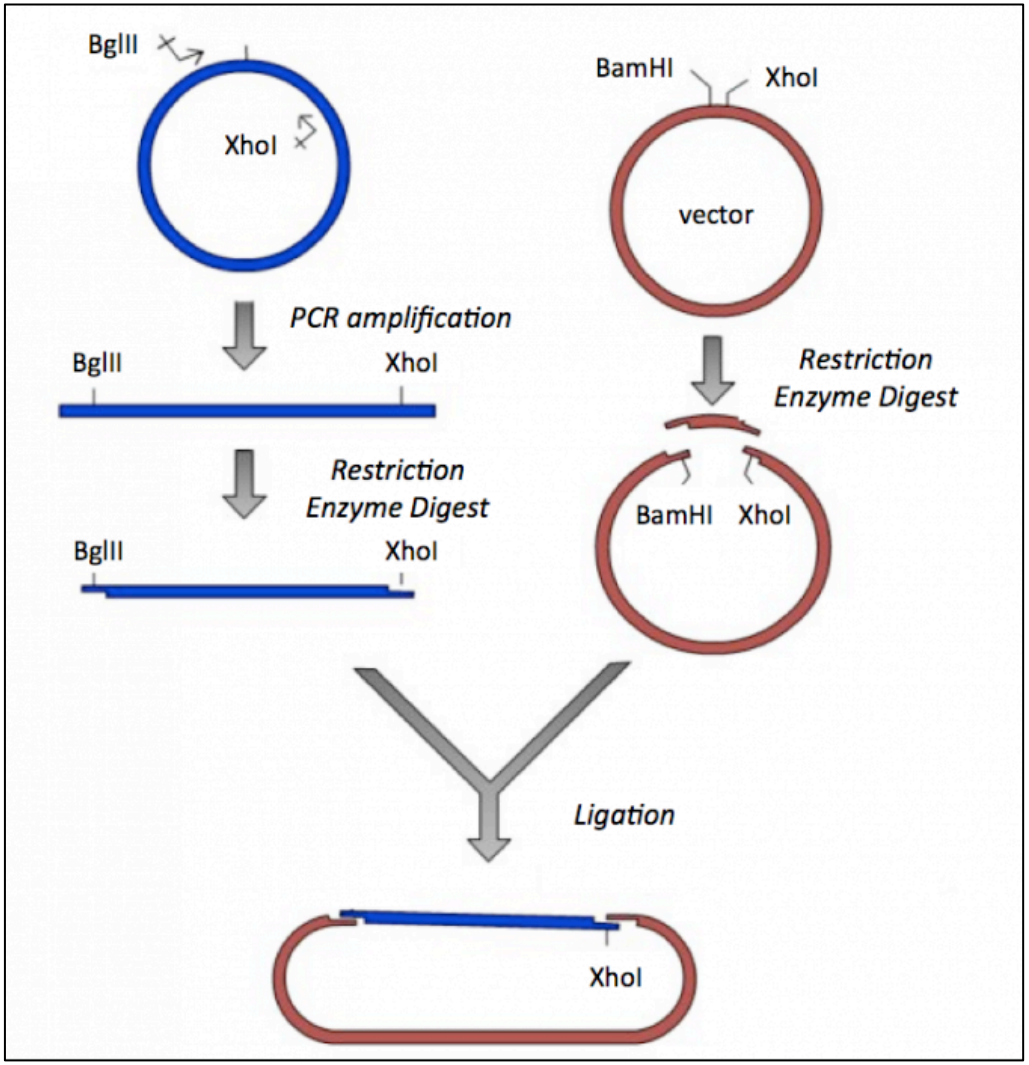
M2D2: Design gRNA for CRISPRi

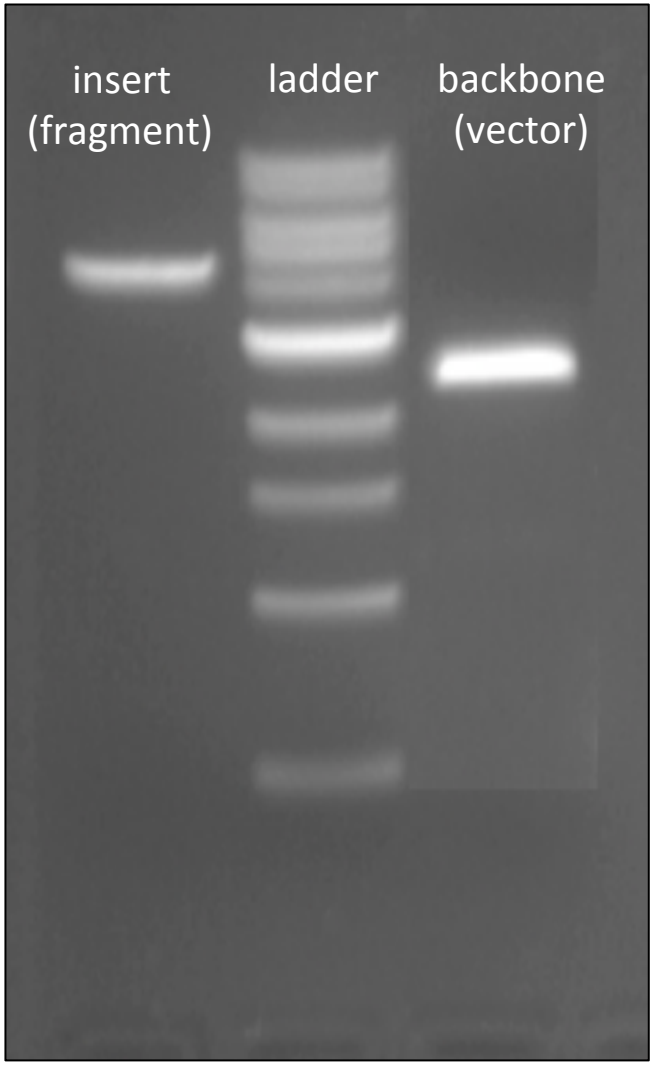
10/17/17

1. Pre-lab Discussion
2. Load digests from M2D1 in agarose gels
3. Mid-lab discussion: gRNA design considerations
4. Select target gene in fermentation pathway
5. Design gRNA for CRISPRi system

