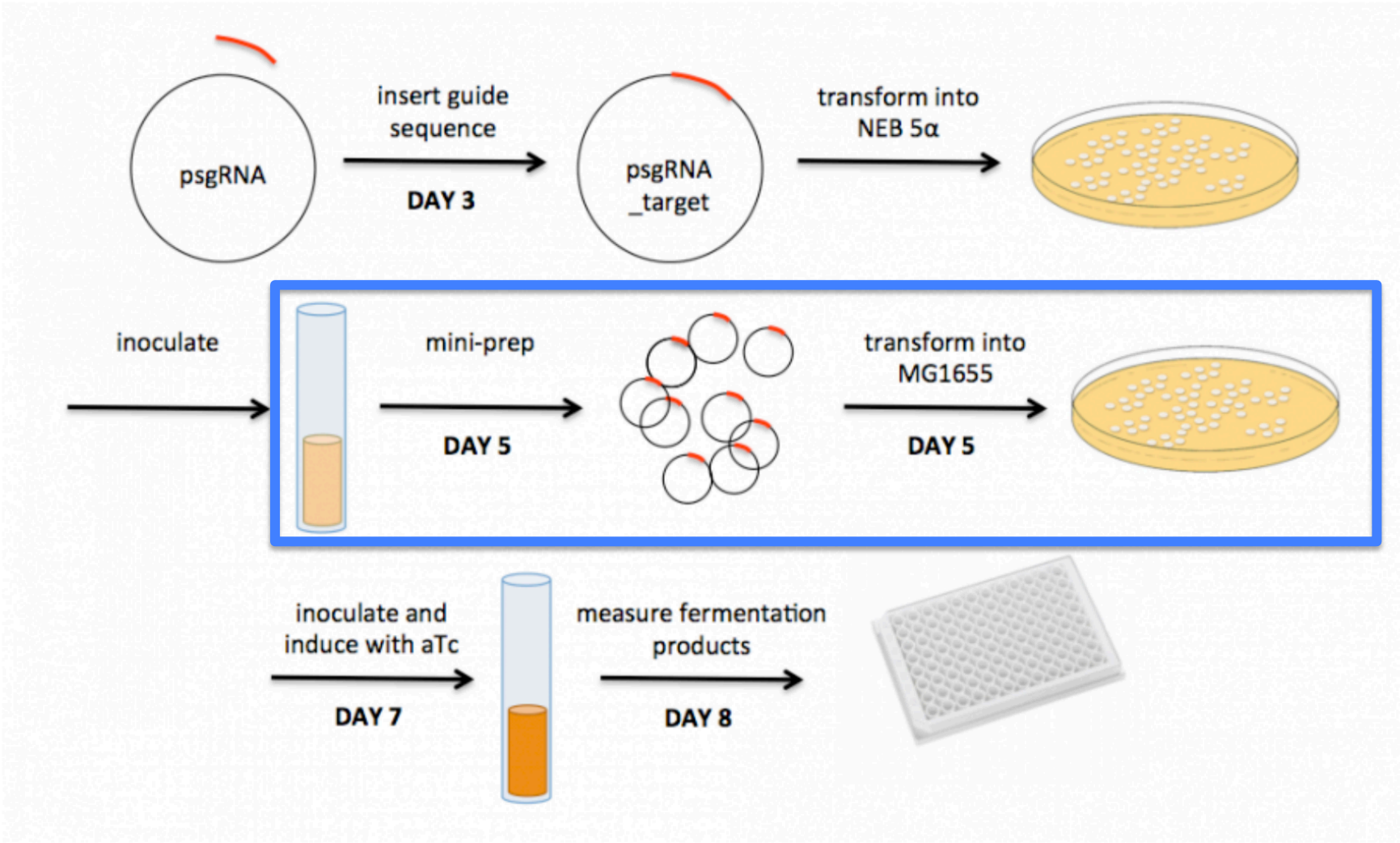
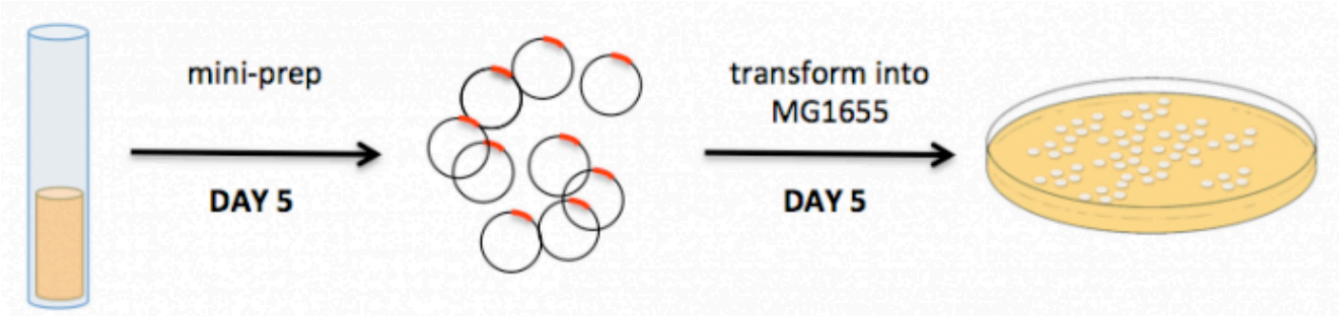
M2D5:
Isolate pgRNA, Co-transform E. coli, Confirm pgRNA sequence

