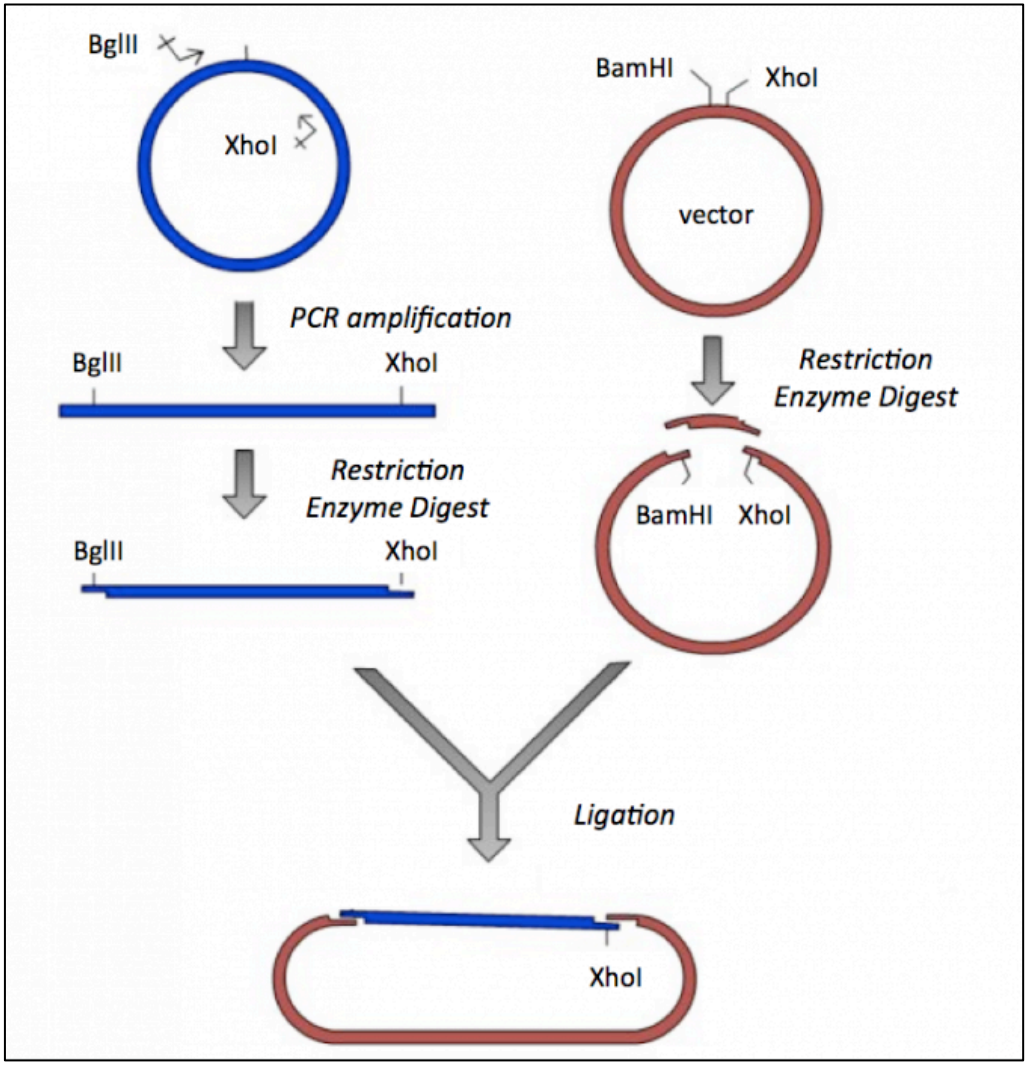
M2D2: Design gRNA for CRISPRi

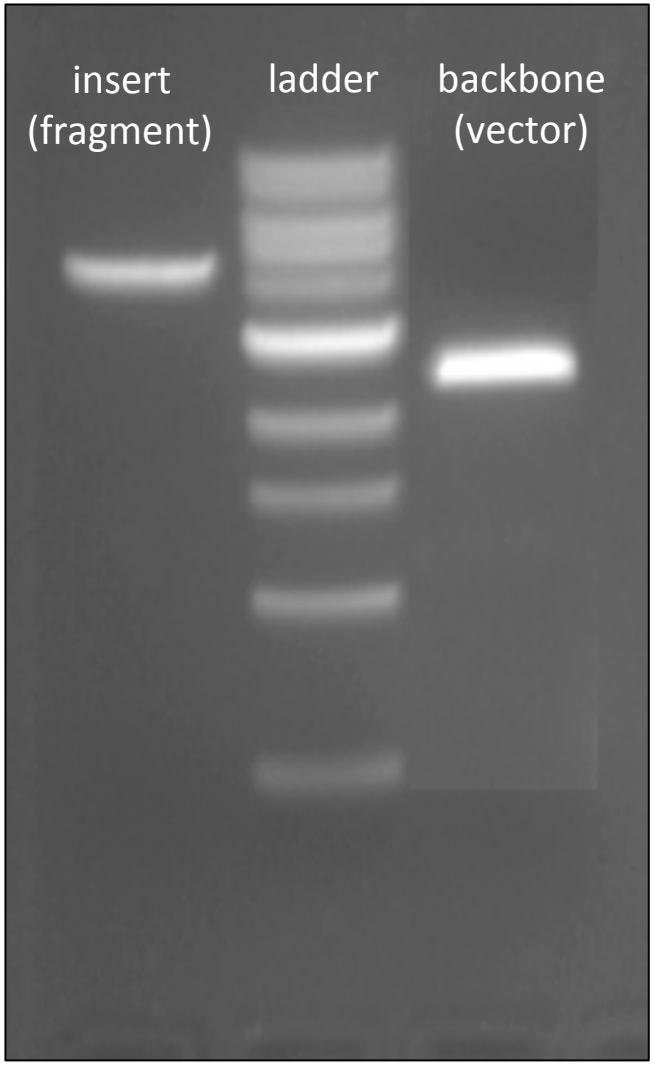
10/18/16

1. Pre-lab Discussion
2. Load digests from M2D1 onto agarose gels
3. Select target protein in fermentation pathway
4. Design gRNA for CRISPRi system
5. Start reading articles for Thursday's in-class discussion

