M2D1: Complete *in silico* cloning of dCas9 & actual confirmation digest

10/13/16

1. Pre-lab Discussion
2. Design primers to dCas9
3. *In silico* PCR amplification, digest, and ligation
4. *Actual* (wet-lab) diagnostic digest of pdCas9

(Almost) done with Mod1!

- Mini-presentation
  - due 10pm on Saturday, October 14
  - You don't have to be exhaustive; tell a focused story
- Data summary
  - ✓ draft due 10pm on Wednesday, October 11
  - receive all comments by Monday, October 16th
  - revision due 10pm on Sunday, October 22nd
- Blog post
  - due 10pm on Monday, October 23

Sign up for journal club (M2D2 homework)

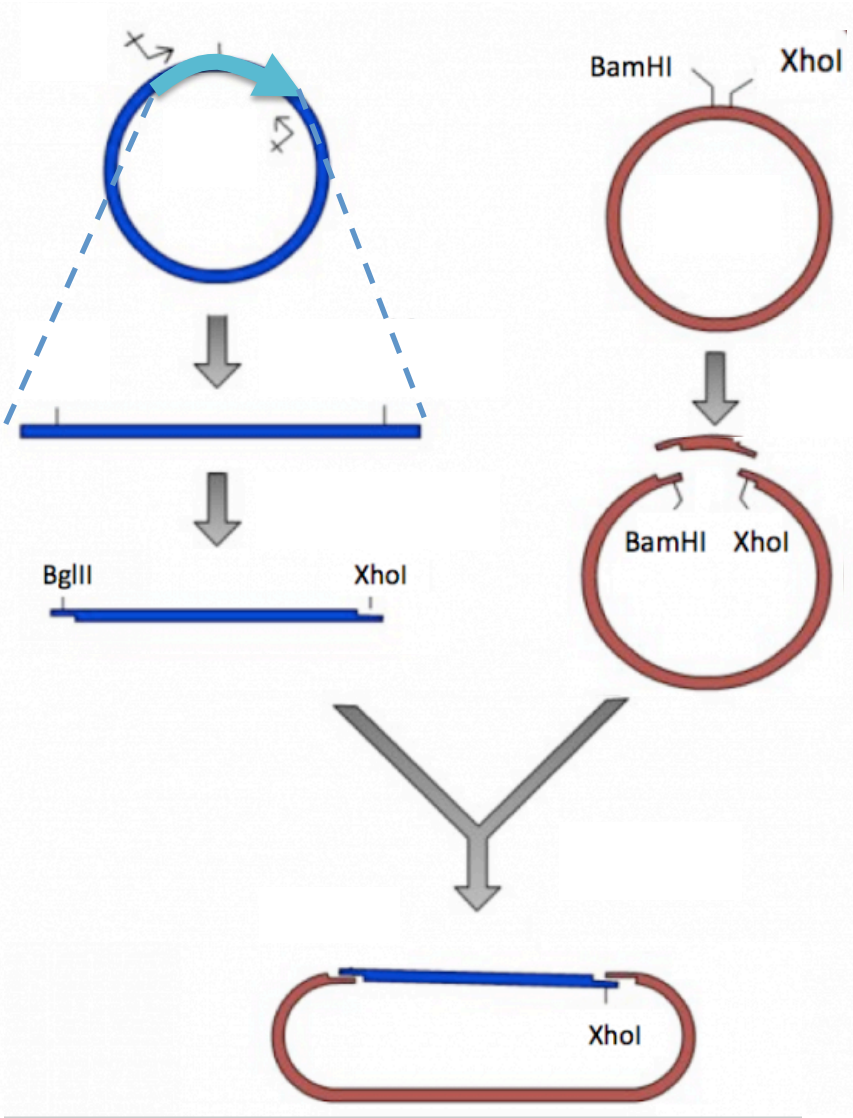
- Sign up on wiki for which day you will present:
  M2D4 (October 25th) or M2D6 (November 1st)
- Pick 1 of 20 papers, or suggest your own
- Reserve paper by adding name next to it **[Bagnall/WF/TeamColor]**
  - First come first serve!
  - Only one T/R and one W/F student per article
  - Don't pick a paper randomly

Slot	Day 4 (T/R)	Day 6 (T/R)	Day 4 (W/F)	Day 6 (W/F)
1				
2				
3				
4				

"insert"
"fragment"
dCas9

"vector"
"backbone"
$p_L$tetO-1

How is DNA engineered?



