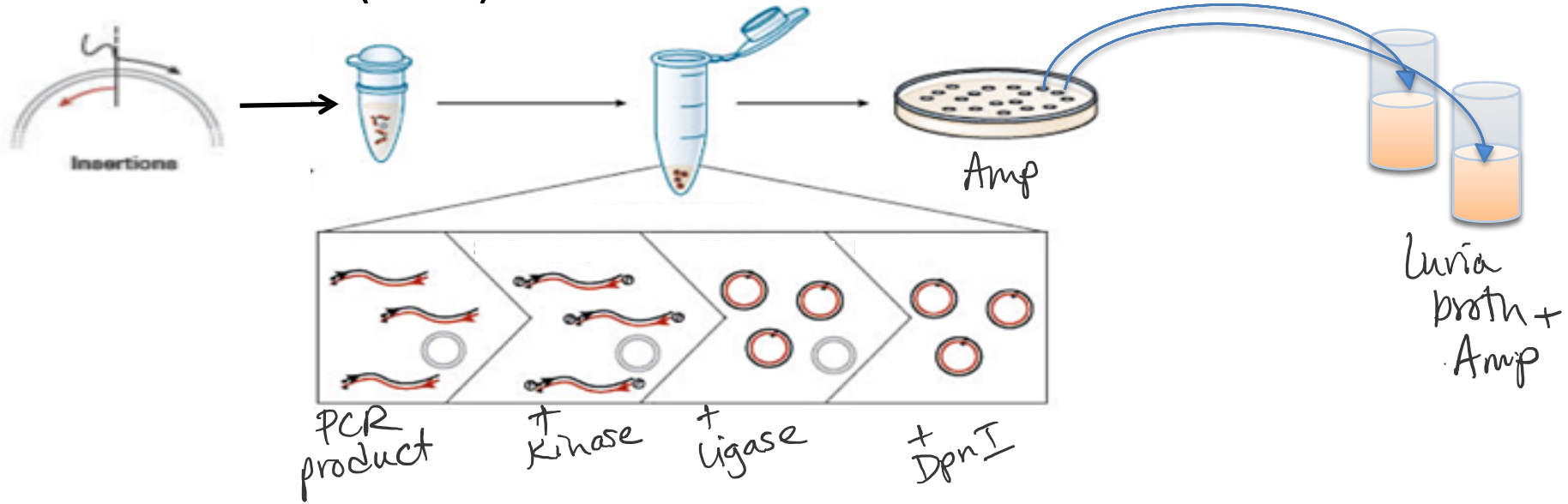
http://engineerbiology.org/wiki/20.109(F18):Module_2

Last time (& while you were away):

Insert gRNA sequence into vector (SDM)

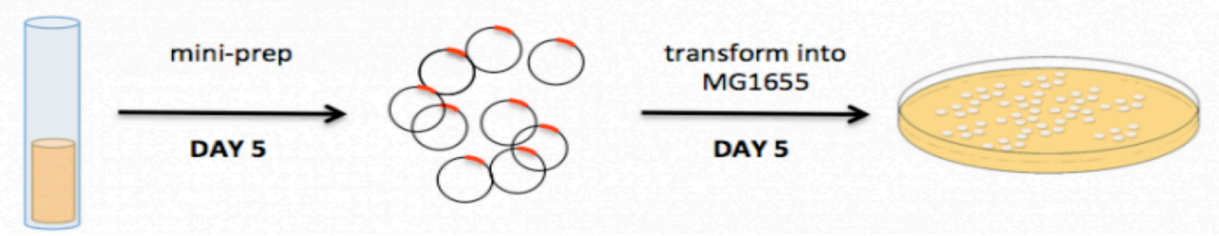
Transform into NEB5α

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 pdCas9 & pgRNA-adhE into MG1655 cells

3.) Submit pgRNA_target for sequencing to confirm product

Mini-prep pgRNA_target clones

- **Goal of mini-prep: purify plasmid from small volume culture**
- Strategy:
  - _lyse_ cells to extract plasmid DNA
  - Separate DNA from _proteins + lipids_
  - Separate plasmid DNA from _genomic / chromosomal_ DNA
  - Purify and collect plasmid from other soluble factors

