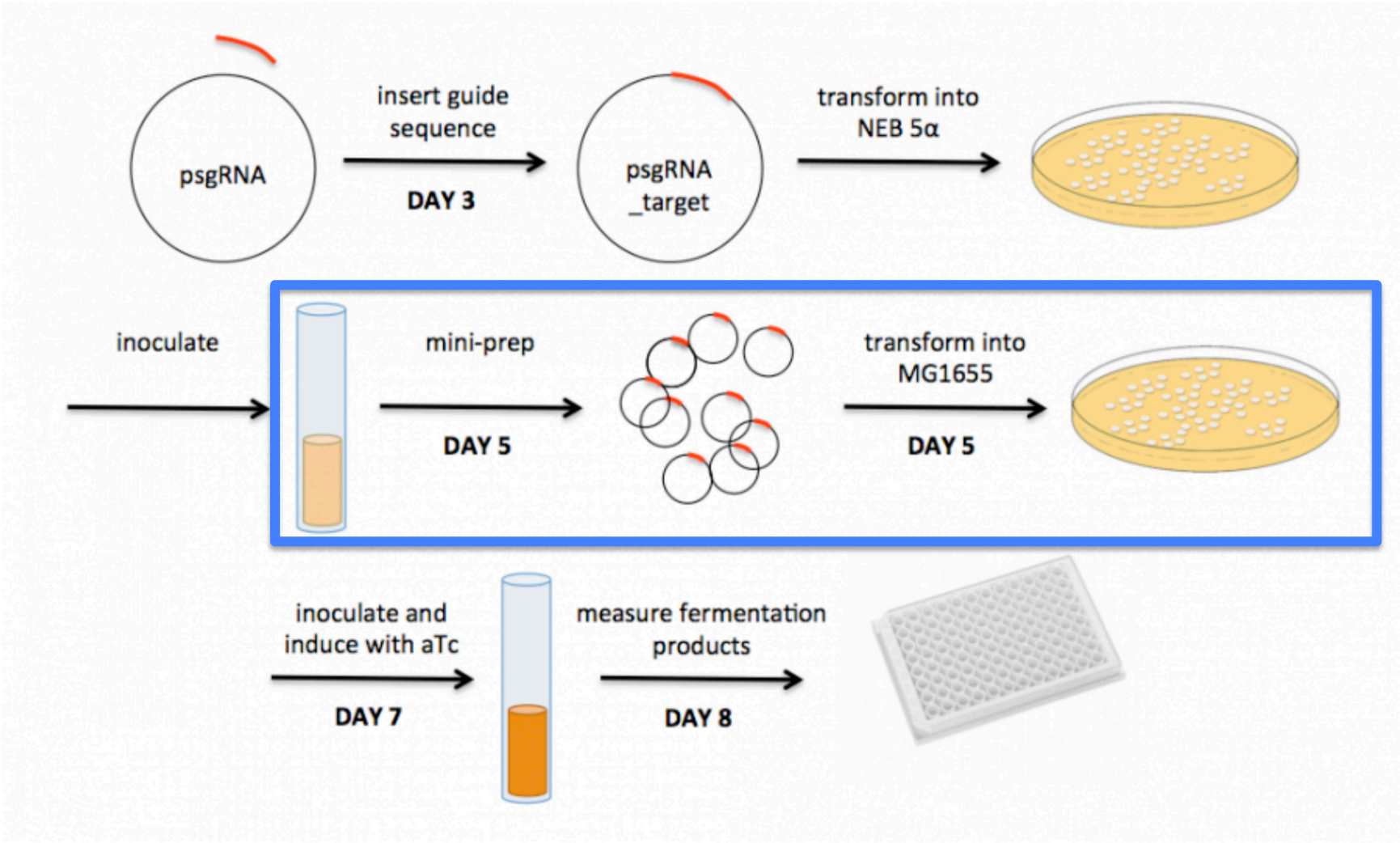
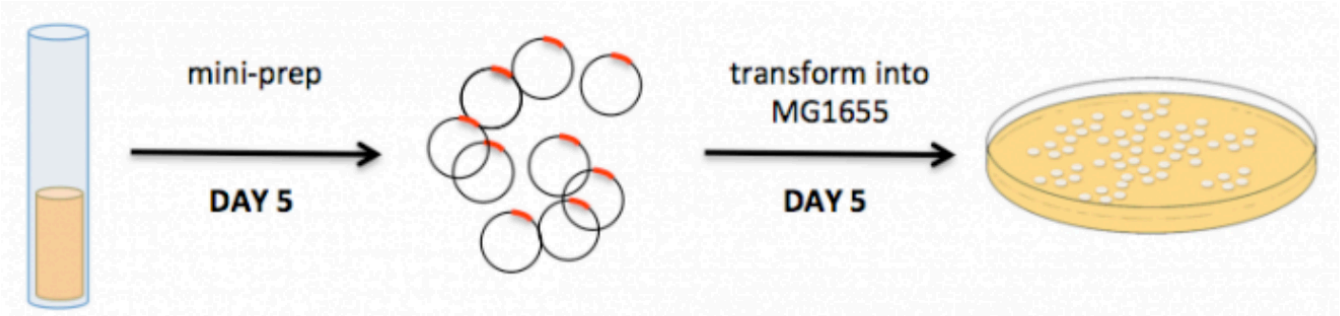
M2D5:
Recover pgRNA, Co-transform E. coli, Confirm pgRNA sequence