1. PCR amplification of DNA:

specific primers to gene of interest

dNTPs

Polymerase

2. Digestion:

Restriction enzymes (endonuclease)
Sticky (or blunt) ends

3. Ligation:

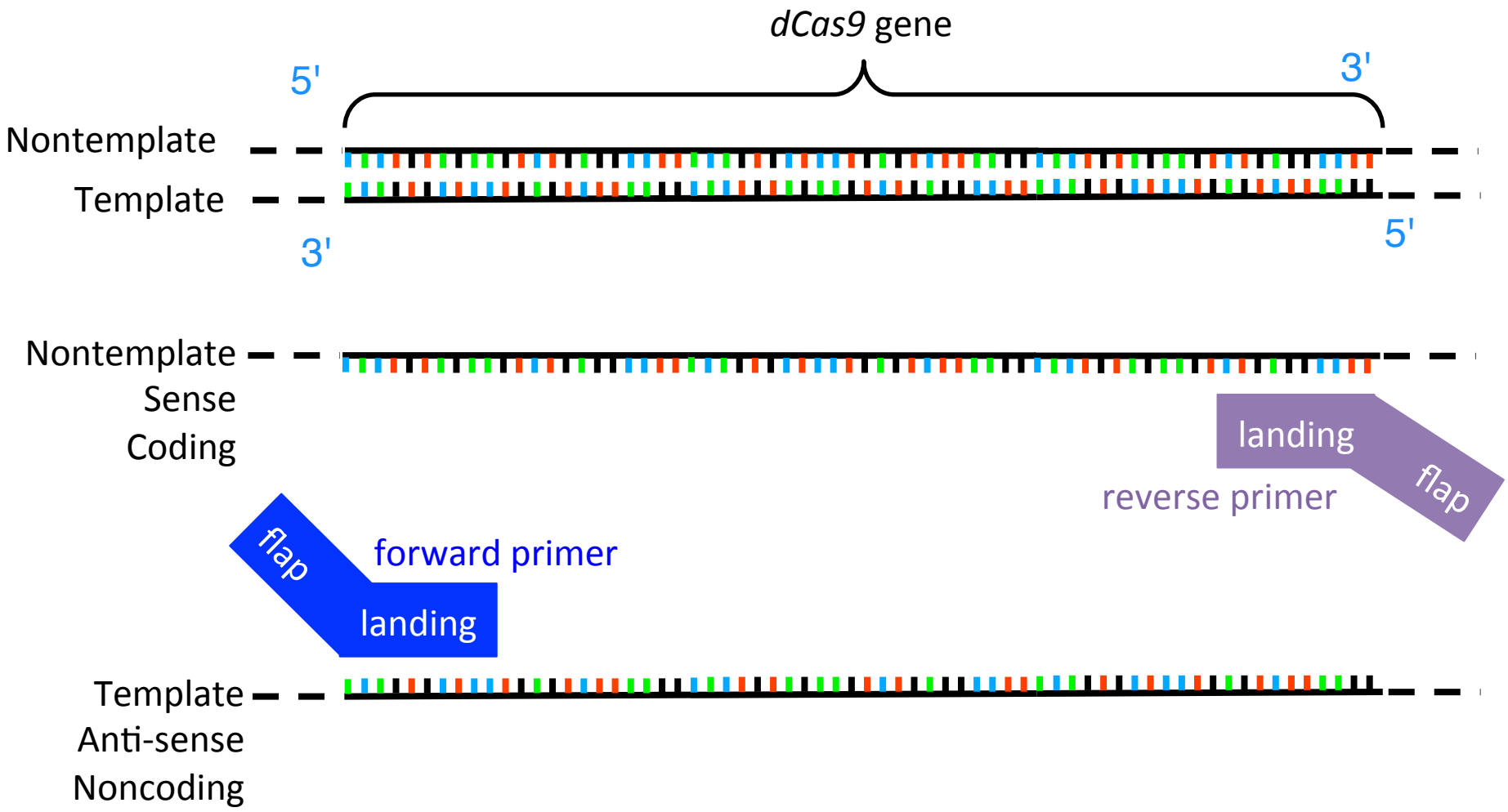