Review:pdCas9 was constructed by ligation



pdCas9 cloning strategy



recovery gel

Goal: Calculate volumes of insert and backbone needed for ligation

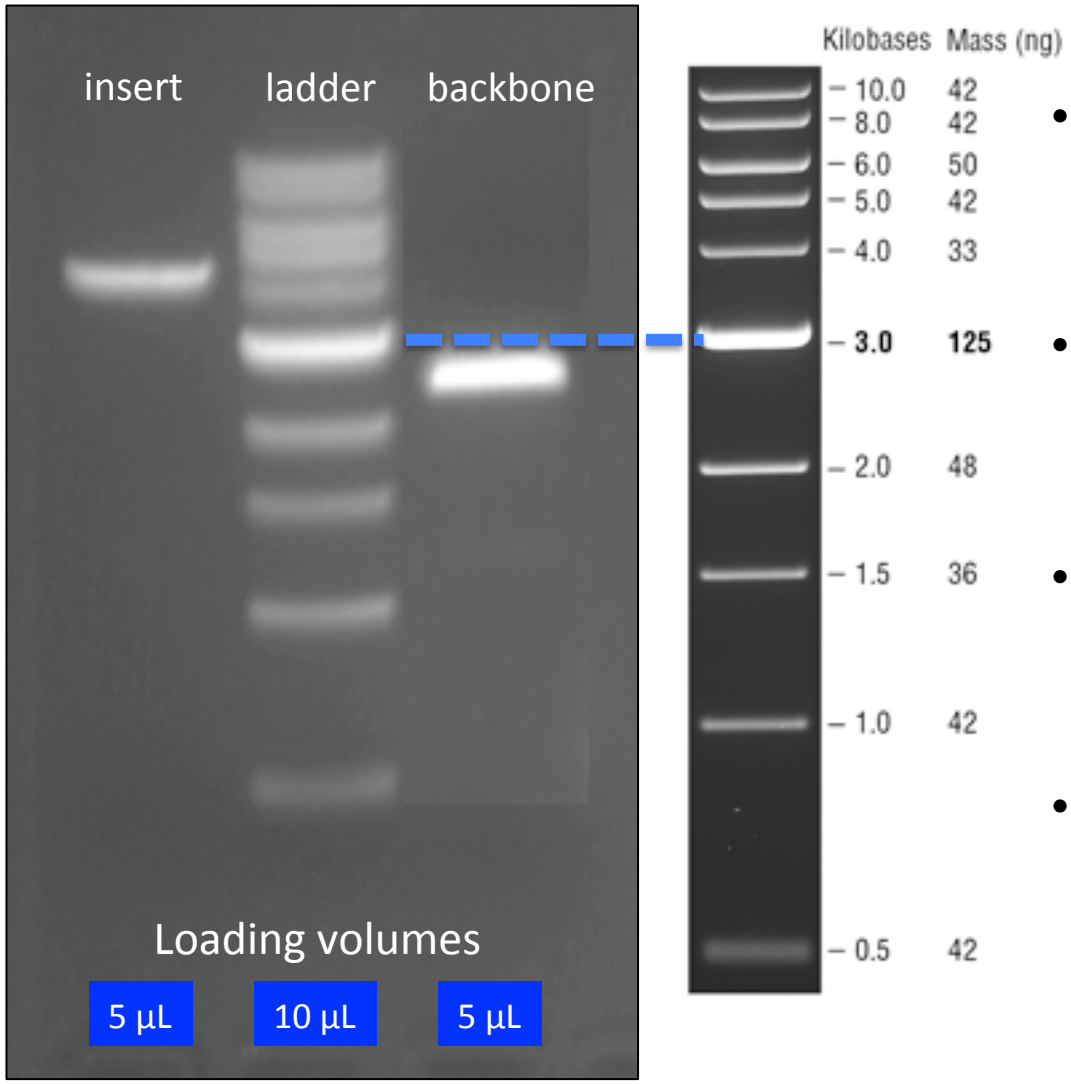
Knowns:

- Need 50-100 ng backbone (from ligase protocol) 1ul backbone
- Backbone: 2592 bp
- Insert: 4113 bp
- Molar mass of base pair(bp) ~660 Daltons =g/(mol*bp)
- Desired molar ratio of insert to backbone is 4:1

Unknowns:

- Concentration of insert: 25ng/ul
- Concentration of backbone: 50ng/ul
- Need to know what **volumes** of backbone and insert to use in ligation reaction:

Use recovery gel to estimate insert and backbone concentrations



- Amount of backbone = **250** ng

  2X intensity

- Amount of insert = **125** ng

  1X intensity

- Concentration of backbone =

  250ng/5ul=50ng/ul

- Concentration of insert =

  125/5=25ng/ul

(concentration) mass of DNA ≠ molar amount of DNA

Calculate 4:1 (insert:backbone) *molar* amounts final volumes for ligation

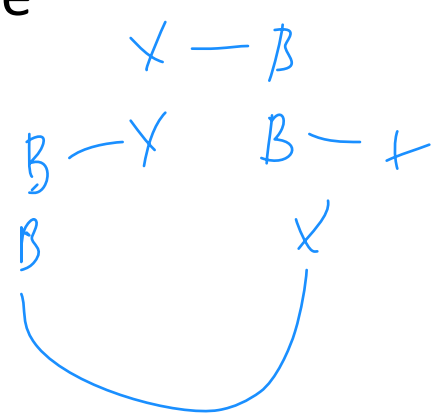
1. Calculate moles of backbone
   - 2592 bp * (660 g / (mol*bp)) = 1.71 x 10^6 g/mol
   - so 50 (5x10^-8g) ng / (1.71 x 10^6 g/mol) = 2.9x10^-14 mol
2. Determine moles of insert needed (4x backbone)
   - 4 x 2.9x10^-14 ~ 1.2 x 10^{-13} mol
   - with 4113 bp * (660 g / (mol*bp)) = 2.7 x 10^6 g/mol
   - so use 1.2 x 10^{-13} mol * 2.7 x 10^6 g/mol ~ 310 ng
3. Calculate volume of backbone and insert needed
   - Backbone: 50 ng/(50 ng/uL) = 1 uL
   - Insert: 310 / (25 ng/µL) = 12.4 µL

Ligation Protocol with T4 DNA Ligase (NEB)

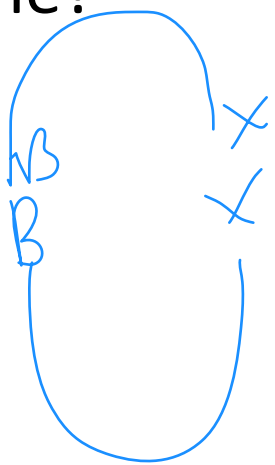
COMPONENT	20 µl REACTION	
T4 DNA Ligase Buffer (10X)*	2 µl	2ul buffer
Vector DNA	50 ng	1ul bkb
Insert DNA		12.4ul insert
Nuclease-free water	to 20 µl	3.6ul H2O
T4 DNA Ligase	1 µl	1ul ligase

Optimal backbone-to-insert ratio

- Ideally, want 4:1 insert:backbone
  - molar ratio, ***not*** mass or volume
- What if too much insert?