M2 experimental overview:



M2D5 experimental overview:



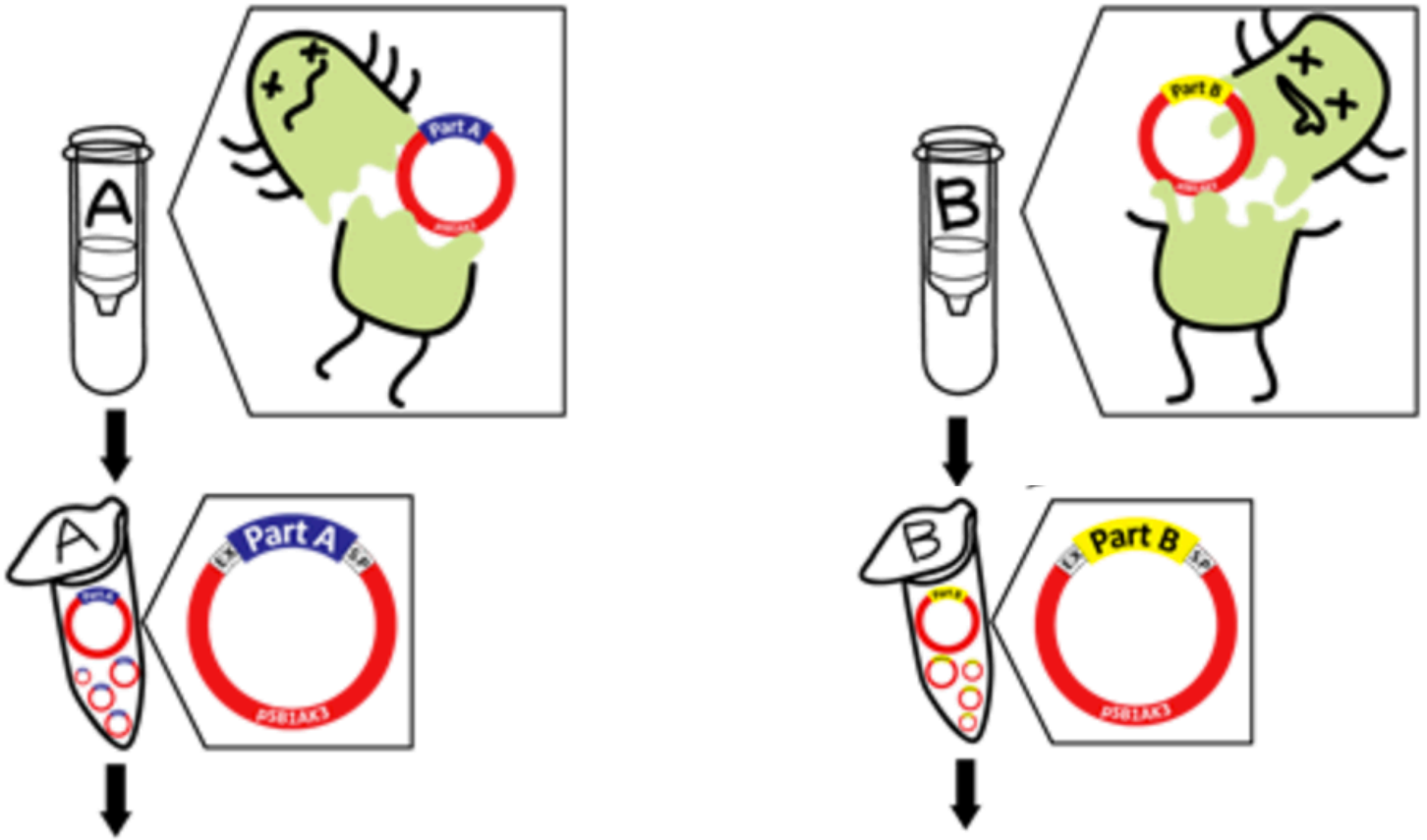
Today's lab goals:

1.) Recover your gRNA plasmid from *E. coli* cultures

2.) Co-transform your plasmid with pdCas9 into MG1655 cells

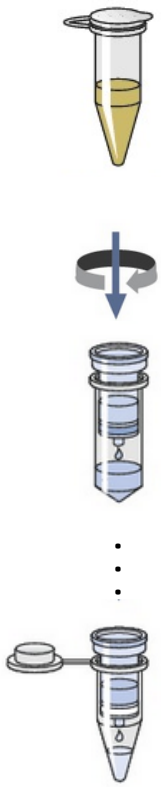
3.) Submit gRNA plasmid for sequencing to confirm product

Mini-preps isolate plasmids from bacteria



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

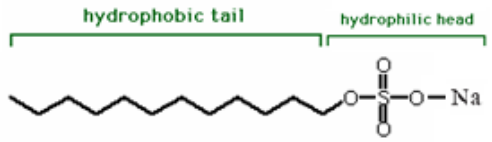
Mini-prep is a standardized process for isolating DNA



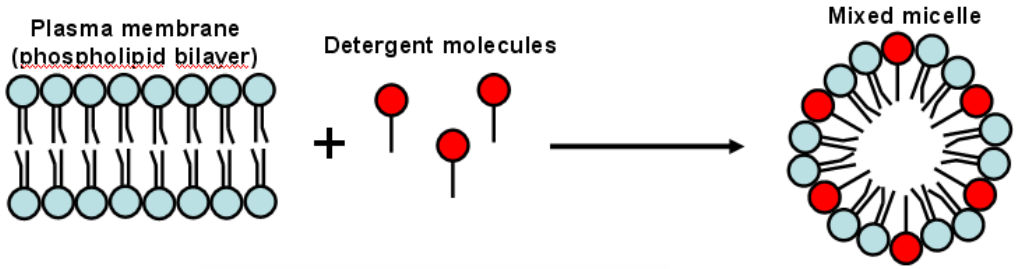
steps	contents	purpose
prepare **P1**	Tris/EDTA buffer RNase	resuspend the cells; weaken membrane EDTA - block DNAse RNAse- degrade RNA
lyse **P2**	SDS **surfactant/detergent** NaOH **alkaline lysis**	solubilize proteins, denature DNA
neutralize N3	acetic acid, chaotropic salt, potassium acetate	renature short DNA precipitate long DNA; protein
spin		
bind	silica column	concentrate DNA
wash PB PE	isopropanol, ethanol	** get rid of *all* ethanol
elute	water, pH 8.0	Elute all DNA off column

Mini-prep: Lyse cells with with SDS/NaOH

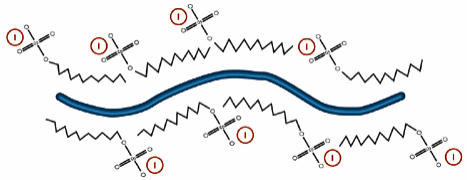
1. Sodium dodecyl sulfate (SDS)



   - dissolves membranes

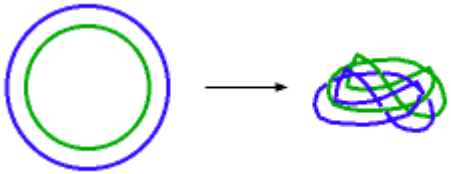


   - binds to and denatures proteins



2. Sodium hydroxide (NaOH)

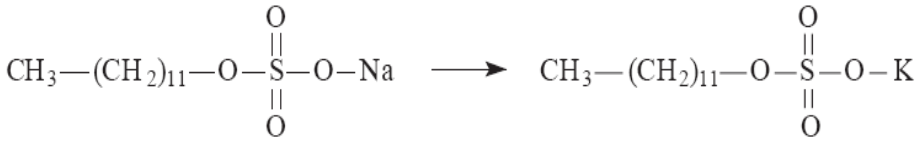
   - denatures DNA



Because plasmids are supercoiled, both DNA strands remain entangled after denaturation