DNA Ligase

Seals backbone
Phosphodiester bonds

1. PCR amplification of DNA

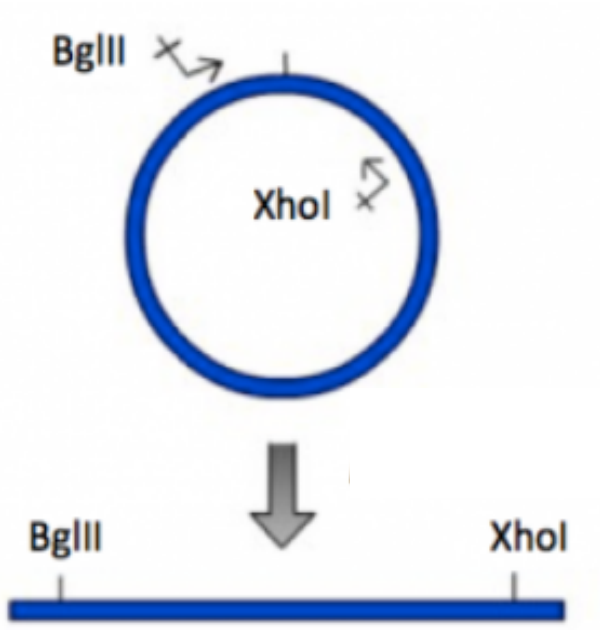
Defining terminology

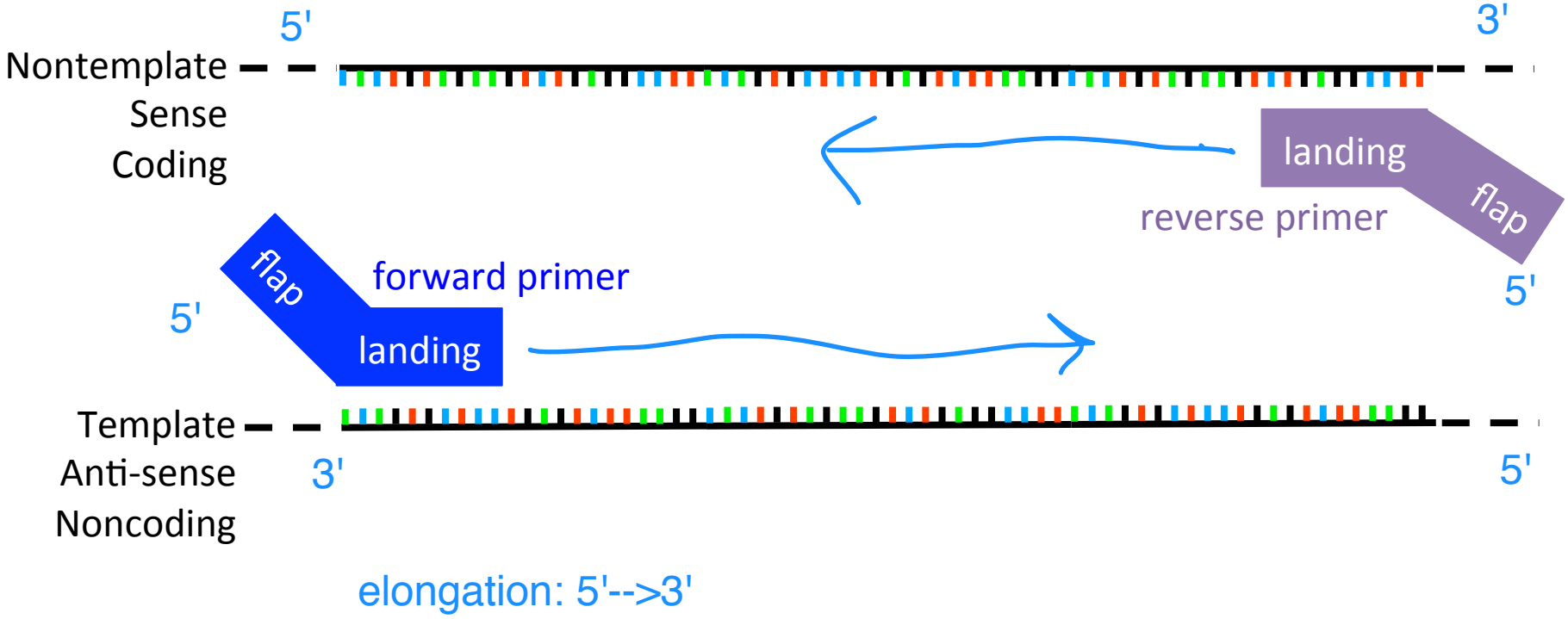
top strand: 5'-->3'



