Happy snow day ?!

M1D1: *in silico* cloning, induce protein expression

02/09/2017

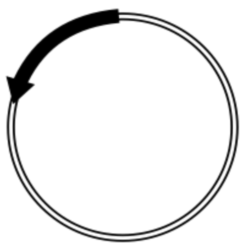
I hope these pre-lab discussion notes help you go through the "pretend cloning" exercises in Part 3 of http://engineerbiology.org/wiki/20.109(S17):In_silico_cloning_and_induction_of_protein_expression_(Day1)

Today in the lab

- Hand in your homework
- Lab orientation quiz

We'll take care of these on M1D2 (Tuesday, 02/14)

- Pre-lab discussion



- "Clone" FKBP12 protein into pRSETb vector plasmid

- Induce FKBP12 protein expression in BL21(DE3)pLysS *E. coli* bacteria

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

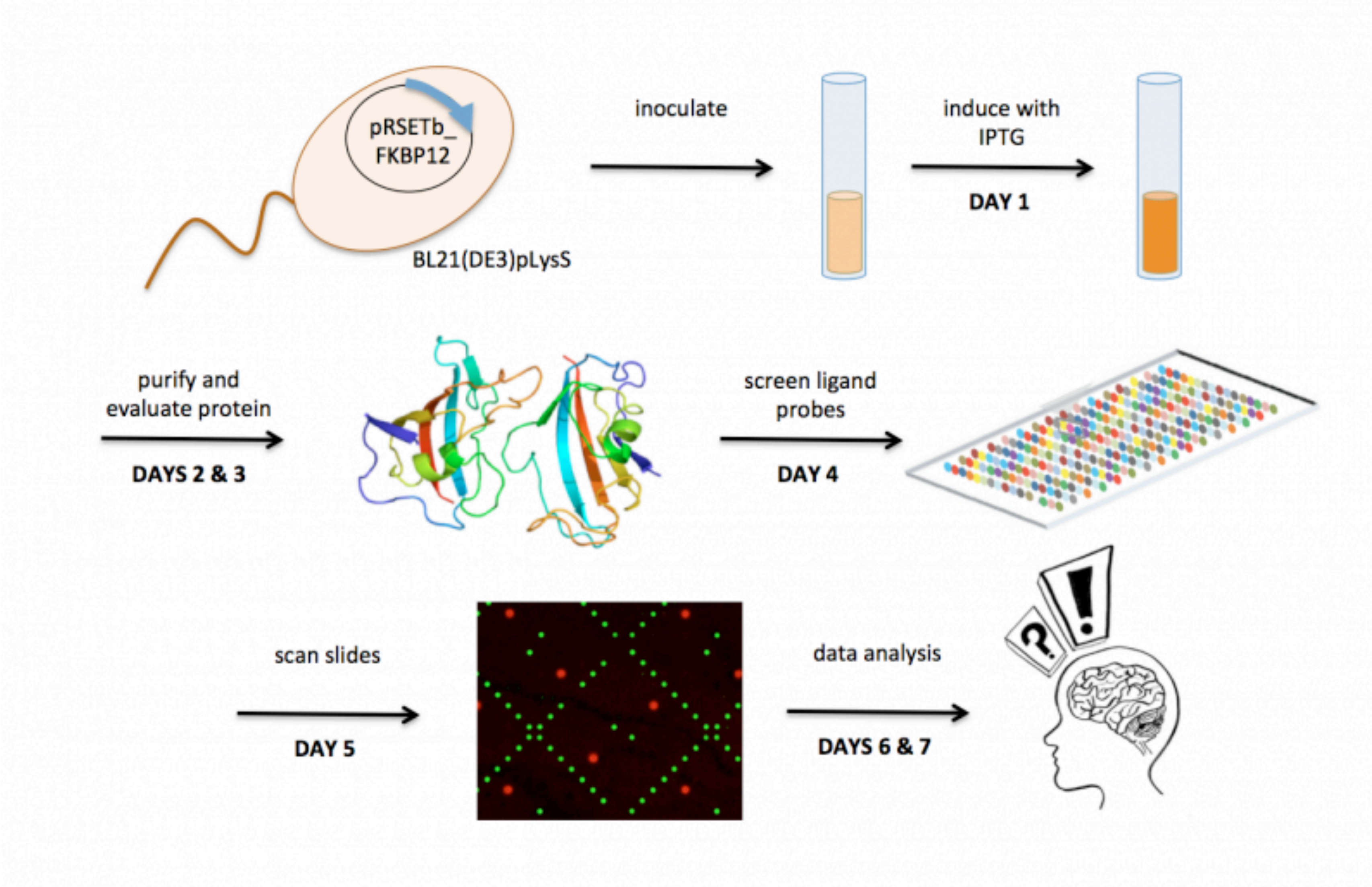
by appointment: nllyell@, lesliemm@, jonas_m@