Mini-prep: Neutralize lysis with acid; add chaotropic salt to promote DNA binding to column

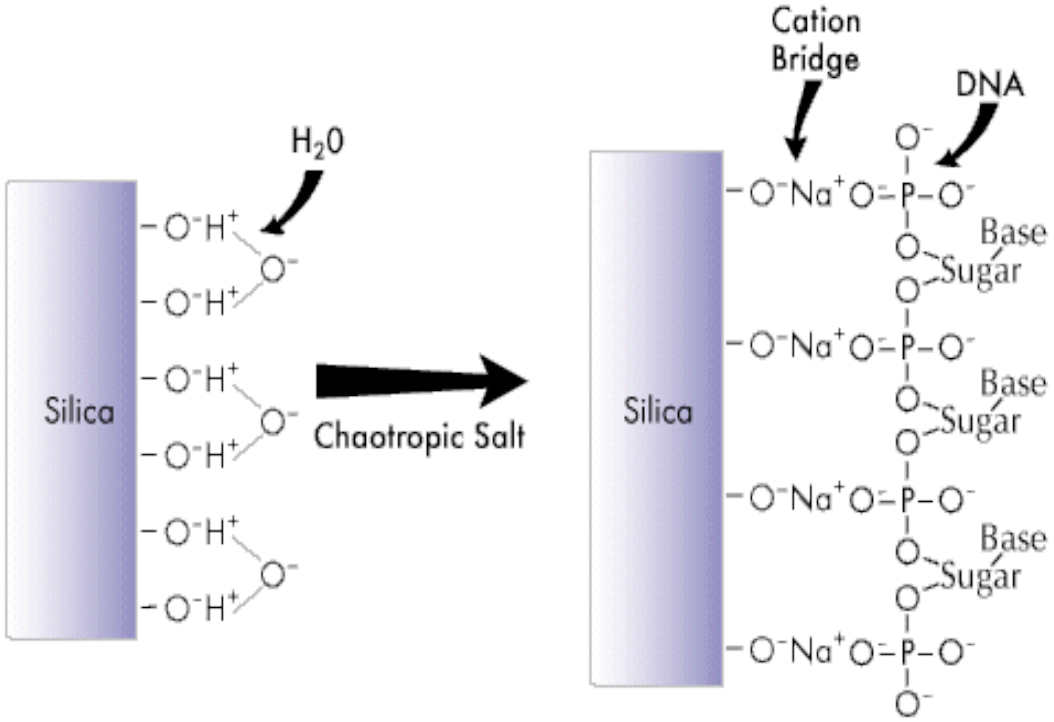
1. Acetic acid / potassium acetate solution
   - neutralizes NaOH (renatures plasmid DNA)
   - converts soluble SDS into insoluble PDS (white fluff)



2. Chaotropic salt
   - facilitates DNA binding to silica

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

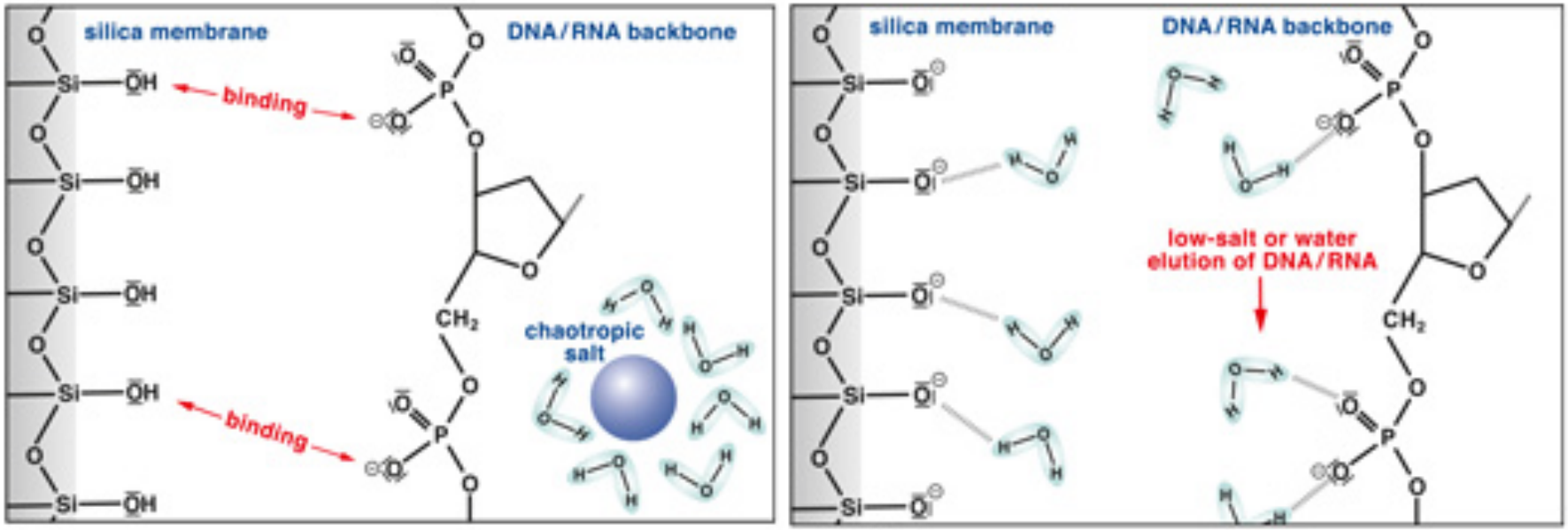
Mini-prep: Bind DNA to silica membrane on column



