M2D1:
in silico cloning

10/14/2016

(Almost) done with M1!

- Mini-presentation
  - due 10pm on Saturday, October 15
  - You don't have to be exhaustive
- Data summary
  - draft due 5pm on Wednesday, October 12
  - revision due 5pm on Monday, October 24
- Blog post
  - due 5pm on Tuesday, October 25
- Sign up for journal club
  - 4-6 students at 1pm on Wednesday, October 26

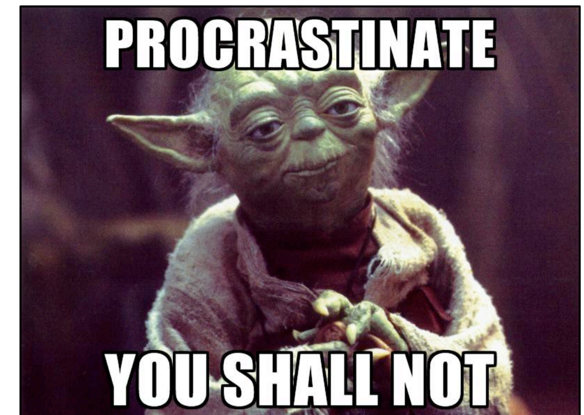
Reflection assignments:

http://be20109f16.blogspot.com/

BE 20.109 Class Blog

Welcome to the 20.109 Class Blog! Our 20.109 Blog is here for MIT's emerging cadre of biological engineers from Course 20. The blog is for your thoughts and work and discoveries in our lab fundamentals class. By capturing your collective experiences in the subject, we hope to learn even more about the work we do -- what's working well and where we need to get better. Please see the first blog post for some important administrative information.

- Due dates
  - M1:October 25 at 5 pm
  - M2: November 20 at 5 pm
  - M3:December 7 at 10 pm
  - one more blog: December 14 at 10 pm
- A few additional notes:
  - Do not publish MIT logo
  - Do not post photographs with names tagged
  - Do not write malicious comments
  - Do not plagiarize

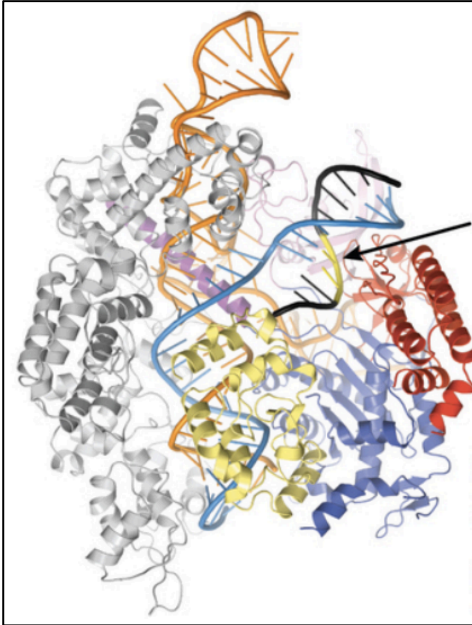


Sign up for journal club

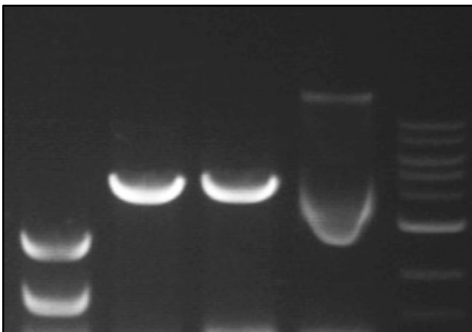
- Pick 1 of 20 papers, or suggest your own
- Present M2D4 (October 26) or M2D6 (November 2)
- Sign up by adding your name next to paper [MJ/WF/Rainbow]
  - first come first serve!
  - only one T/R and one W/F student per article

Slot	Day 4 (T/R)	Day 6 (T/R)	Day 4 (W/F)	Day 6 (W/F)
1				
2				
3				
4				
5				
6				

