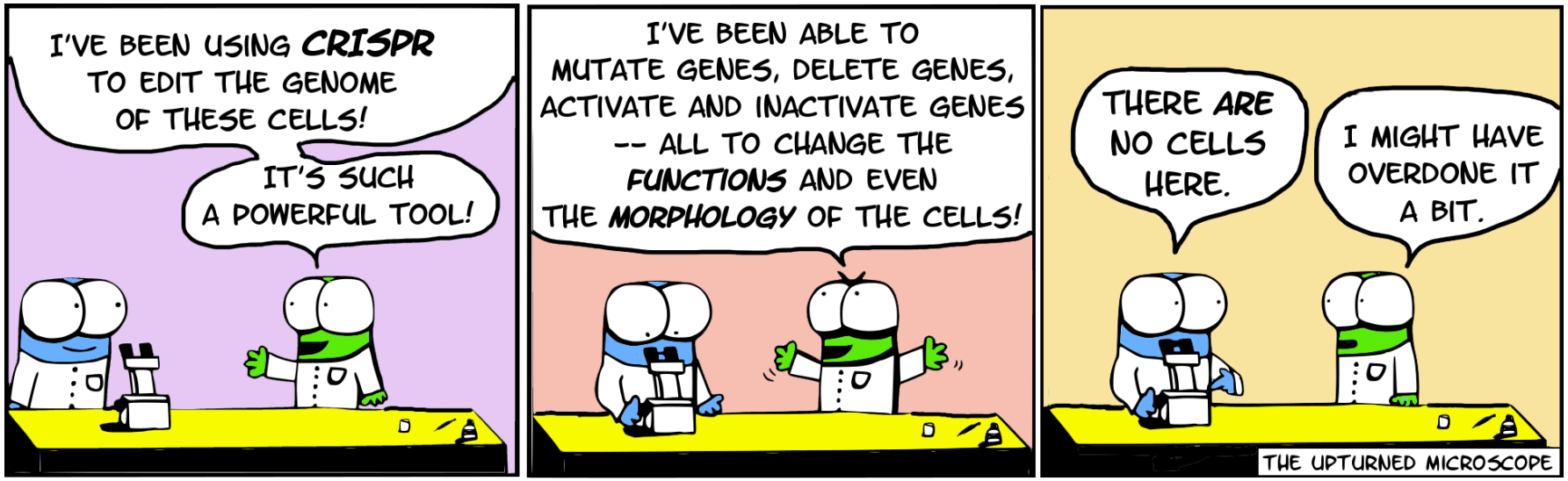
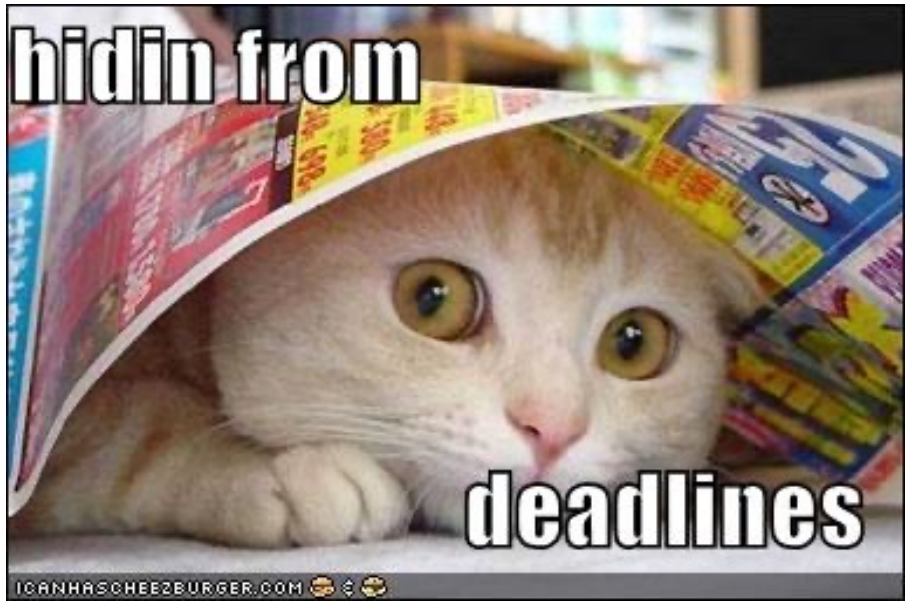
M2D1: Complete *in silico* cloning of dCas9 expression plasmid

1. Prelab
2. *In silico* cloning
3. Set up confirmation digest reaction by 4:30pm



(Almost) done with Mod 1!

