

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
Tues (10/30)	Journal Club 2
Thurs (11/1)	Comm Lab Workshop
Friday (11/2)	Blogpost due
Mon (11/12)	M2 Research Article Due

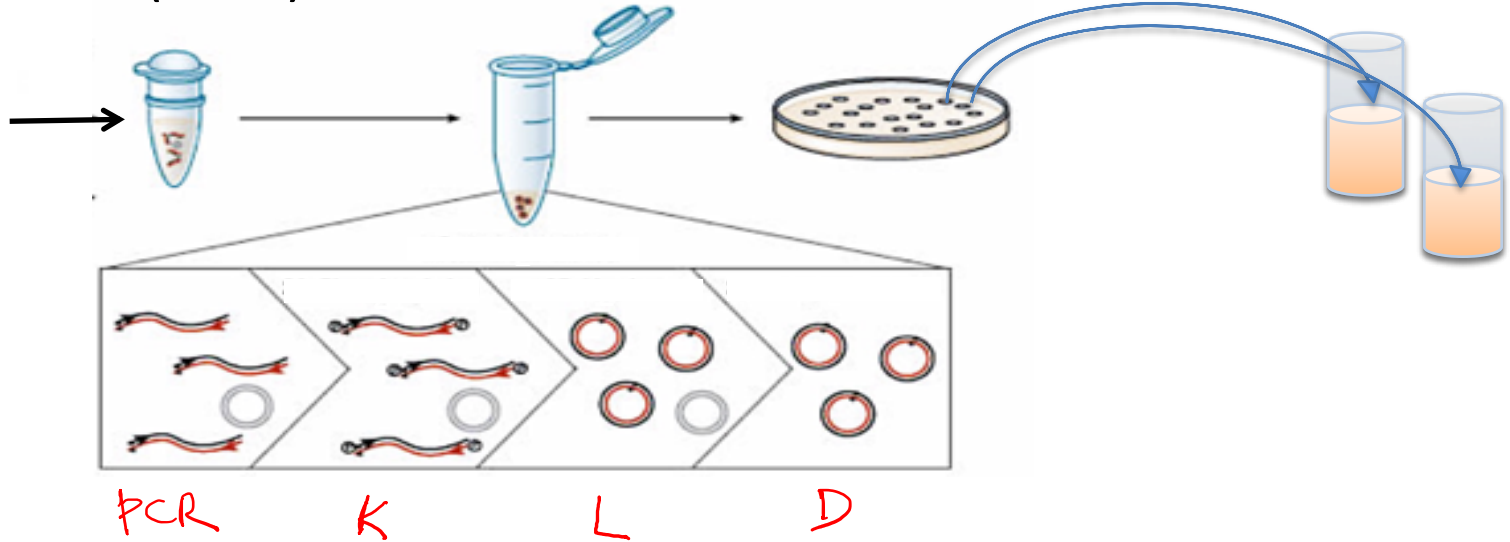
Last time (& while you were away):

Insert gRNA sequence into
vector (SDM)