Today in lab



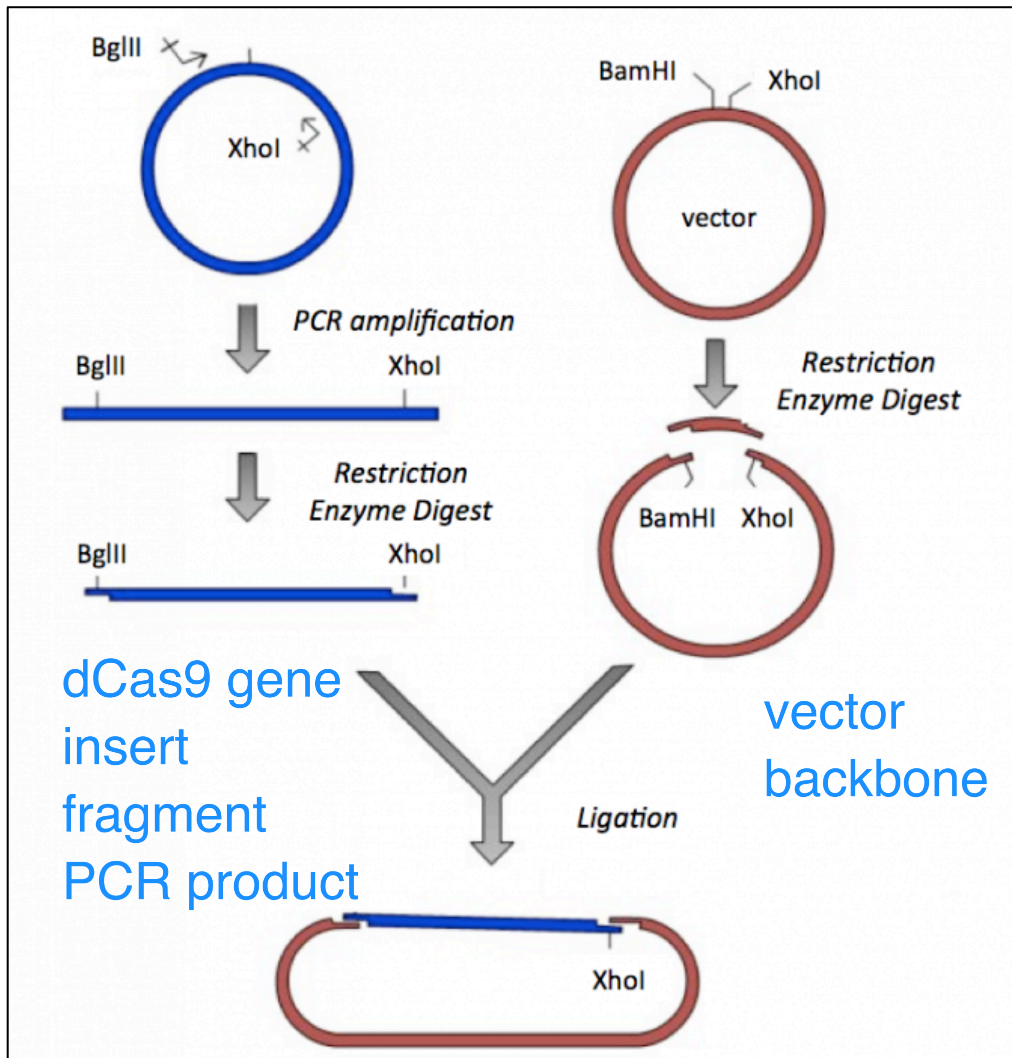
- Reproduce *in silico* (in Benchling) the cloning of pdCas9 *
  - PCR amplification of dCas9 insert
  - digestion of vector by restriction enzymes
  - ligation



- Set up *(for real)* confirmation digests for agarose gel electrophoresis
  - pdCas9 cut by restriction enzymes

* Cas9–sgRNA–DNA complex from *Nature* **513**, 569–573 (2014)

How do we engineer DNA?



