M1D1:*in silico* cloning, induce protein expression

02/10/2017

1. Lab Orientation Quiz
2. Subculture BL21 *E. coli*
3. Prelab Discussion
4. Complete *in silico* cloning exercise
5. Induce protein expression in BL21 *E. coli*

Office hours

Noreen Lyell

- M 2-5
- in 16-317

Leslie McClain

- T 9:30-11
- in 56-341c

Maxine Jonas

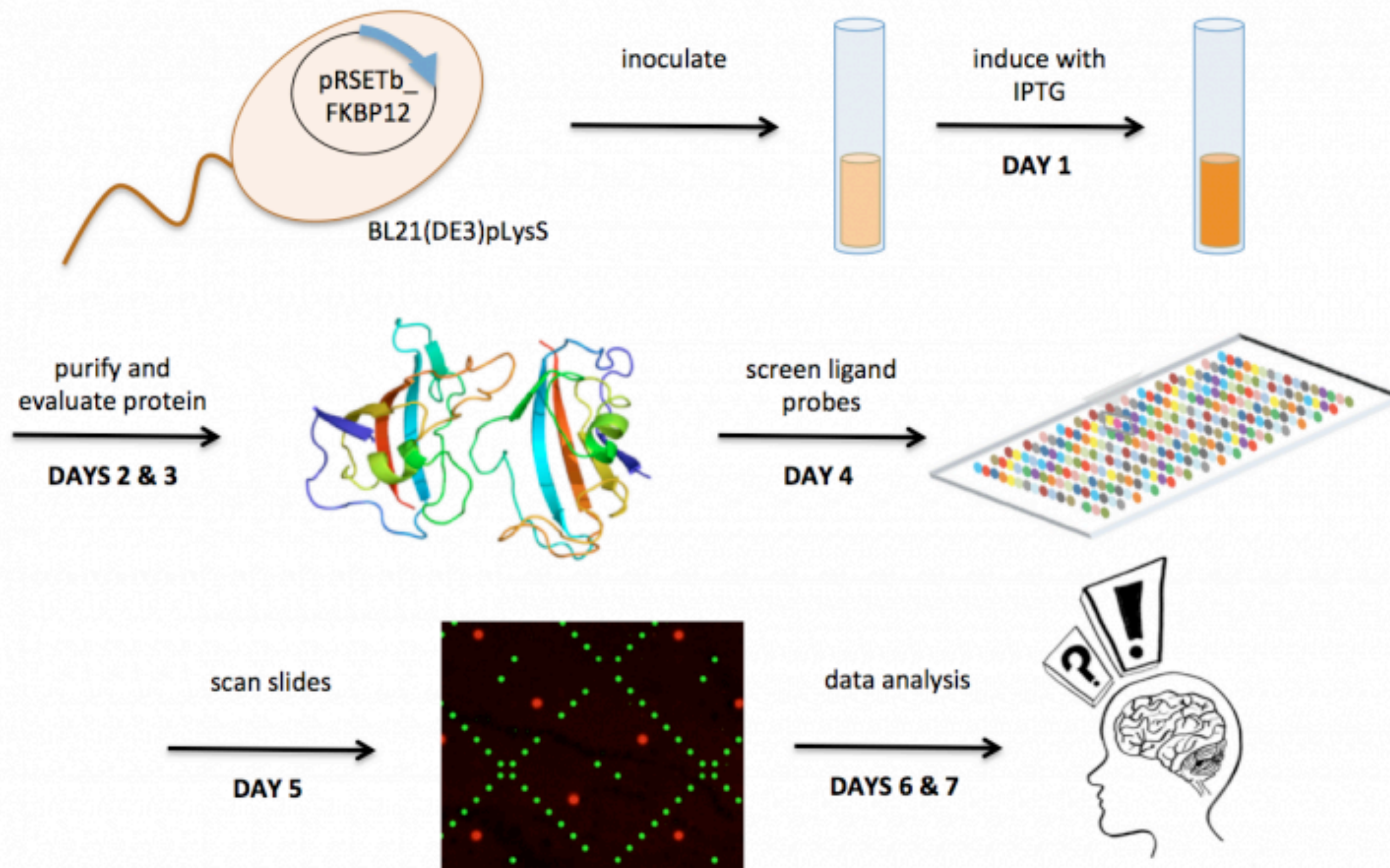
- R 9:30-11
- in 16-239

by appointment: nllyell@, lesliemm@, jonas_m@

M1 major assignments

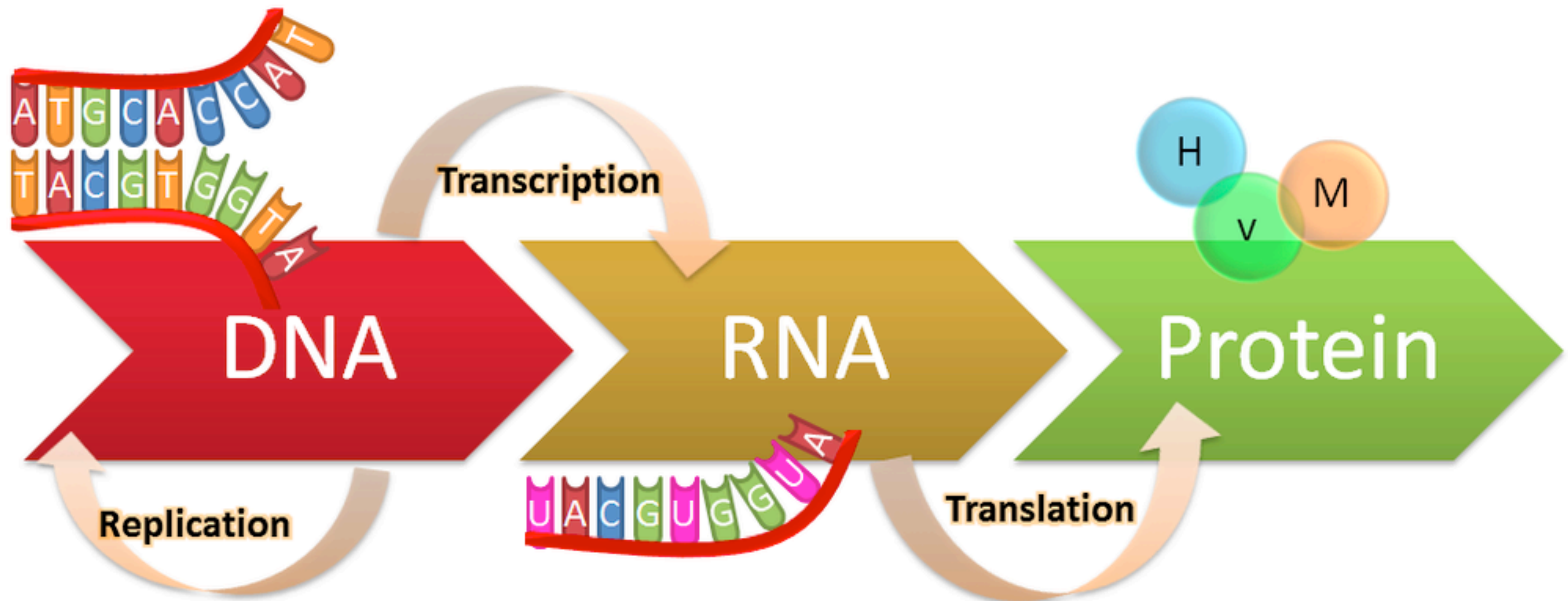
- Data summary (15%)
  - in teams, on Stellar
  - draft due 03/10, final revision due 03/27
  - bullet points, .PPTX