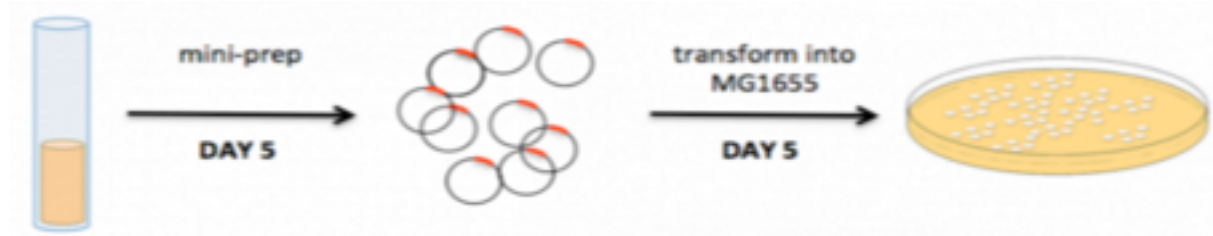


Transform
into NEB5 α

Inoculate liquid
cultures



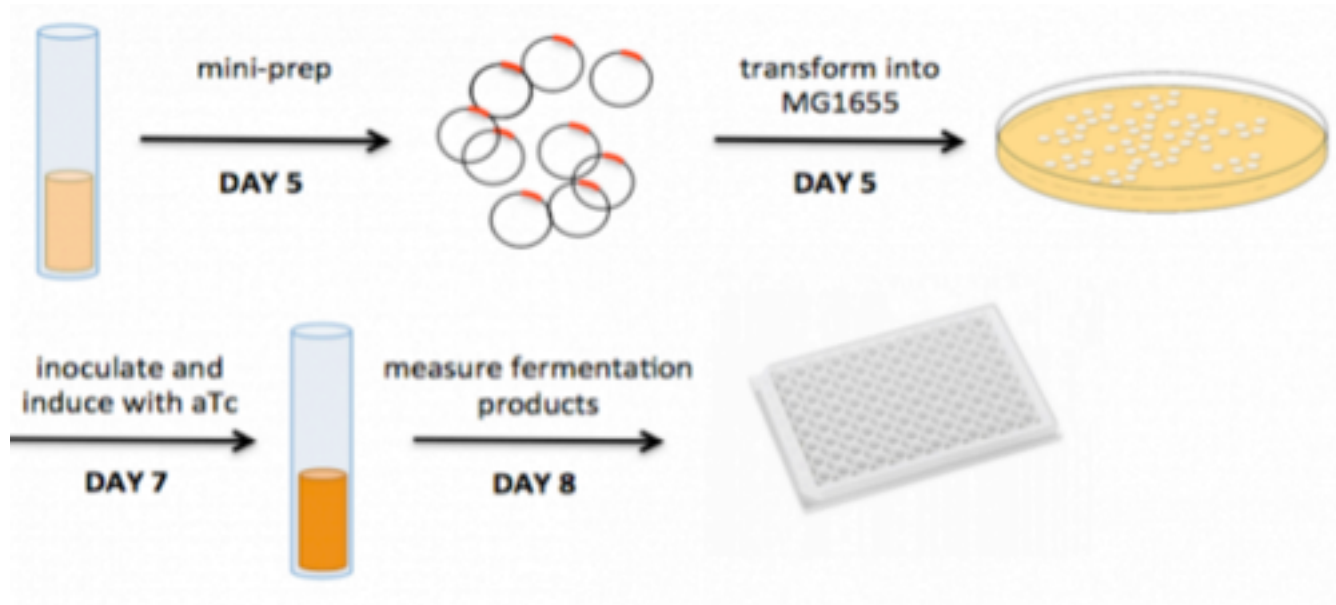
Today: confirm psgRNA_target



- 1.) Isolate pgRNA_target from *E. coli* cultures (mini-prep)
- 2.) Co-transform pd Cas9 & pgRNA_target into MG1655 cells
- 3.) Submit pgRNA_target for sequencing to confirm product

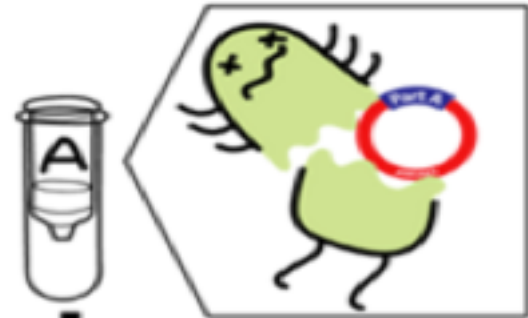
gRNA = sgRNA ; pgRNA = psgRNA

Today: confirm psgRNA_target



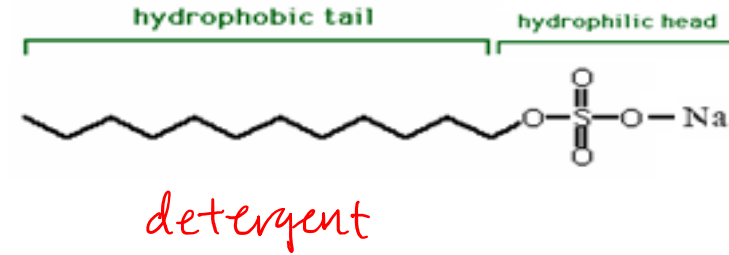
Mini-prep pgRNA_target clones

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



Prepare and lyse cells

- Prepare cells (P1)
 - Tris/EDTA buffer *Weakens cell envelope, blocks DNase*
 - Rnase *degrades RNA*
- Lyse cells (P2)
 - Sodium dodecyl sulfate (SDS)
 - *dissolves lipid membranes*
 - *denatures proteins*
 - Sodium hydroxide (NaOH)
 - *alkaline lysis*
 - *denature DNA*