Review:pdCas9 was constructed by ligation

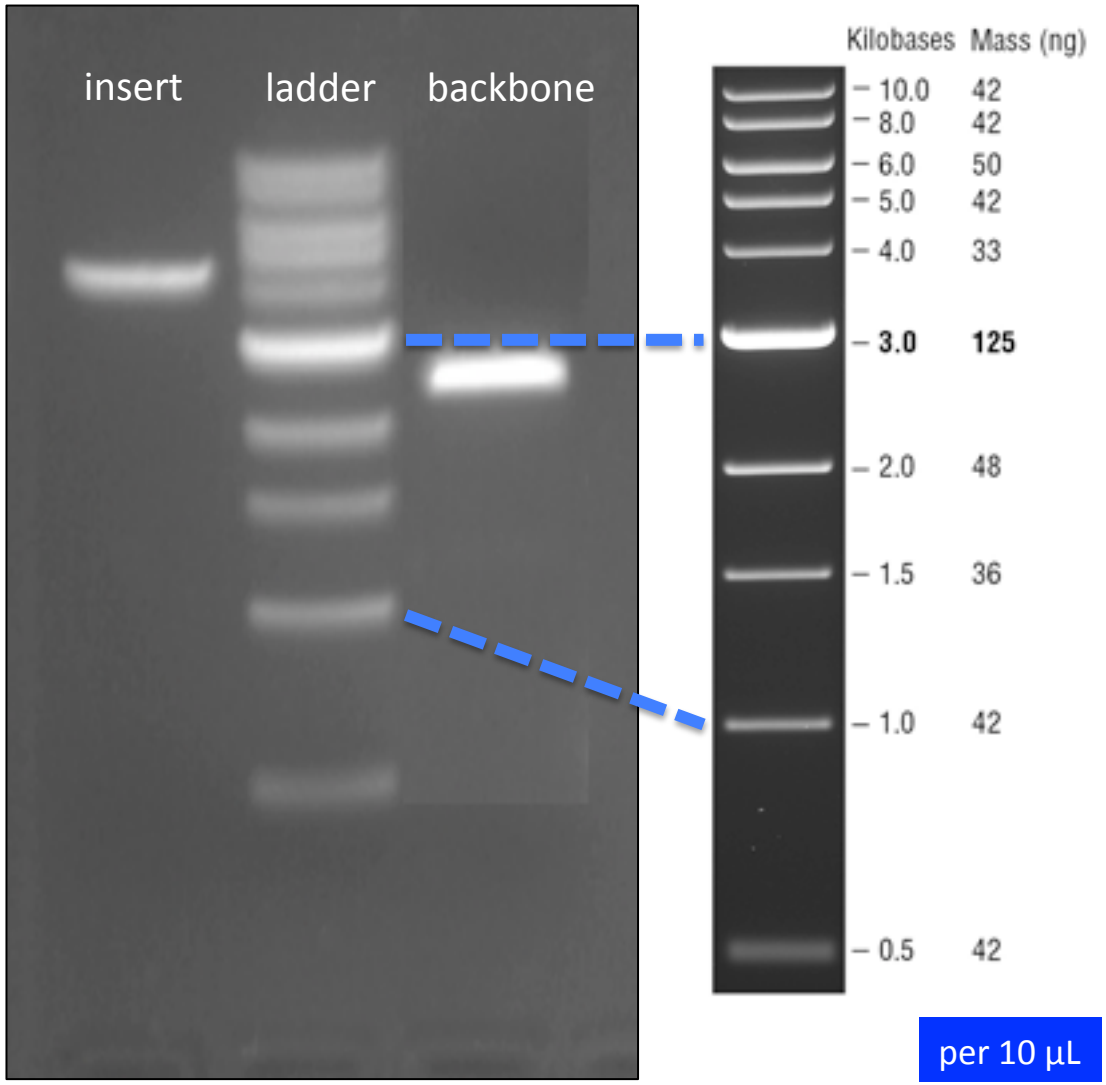


pdCas9 cloning strategy



recovery gel

For ligation, mix 1:4 *molar* backbone : insert



- Assuming
  - 10 µL of ladder loaded,
  - 5 µL of double digests,
- amount of backbone =
  375 ng 3X 125
- amount of insert =
  250 ng 2X125

➢ but mass of DNA ≠ molar amount of DNA

Calculate the 1:4 *molar* amounts for ligation

base pair=660Da=660g/mol, solve for moles

1. From recovery gel, estimate
   - backbone: 375 ng / 5 µL = 75 ng/µL BKB
   - insert: 250 ng / 5 µL = 50 ng/µL INS

2. Determine volume of backbone needed
   - 50-100 ng, choose 50 ng, *i.e.* 0.67 µL

   50-100ng suggestion from the ligase protocol

3. Calculate moles of backbone
   - 2636 bp * (660 g / (mol*bp)) = 1.74 x 10^6 g/mol MW BKB
   - so 50 ng / (1.74 x 10^6 g/mol) = 2.9x10^-14 mol

   5x10^-8g

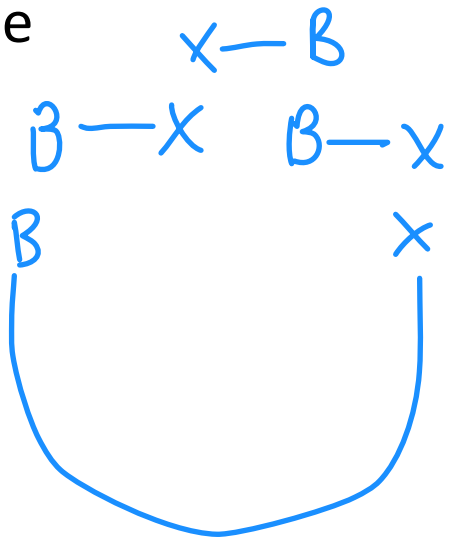
4. Determine moles of insert needed (4x BKB)
   - 4 x 2.9x10^-14 ~ 1.15 x 10^{-13} mol what we want, insert
   - with 4107 bp * (660 g / (mol*bp)) = 2.7 x 10^6 g/mol MW INS
   - so use 1.15 x 10^{-13} mol * 2.7 x 10^6 g/mol ~ 310ng

   ng/(g/mol)=mol

5. Calculate volume of insert needed
   - 310ng / (50 ng/µL) =6.2 µL plus 0.67ul backbone, ligase and ligase buffer

Optimal backbone-to-insert ratio

- ideally, want 1:4 backbone : insert
  - molar ratio, ***not*** mass or volume



- What if too much insert?