- PCR amplification
  - Kary Mullis 1993

  polymerase chain reaction
  amplify DNA
  - insertion
  - mutation

- Restriction enzymes

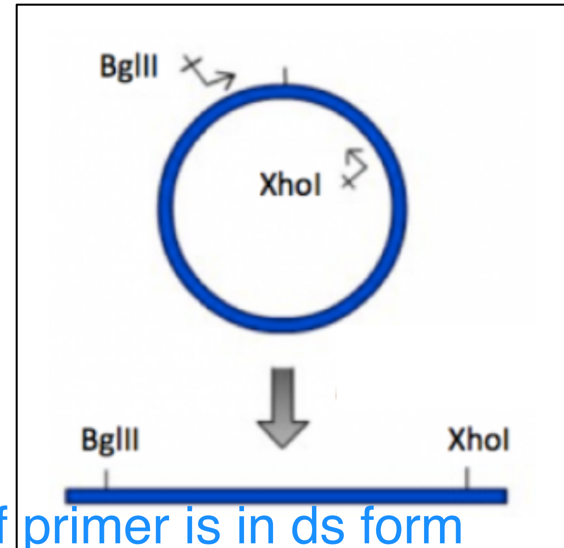
  endonucleases
  cut & paste

- Ligase

Using PCR to generate *dCas9* flanked by restriction enzyme recognition sites

3 major steps in each cycle:

- Melt
  - 95 °C
  - cut hydrogen bonds
- Anneal
  - T_m(primer) = temperature at which 50% of primer is in ds form
  - T_{anneal} ~ T_m(primer) – 5°C
    > 50-55 C
- Extend
  - 72 °C (for Taq)
  - 1 min / 1000 bp



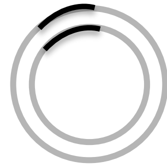
✧ Primers
- specificity
- option to add base pairs, *e.g.* endonuclease recognition sequence

Leslie's favorite PCR animation

learn.genetics.utah.edu

How many PCR cycles until only your amplicon?

Cycle 1



dsDNA

dCas9

melt

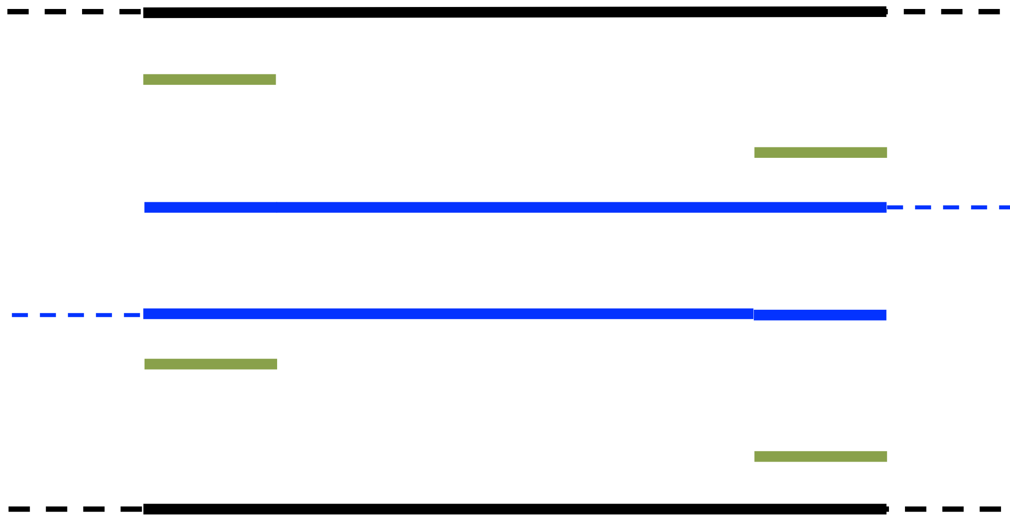
anneal

extend

Cycle 2

(melt)