- Mini-presentation (5%)
  - individual, video via Gmail
  - due 03/18
- Lab quizzes (extra credit on homework grade)
  - M1D3, M1D5, and M1D7
- Notebook (5% total)
  - one day will be collected and graded by Rob on M1D7
- Blog: http://be20109s17.blogspot.com/ (participation: 5% total)
  - by 04/03

Overview of
"M1: High-throughput ligand screening"



The central dogma

- To study interactions of FKBP12 *protein*,
  - first *make* FKBP12 protein
  - by having the *Fkbp12 gene* transcribed and translated

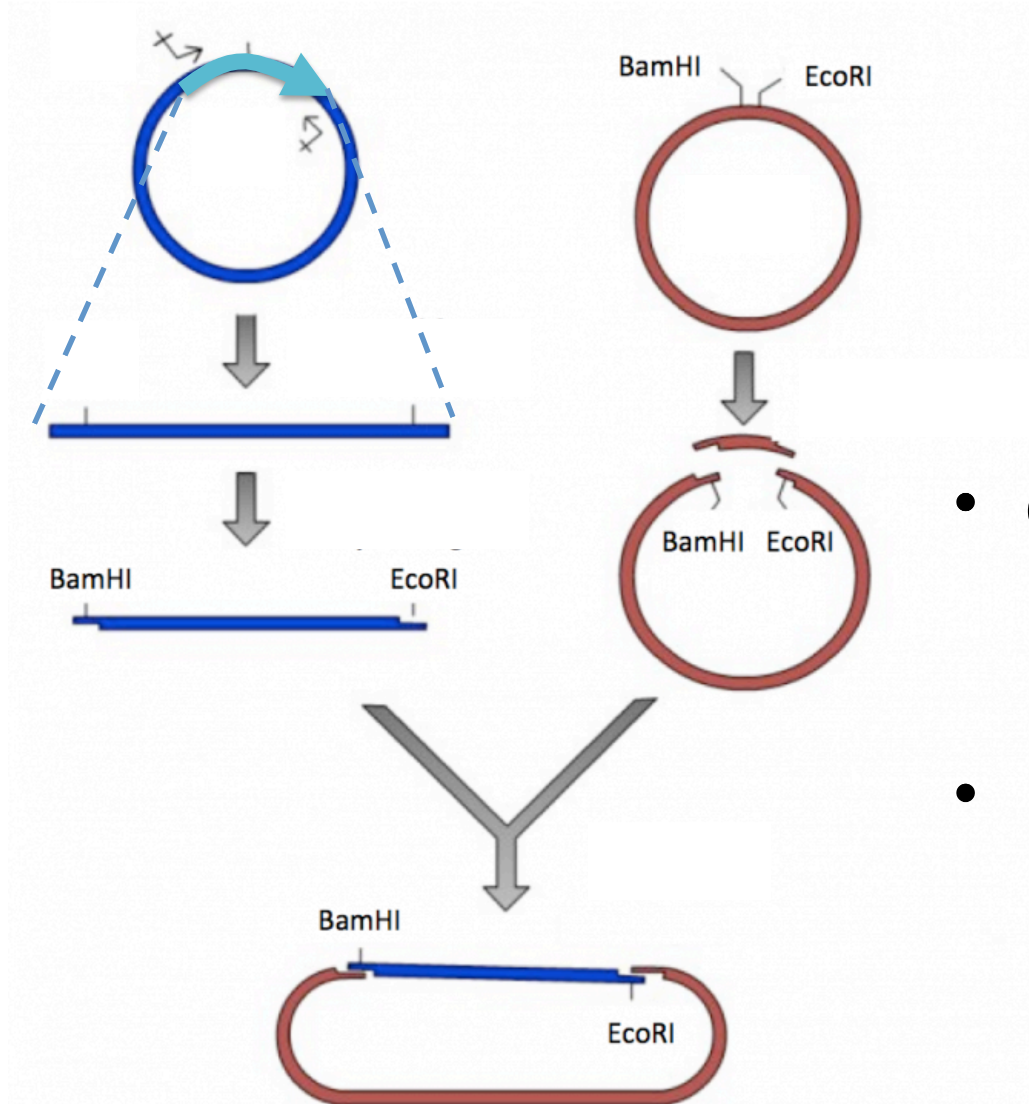


http://genius.com/Biology-genius-the-central-dogma-annotated

How is DNA engineered?