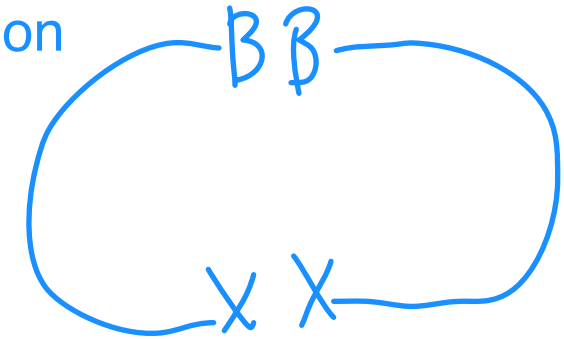
  100X

  tandem inserts
  unsuccessful ligation

- What if too much backbone?

  100X

  giant backbone
  unsuccessful ligation



Separate DNA by gel electrophoresis

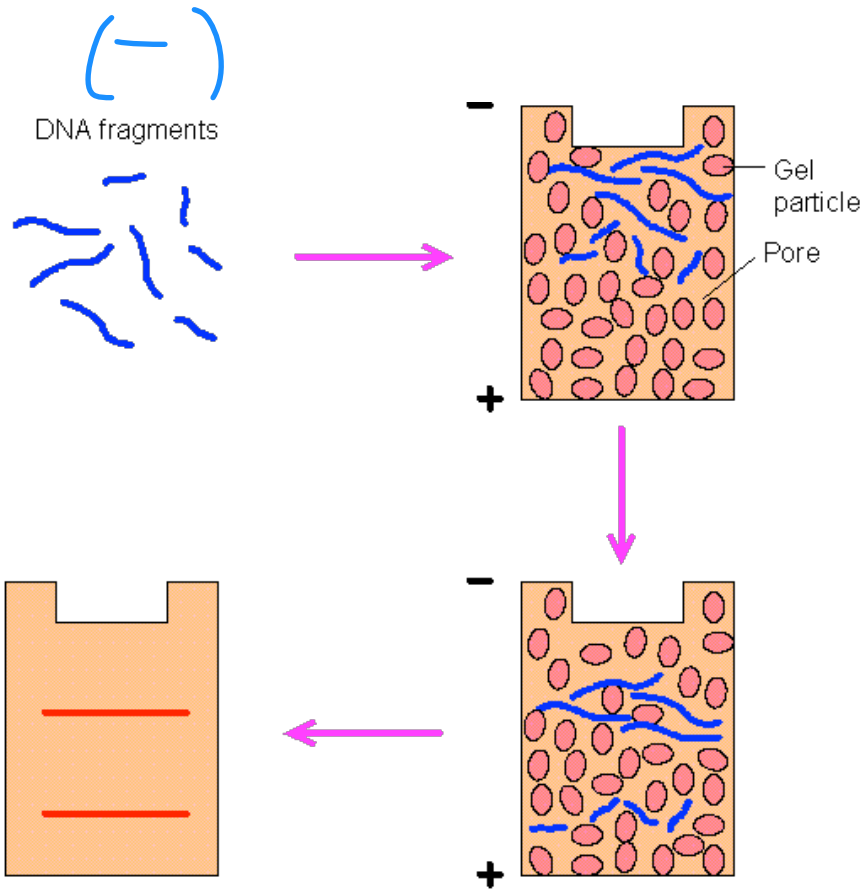
- Agarose gel electrophoresis

  – driving force:

  charge

  – separates DNA by:

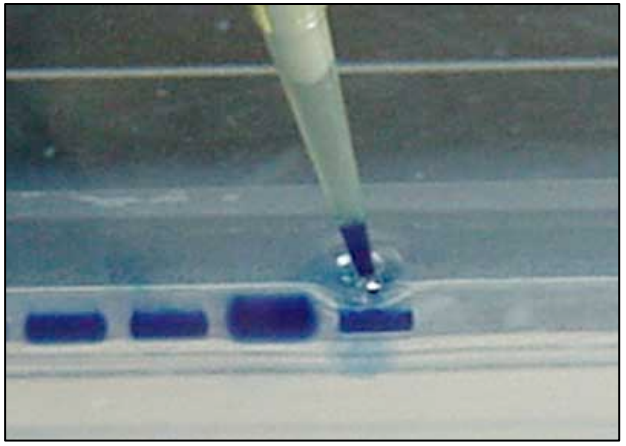
  size



Visualize DNA + save a picture!

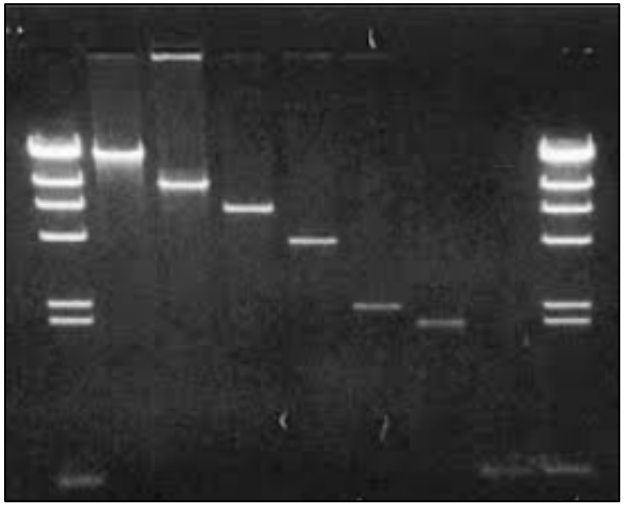
- DNA Loading dye (6X):