don't
hesitate !

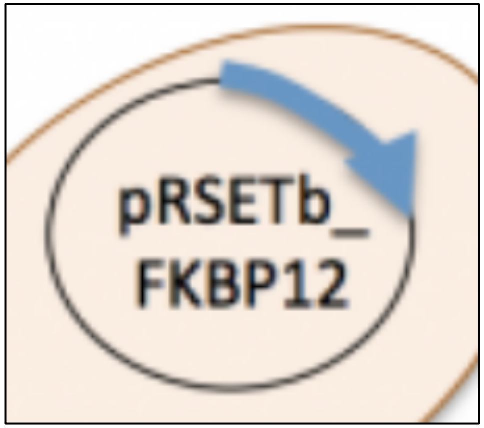
M1 major assignments

- 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

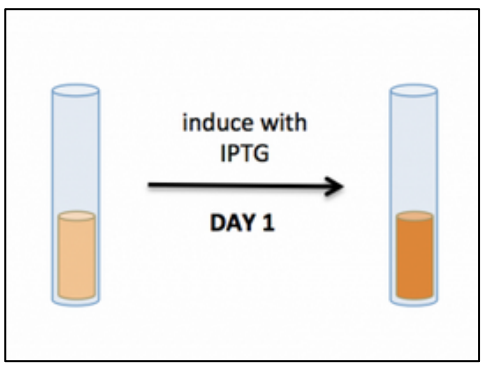
Overview of
"M1: High-throughput ligand screening"