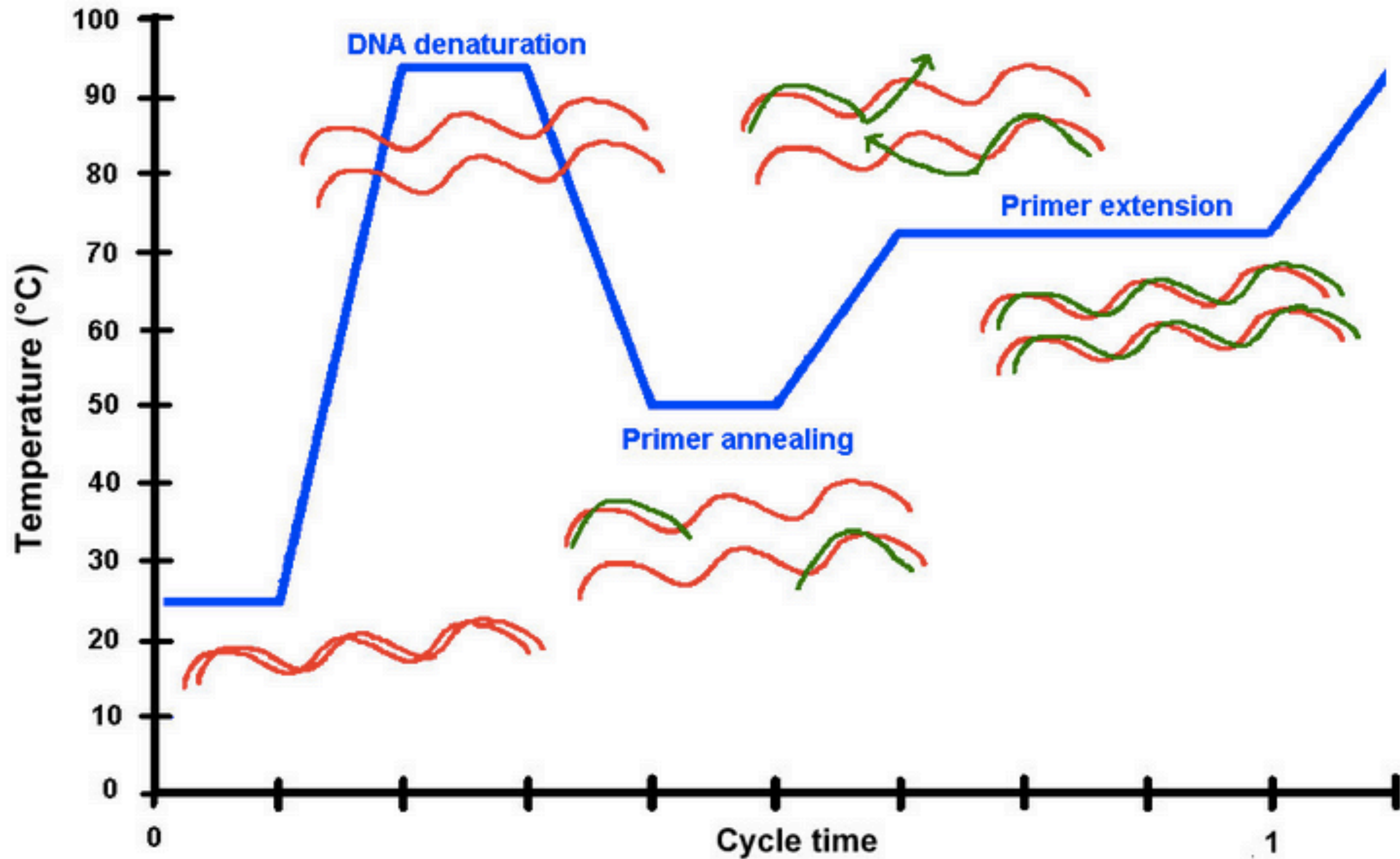
insert
fragment
Fkbp12

vector
backbone
pRSETb



- amplification of DNA:
  - **Polymerase Chain Reaction**
    - Nobel prize, Kary Mullis 1993
  - primers
    ssDNA complementary to gene of interest
- digestion:
  - **restriction enzymes endonucleases**
- ligation:
  - **ligase: seals phosphodiester bond**

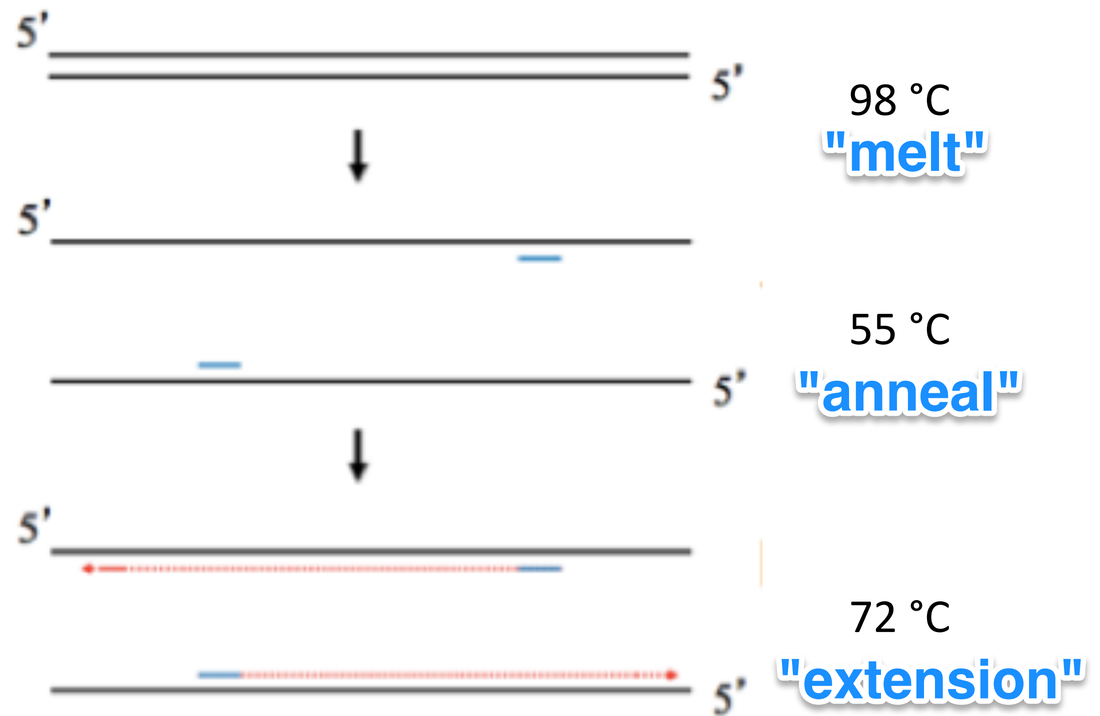
Polymerase chain reaction (PCR): 1 cycle



splice-bio.com

PCR ingredients and cycling conditions

PCR ingredients
high fidelity polymerase
template DNA
forward and reverse primers
dNTPs
buffers/Mg^{2+}
H_2O