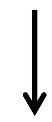
anneal



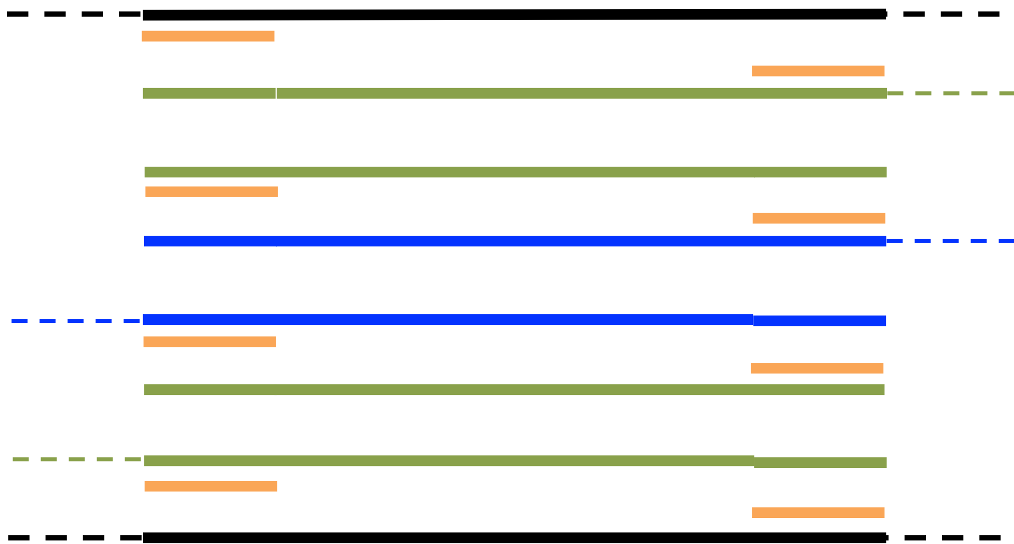
extend

Cycle 3

(melt)