bromophenol blue: dye migrates ~500bps
glycerol: increase viscosity



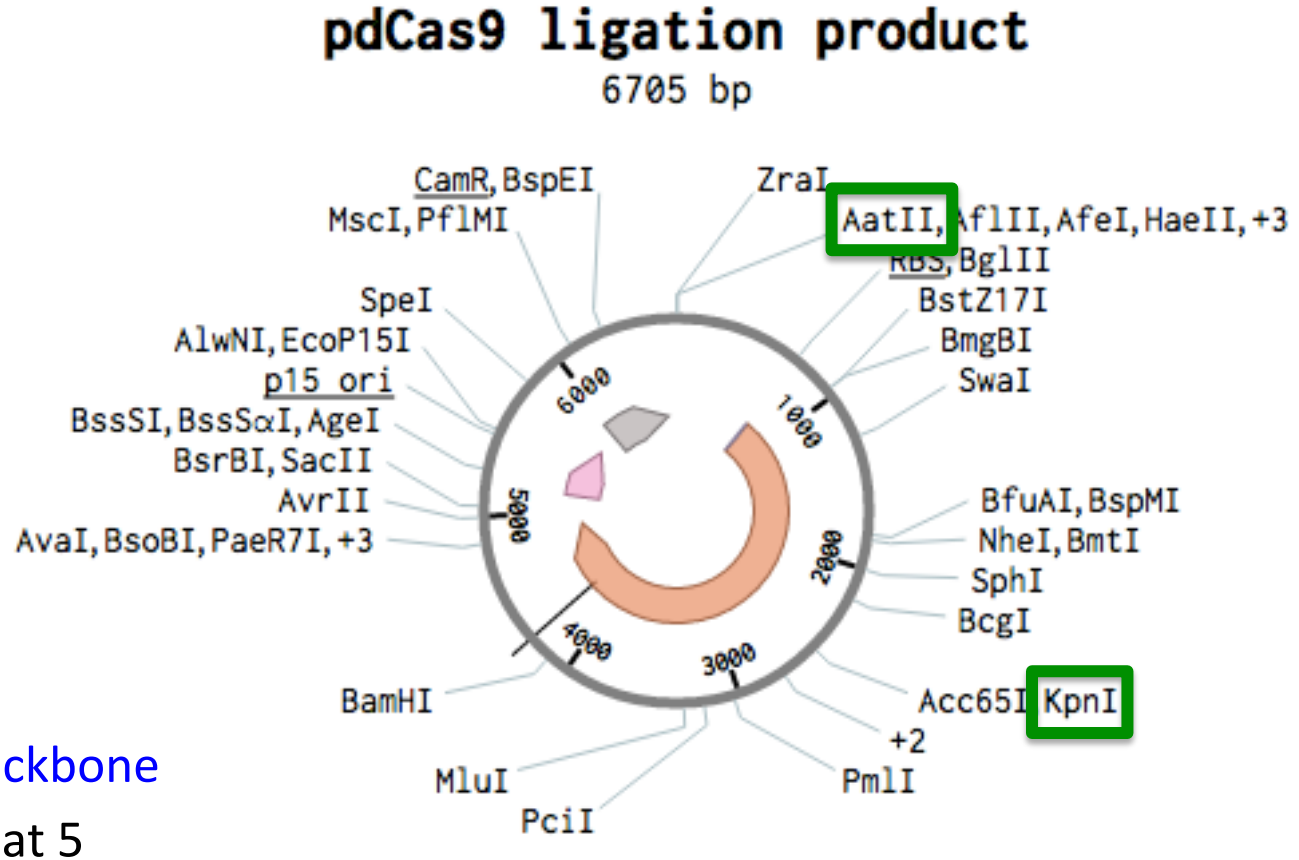
- Sybr-Safe DNA stain:

DNA intercalator
visualize by UV or blue light



➢ Safety : wear nitrile gloves

Confirmation digest example



- Goal:
  - 1 cut only in backbone

    AatII cuts at 5
  - 1 cut only in insert

    KpnI-HF cuts at 2517
- Fragments distinguishable? yes: 2512 bp *vs.* 4193 bp
- Compatible? yes: in CutSmart buffer

Confirmation digest example

- Goal:
  - 1 cut only in backbone

    AatII cuts at 5
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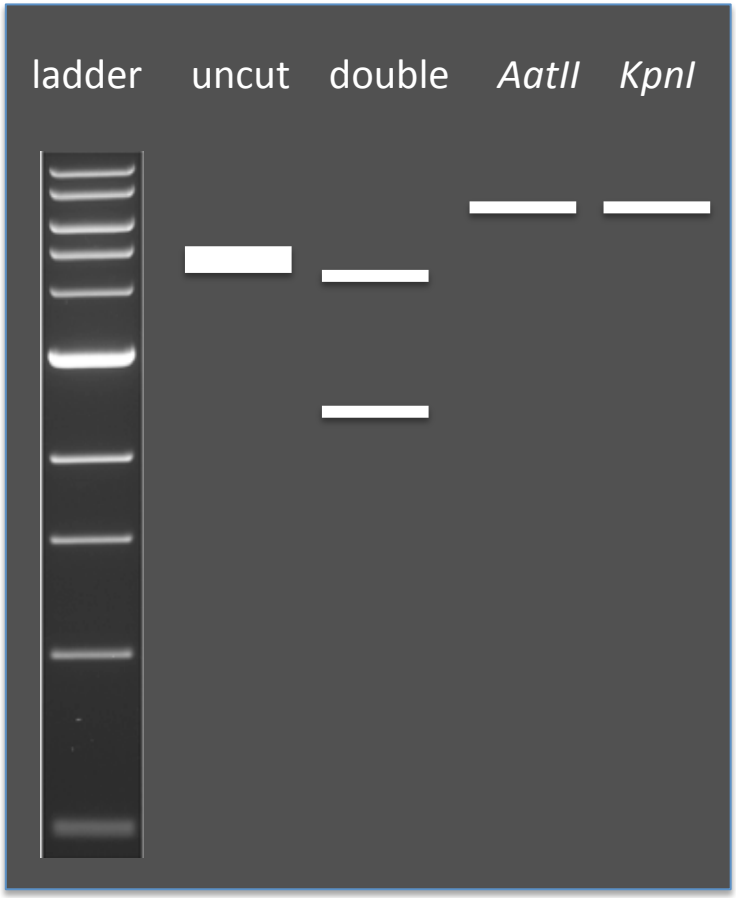
    KpnI-HF cuts at 2517
- Fragments distinguishable?
- Compatible?

- Expectation:

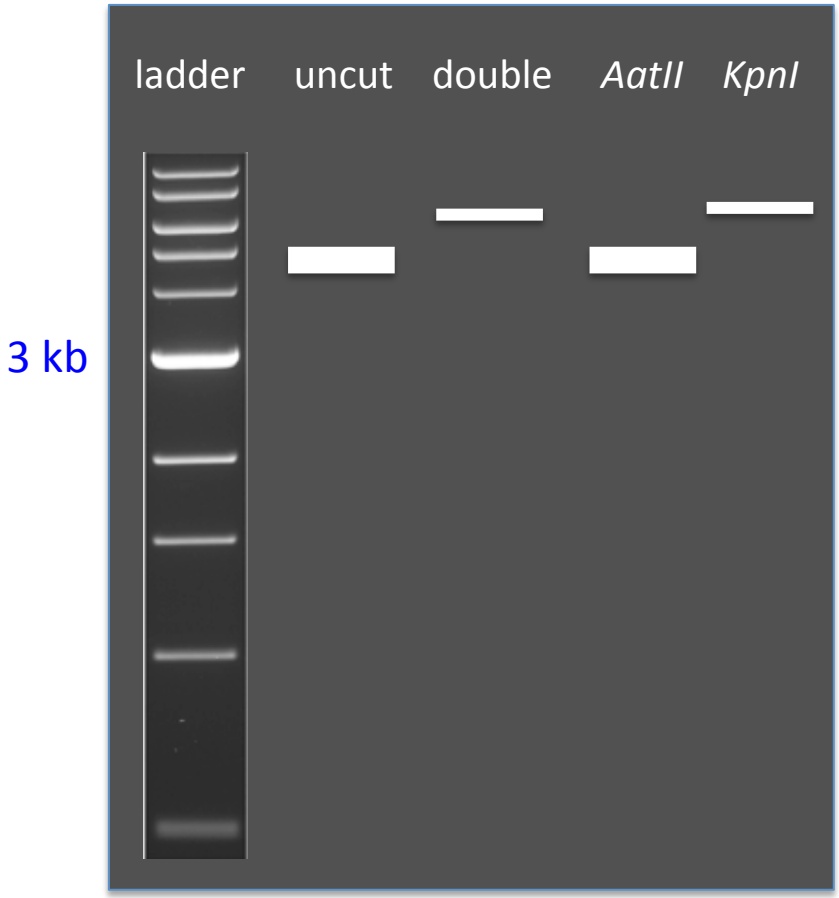


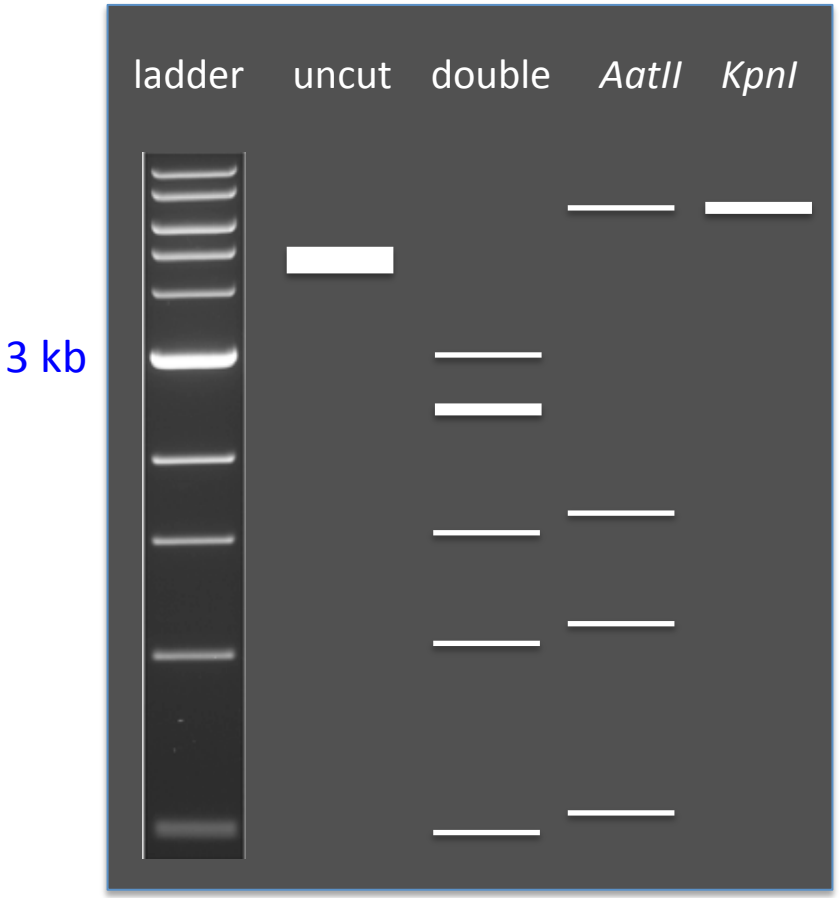
yes: 2512 bp *vs.* 4193 bp

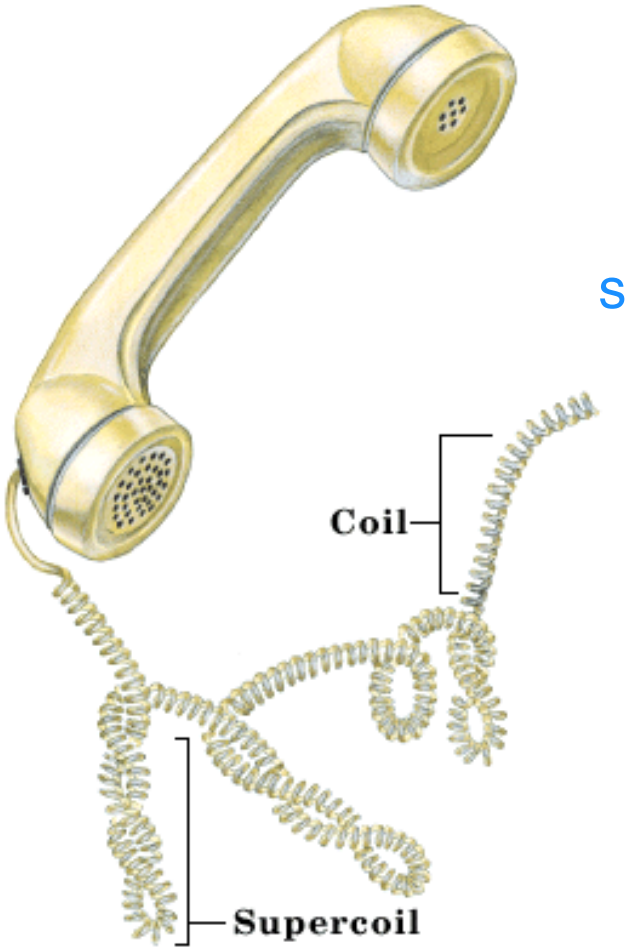
yes: in CutSmart buffer

Confirmation digest – Why do we run single cut and uncut controls?