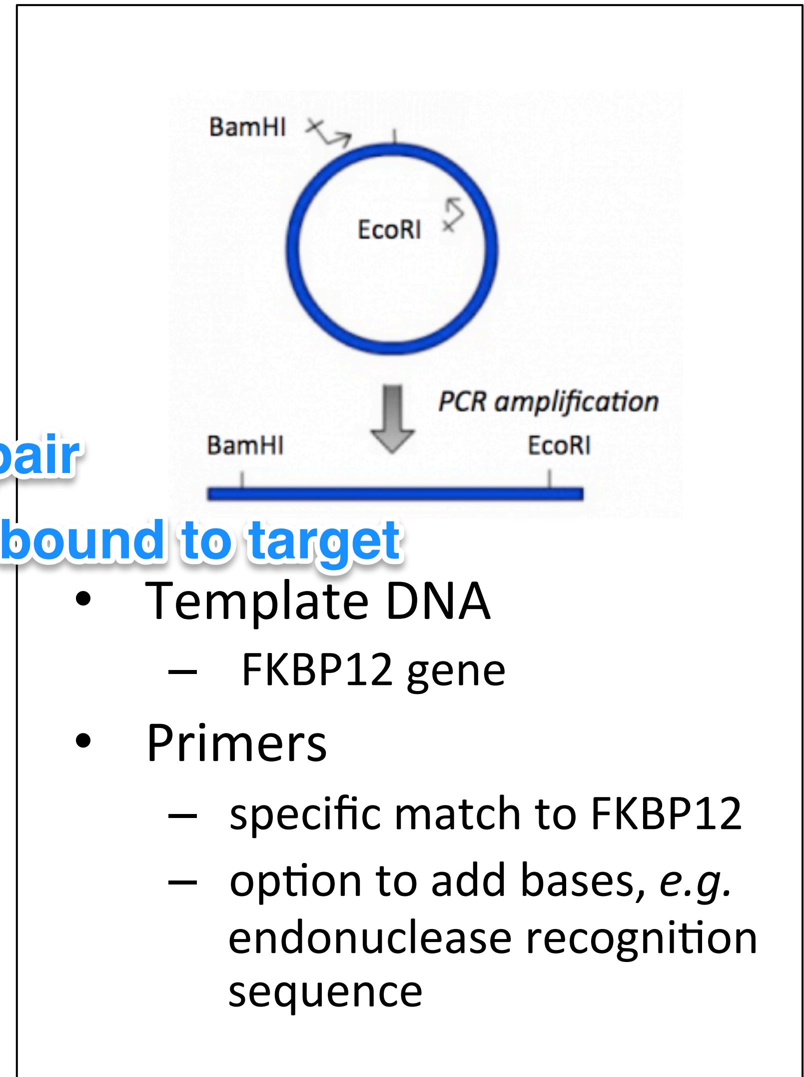
25 cycles

Using PCR to generate *FKBP12* product

3 major steps in the PCR cycle: which temperature and why?

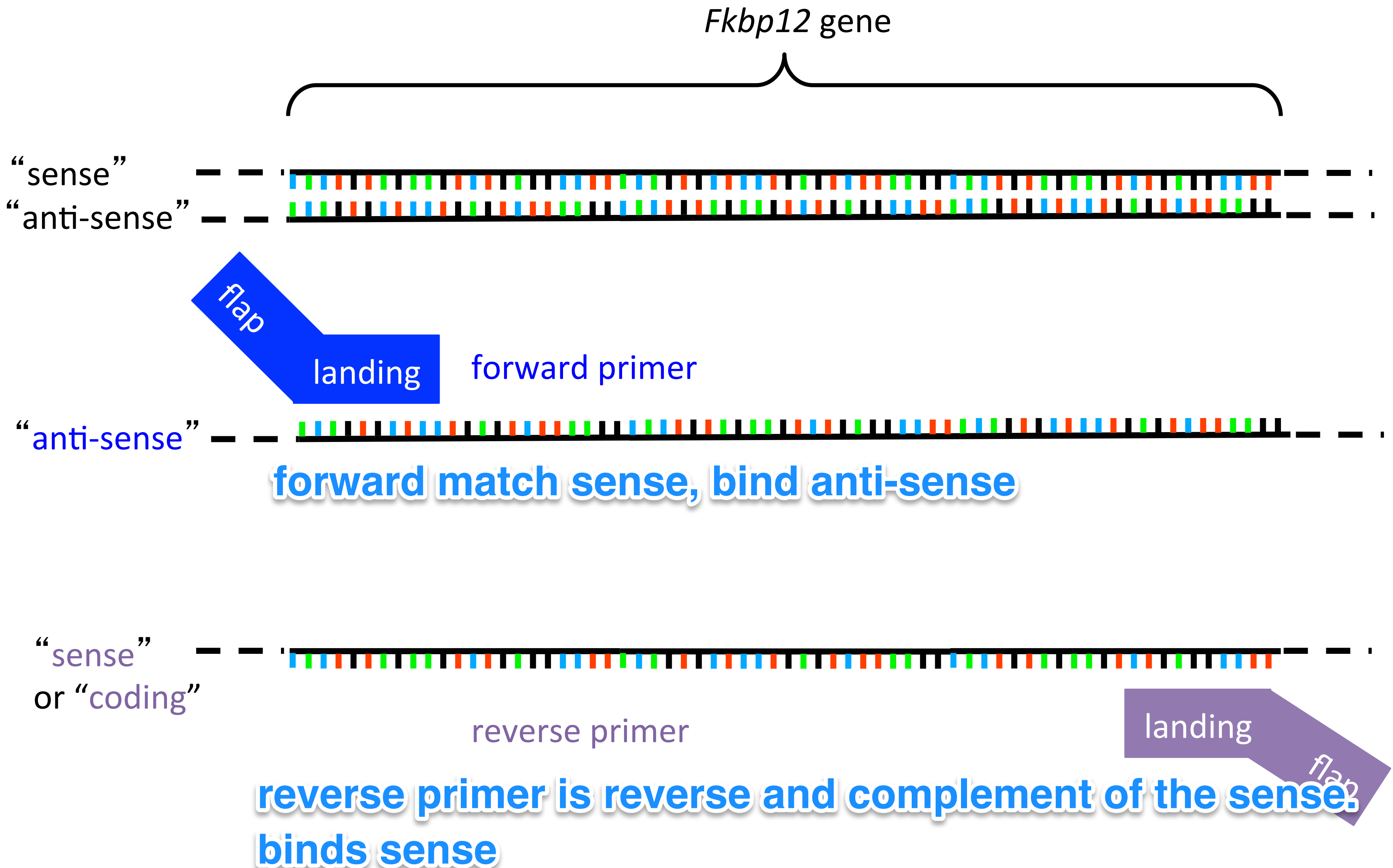
- Melt
  - 98 °C
  - breaking hydrogen bonds
- Anneal adjusted for each primer pair
  - T_m(primer) = 50% of primers are bound to target
  - $T_{anneal} \sim T_m$(primer) – 5°C
- Extend
  - 72 °C (for Taq)
  - 1 min / 1000 bp