- What if too much backbone?



Separate DNA by gel electrophoresis

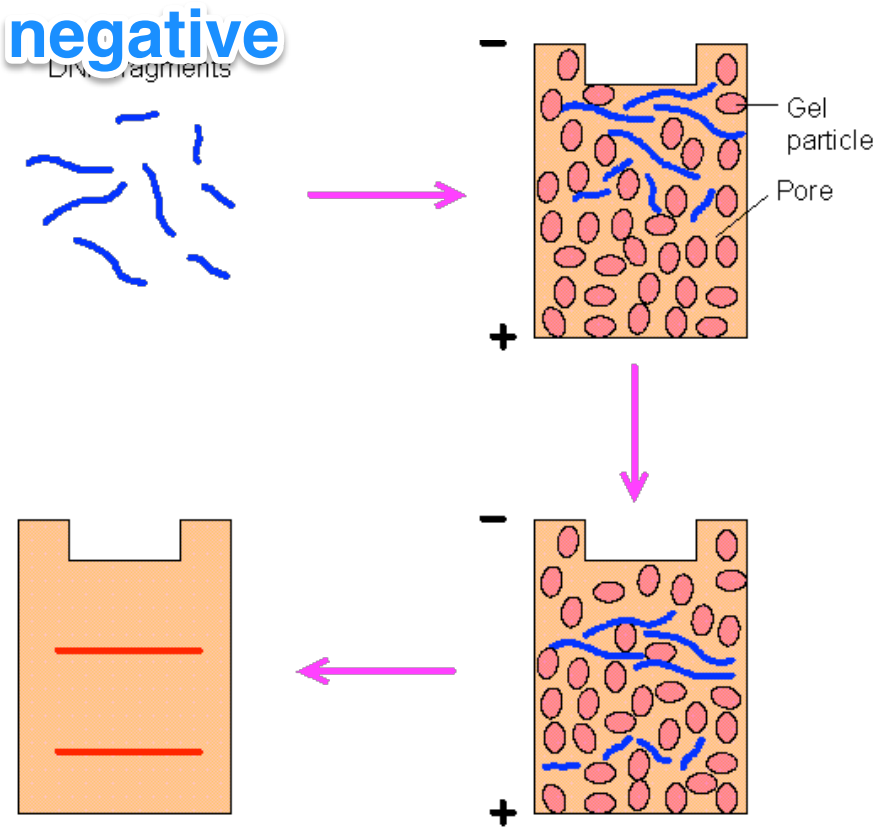
- Agarose gel electrophoresis

  – driving force:

  charge, electric field

  – separates DNA by:

  size



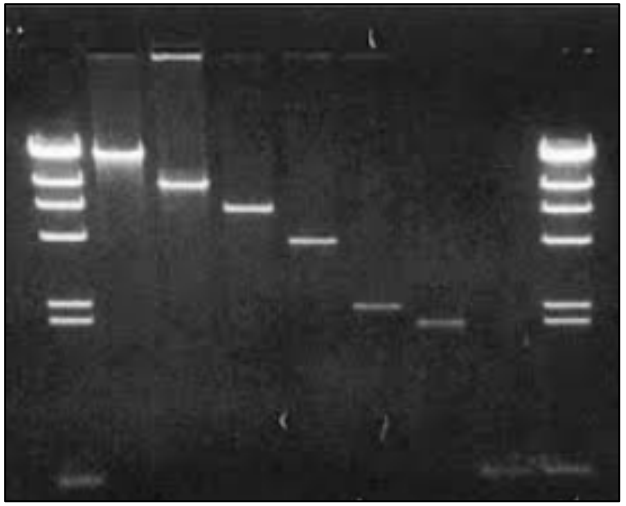
Visualize DNA + save a picture!

- DNA Loading dye (6X):

bromophenol blue: small molecule run at 500bps
glycerol: increase viscosity, DNA sinks

- Sybr-Safe DNA stain:

DNA intercalator
visualize via UV or blue light

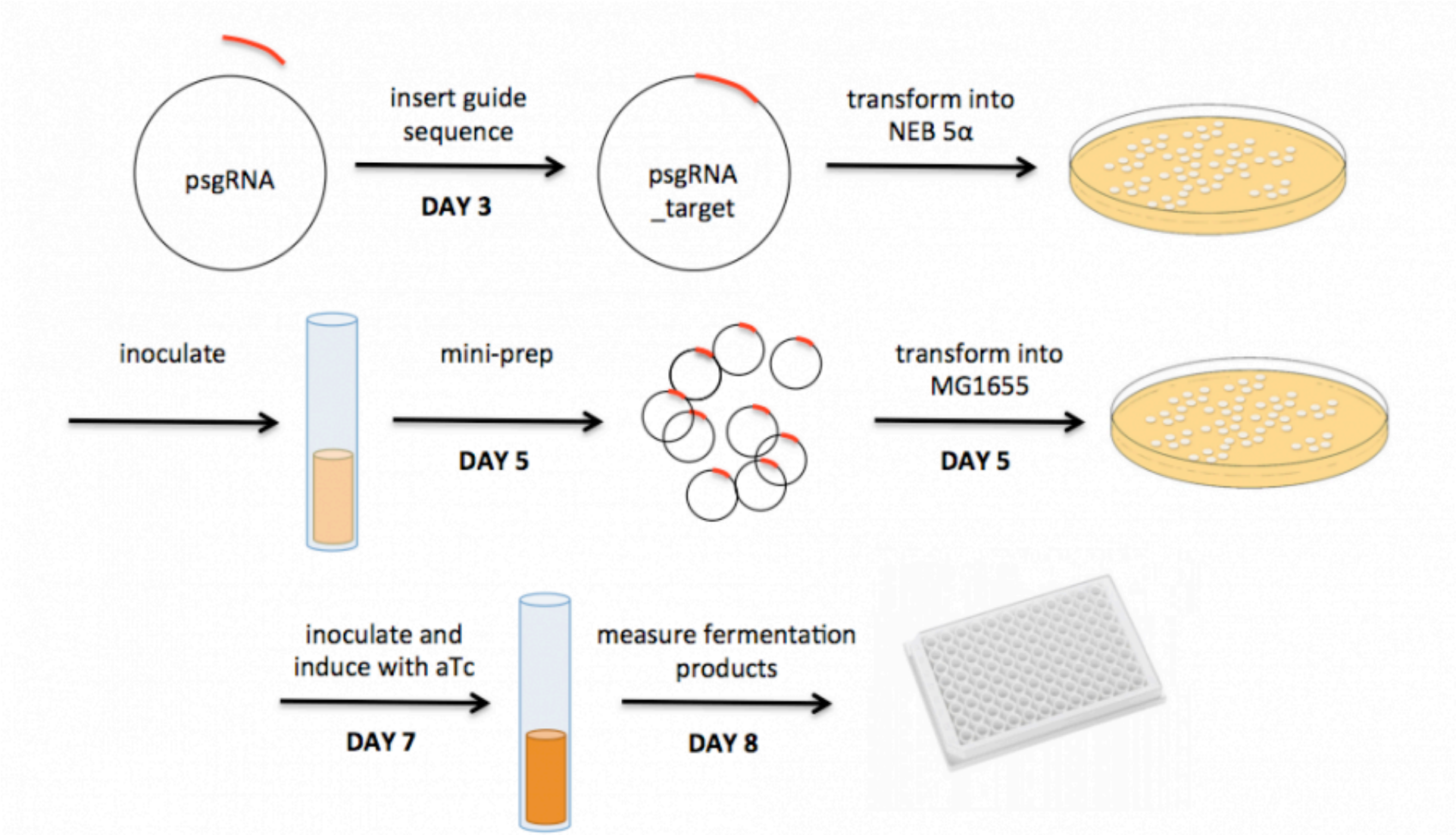


➢ Safety : wear nitrile gloves

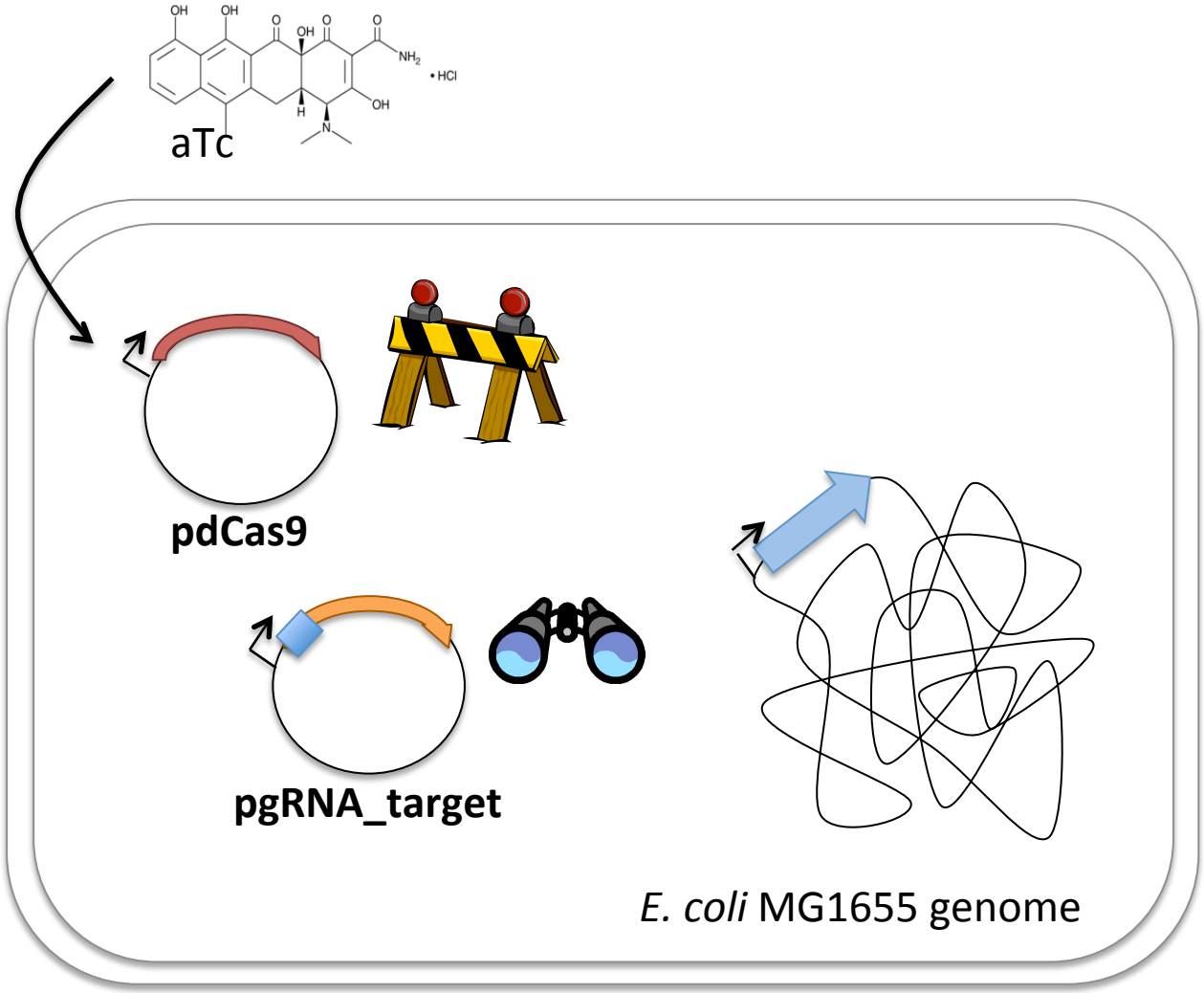
Today in lab…

1. Pick up digest from the front bench and prepare samples to be loaded on the agarose gels
2. Four groups at a time can load their samples and we will start gels after 2 groups per gel are loaded
3. Regroup for another short discussion before moving onto Part 2