Today in lab



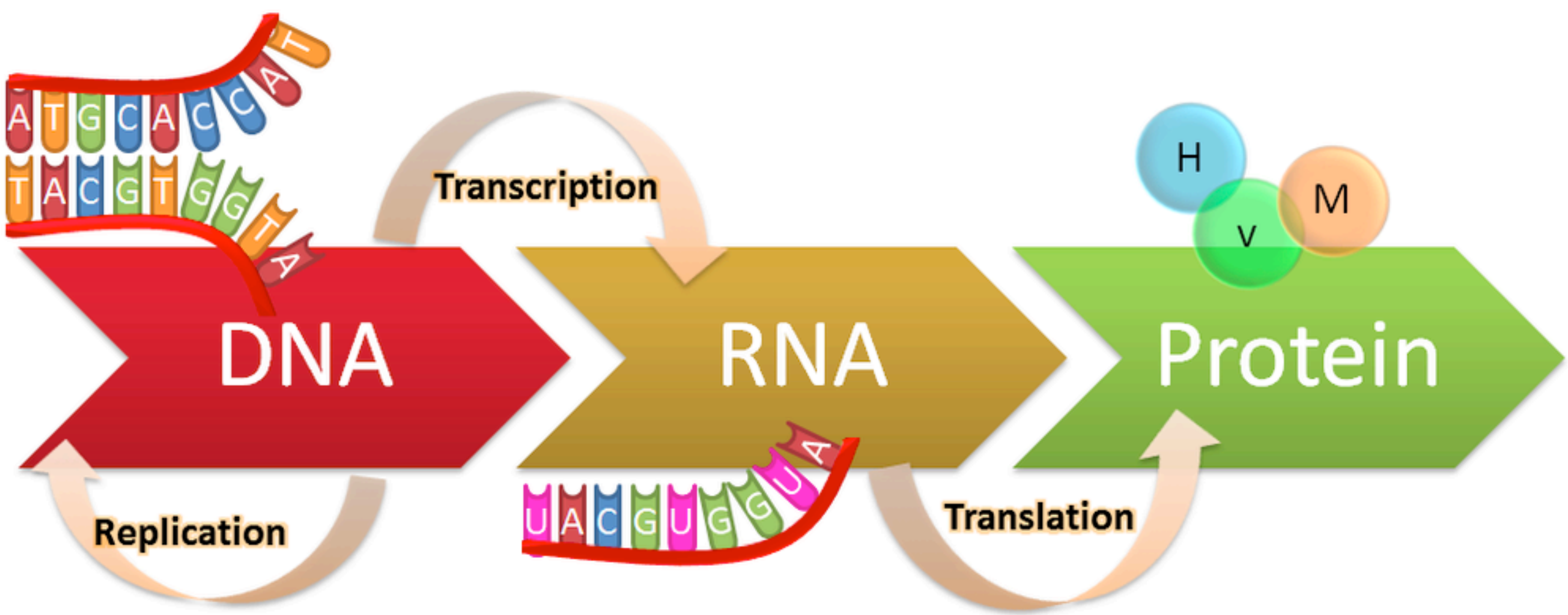
- Reproduce *in silico* the cloning of pRSETb-FKBP12
  - *A Plasmid Editor* (APE)
  - PCR amplification of *Fkbp12* **insert**
  - digestion of pRSETb **vector** by endonucleases
  - ligation



- Induce expression of FKBP12 protein
  - in BL21(DE3)pLysS *E. coli* cells
  - using IPTG
  - More about these acronyms on M1D2!