rate of extension of the polymerase



always 5' to 3'

How do you design primers?



Primer design guidelines

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

GC=3 H bonds
AT= 2 Hbonds

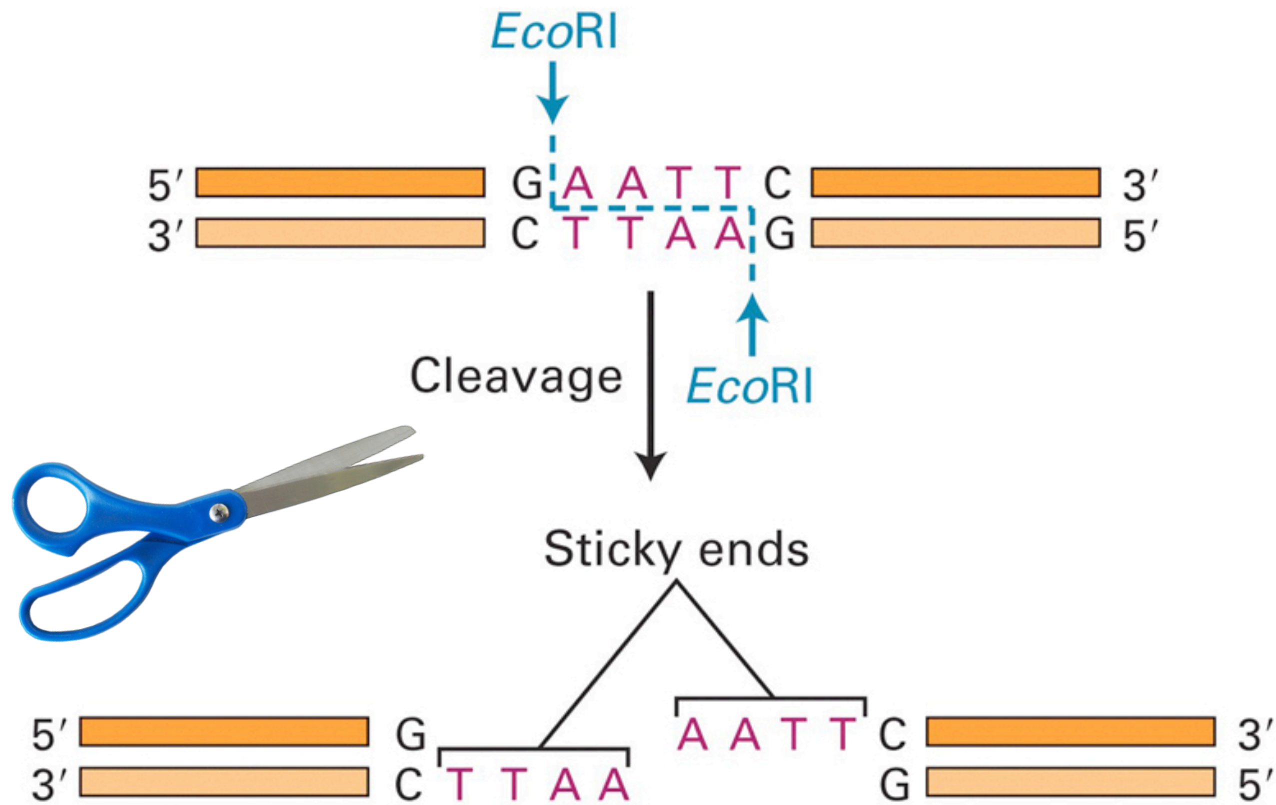
- T_m(primer)
  - < 65 °C

- Specificity
  - is primer complementary to other loci of the plasmid?
- Secondary structure
  - hairpins
  - complementation
- Repetitive sequence
  - di-nucleotides < 4 tatatatata
  - runs < 4 bp tttttttttt