- Data summary due: **Saturday March 12 at 10pm**
  - Additional office hours announced soon
- Revision (if you want) due: **Sunday March 20 at 10pm**



Experimental design for Mod 1: Localization

Localization:

- How does this connect to drug discovery and TDP43?

- Aberrant localization to cytoplasm has been reported to promote cytoplasmic aggregation of TDP43
  - Is it too late for treatment by the time we get to aggregates?
  - Could be valuable to target an early step in that process
  - Look for a small molecule that can regulate TDP43 localization

- Does your small molecule have any effect on basal localization of TDP43?

Data Summary Questions?

Mod 2 Due Dates
(because we haven't given you enough to think about…)

- Journal Club presentation (15%)
  - Individual
  - Presentations on 3/29 & 3/31
- Research article (20%)
  - Individual
  - due 4/23
- Laboratory quizzes (collectively 5%)
  - M2D4 and M2D7
- Notebook (collectively 5%)
  - one entry will be graded in detail by Christine
- Blog (part of 5% Participation)
  - due 4/1 & 4/25 via Slack channel

Mod 2: Metabolic engineering using CRISPRi

- **Research goal: Increase the yield of commercially valuable byproducts in *E.coli* using CRIPSRi technology to target genes involved in mixed-acid fermentation pathway.**

- Today we are generating a plasmid to express dCas9, a key component of our CRISPRi system

- We are also learning about how our CRISPRi system was designed so that we can employ it most effectively

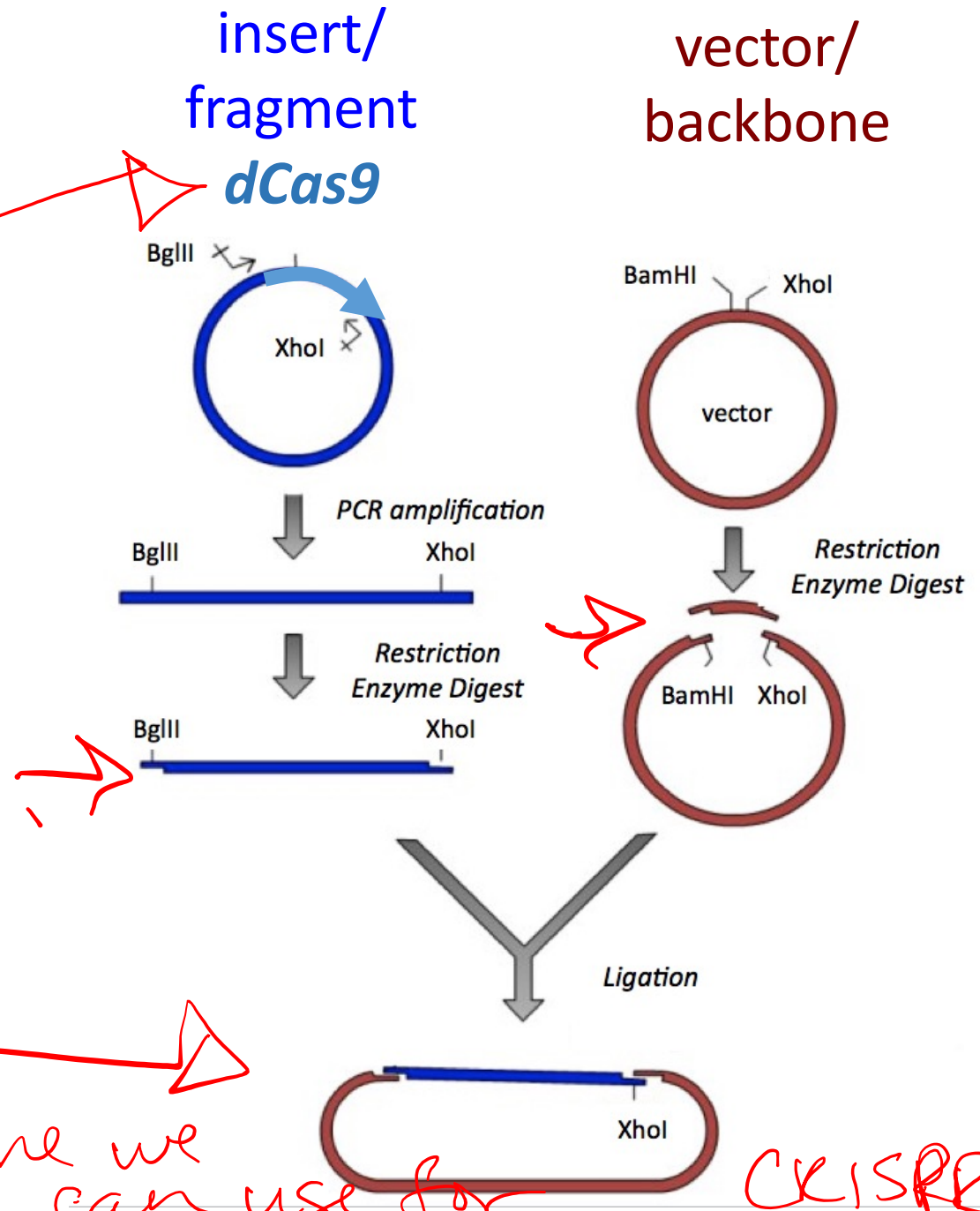
- Next time we will learn about the metabolic pathways we are targeting and design tools to manipulate genes in those pathways to produce ethanol or acetate