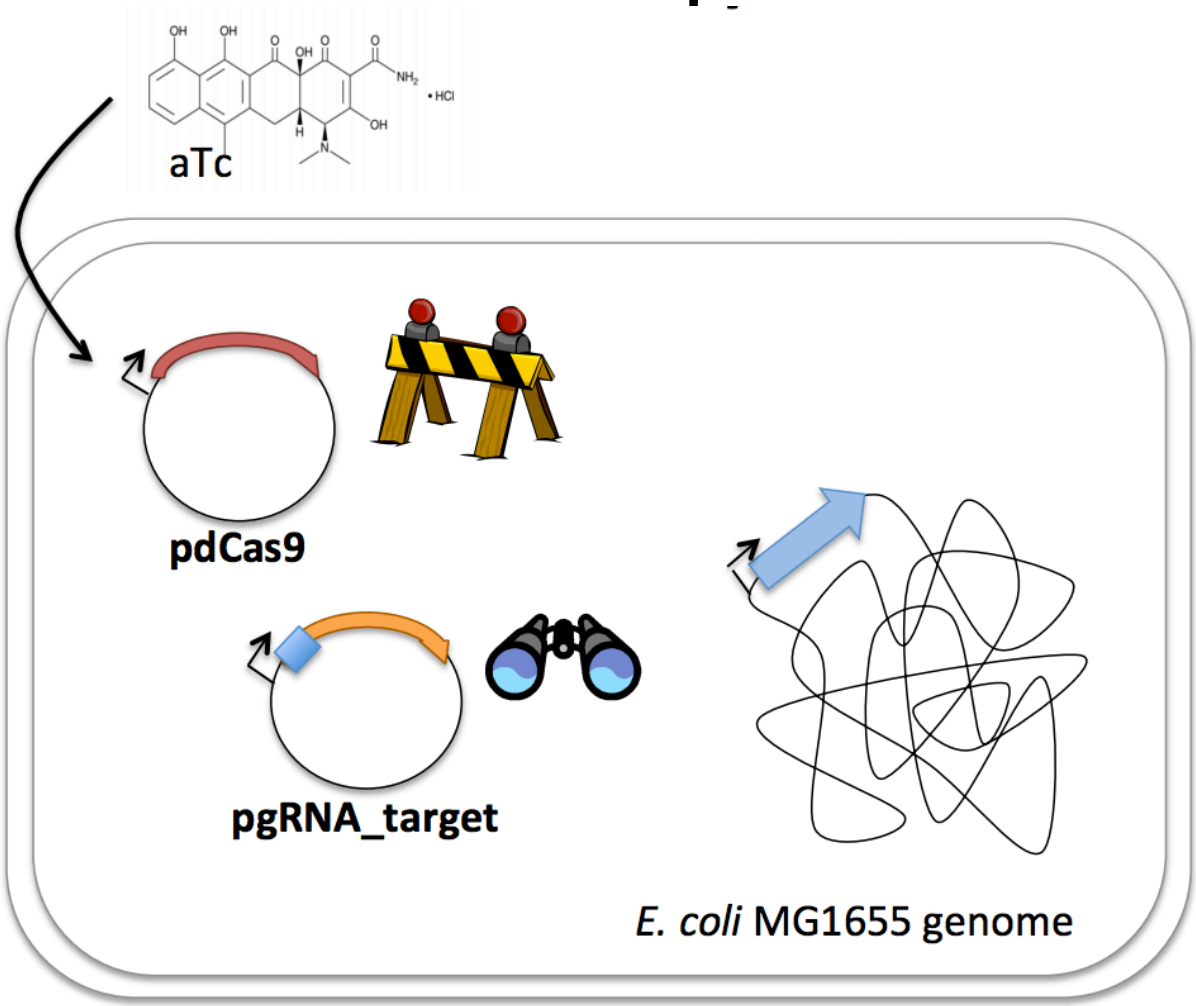
- Washes with PB and PE
  - remove residual contaminants (eluent)
  - maintain DNA onto column