1. PCR amplification of DNA
Designing primers

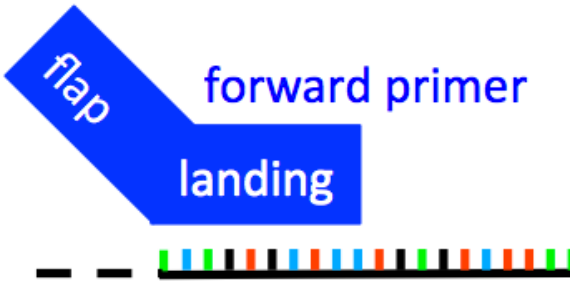
- **Landing sequence**: match to dCas9
- **Flap sequence**: contains endonuclease recognition sequence and junk DNA





1. PCR amplification of DNA

Primer design guidelines



- Length
  - 17-28 base pairs
  - long enough to be specific, short enough for easy annealing
- GC content
  - 40-60%
  - GC clamp at ends

AT: 2 H bonds
GC: 3 H bonds

- T_m(primer)
  - < 65 °C
- Avoid secondary structures
  - hairpins
  - complementation w/in primer sequence
- Avoid repetitive sequences
  - Max of 4 di-nucleotide repeats (ex. ATATAT)
  - Max of 4 bp in a run (ex. GATGGGG)

1. PCR amplification of DNA

Three major PCR steps—which temperature & why?