10/27/2016 and 10/28/2016

M2 experimental overview:



M2D5 experimental overview:



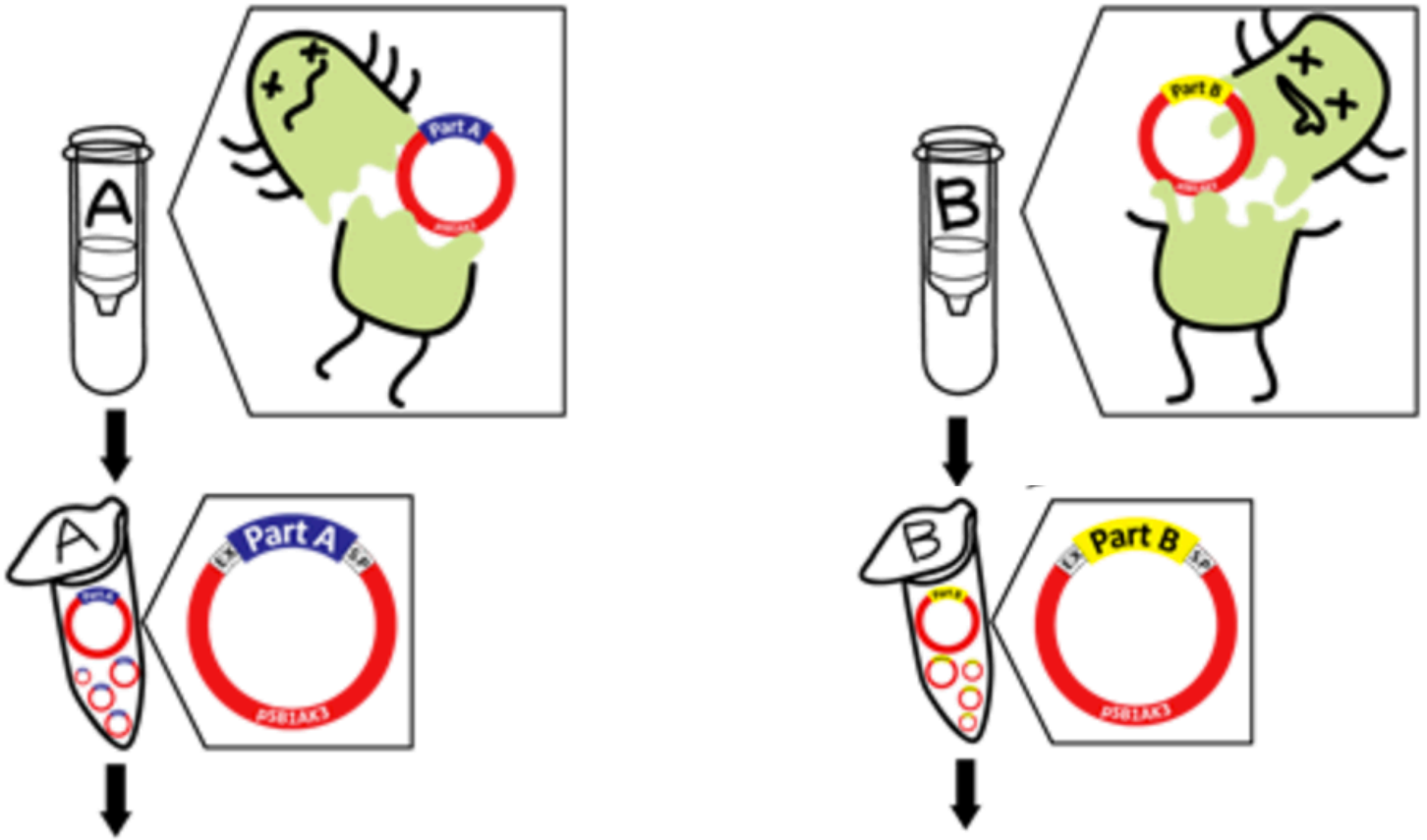
Today, you have <u>three</u> goals:

1.) Isolate 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

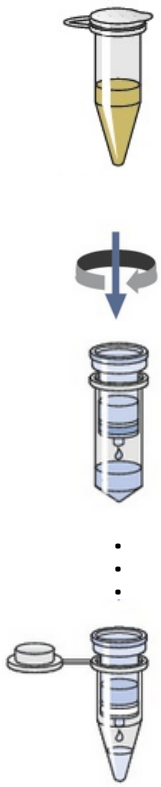
Mini-preps isolate plasmids from bacteria



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

Mini-prep to isolate DNA

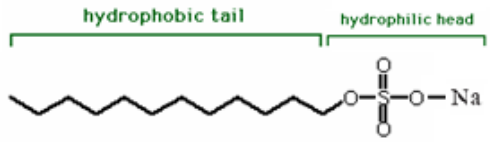
Qiaprep kit (Qiagen)



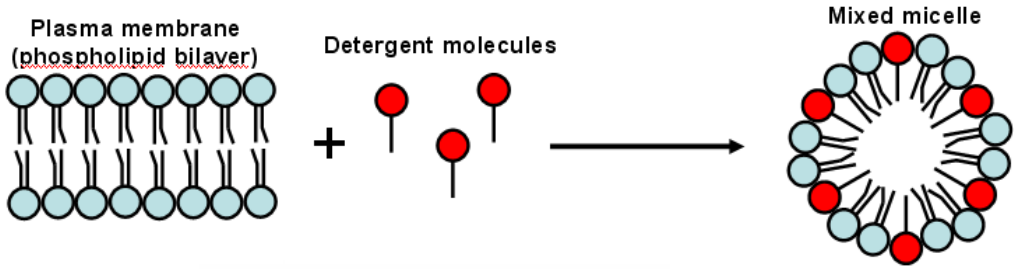
steps	contents	purpose
prepare P1	Tris/EDTA buffer RNase	Resuspend cells, EDTA (block DNase), RNase down RNA
lyse P2	SDS NaOH	solubilize proteins, denature DNA
neutralize N3	acetic acid, chaotropic salt, potassium acetate	
spin	separates soluble from	the insoluble
bind	silica column	concentrate DNA
wash	ethanol	** get rid of *all* ethanol
elute	water, pH 8.0	Elute all DNA off column

Review of mini-prep: Lysis with SDS/NaOH

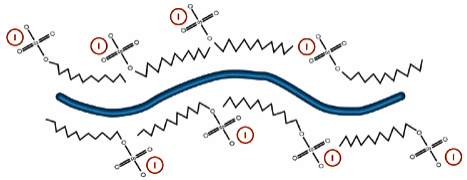
1. Sodium dodecyl sulfate (SDS)



   - dissolves membranes

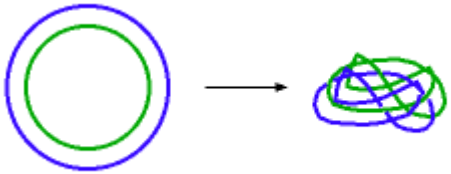


   - binds to and denatures proteins



2. Sodium hydroxide (NaOH)

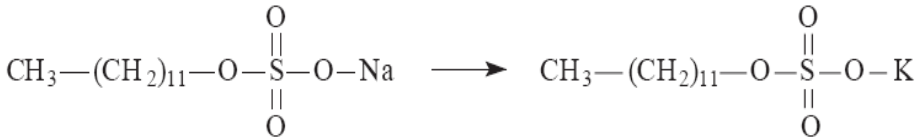
   - denatures DNA



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

Review of mini-prep: Neutralization

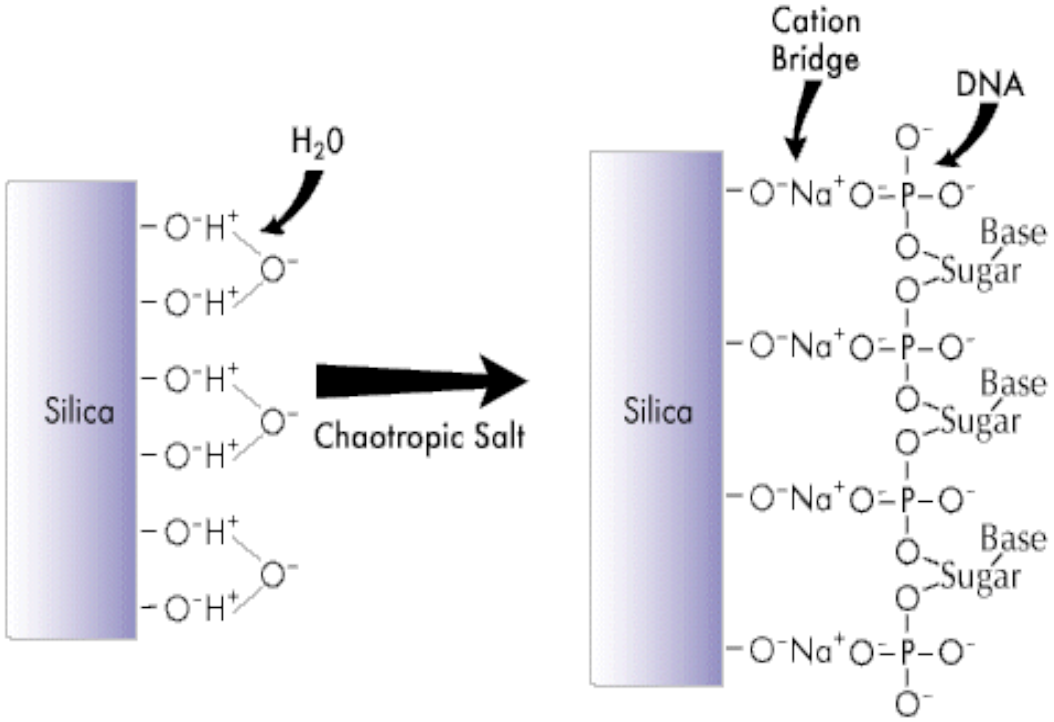
1. Acetic acid / potassium acetate solution
   - neutralizes NaOH (renatures plasmid DNA)
   - converts soluble SDS into insoluble PDS (white fluff)