Mini-prep: Elute DNA off column with water

- Water competes DNA off of column



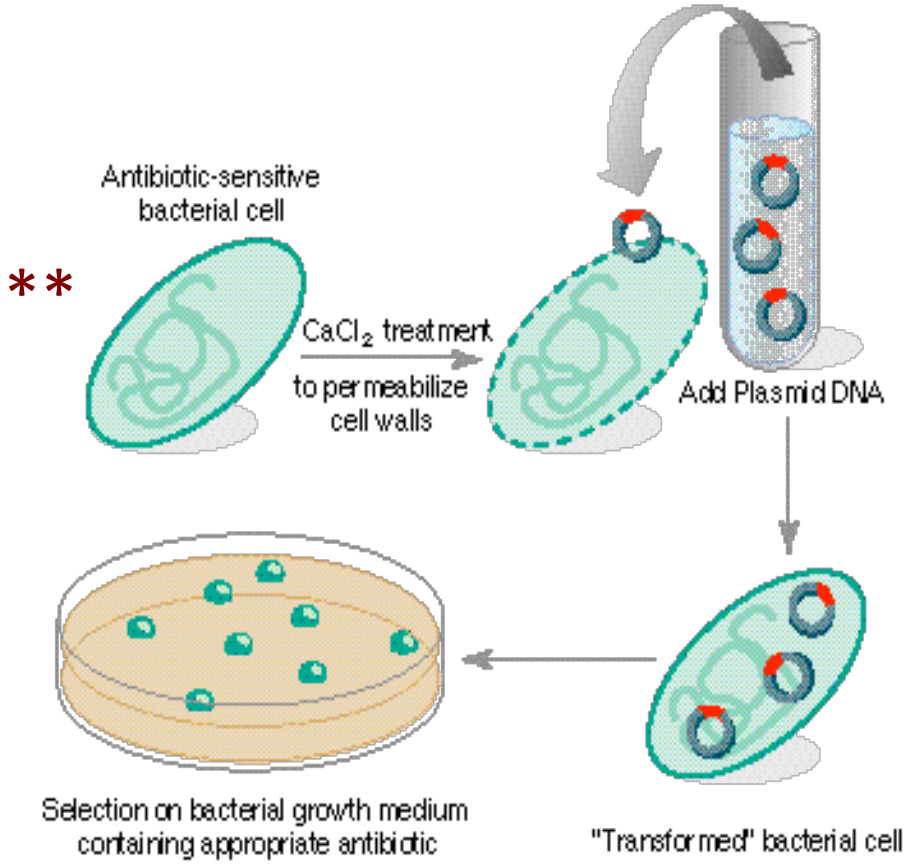
Your CRISPRi system uses two plasmids



- Target gene
- pgRNA_target
- pdCas9

Noreen's M2 lecture slides

Transform MG1655 competent cells with both plasmids



- made **competent** by $CaCl_2$
  - Ca^{2+} ions attract both DNA backbone and lipopolysaccharides (LPS) negative core
  - heat shock
- in exponential growth phase
  - OD_{600} = 0.4-0.8
- handle very gently, or will lyse
  - *on ice* all the time, and with chilled solutions **
  - not vortexed **

Antibiotic resistance selects for MG1655 co-transformed with both pdCas9 and pgRNA