Mod 2 experimental overview



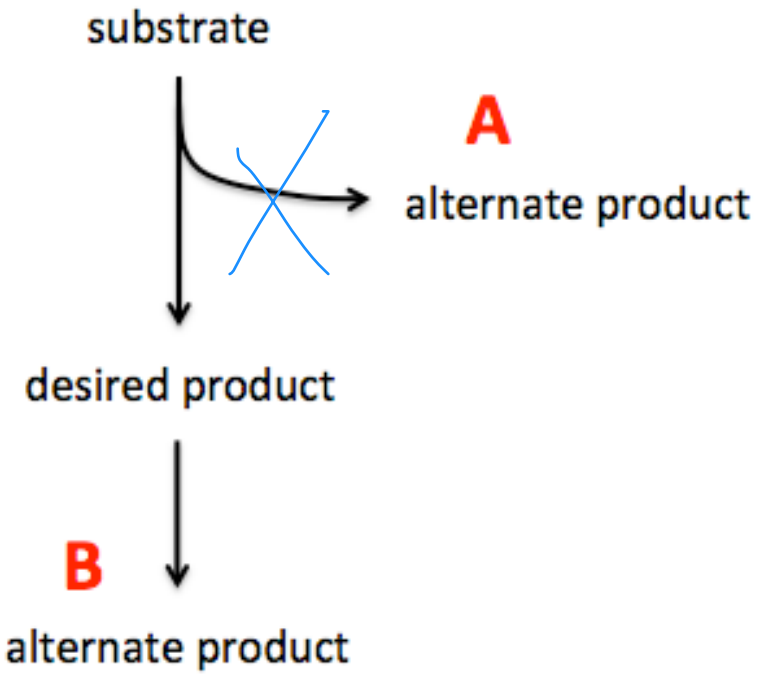
CRISPRi system overview



- Target gene
  increase ethanol or acetate production
- pgRNA_target
  express gRNA to specific gene
- pdCas9
  block of expression

Noreen's lecture M2D1

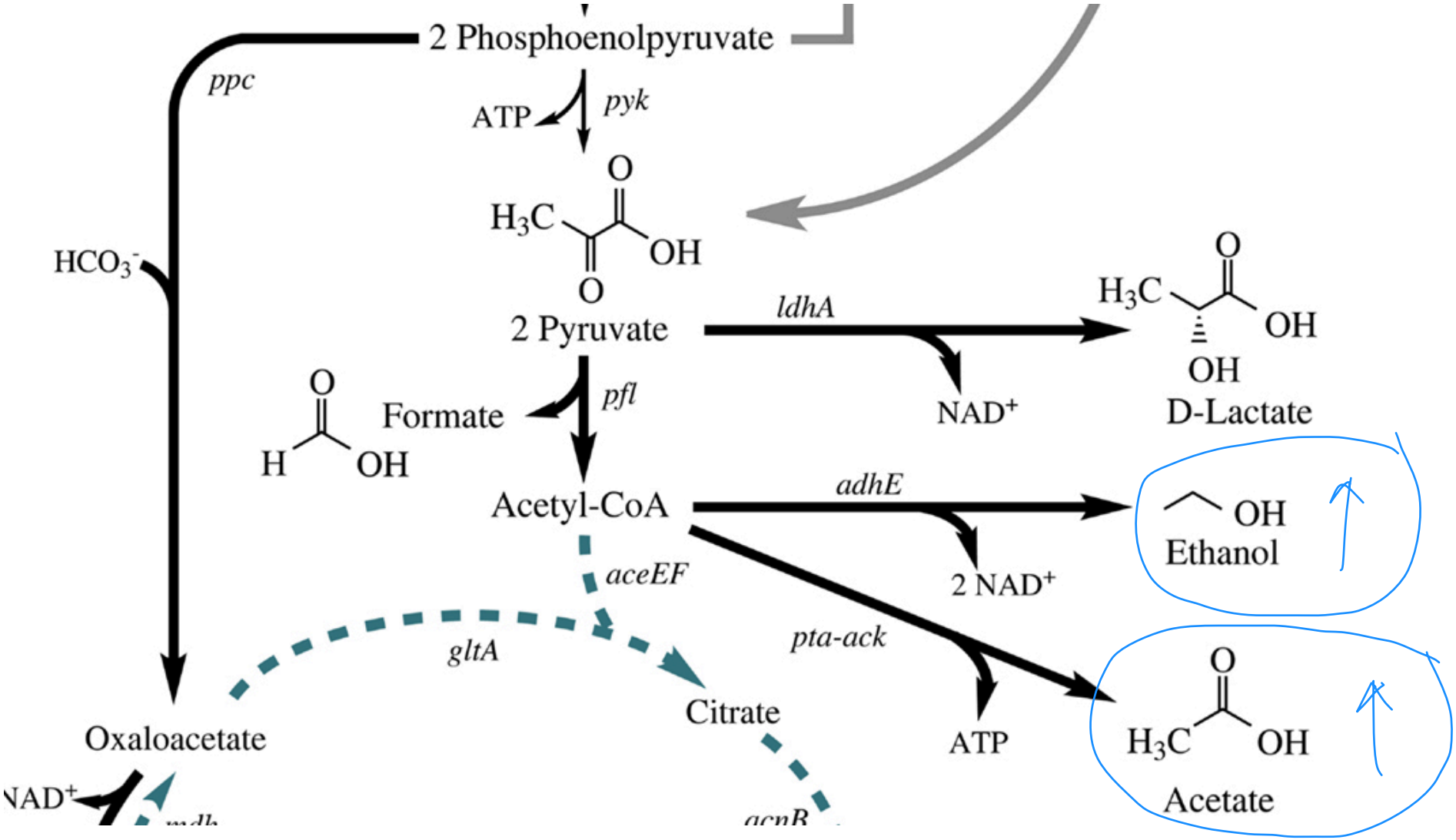
How would you increase yield of the desired product?