2. Chaotropic salt
   - facilitates DNA binding to silica

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

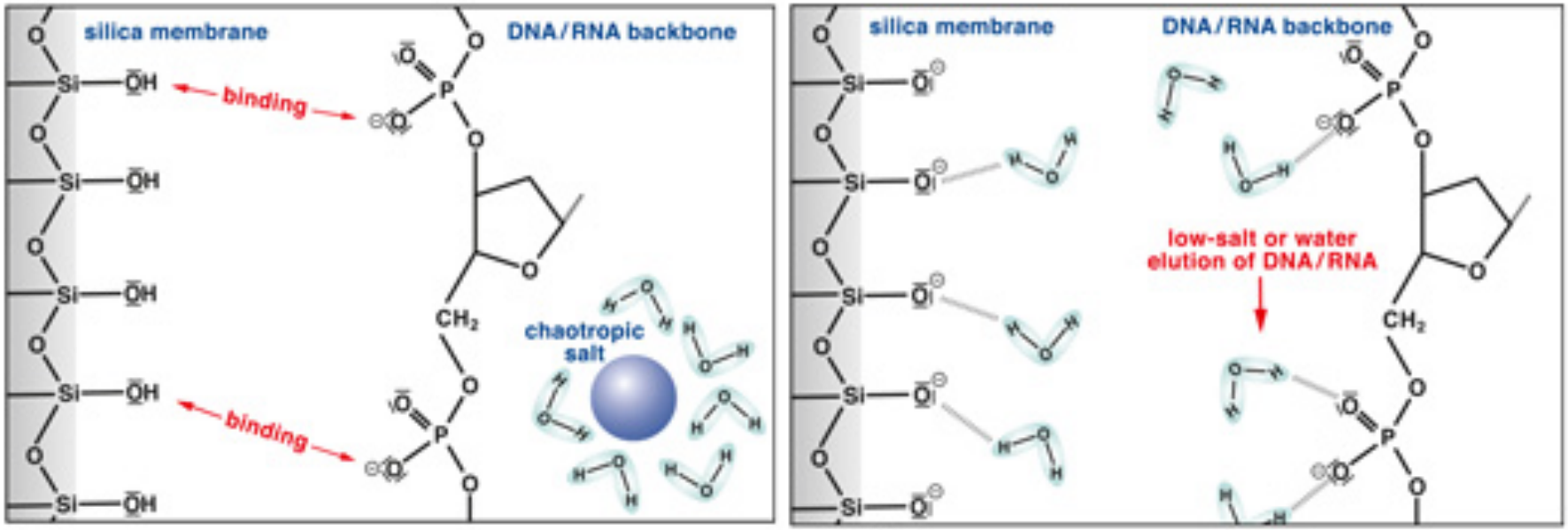
Review of mini-prep: Bind DNA to column



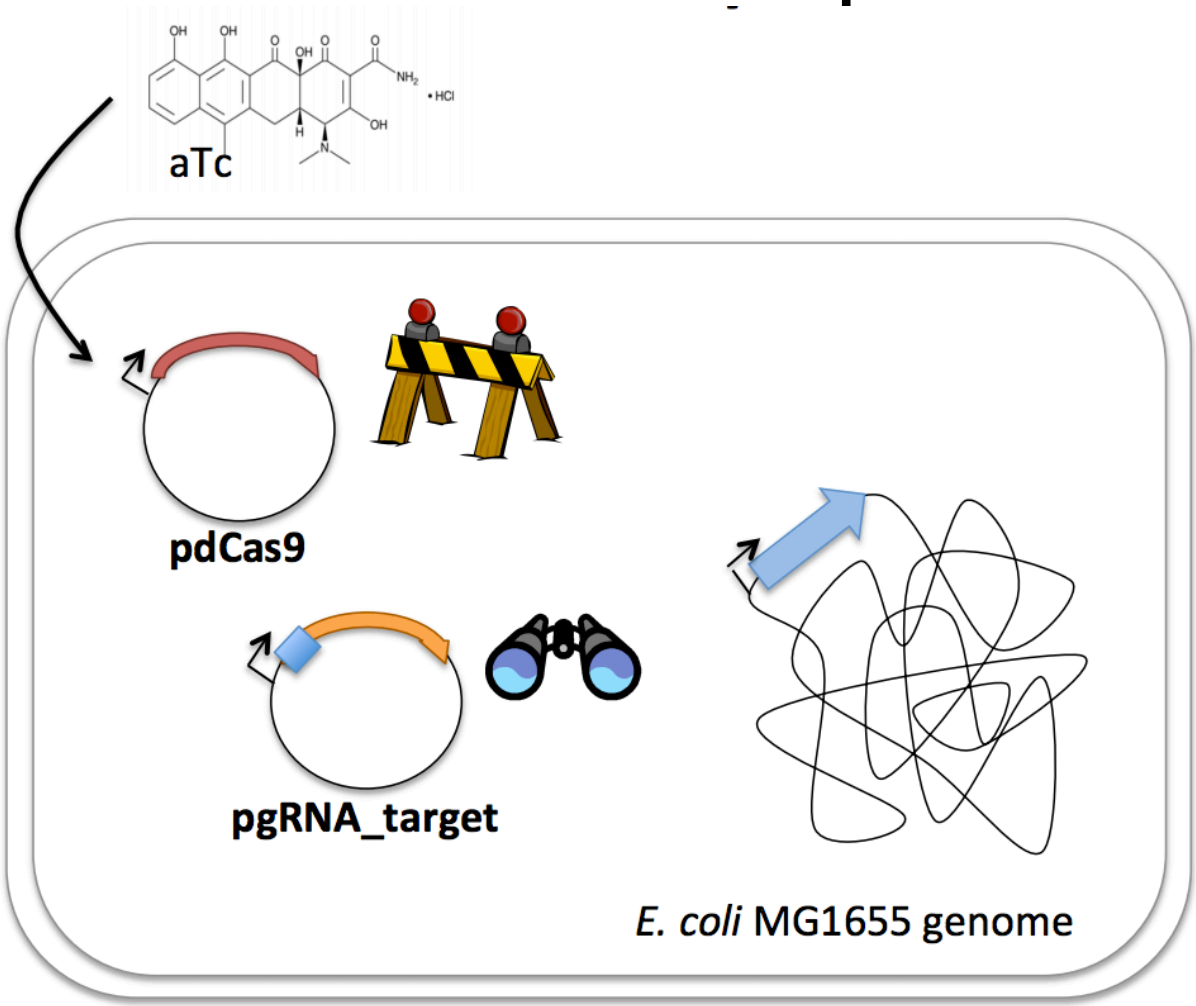
- Washes with PB and PE