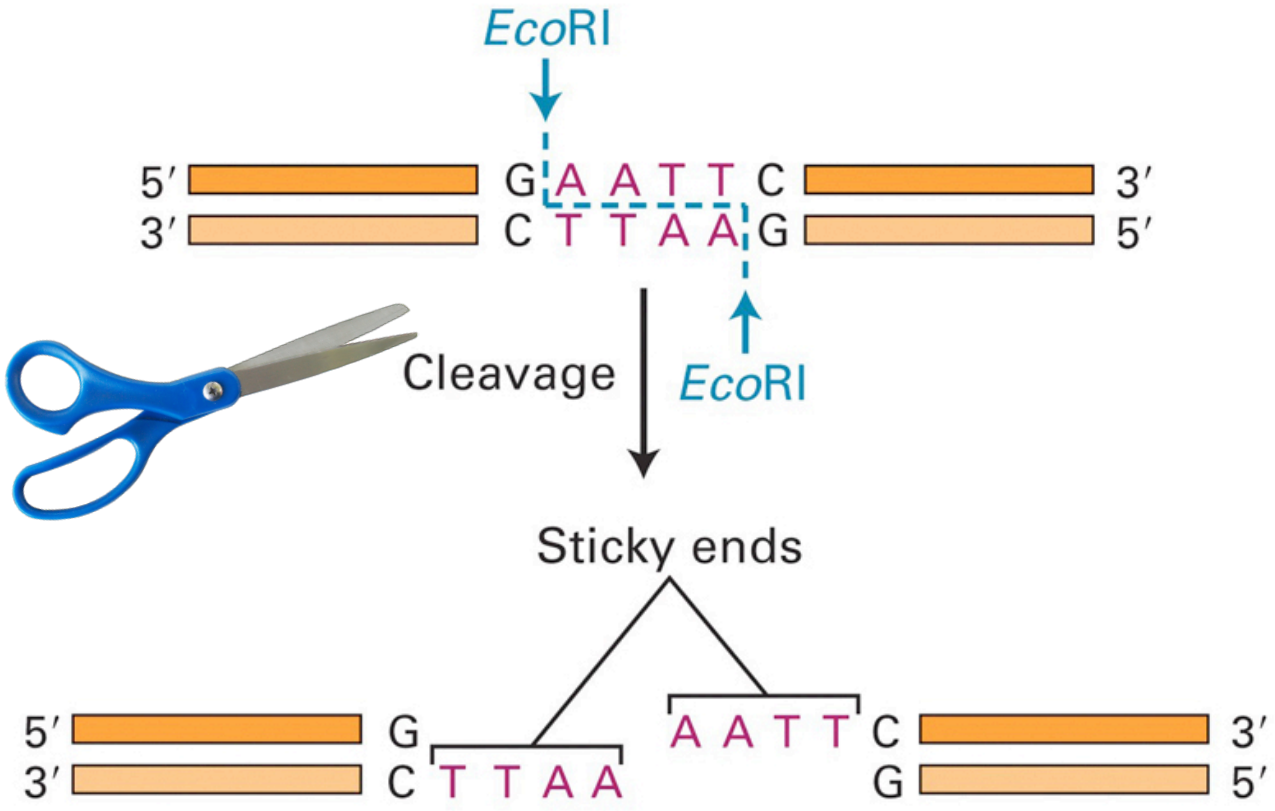
- Melt
  - 95 °C
  - break hydrogen bonds
- Anneal depends on primer sequence
  - T_m(primer) = 1/2 primer annealed to target
  - $T_{anneal} \sim T_m(primer) - 5°C$
- Extend
  - 72 °C (for Taq)
  - 1000 bases/min