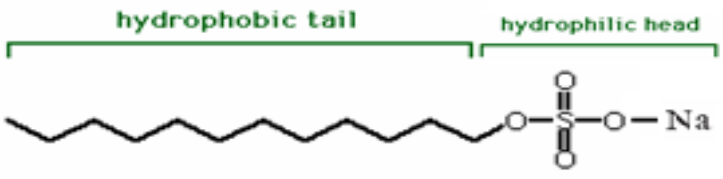
Prepare and lyse cells

- Prepare cells (P1)
  - Tris/EDTA buffer
  - Rnase
- Lyse cells (P2)
  - Sodium dodecyl sulfate (SDS)
  - Sodium hydroxide (NaOH)

weakens outer membrane, block DNase

degrades RNA

detergent



disrupt lipid membrane
denatures proteins

lysis via alkaline conditions
denature all DNA

Neutralization (N3)

- Acetic acid / potassium acetate solution
  - Neutralizes pH ⇒ small plasmid (circular) DNA renatures
  - Converts soluble SDS into insoluble PDS (Potassium dodecyl sulfate, white fluff)

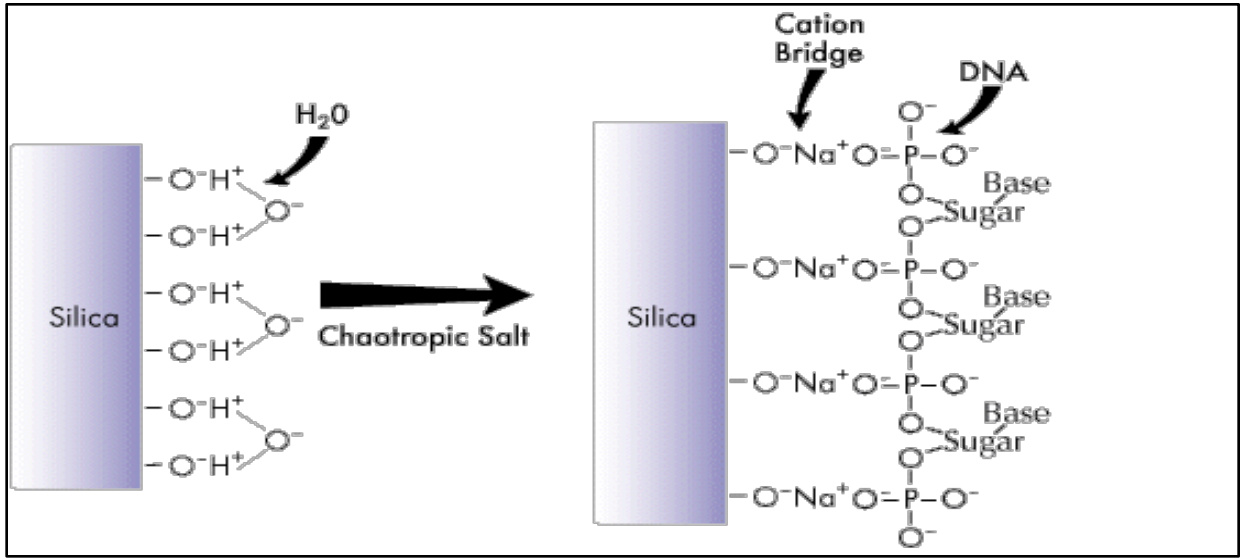
precipitate protein lipid, genomic DNA

- Chaotropic salt disrupt bonds between DNA + H_2O
  - Will help facilitates DNA binding to silica column in next step

➢ After 10min centrifugation
  - supernatant: plasmid (and soluble cellular constituents)
  - pellet: PDS, lipids, proteins, chromosomal DNA