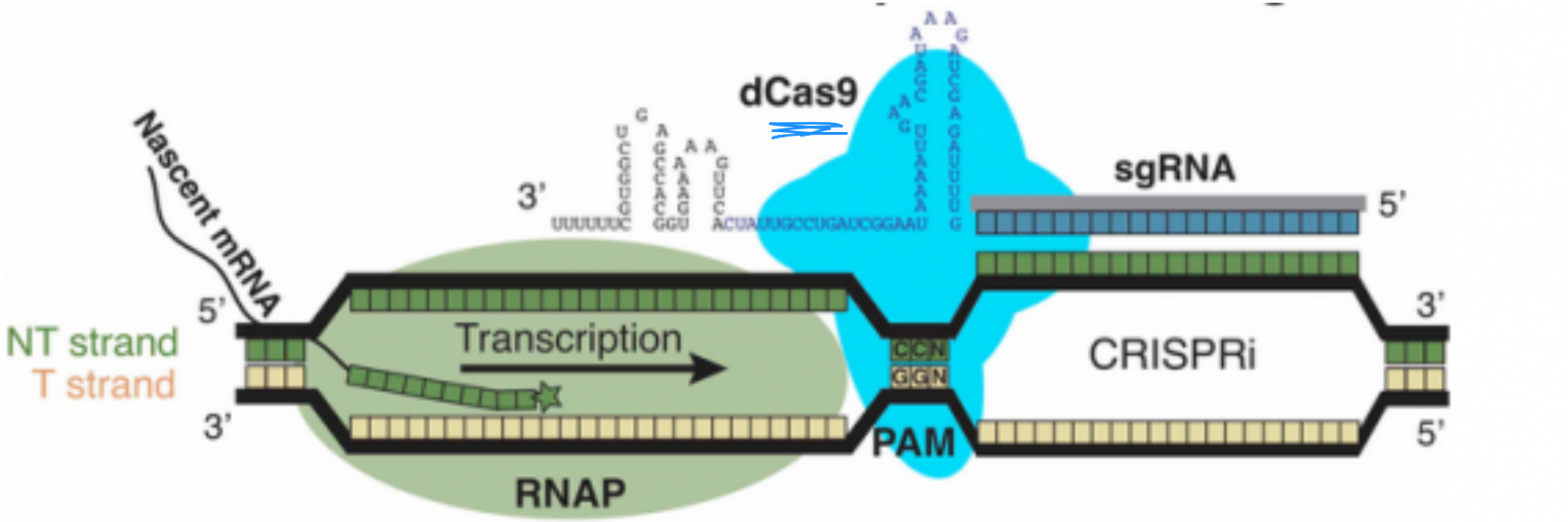
HYPOTHESIS:

We hypothesize that inhibiting production of enzymeA will decrease alternative product A and increase desired product.

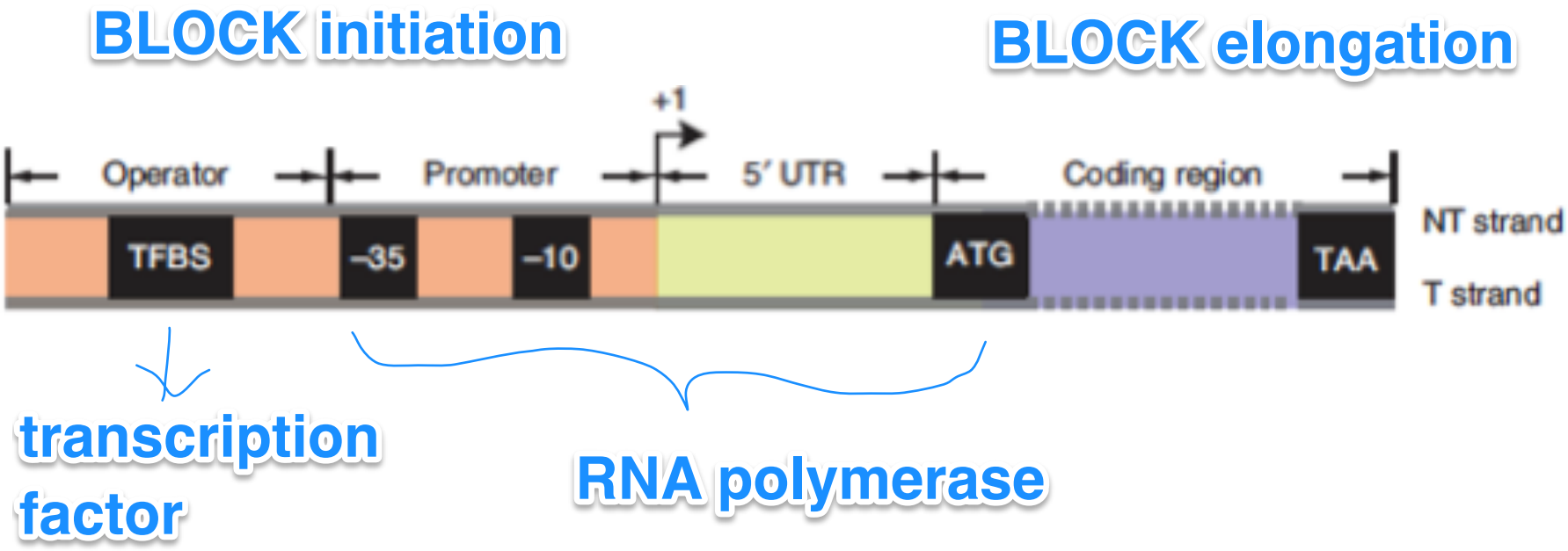
Using CRISPRi manipulate the *E. coli* fermentation pathway