Leslie's favorite PCR animation

http://learn.genetics.utah.edu/content/labs/pcr/

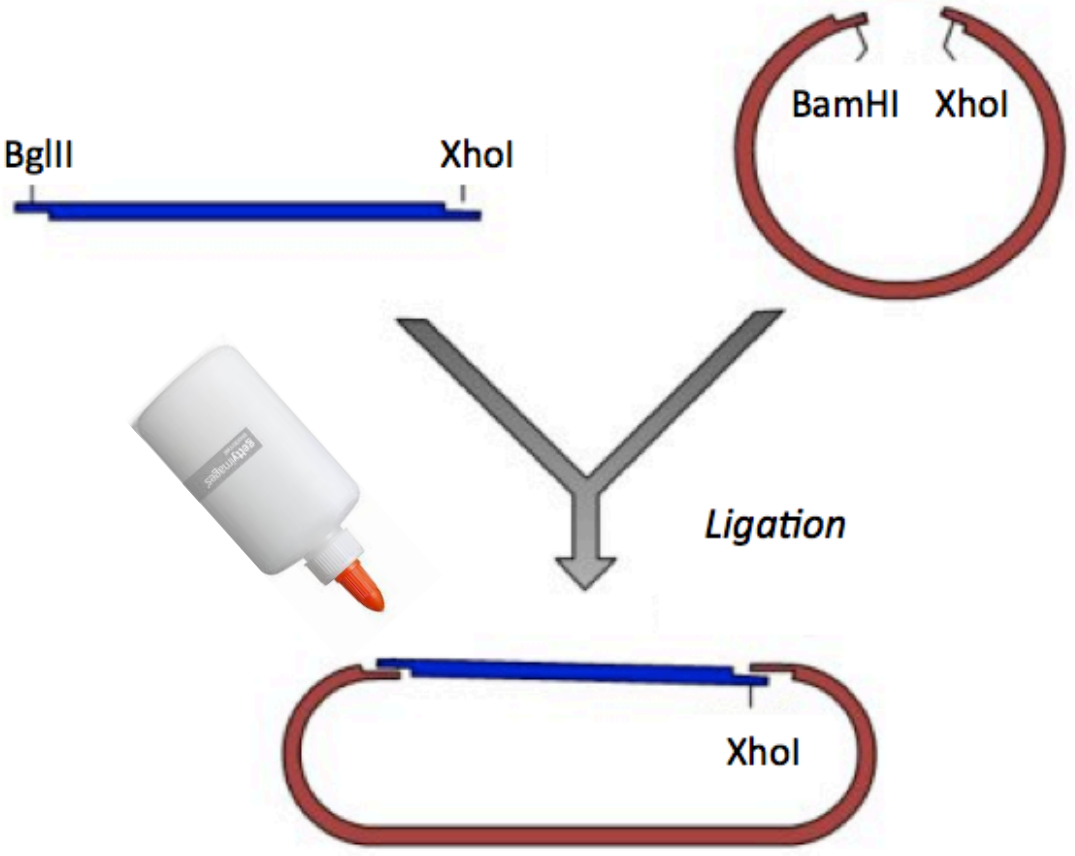
2. Digestion

Restriction endonucleases create sticky ends on dCas9 insert and plasmid backbone