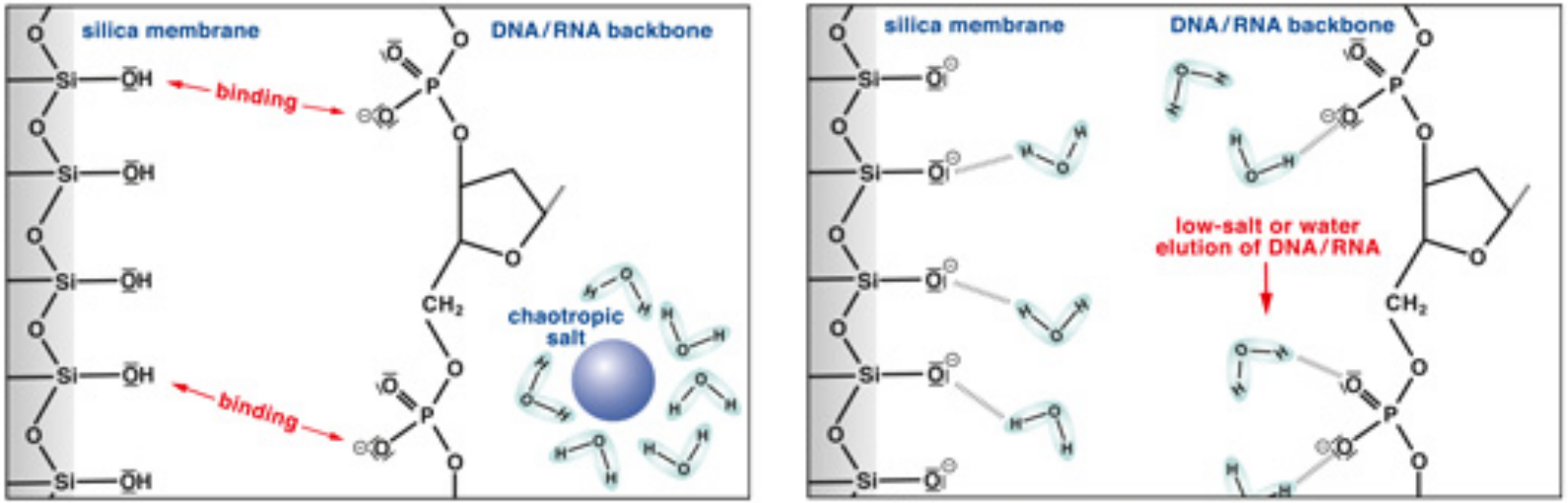
Bind DNA to column

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

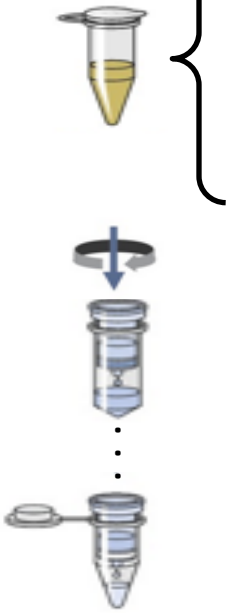


Elute DNA with water (pH 8)

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



Summary of mini-prep to isolate DNA

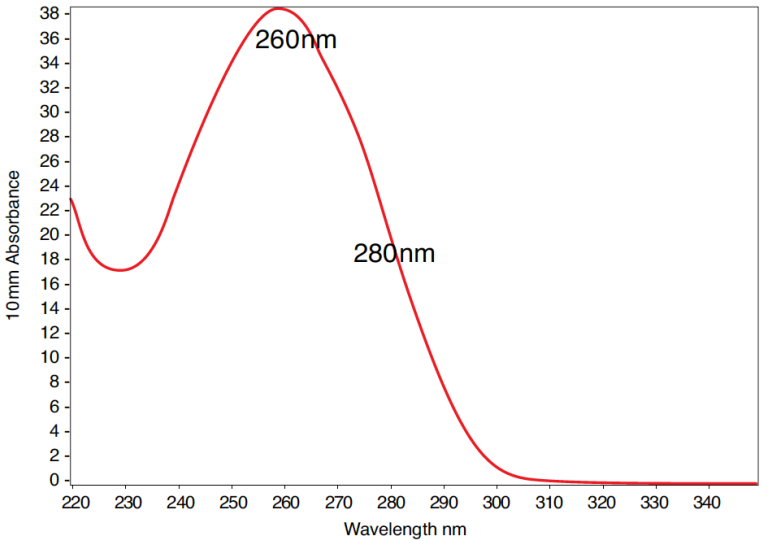


Steps	Contents	Purpose
Prepare (P1)	Tris/EDTA buffer RNase	Resuspend cells, weaken membrane, EDTA blocks DNase, RNase degrades RNA
Lyse (P2)	SDS (detergent) NaOH (alkaline lysis)	solubilize proteins, denature DNA
Neutralize (N3)	Acetic acid, chaotropic salt, potassium acetate	Renature short DNA (plasmid),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