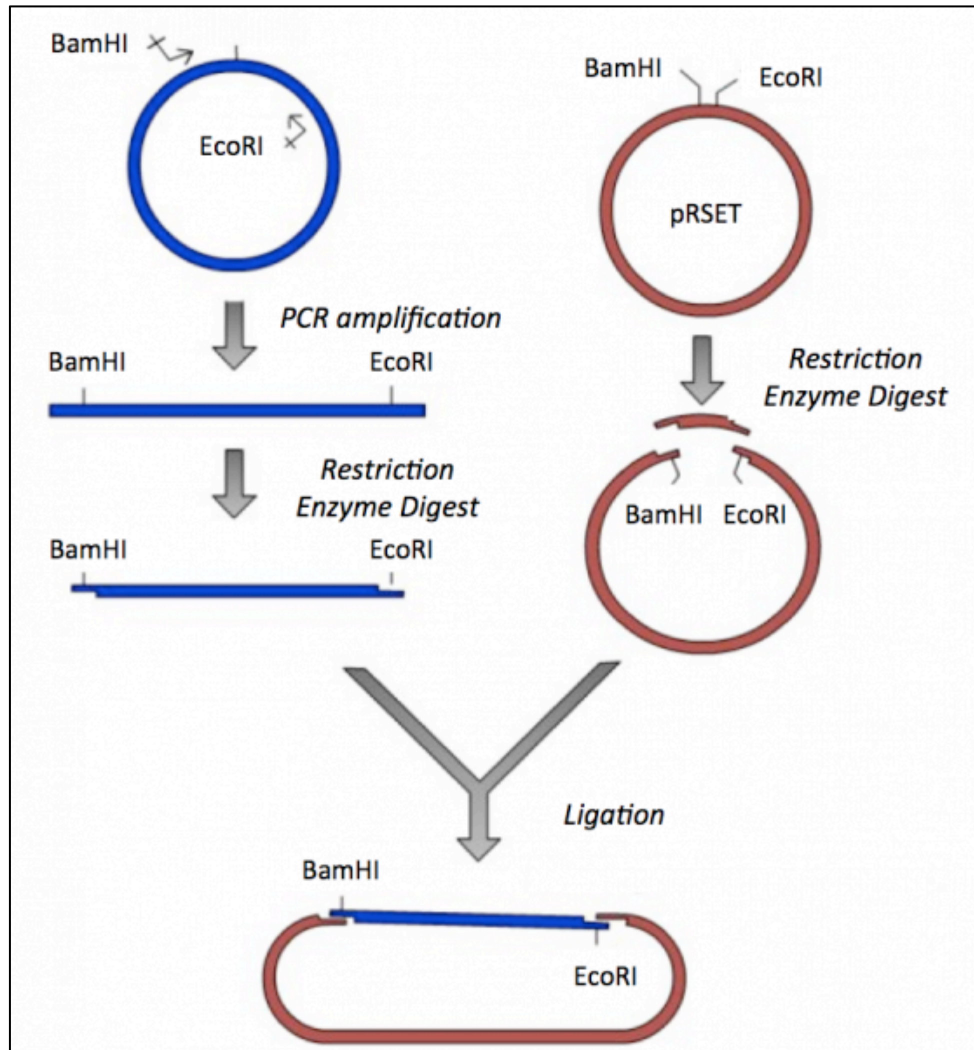
University of Utah: PCR animation

learn.genetics.utah.edu

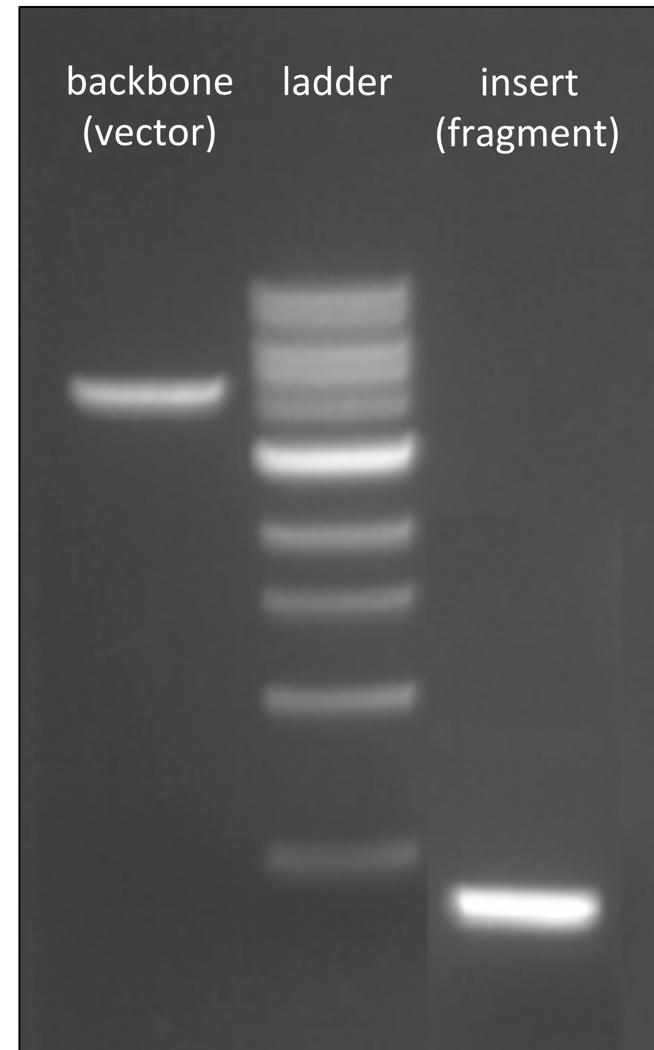
Digestion: FKBP12 insert and pRSETb backbone