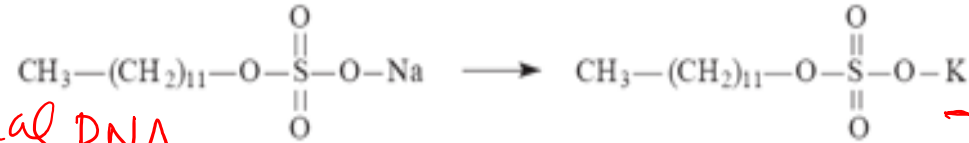


Neutralization (N3)

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

precipitates with

proteins, lipids, chromosomal DNA

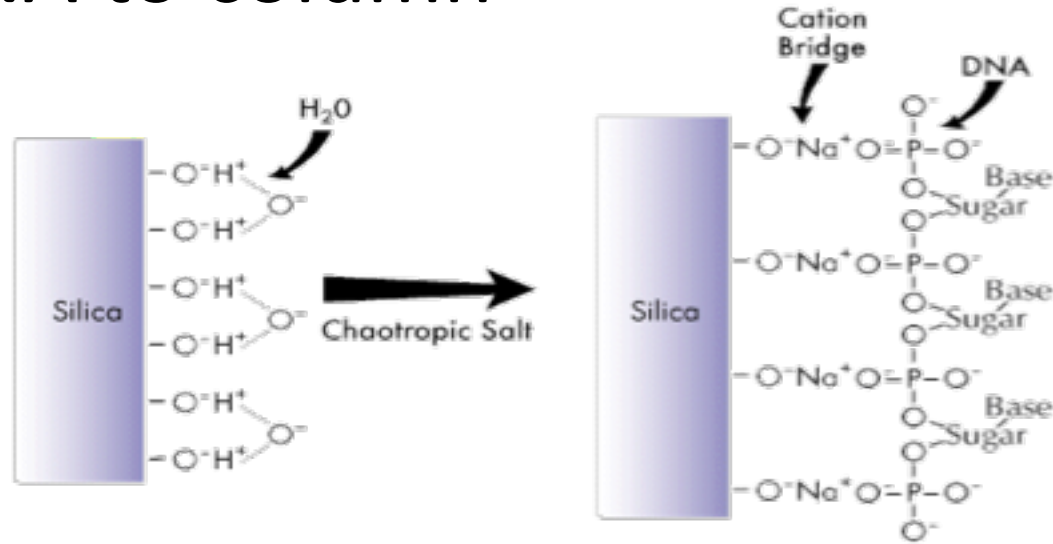


- Chaotropic salt
 - facilitates DNA binding to silica

➤ After centrifugation

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

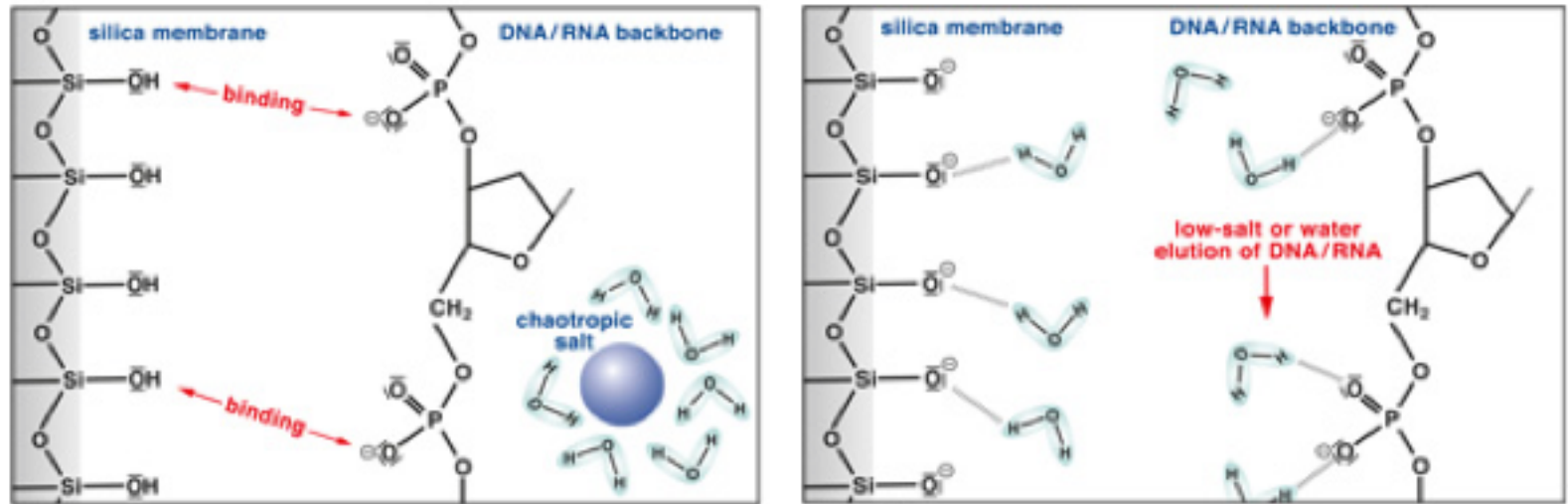
Bind DNA to column




- 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