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

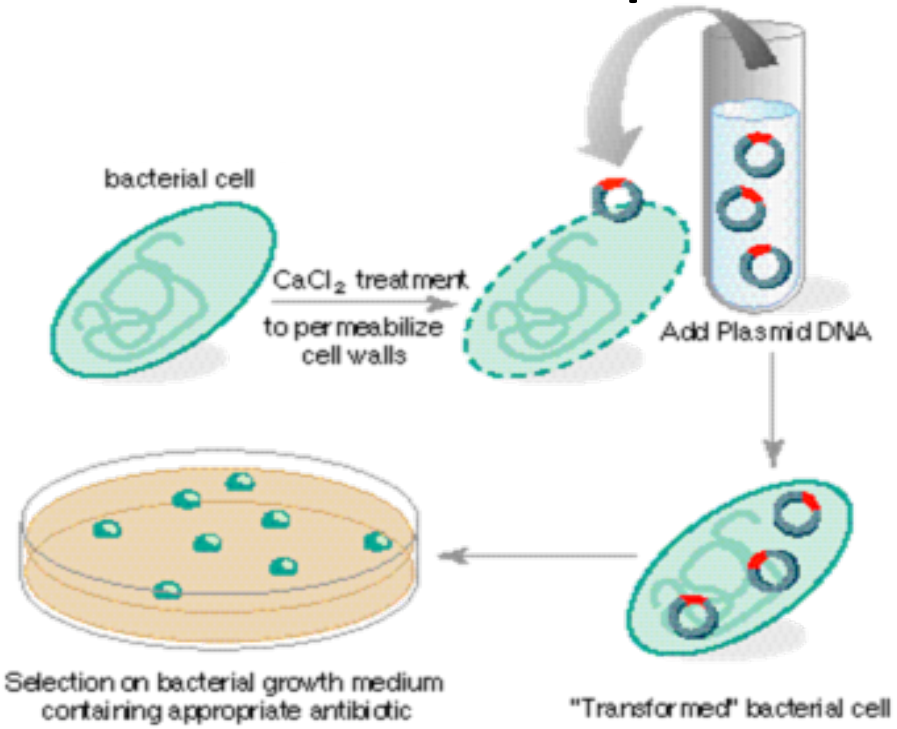
Measure DNA concentration with NanoDrop spectrophotometer

- Nucleic acids absorb 260 nm light



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

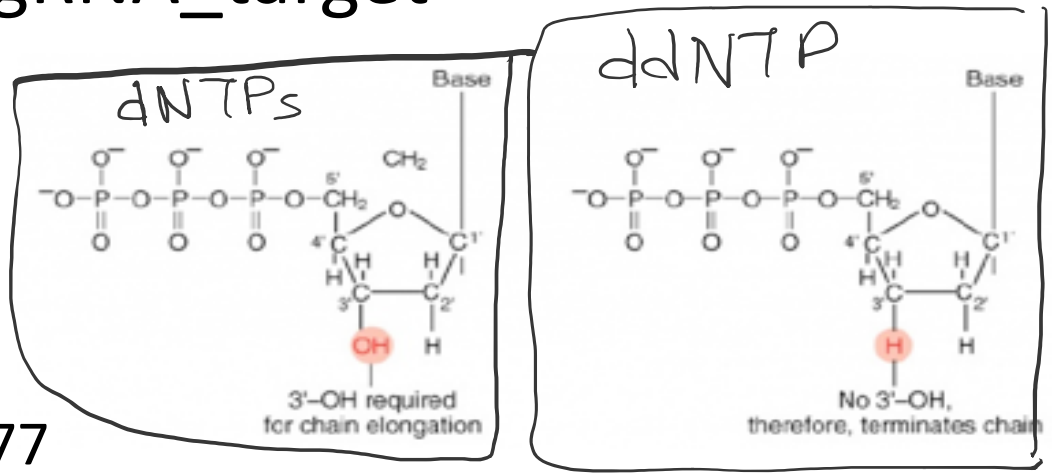
Heat shock competent cells for transformation



- MG1655 made chemically **competent** by $CaCl_2$
  - Bacteria in exponential growth phase
  - Ca^{2+} ions attract both Lipopolysaccharide membrane and DNA
- Heat shock competent cells with plasmids
  - Potentially alters membrane to allow plasmid entry
- Double transformed MG1655 should be resistant to
  - Chloramphenicol via pdCas9
  - Ampicillin via pgRNA-gene