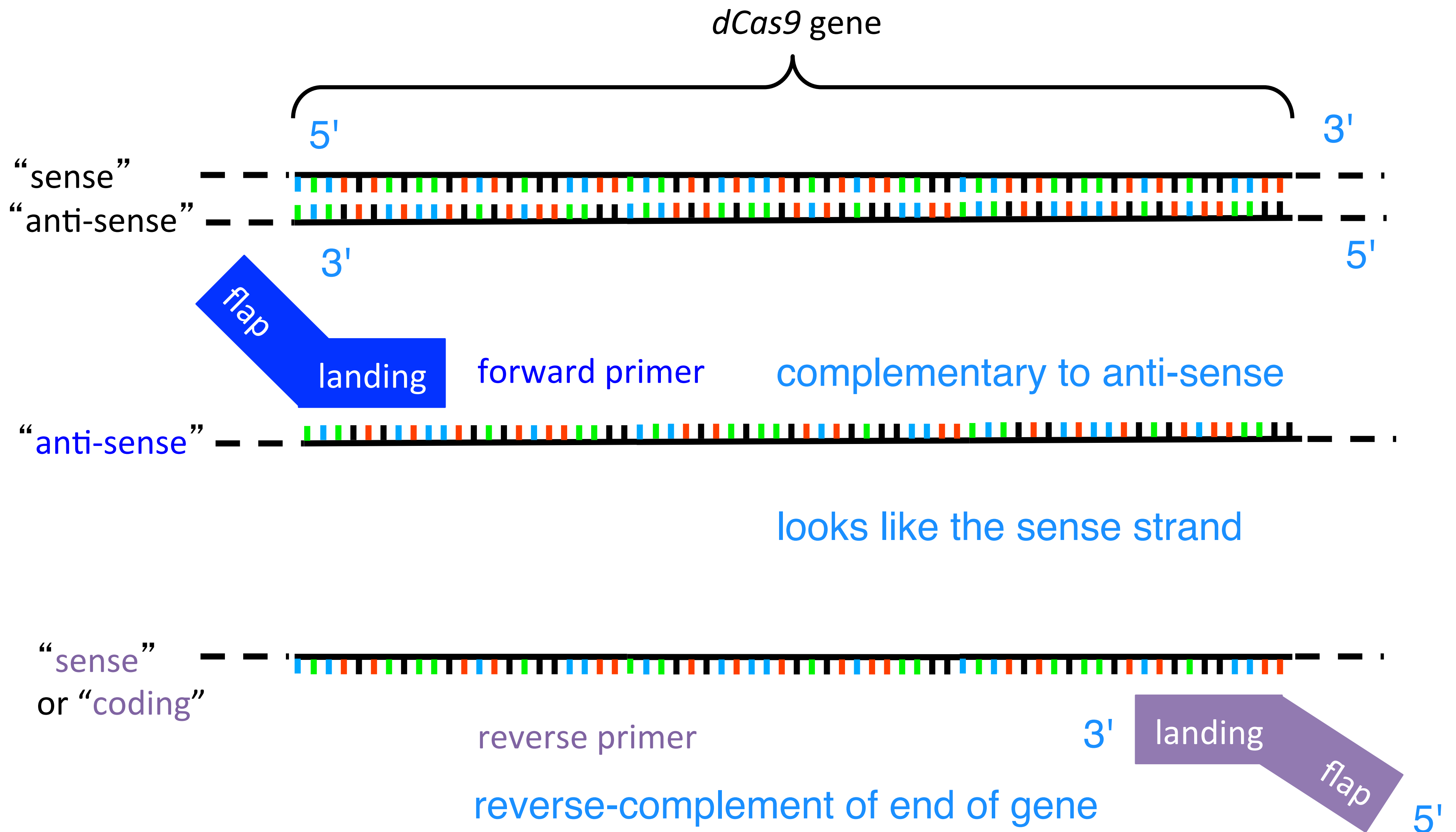
anneal



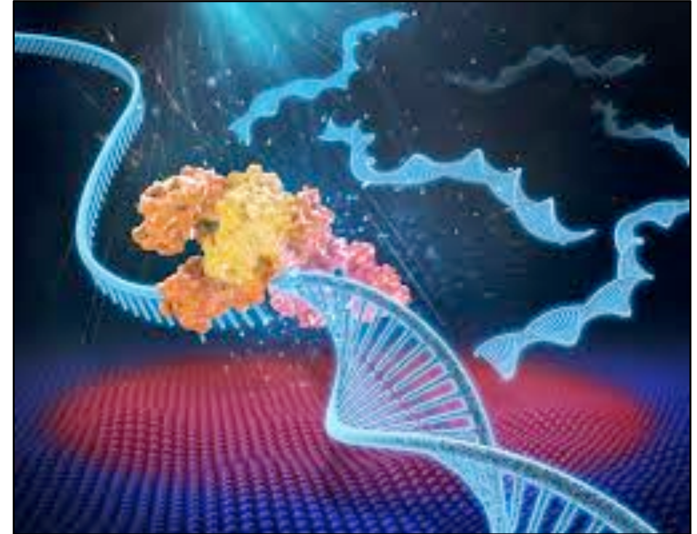
extend

* PCR product

How do you design primers?