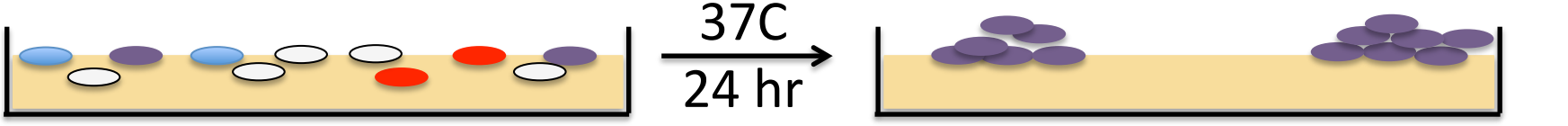


Plate transformation mix on double antibiotic plates

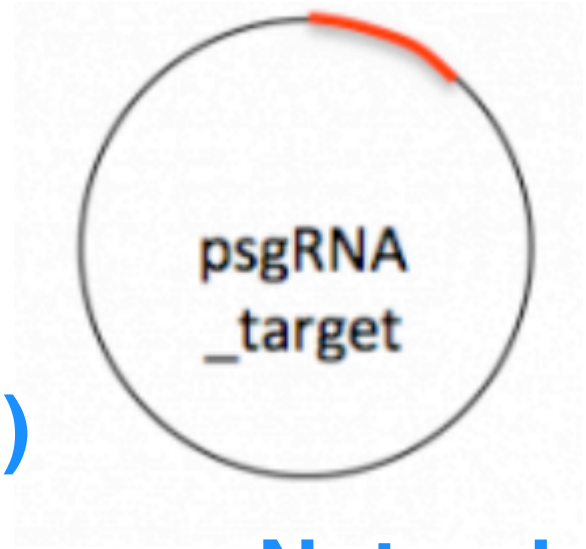
Selecting for transformants that received both plasmids

pdCas9 confers resistance to: **chloramphenicol**

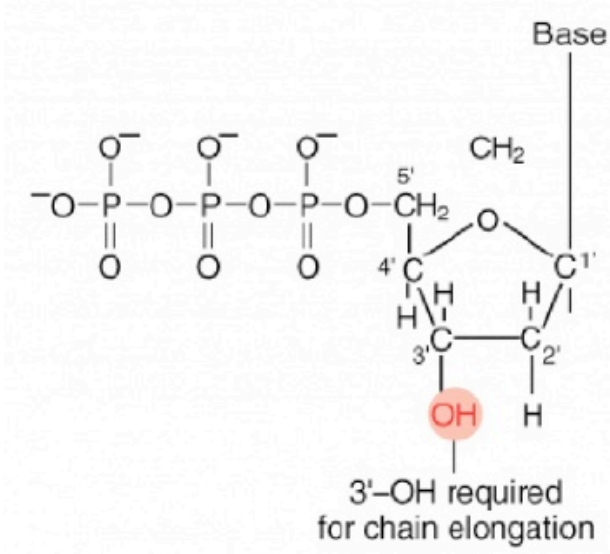
pgRNA confers resistance to: **ampicillin**

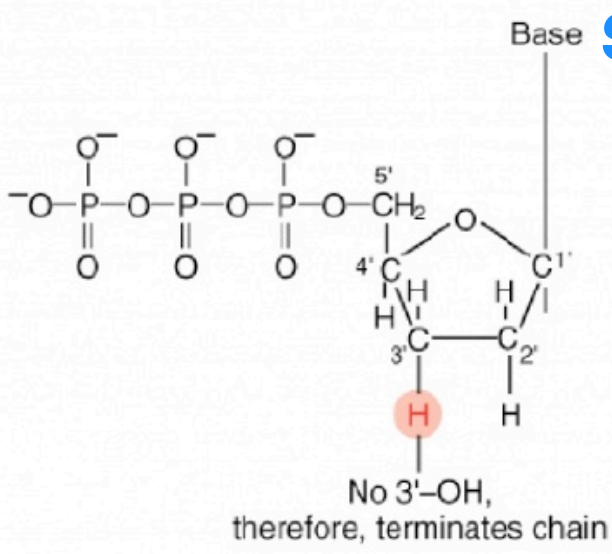
Sequencing lets us ask: Do we have the intended pgRNA?