  - remove residual contaminants (eluent)
  - maintain DNA onto column

Review of mini-prep: Elution with water

- Water competes DNA off of column



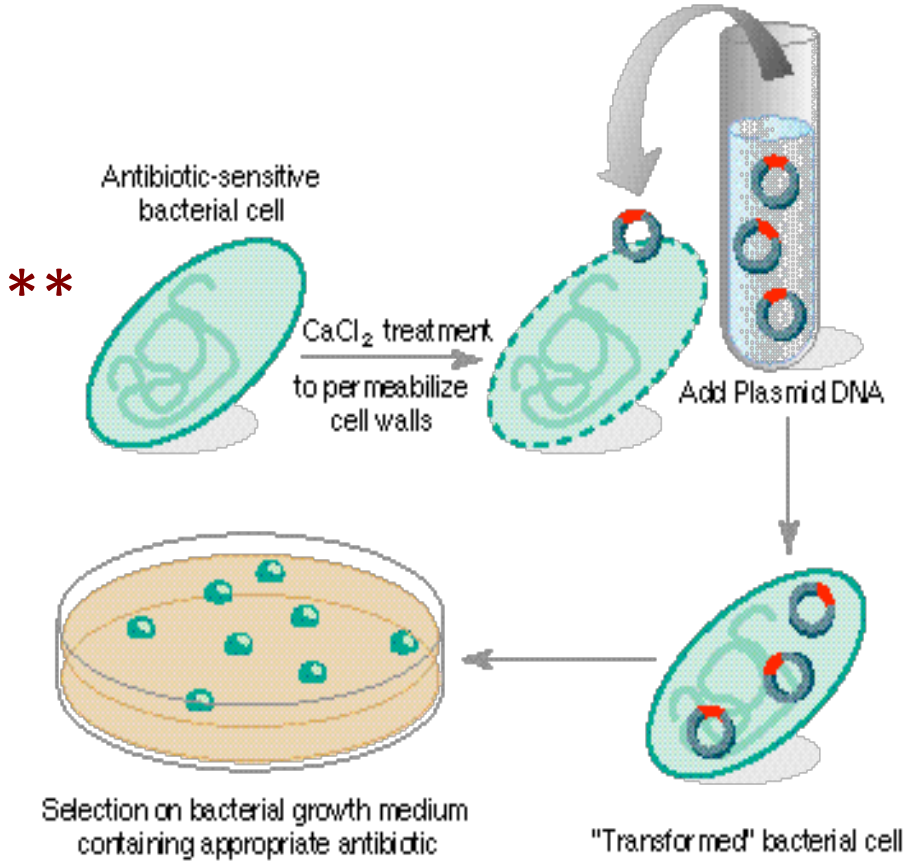
Your CRISPRi system requires the use of two plasmids