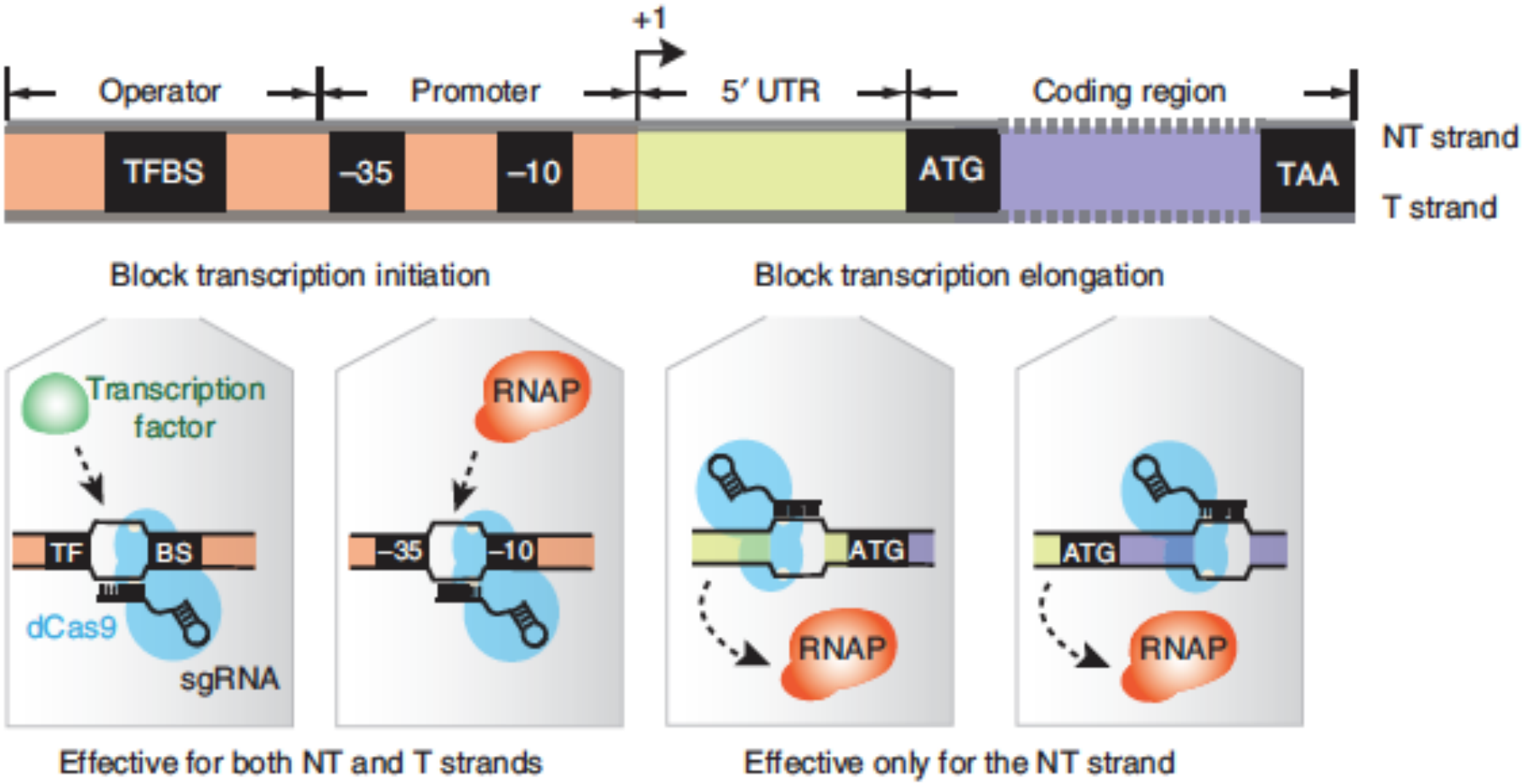
CRISPRi system can block the RNA polymerase



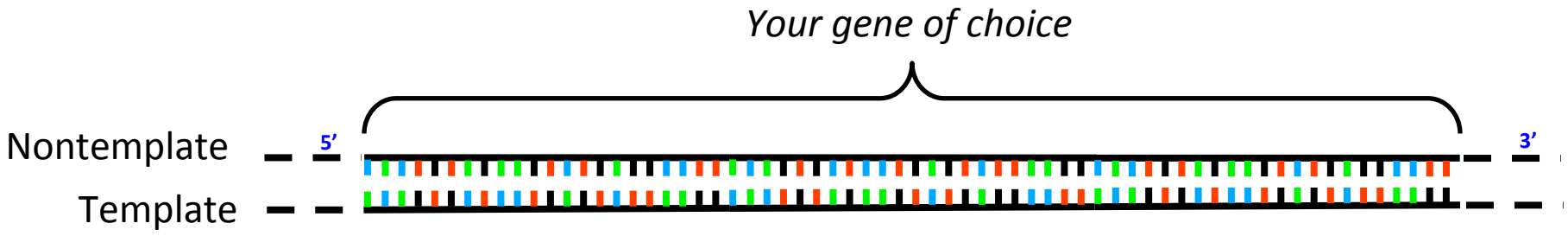
Let's review binding partners:



Which region of the gene will you target?

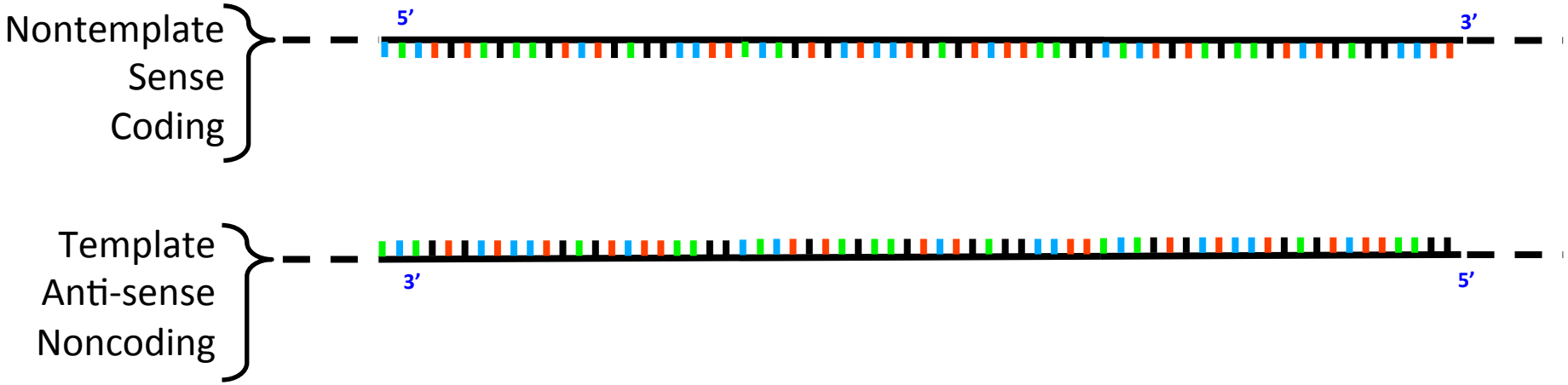


Design of gRNA for CRISPRi system



(1) If you target the template DNA strand, the gRNA sequence will be the same as the transcribed sequence.

(2) If you target the nontemplate strand, the gRNA sequence will be the reverse-complement of the transcribed sequence.