- What if we observe:



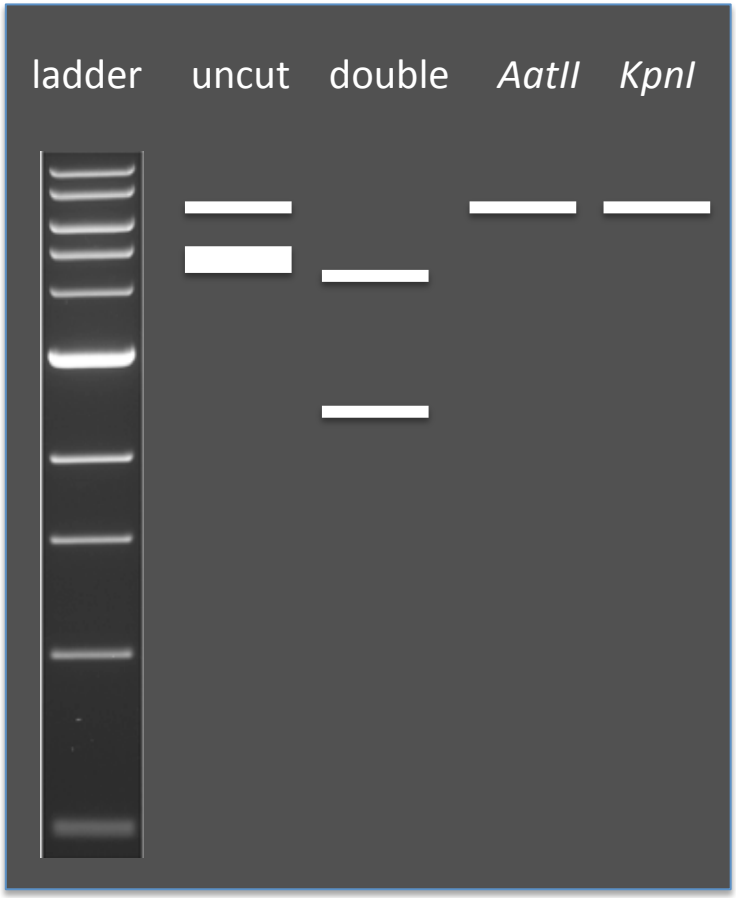




nicked

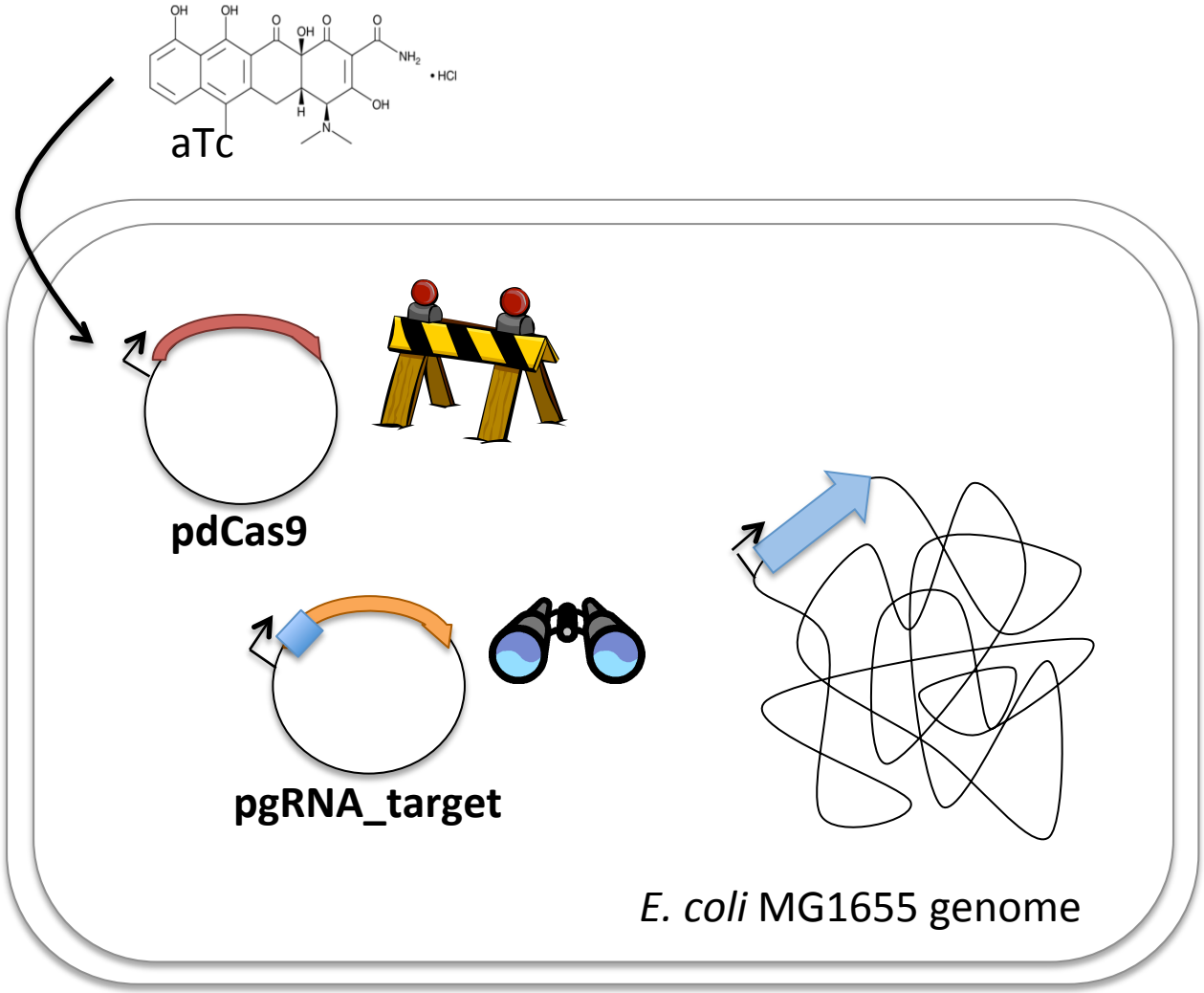
supercoiled

3 kb



http://chemistry.umeche.maine.edu, DNA and the Double Helix

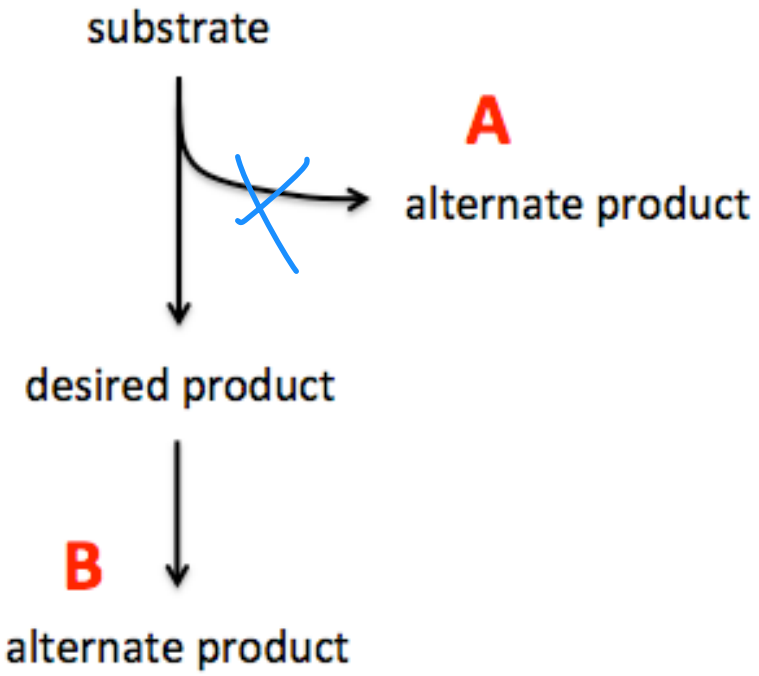
CRISPRi system overview



- Target gene
  increase ethanol or lactate
- pgRNA_target
  guide RNA, target to specific genomic seq.
- pdCas9
  dead scissors