- Target gene
- pgRNA_target
- pdCas9

Noreen's M2 lecture slides

Transform MG1655 competent cells with both plasmids



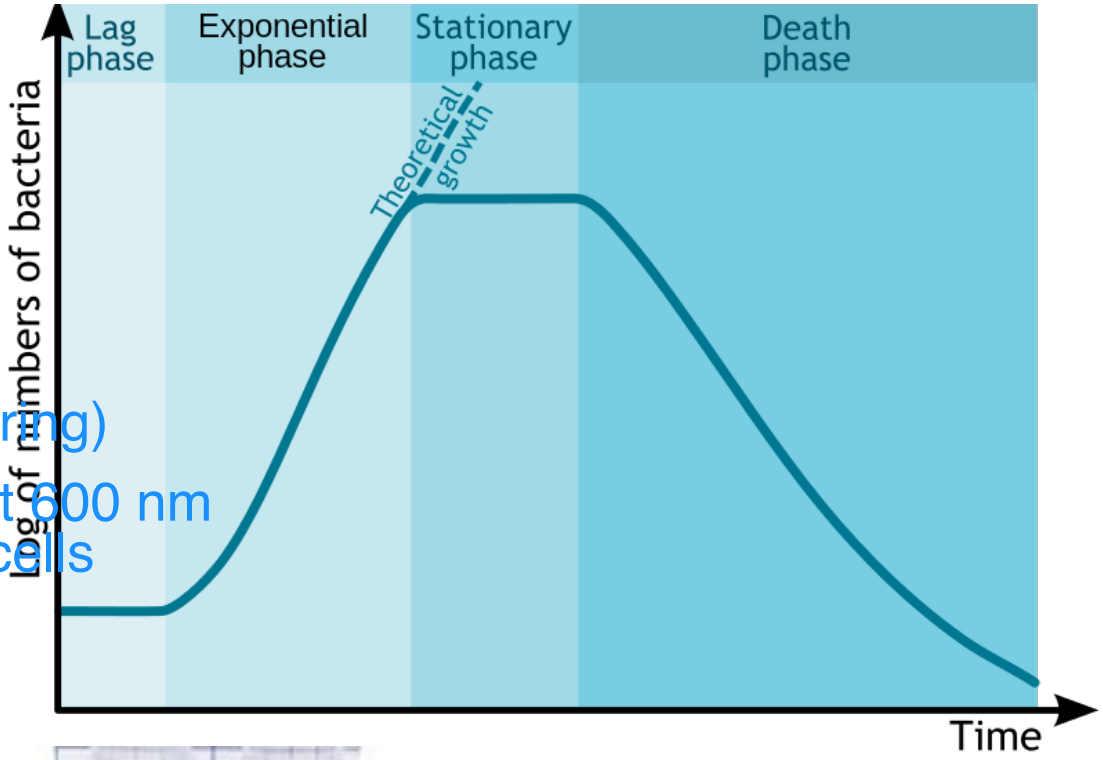
- made **competent** by $CaCl_2$
  - Ca^{2+} ions attract both DNA and inner core of LPS (lipopolysaccharide)
  - heat shock
- in exponential growth phase
  - OD_{600} = 0.4-0.8
- handle very gently, or will lyse
  - *on ice* all the time, and with chilled solutions **
  - not vortexed **

A few brief notes on *E. coli* growth curve

Measuring at 600 nm!