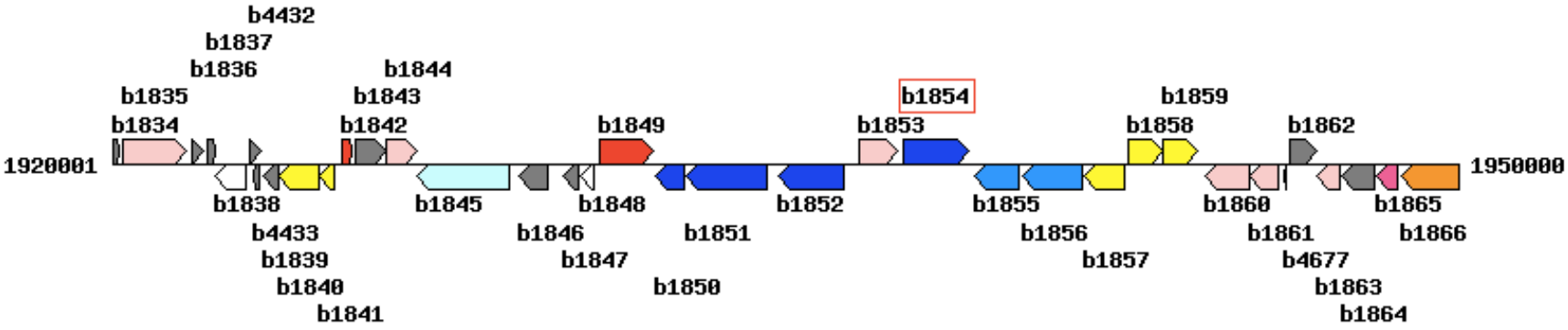
Practically: Using KEGG Database

Position	1937649..1939091 Genome map
AA seq	480 aa AA seq DB search MSRRLRRTKIVTTLGPATDRDNNLEKVIAAGANVVRMNFSHGSPEDHKMRADKVREIAAK LGRHVAILGDLQGPKIRVSTFKEGKVFLNIGDKFLLDANLGKGEGDKEKVGIDYKGLPAD VVPGDILLLDDGRVQLKVLEVQGMKVFTEVTVGGPLSNNKGINKLGGGLSAEALTEKDKA DIKTAALIGVDYLAVSFPRCGEDLNYARRLARDAGCDAKIVAKVERAEAVCSQDAMDDII LASDVVMVARGDLGVEIGDPELVGIQKALIRRARQLNRAVITATQMMESMITNPMPTRAE VMDVANAVLDGTDAVMLSAETAAGQYPSETVAAMARVCLGAEKIPSINVSKHRLDVQFDN VEEAIAMSAMYAANHLKGVTAIITMTESGRTALMTSRISSGLPIFAMSRHERTLNLTALY RGVTPVHFDSANDGVAAASEAVNLLRDKGYLMSGDLVIVTQGDVMSTVGSTNTTRILTVE
NT seq	1443 nt NT seq +upstream 0 nt +downstream 0 nt atgtccagaaggcttcgcagaacaaaaatcgttaccacgttaggcccagcaacagatcgc gataataatcttgaaaaagttatcgcggcgggtgccaacgttgtacgtatgaacttttct cacggctcgcctgaagatcacaaaatgcgcgcggataaagttcgtgagattgccgcaaaa ctggggcgtcatgtggctattctgggtgacctccaggggcccaaaatccgtgtatccacc tttaaagaaggcaaagttttcctcaatattggggataaattcctgctcgacgccaacctg ggtaaaggtgaaggcgacaaagaaaaagtcggtatcgactacaaaggcctgcctgctgac gtcgtgcctggtgacatcctgctgctggacgatggtcgcgtccagttaaaagtactggaa gttcagggcatgaaagtgttcaccgaagtcaccgtcggtggtcccctctccaacaataaa ggtatcaacaaacttggcggcggtttgtcggctgaagcgctgaccgaaaaagacaaagca gacattaagactgcggcgttgattggcgtagattacctggctgtctccttcccacgctgt ggcgaagatctgaactatgcccgtcgcctggcacgcgatgcaggatgtgatgcgaaaatt gttgccaaggttgaacgtgcggaagccgtttgcagccaggatgcaatggatgacatcatc ctcgcctctgacgtggtaatggttgcacgtggcgacctcggtgtggaaattggcgacccg gaactggtcggcattcagaaagcgttgatccgtcgtgcgcgtcagctaaaccgagcggta atcacggcgacccagatgatggagtcaatgattactaacccgatgccgacgcgtgcagaa gtcatggacgtagcaaacgccgttctggatggtactgacgctgtgatgctgtctgcagaa actgccgctgggcagtatccgtcagaaaccgttgcagccatggcgcgcgtttgcctgggt gcggaaaaaatcccgagcatcaacgtttctaaacaccgtctggacgttcagttcgacaat gtggaagaagctattgccatgtcagcaatgtacgcagctaaccacctgaaaggcgttacg gcgatcatcaccatgaccgaatcgggtcgtaccgcgctgatgacctcccgtatcagctct ggtctgccaattttcgccatgtcgcgccatgaacgtacgctgaacctgactgctctctat cgtggcgttacgccggtgcactttgatagcgctaatgacggcgtagcagctgccagcgaa gcggttaatctgctgcgcgataaaggttacttgatgtctggtgacctggtgattgtcacc cagggcgacgtgatgagtaccgtgggttctactaataccacgcgtattttaacggtagag taa

NT here means nucleotide sequence

Practically: Using KEGG Database

Genome Map



M2D3 HW: Figure/Caption/Results

- figure= agarose gel image with title and caption
  - don't add drawings/modification on top of image, next to image is sufficient
- results **paragraph** should follow below figure and results subsection title (take-home message)
- minimize interpretation in the results section in Mod2 report (separate discussion section; *M2D5HW*)
  - State the result of the experiment without discussing conclusions drawn from experiment
- figure/text expectations same as Mod1 report, formatting major difference
  - all text in paragraph form

In lecture discussion Thursday

Multiple Gene Repression in Cyanobacteria Using CRISPRi

Lun Yao, Ivana Cengic, Josefine Anfelt, and Elton P. Hudson*

KTH—Royal Institute of Technology, Division of Proteomics and Nanobiotechnology, Science for Life Laboratory, Stockholm SE-171 21 Sweden

Supporting Information

ABSTRACT: We describe the application of clustered regularly interspaced short palindromic repeats interference (CRISPRi) for gene repression in the model cyanobacterium *Synechcocystis* sp. PCC 6803. The nuclease-deficient Cas9 from the type-II CRISPR/Cas of *Streptrococcus pyogenes* was used to repress green fluorescent protein (GFP) to negligible levels. CRISPRi was also used to repress formation of carbon storage compounds polyhydroxybutryate (PHB) and glycogen during nitrogen starvation. As an example of the potential of CRISPRi for basic and applied cyanobacteria research, we simultaneously knocked down 4 putative aldehyde reductases and dehydrogenases at 50−95% repression. This work also demonstrates that tightly repressed promoters allow for inducible and reversible CRISPRi in cyanobacteria.