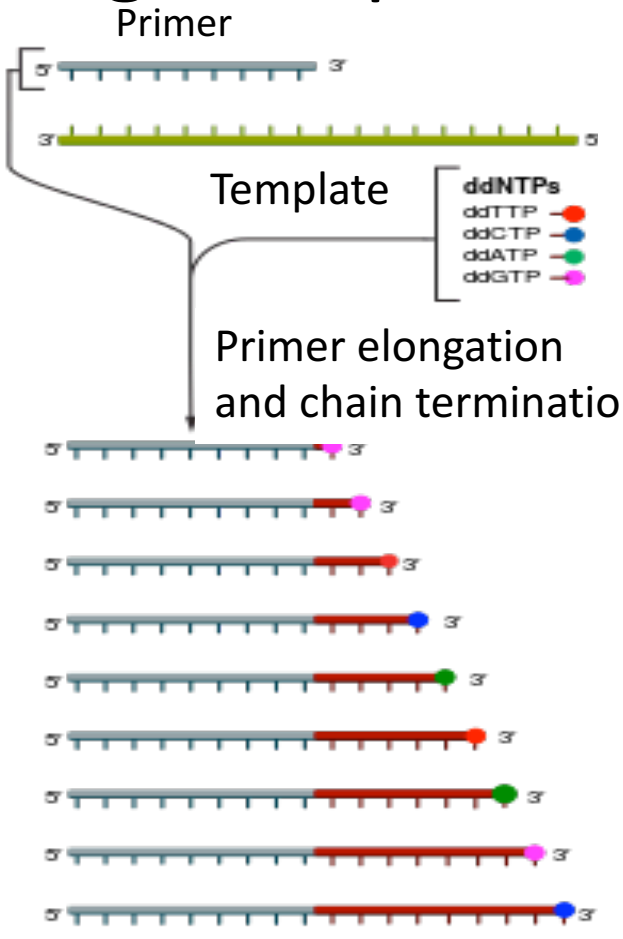
Use sequencing to determine if we have the intended plasmid with gRNA_target

- Diagnostic digests check size



- Sanger DNA Sequencing, 1977
  - di-deoxynucleotides terminate elongation
  - good to have both forward and reverse coverage
    - 2X coverage of ligation sites
    - ~1000 bases "good" sequence

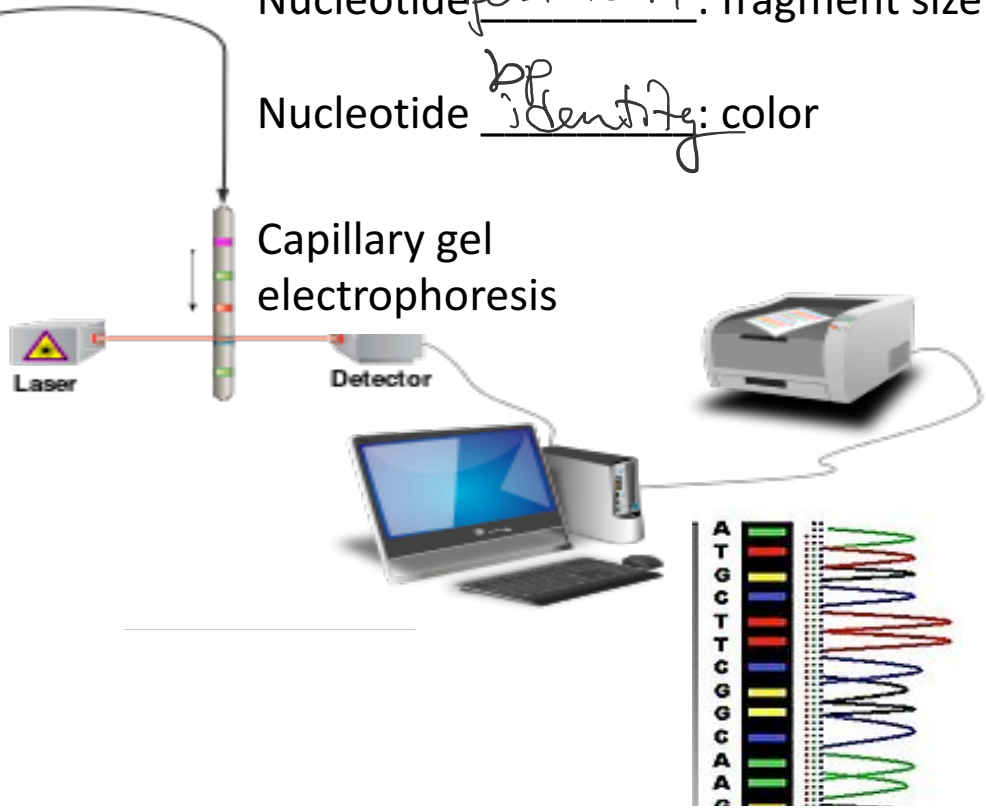
Sanger sequencing by Genewiz



Nucleotide position: fragment size

Nucleotide bp identity: color

Capillary gel electrophoresis



Chromatogram

Tips to write Methods (due M2D7)

- **Methods completed individually and included in your Mod2 Research article**
  - M2D1 through M2D5 (leave out M2D1) in silico cloning
  - 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
  - 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]

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

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

and samples put on PCR machine.

Improving your Methods [2]

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

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

- In downtime:
  - Incorporate Mod1 edits
  - Ask questions about journal club paper
  - Work on M2D7 homework