pRSETb-FKBP12 was constructed by ligation

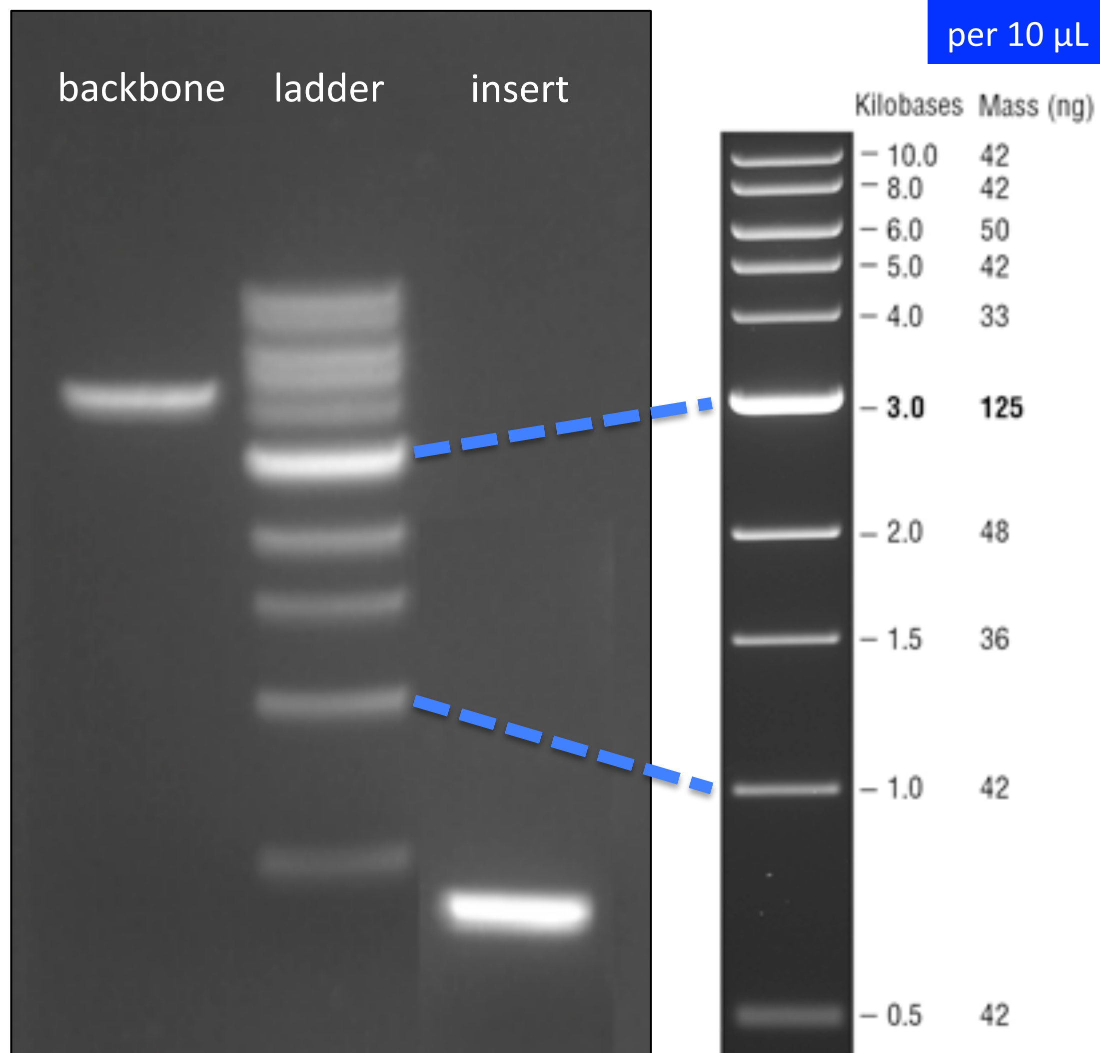


pRSETb-FKBP12 cloning strategy



recovery gel

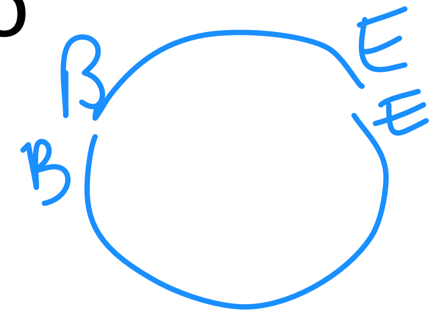
For ligation, want 1:4 *molar* backbone : insert, first must estimate DNA mass



- Assuming
  - 10 µL of ladder loaded,
  - 5 µL of *BamHI-EcoRI* double digest loaded,
- amount of backbone = 100 ng
- amount of insert = 200 ng

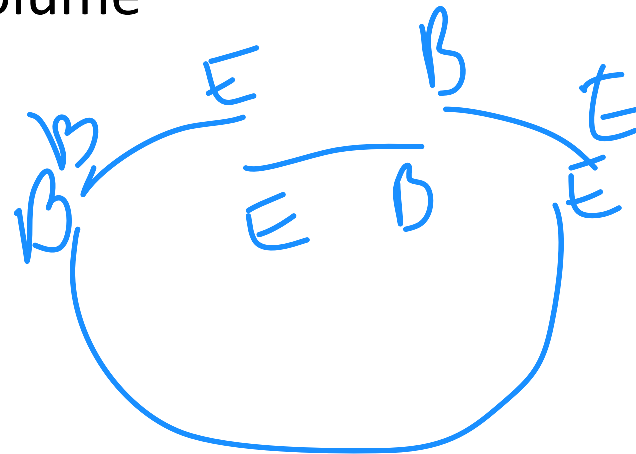
➢ but mass of DNA ≠ molar amount of DNA **(see handout)**

Optimal backbone-to-insert ratio



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

- What if too much insert?



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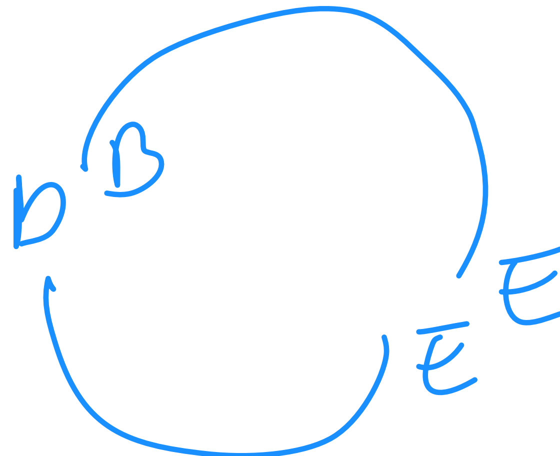
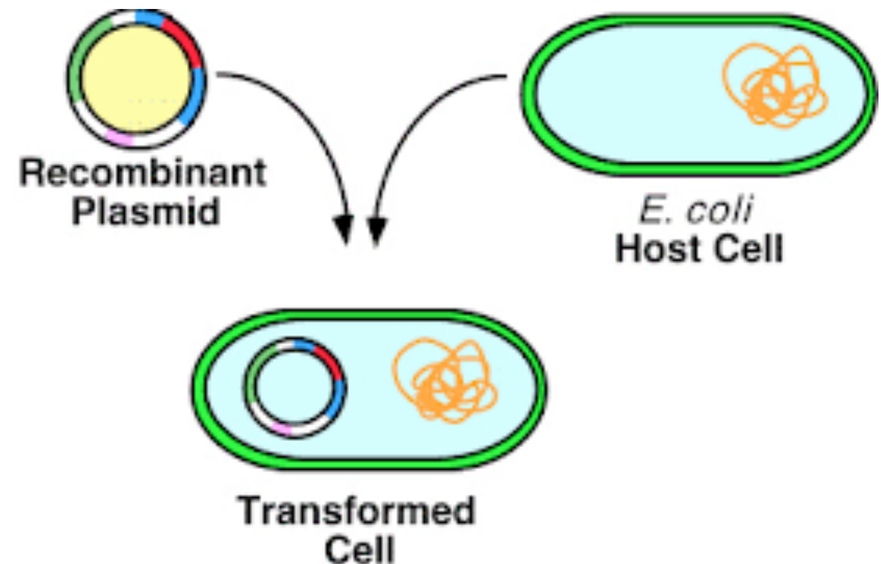