Noreen's lecture M2D1

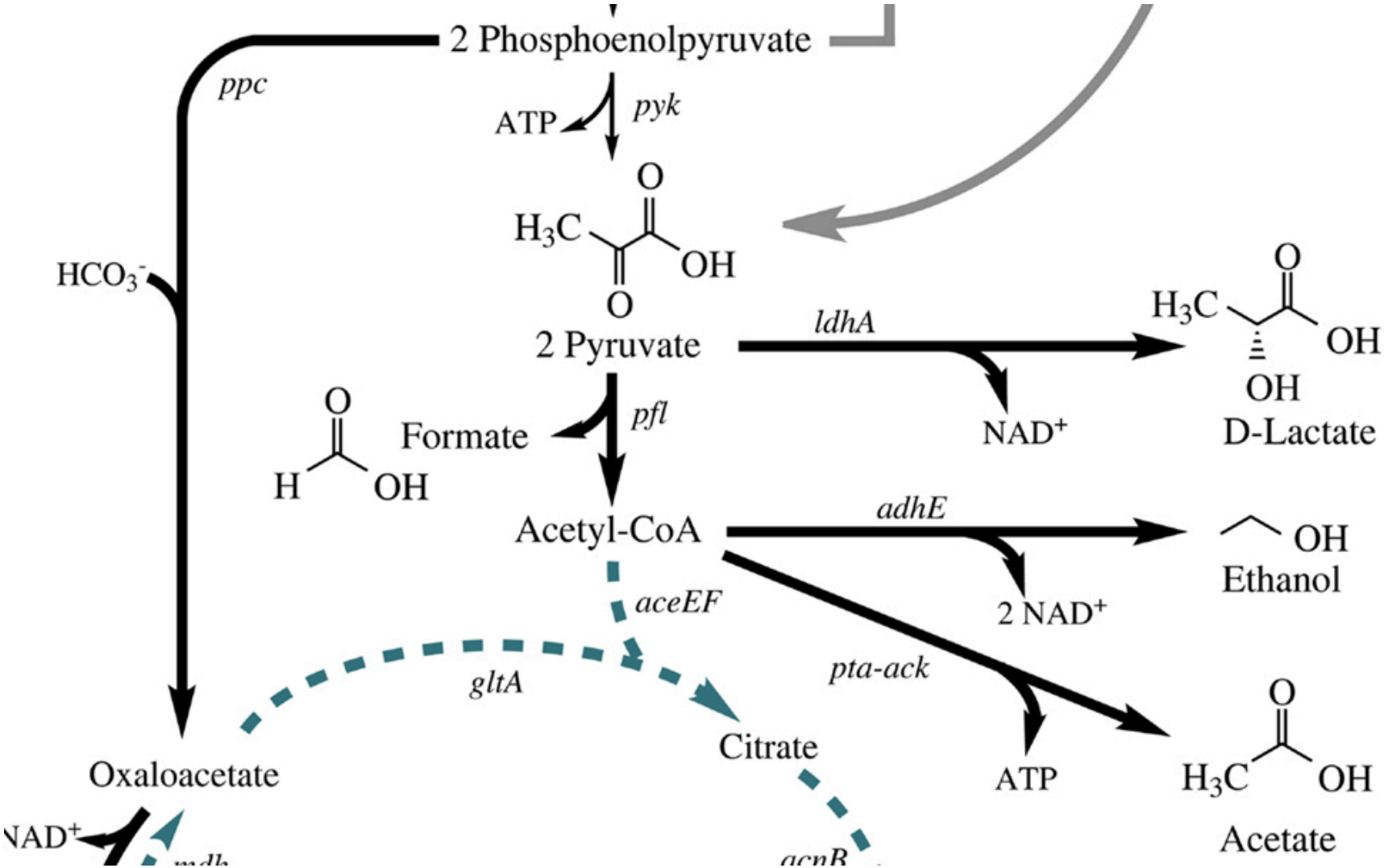
How would you increase yield of the desired product?



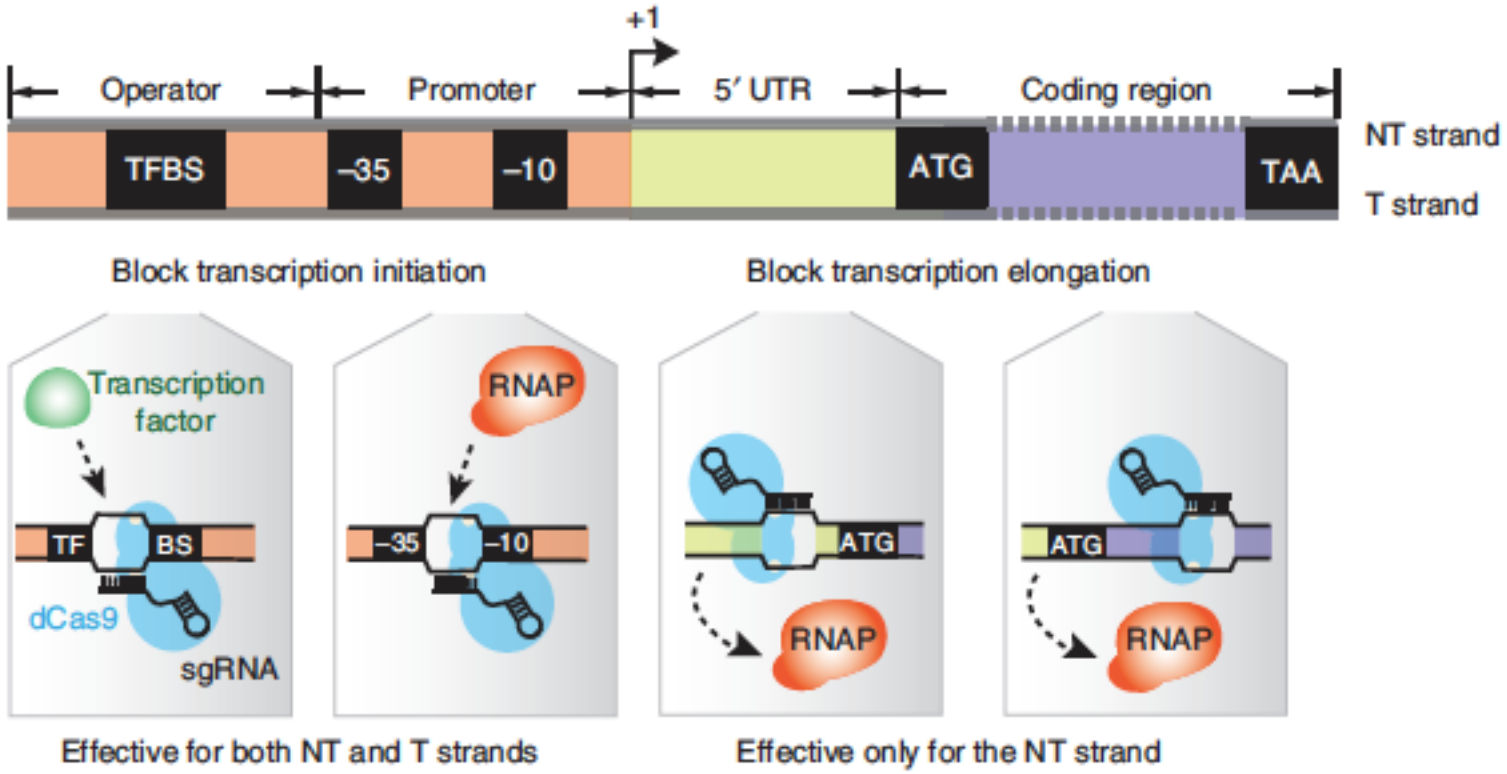
HYPOTHESIS:

We hypothesize that inhibiting production of enzyme A will decrease alternate product A and increase desired product.

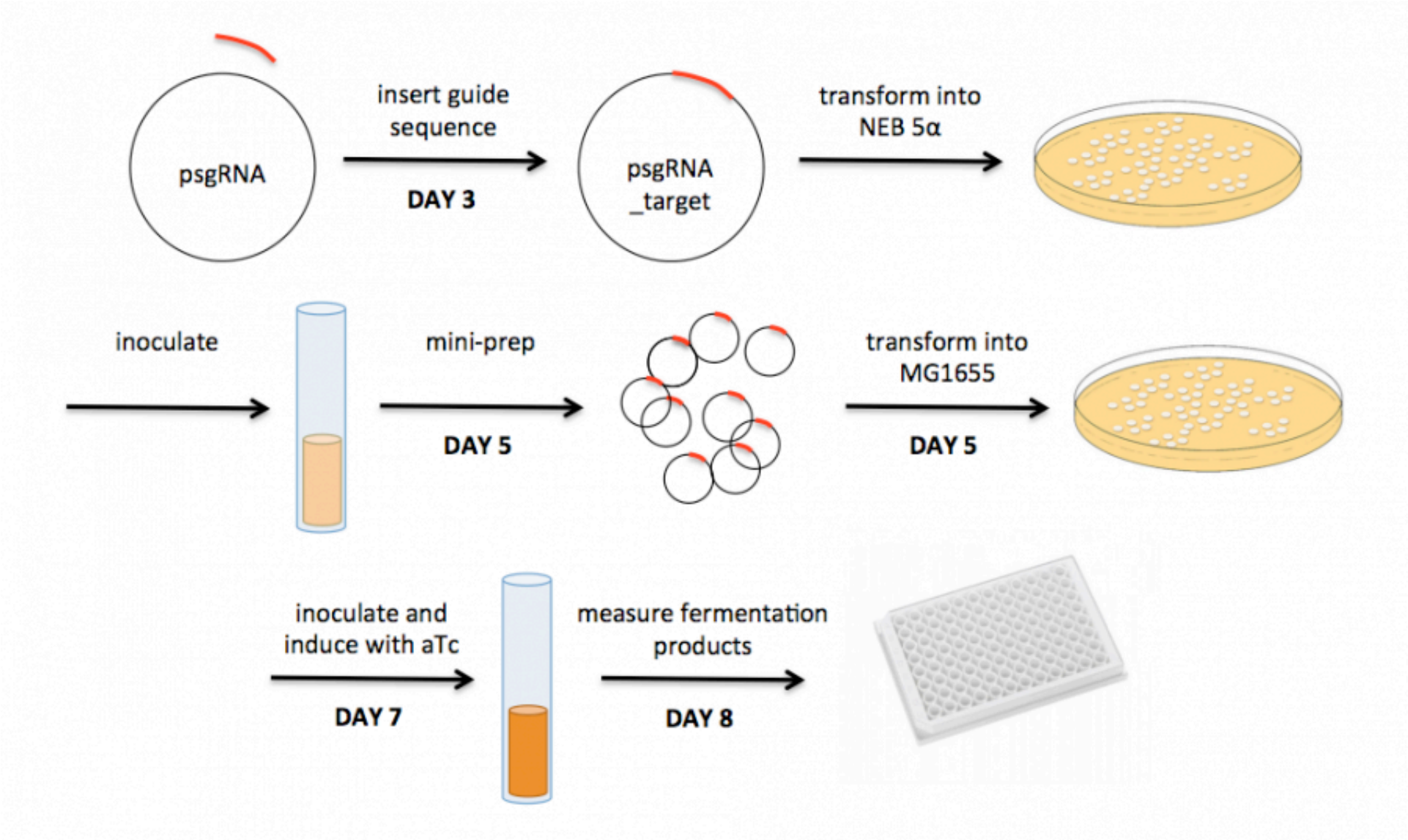
Using CRISPRi manipulate the *E. coli* fermentation pathway



Design of gRNA for CRISPRi system



M2 experimental overview



Today in lab…

- Run dCas9 confirmation digests on agarose gel
- Develop your Mod2 hypothesis and select *E. coli* fermentation pathway protein to target
- Design gRNA to target for CRISPRi system
- Read before M2D3:

ACS Synthetic Biology

Research Article

pubs.acs.org/synthbio

CRISPR Perturbation of Gene Expression Alters Bacterial Fitness under Stress and Reveals Underlying Epistatic Constraints

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M2D3 HW: Figure/Caption/Results

- all text in paragraph form
- result subsections need titles (take-home message)
- minimize interpretation in the results section in Mod2 report (separate discussion section; *M2D5HW*)
  - present data with guiding framework in an unbiased way
- figure/text expectations same as Mod1 report, formatting major difference