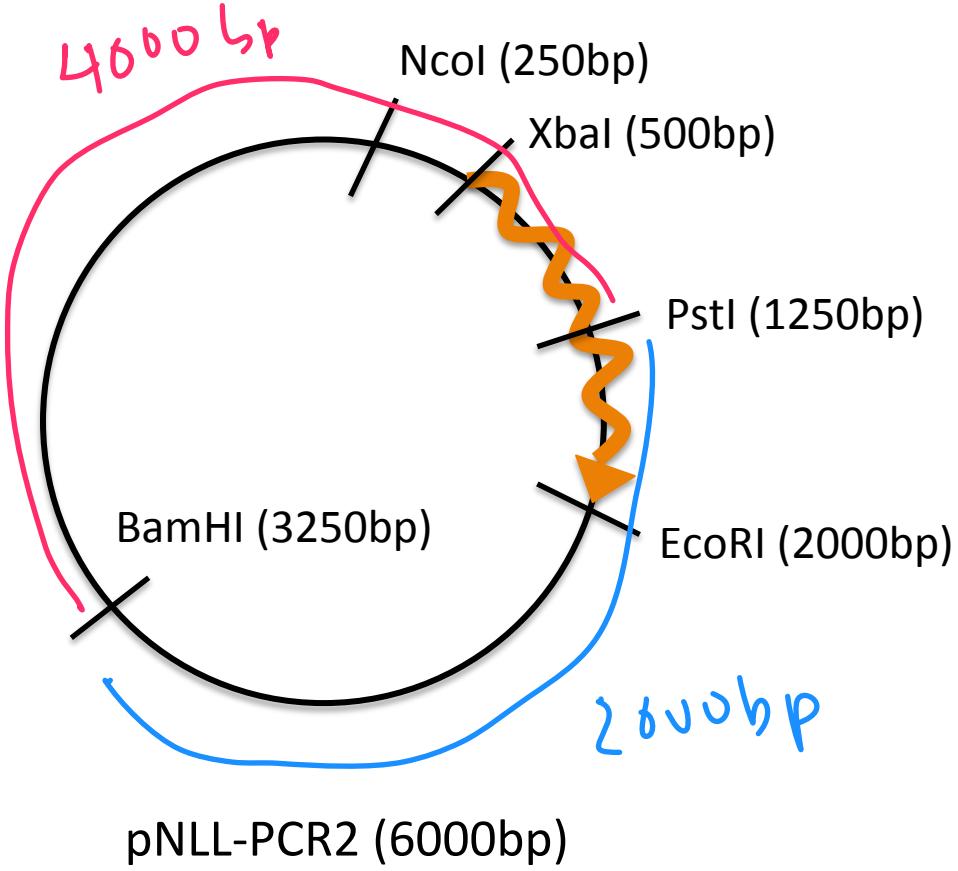
3. Ligation

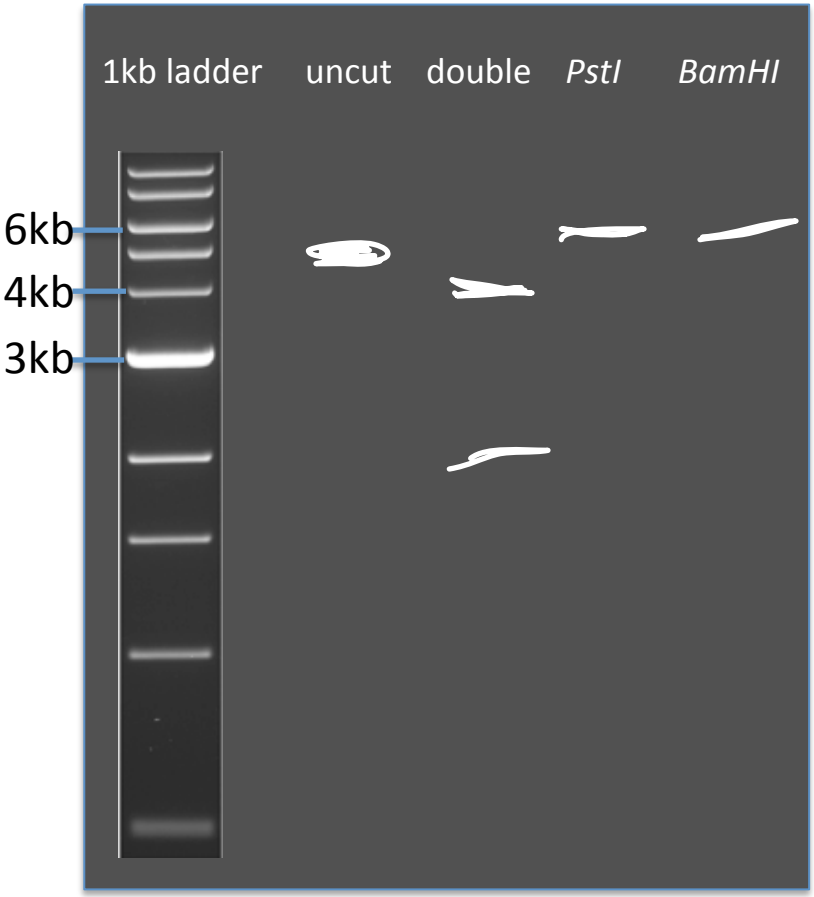
Insert dCas9 into expression vector (backbone) to create new plasmid (pdCas9)



Confirmation digest considerations

- Do you have access to the enzymes?
- Are the two enzymes compatible?
- Are fragments easily distinguished on an agarose gel?





Today in lab

1. Reproduce *in silico* (in Benchling) the cloning of pdCas9
   - Design primers that would amplify the gene dCas9
   - Depict PCR amplification product
   - Digestion of dCAS9 PCR product and vector by restriction enzymes
   - Ligation of insert and vector ** at 2:30pm we will all work through this calculation together

For now, skip Part 3: #1-6

2. Set up confirmation digests of pdCas9 for agarose gel electrophoresis
   - Choose restriction enzymes for diagnostic digest
   - Calculate volumes of digest components
   - Set-up digest and leave overnight at 37°C