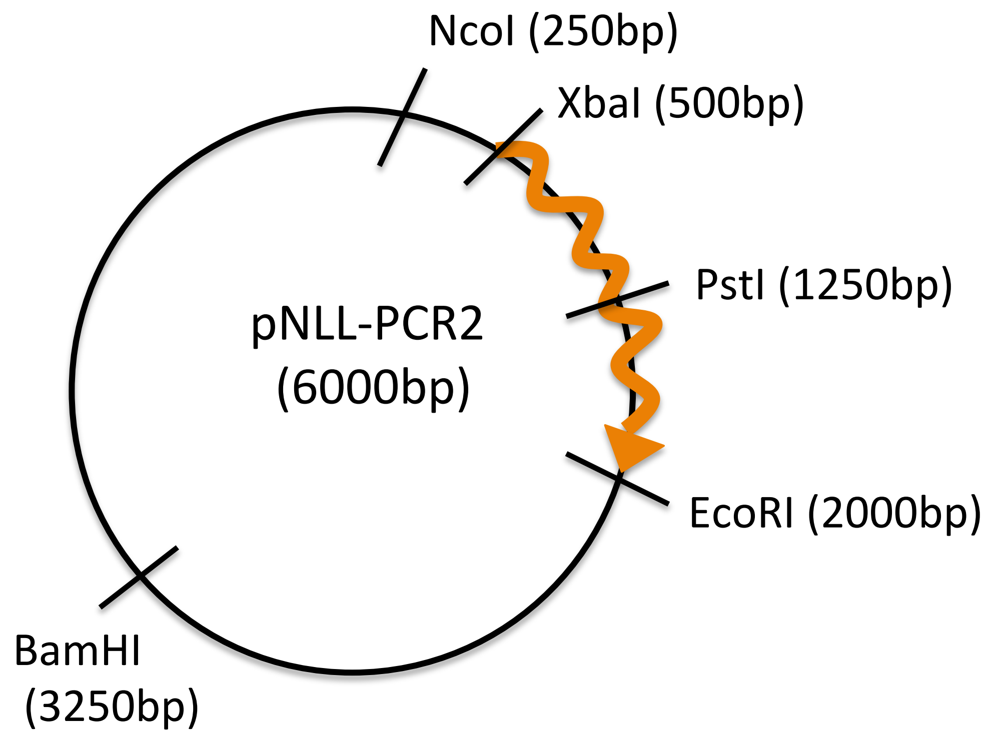
How do we confirm our product?

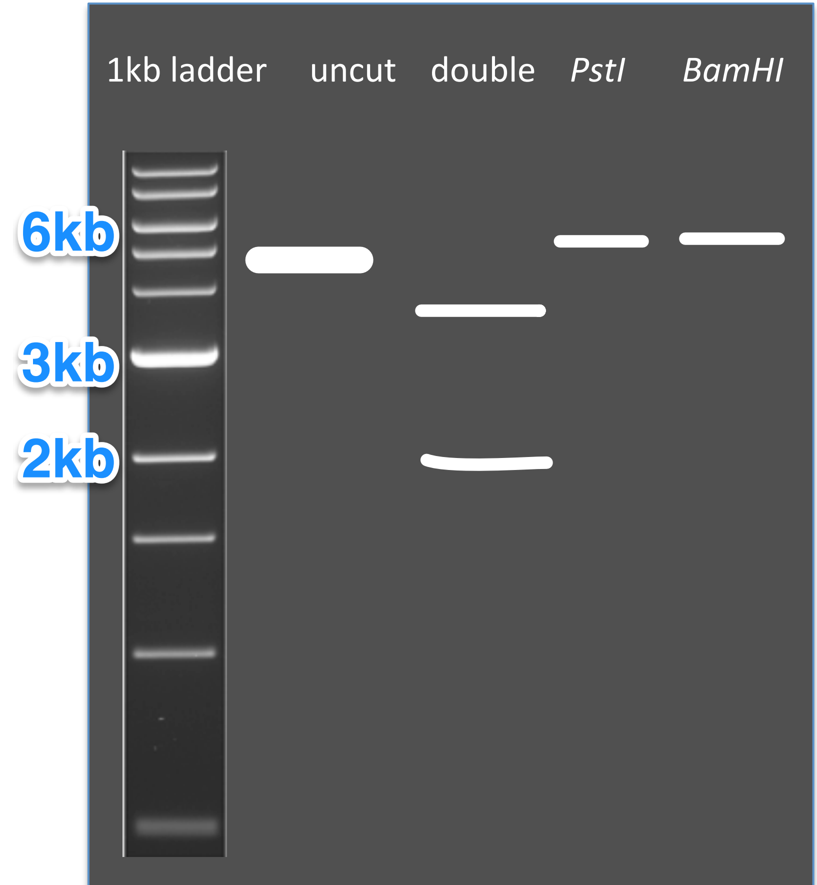


1. Transformation
   - competent cells
   - incubation
   - heat shock
   - recovery
   - selection by antibiotics resistance pRSETb=ampicillin
2. Purification (mini-prep)
   - separate plasmid from host (chromosomal) DNA
3. Digestion +sequencing
   - again
   - by *different* restriction enzymes

Confirmation digest considerations

- GOAL: 1 cut un backbone and 1 cut in insert
- Are the two enzymes compatible (same buffer)?
- Are fragments easily distinguished on an agarose gel?





Today in lab:

1. Complete *in silico* cloning exercise
2. Check growth phase of BL21 (start this no later than 4:40pm)
3. Induce protein expression in BL21 *E. coli*

*Make sure to keep notes in Benchling