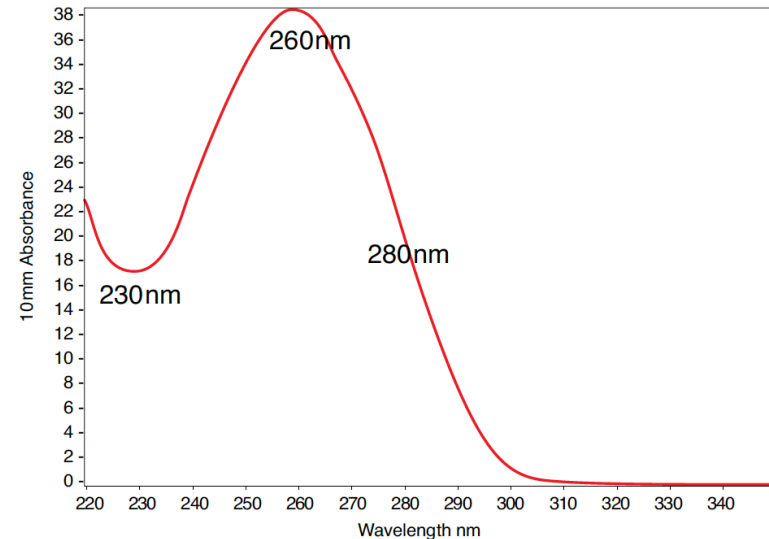
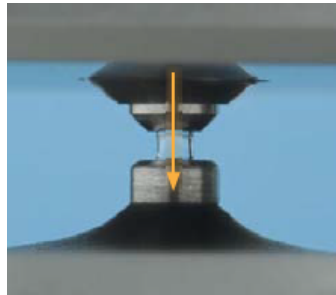
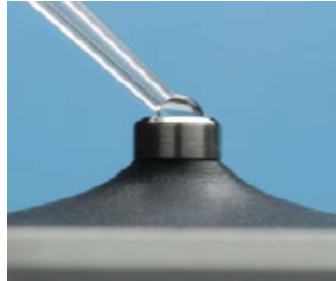


Steps	Contents	Purpose
Prepare (P1)	Tris/EDTA buffer RNase	Resuspend cells, weaken membrane, EDTA blocks DNase, RNase degrades RNA
Lyse (P2)	SDS (detergent) NaOH (alkaline lysis)	solubilize proteins, denature DNA
Neutralize (N3)	Acetic acid, chaotropic salt, potassium acetate	Renature short DNA, precipitate 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