Cloning of pdCas9:

1. PCR amplification of DNA:

2. Digestion:

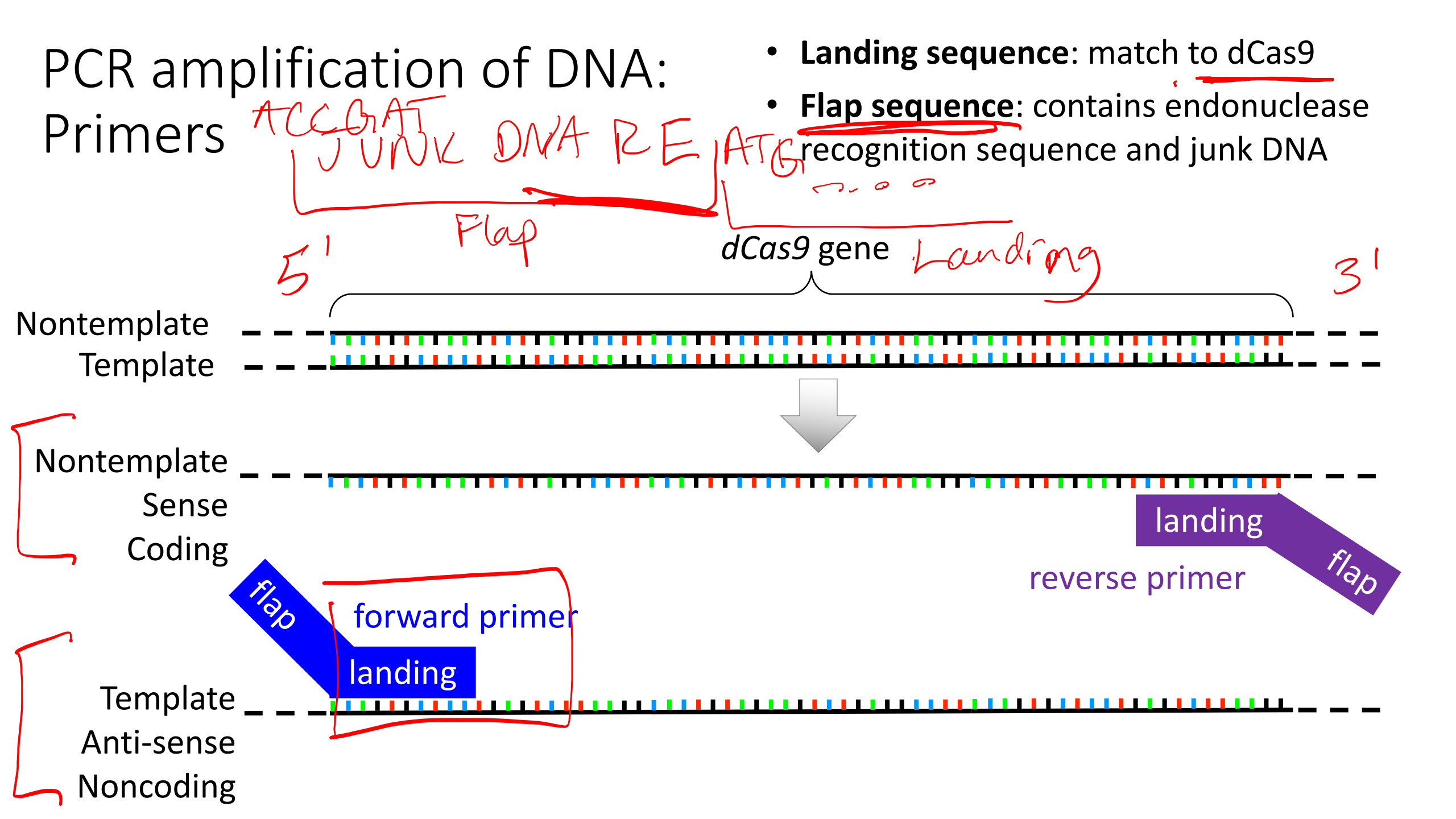
3. Ligation:



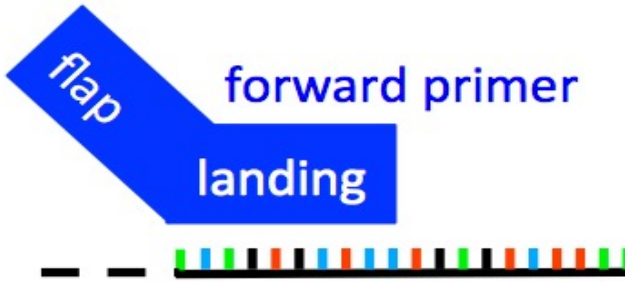
Moving dCas9 gene from one plasmid into one we can use for CRISPR!

PCR amplification of DNA: Primers

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



Primer design guidelines

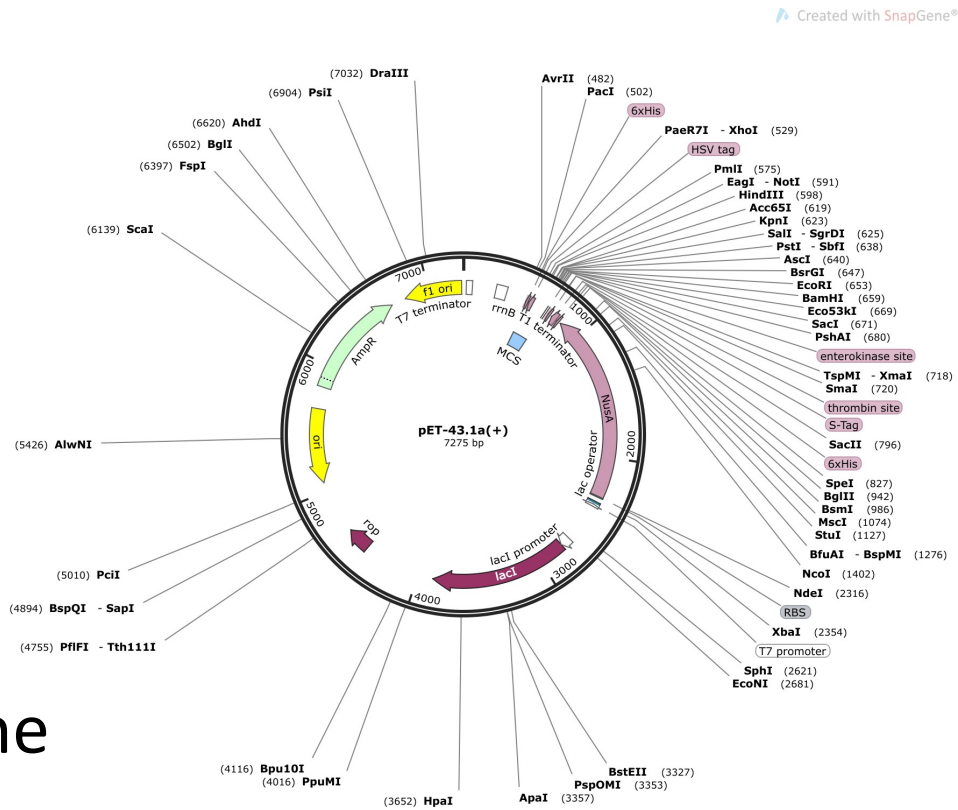


- Length: 17-28 base pairs
- GC content: 40-60%
  - GC has 3 hydrogen bonds; AT has 2 hydrogen bonds
  - GC clamp at ends
- T_m(primer) < 65 °C

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

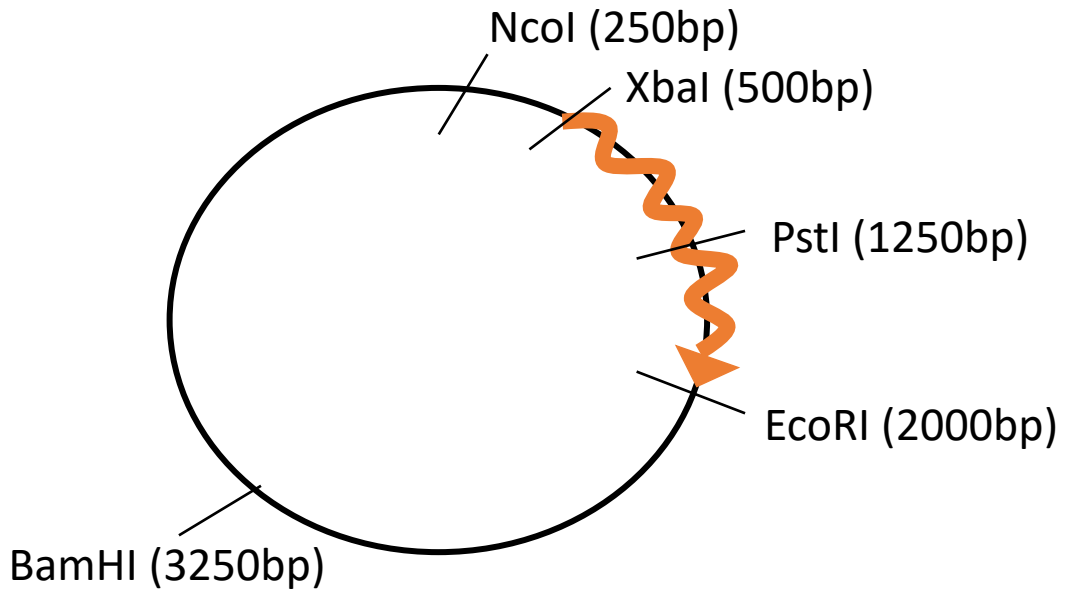
In silico cloning in SnapGene

- Work through the steps of cloning without the hours of incubations
  - Primer design and PCR amplification of insert
  - Restriction enzyme digest of insert and backbone
  - Ligation of insert into new backbone
- Generate plasmid map of dCas9 in new vector
- The plasmid you generate *in silico* is the same one we generated in the lab
  - Want to test that we generated the correct plasmid



Use a confirmation digest to test correct plasmid construction

- Use 2 restriction enzymes to cut the plasmid
  - Different from REs used to cut insert and backbone
  - One RE cuts only insert
  - One RE cuts only vector
- Can infer correct insertion of insert into backbone
- Considerations when choosing restriction enzymes:
  - Do you have access to the enzymes? wiki
  - Are the two enzymes compatible? - NEB
  - Are the resulting fragments easily distinguished on an agarose gel?



pNLL-PCR2 (6000bp)

For today

- Complete cloning and generate plasmid map of pdCas9
- Set up confirmation digest of pdCas9 based on plasmid map
  - Begin by 4:30pm
  - Printed maps available at front bench if needed

For M2D2

- Select article for Journal Club
- Sign up on wiki using instructions on the sign up page
  - Only one person per section can sign up for an article
  - First come first serve
- Write summary of why article is interesting, based on abstract and introduction