Ligation calculation

Goal: Calculate volumes of insert and backbone needed for ligation

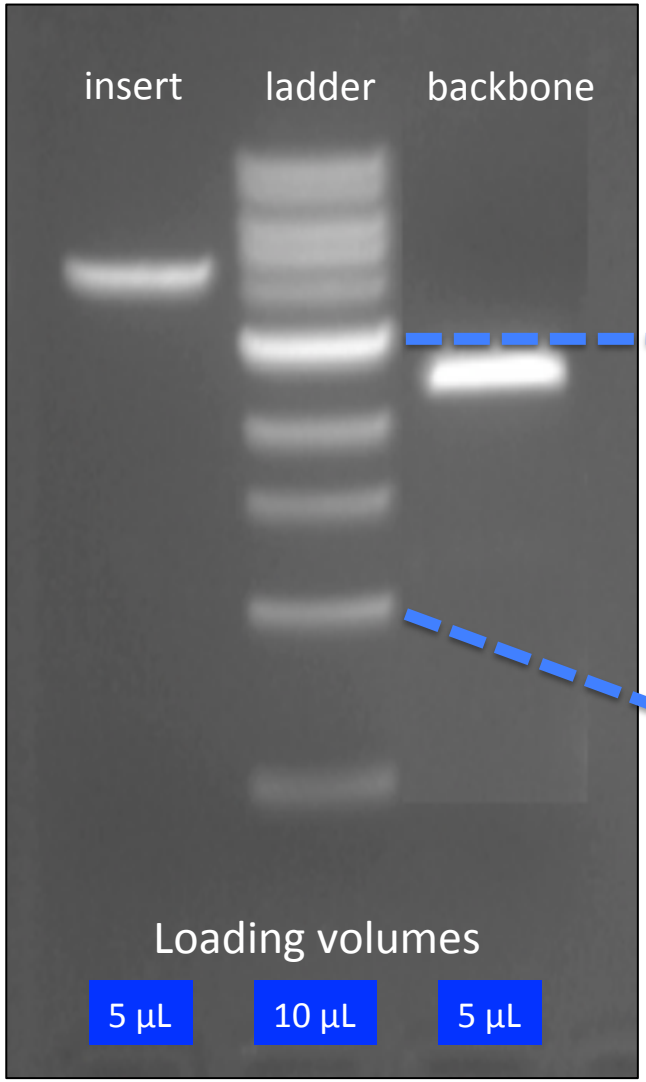
Knowns:

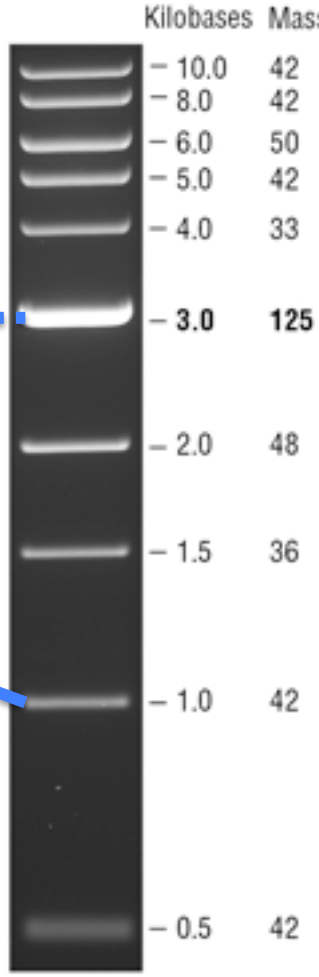
- Need 50-100 ng backbone
- Backbone: 2592 bp
- Insert: 4113 bp
- Molar mass ~660g/(mol*bp)
- Desired molar ratio of insert to backbone is 4:1

Missing information needed to know what volumes of backbone and insert to use:

volume concentration of DNA

Use recovery gel to estimate insert and backbone concentrations





- Amount of backbone = 200 ng
- Amount of insert = 100 ng
- Concentration of backbone = 200 ng/ 5uL = 40 ng/uL
- Concentration of insert = 100 ng/ 5uL = 20 ng/uL

mass of DNA ≠ molar amount of DNA

Goal: Calculate volumes of insert and backbone needed for ligation

Knowns:

- Need 50-100 ng backbone
- Backbone: 2592 bp
- Insert: 4113 bp
- Molar mass ~660g/(mol*bp)
- Desired molar ratio of insert to backbone is 4:1
- Concentration of insert: 20 ng/uL
- Concentration of backbone: 40 ng/uL

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 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 ~ 1.2x 10^{-13} mol
   - with 4113 bp * (660 g / (mol*bp)) = 2.7 x 10^6 g/mol
   - so use ~~1.15~~ 1.2 x 10^{-13} mol * 2.7 x 10^6 g/mol ~ 310ng

3. Calculate volume of backbone and insert needed
   - Backbone: 50 ng/(40 ng/uL) = 1.25 uL
   - Insert: 310ng / (20 ng/µL) = 15.5 µL

scale down: 1 uL backbone, 12.4 uL insert

Optimal backbone-to-insert ratio

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

- What if too much insert?

  multiple inserts daisy-chained, inserted into backbone

- What if too much backbone?

  backbone ligating to other backbone