Primer design guidelines



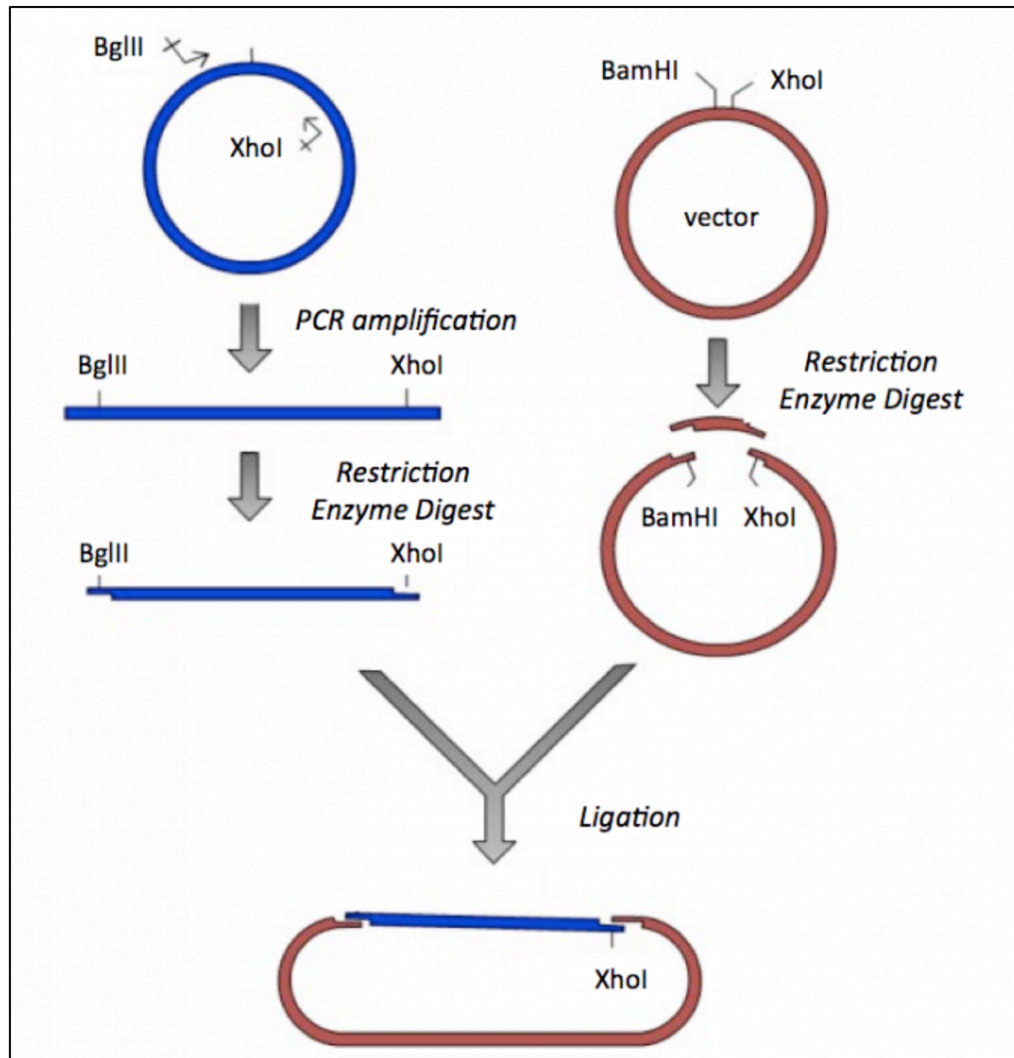
- Length
  - 17-28 base pairs
  - specificity
- GC content
  - 40-60%
  - GC clamp at ends