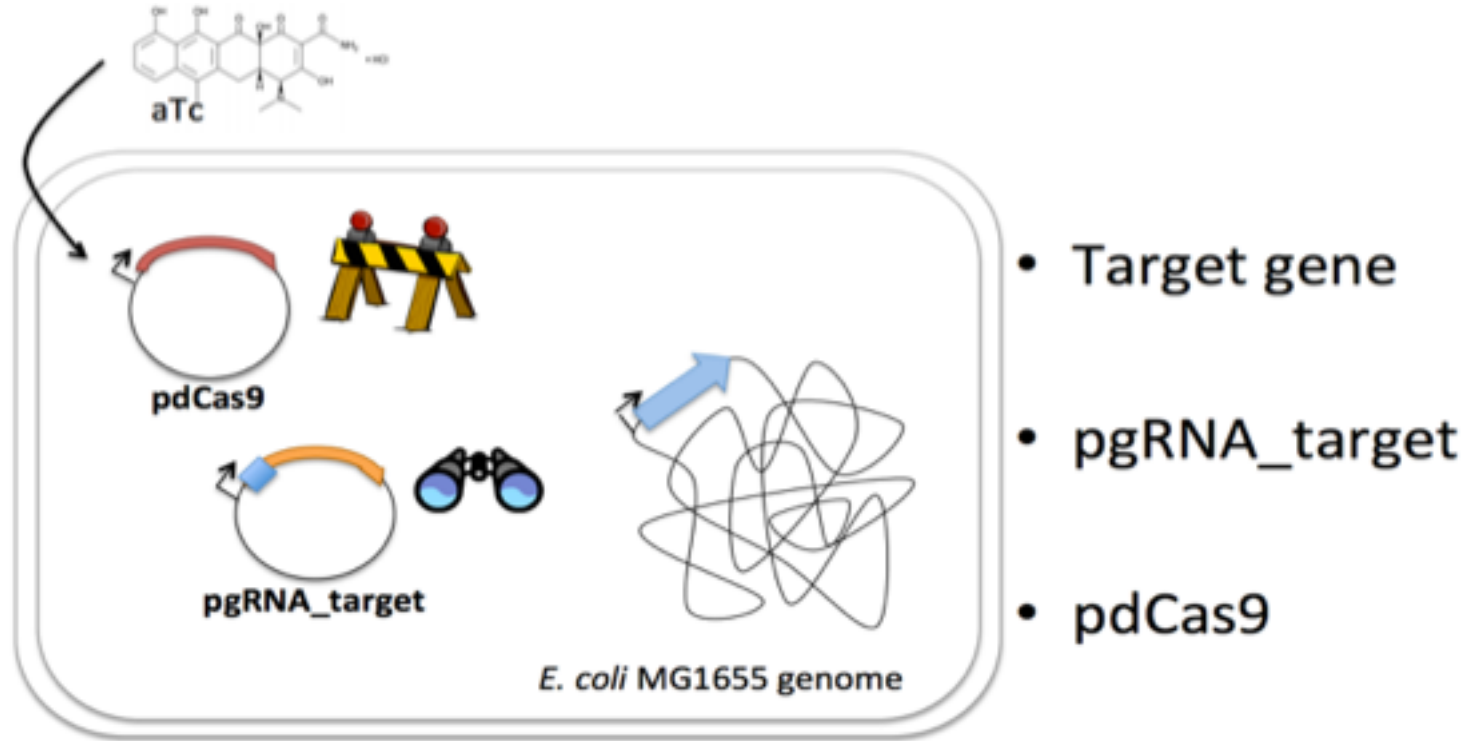
*Note: All liquid waste should be collected in conical tube, never aspirated