- Diagnostic digests checks size
- Sequencing
  - good to have both Forward and Reverse primers (but one at a time!)
    - more coverage (1kb)
    - double-check
  - di-deoxynucleotides terminate elongation

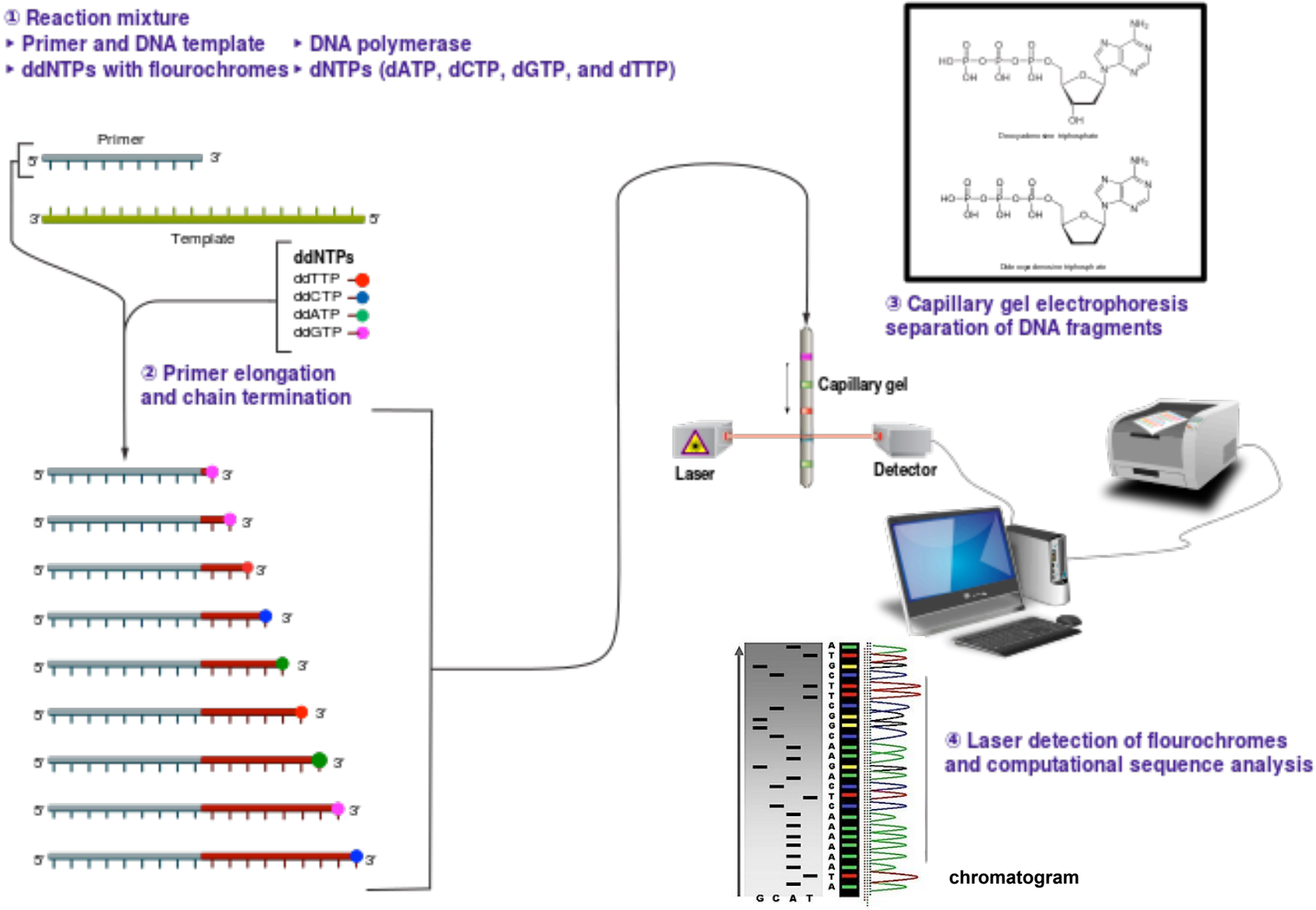


Note: do separately





Automated Sanger sequencing by Genewiz is fast and cheap (by scientific standards)



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]

IdahA_gRNA_F(5' GTTAG...3')

pgRNA

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.

1ng/ul

1X

(98C for 10sec...

Improving your Methods [2]

Include:
genotype
growth phase (exponential vs. saturation/overnight
volume (1.5ml)

A liquid bacteria culture was pelleted and the DNA was purified

using a Qiagen kit.(Manufacturers information!)

Elution step (30ul of H2O pH8) different from kit so include this information (situation where mentioning a volume and water ok.)

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)