- exponential phase
  - binary fission
  - OD600 ~ 0.4 - 0.8
  - machinery ready

- OD ≠ absorbance
  - turbidity, (light scattering)
  - Cells don't absorb at 600 nm
  - UV is damaging to cells



low OD

High OD (very turbid)

MG1655 needs to be co-transformed with both pdCas9 and pgRNA

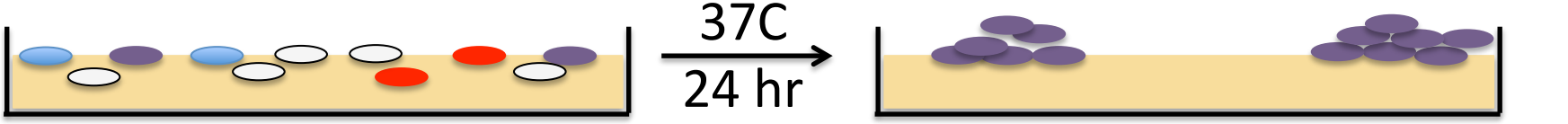


Plate transformation mix on double antibiotic plates

Selecting for transformants that received both plasmids

pdCas9 confers resistance to:

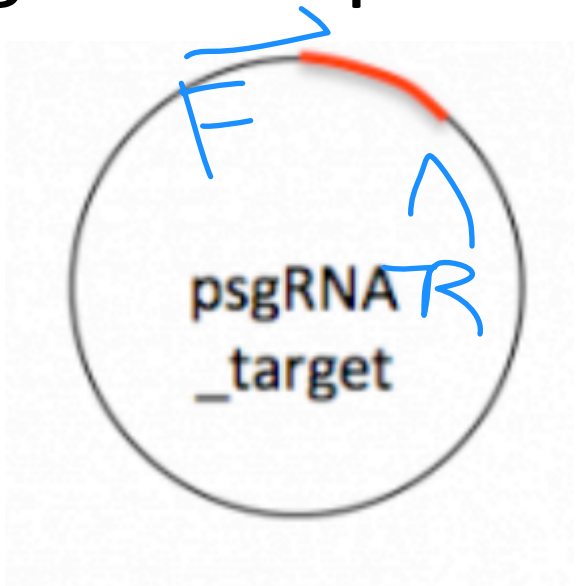
chloramphenicol

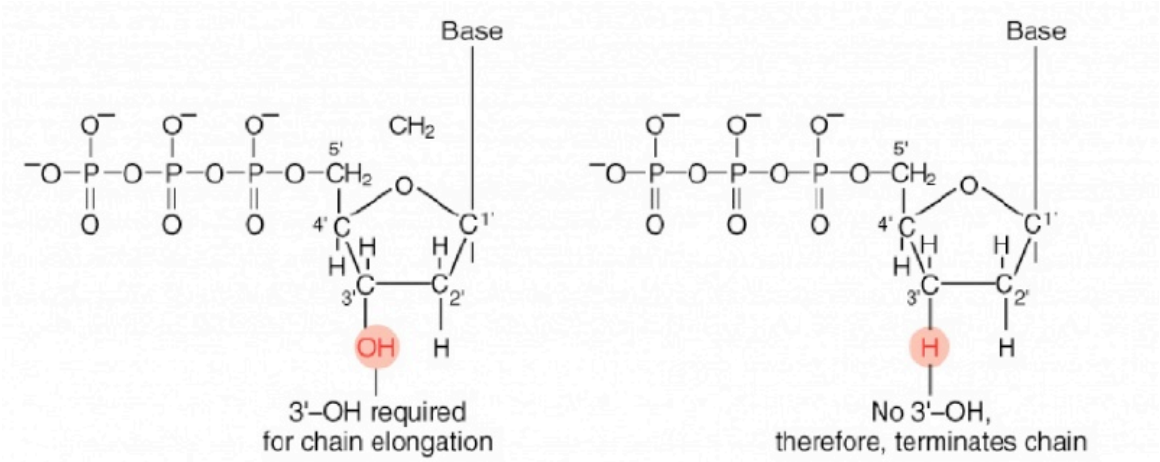
pgRNA confers resistance to:

ampicillin

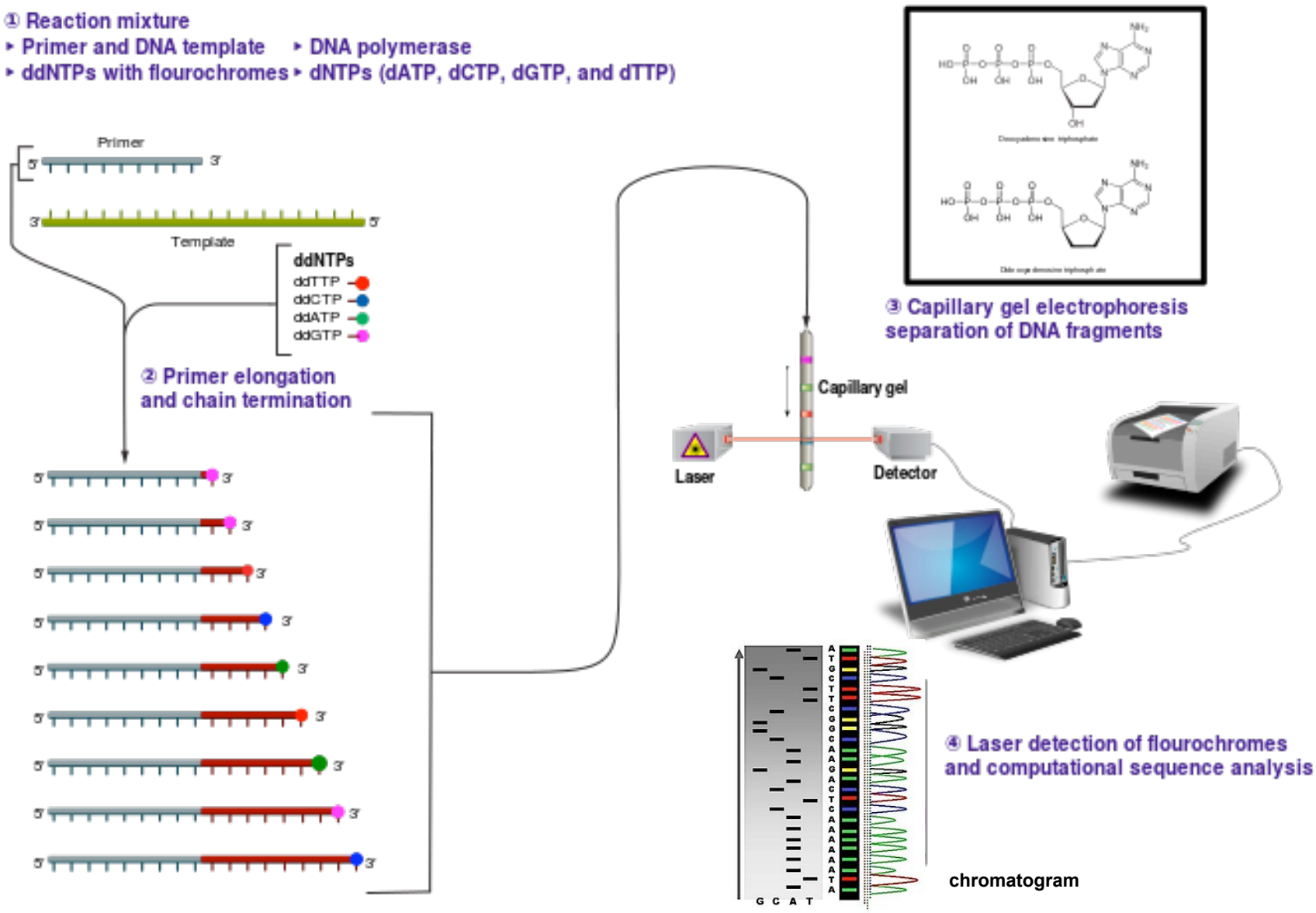
Do we have the intended gRNA sequence?

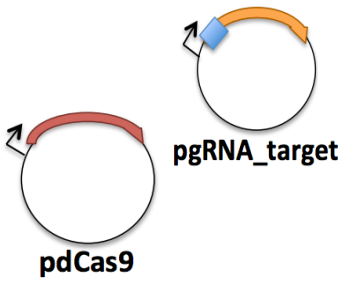
- Diagnostic digests
- Sequencing
  - good to have both F and R primers
    - double check
    - for better coverage
  - di-deoxynucleotides terminate elongation



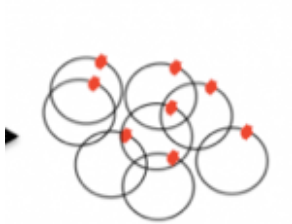


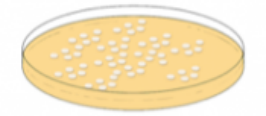
Sanger sequencing by Genewiz





Tips to write Methods (due M2D7)





- Use subsections with descriptive titles
  - Put in logical order
  - Begin with topic sentence to introduce purpose
- Use clear and concise full sentences
  - Eliminate tables and lists
  - Passive voice expected
- Use the most flexible units
  - Write concentrations (when known) rather than volumes
- Eliminate ‘109 specific details
  - Do not include details about tubes and water!
  - Assume reader has some microbiology 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.

A tube without template was prepared and labeled control.

-Final concentrations of DNA
-No volumes (i.e. for master mix)
-No tubes, no water
-What Master mix? (manufacturer, city, etc.)
-Sequences of primers (5' - NNN- 3')
-Don't need to say that you did a "control"
-What is your template
-What were the thermocycler conditions (in sentence form)

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] ***Site-directed mutagenesis and amplification of the pRSET-IPC plasmid***

Introductory topic sentence. SDM (including KLD, transformation in NEB5α)

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.

Confirmation of intended mutation by sequencing (Genewiz, Cambridge, MA)

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