Measure DNA concentration with NanoDrop spectrophotometer

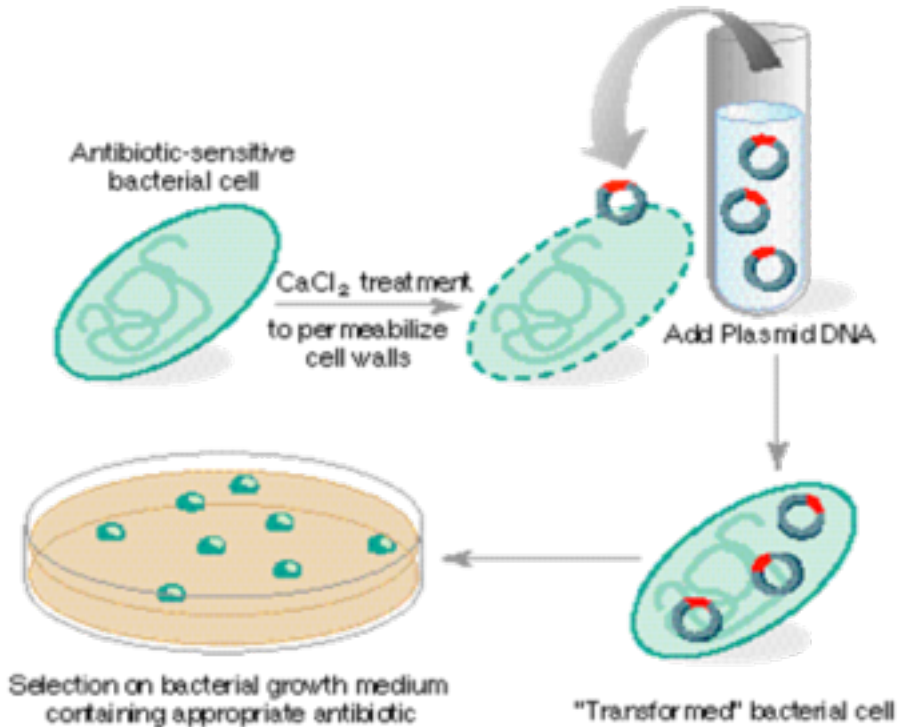
- Nucleic acids absorb 260 nm light



Transform CRISPRi system (two plasmids) into MG1655

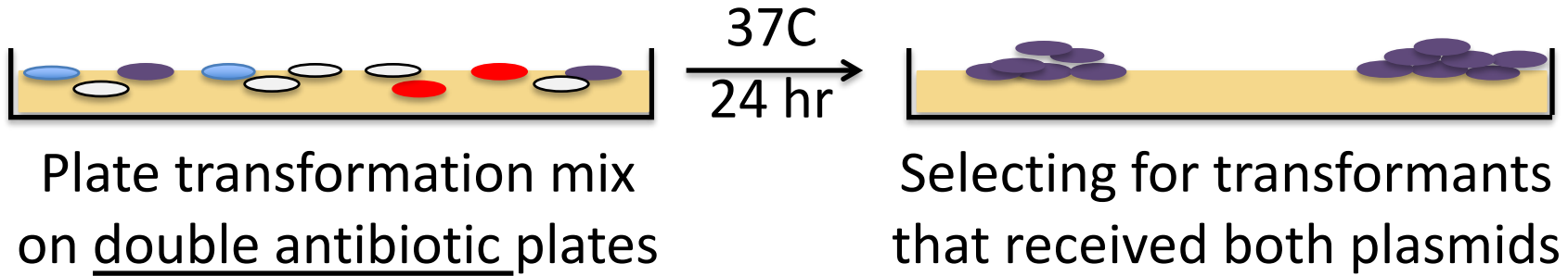


Heat shock competent cells for transformation



- MG1655 made chemically **competent** by CaCl₂
 - In exponential growth phase
 - OD₆₀₀ = 0.4-0.8
 - Ca²⁺ ions attract both DNA backbone and Lipopolysaccharide inner core
 - 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



pdCas9 confers resistance to:



chloramphenicol (cam)

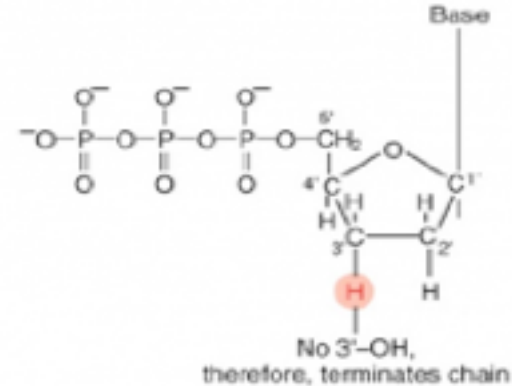
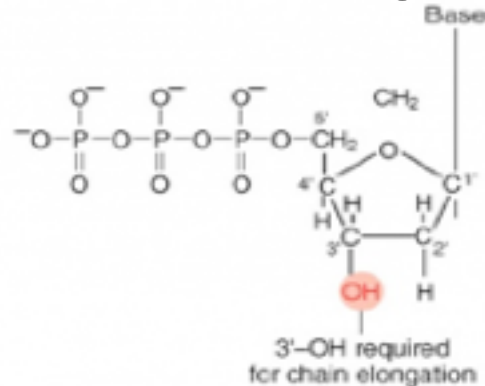
pgRNA confers resistance to:



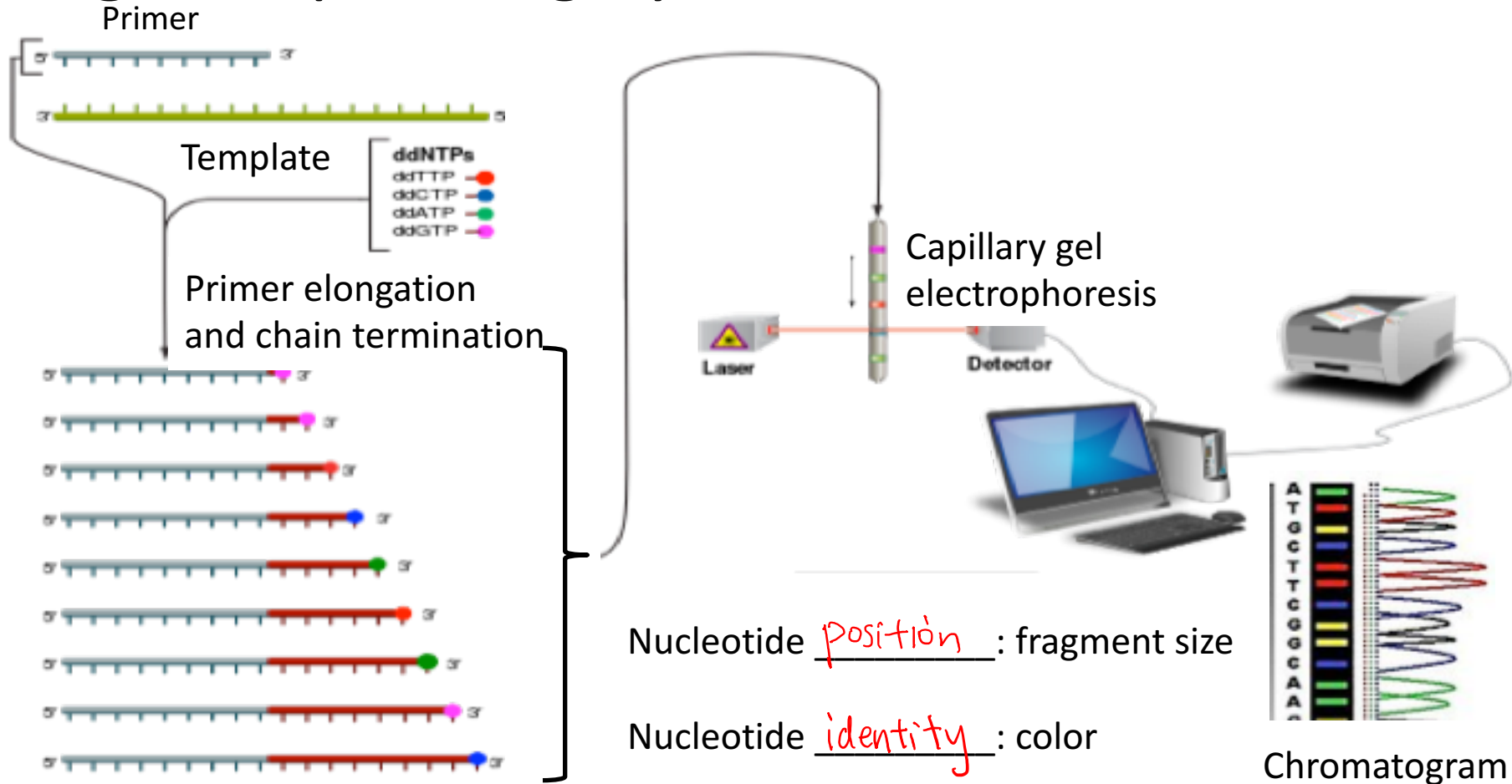
ampicillin (amp)

Use sequencing to determine if we have the intended psgRNA_target

- Diagnostic digests check size
- Sequencing
 - good to have both forward and reverse primers
 - more coverage (~1 kb)
 - double check
 - di-deoxynucleotides terminate elongation



Sanger sequencing by Genewiz



Tips to write Methods (due M2D7)

- **Methods completed individually and included in your Mod2 Research article**
 - M2D2 through M2D5 (leave out M2D1)
 - Using the phrase “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
final concentration (ng/μL)
Template DNA (5 μL) and primers were mixed with 20 μL of
1x
~~2.5X~~ Master Mix in a PCR tube. ~~Water~~ was added to 50 μL *remember to add Cas9 handle*
gRNA-ldha-F (5' GTCAG...3')
and samples put on PCR machine. *what conditions? (98°C for 10 sec...)*

Improving your Methods [2]

genotype, growth phase

A liquid bacteria culture was pelleted and the DNA was purified using a Qiagen kit. (manufacturer info)

- You may say "used Qiagen kit" for miniprep only, but be sure to mention elution step (H₂O, pH 8) as it differs from manufacturer specification.

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)