    stability
- T_m(primer)
  - < 65 °C

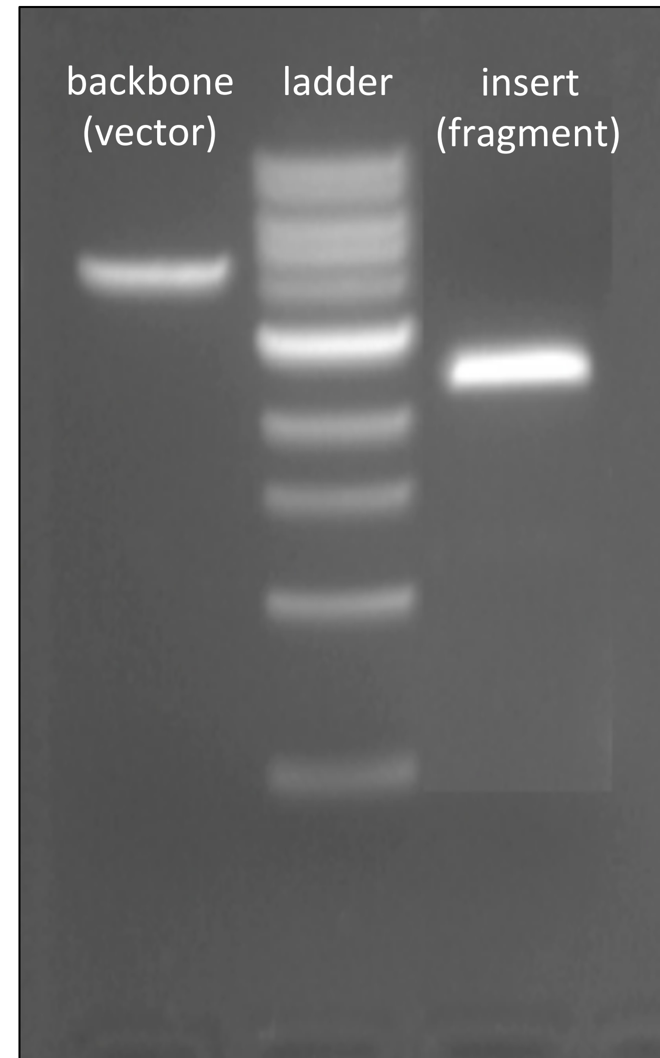
    but not too low either!
    Tm(primer) > 55 C

- Secondary structure
  - hairpins
  - complementation
- Repetitive sequence
  - di-nucleotides < 4
  - runs < 4 bp

pdCas9 was constructed by ligation

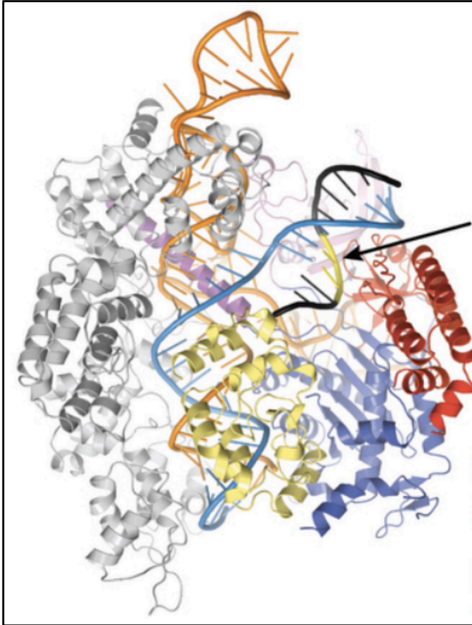


pdCas9 cloning strategy



recovery gel

Today in lab



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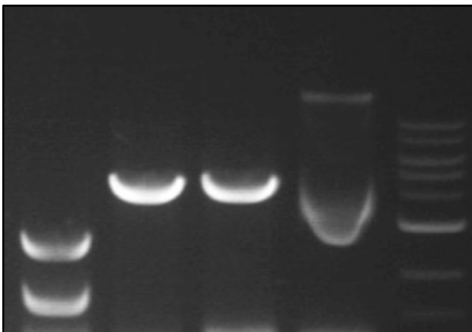

- Set up confirmation digests for agarose gel electrophoresis
  - pdCas9 cut by restriction enzymes

start by 3:45pm

in this order:
- water
- buffer
- enzymes
- pdCas9

* Cas9–sgRNA–DNA complex from *Nature* **513**, 569–573 (2014)

Confirmation digest

- Goal: 1 cut only in backbone + 1 cut only in insert
- Are fragments easily distinguished on an agarose gel? not the same size, not too small (< 500 bp)
- Do you have access to the enzymes?
- Are the two enzymes compatible?
  work in same buffer