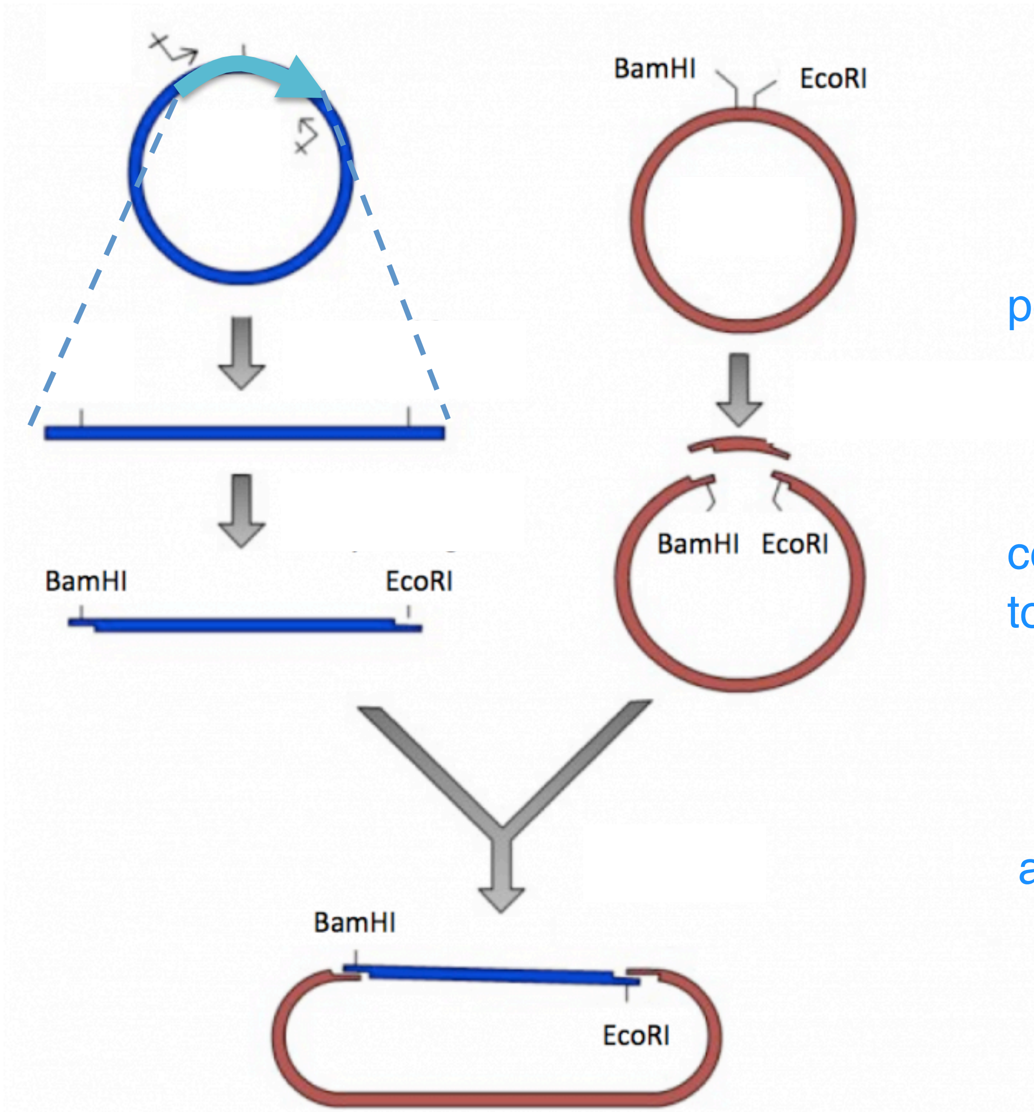
The central dogma

- 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 insert fragment *Fkbp12*

vector backbone **pRSETb**



How is DNA engineered?

- amplification
  - PCR:

polymerase chain reaction

  - Kary Mullis 1993
  - primers

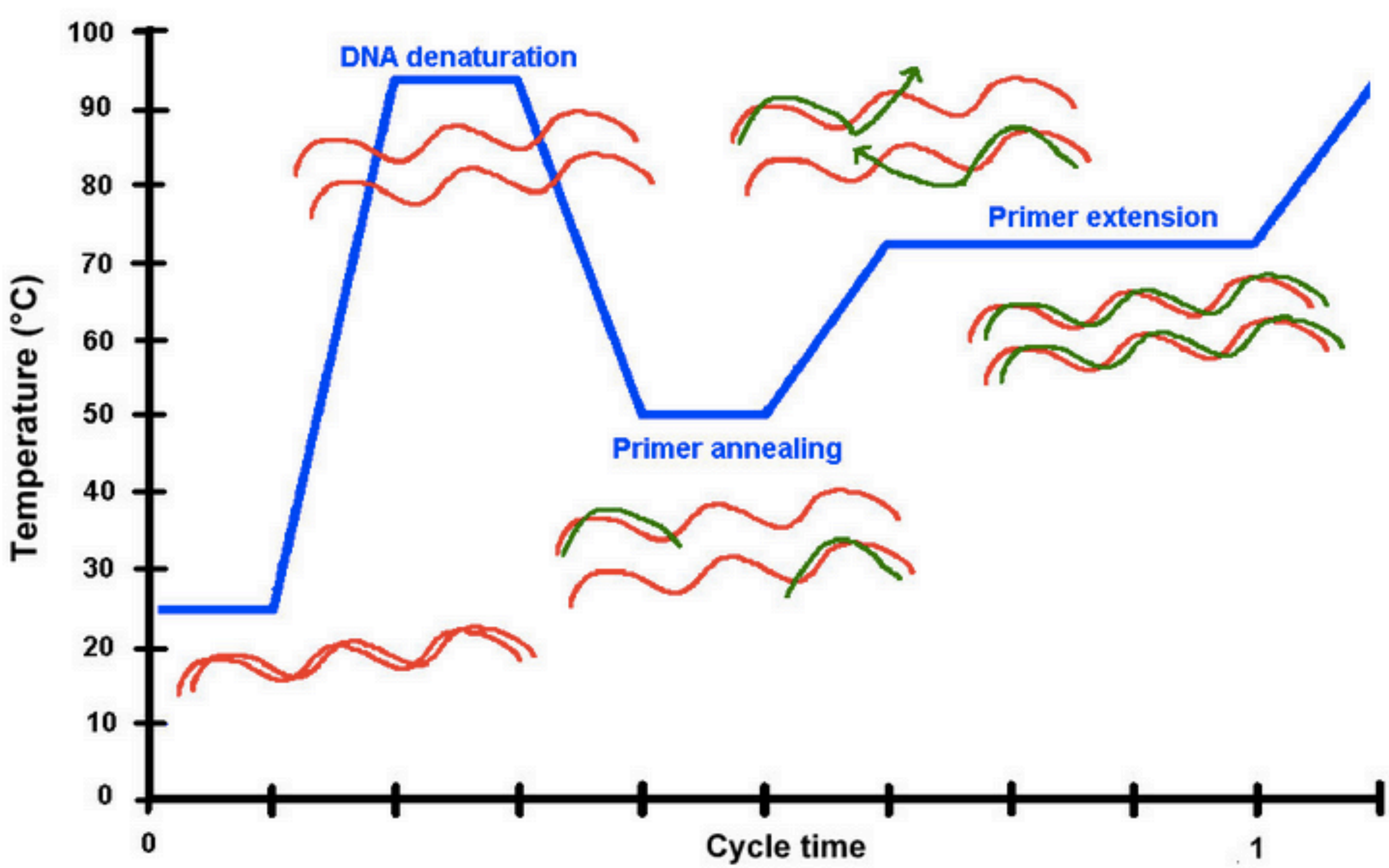
complementary to the beginning and to the end of your gene of interest

- digestion
  - restriction enzymes

also called "endonucleases"

- ligation

Polymerase chain reaction (PCR): 1 cycle

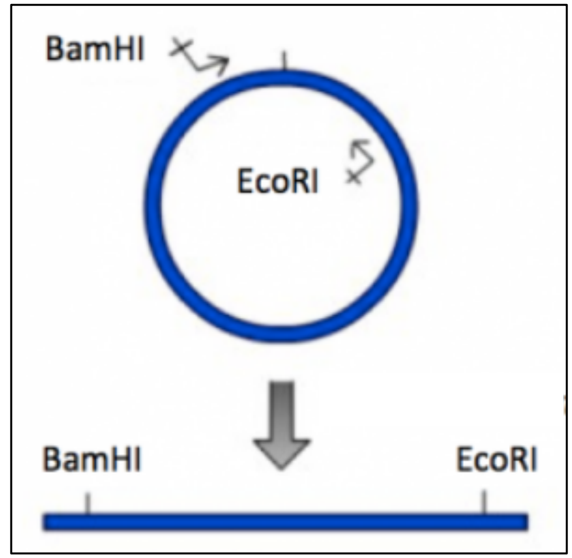


splice-bio.com

Using PCR to generate FKBP12 *gene* flanked by restriction enzyme recognition sites

3 major steps in each cycle:

- Melt
  - 95 °C
  - break hydrogen bonds
- Anneal
  - T_m(primer) = half is annealed
  - $T_{anneal} \sim T_m(\text{primer}) - 5°C$
- Extend
  - 72 °C (for Taq*)*
  - 1 min / 1000 bp



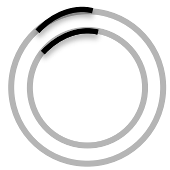
✧ Primers

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

Make sure you understand and can reproduce yourself the next 3 slides :

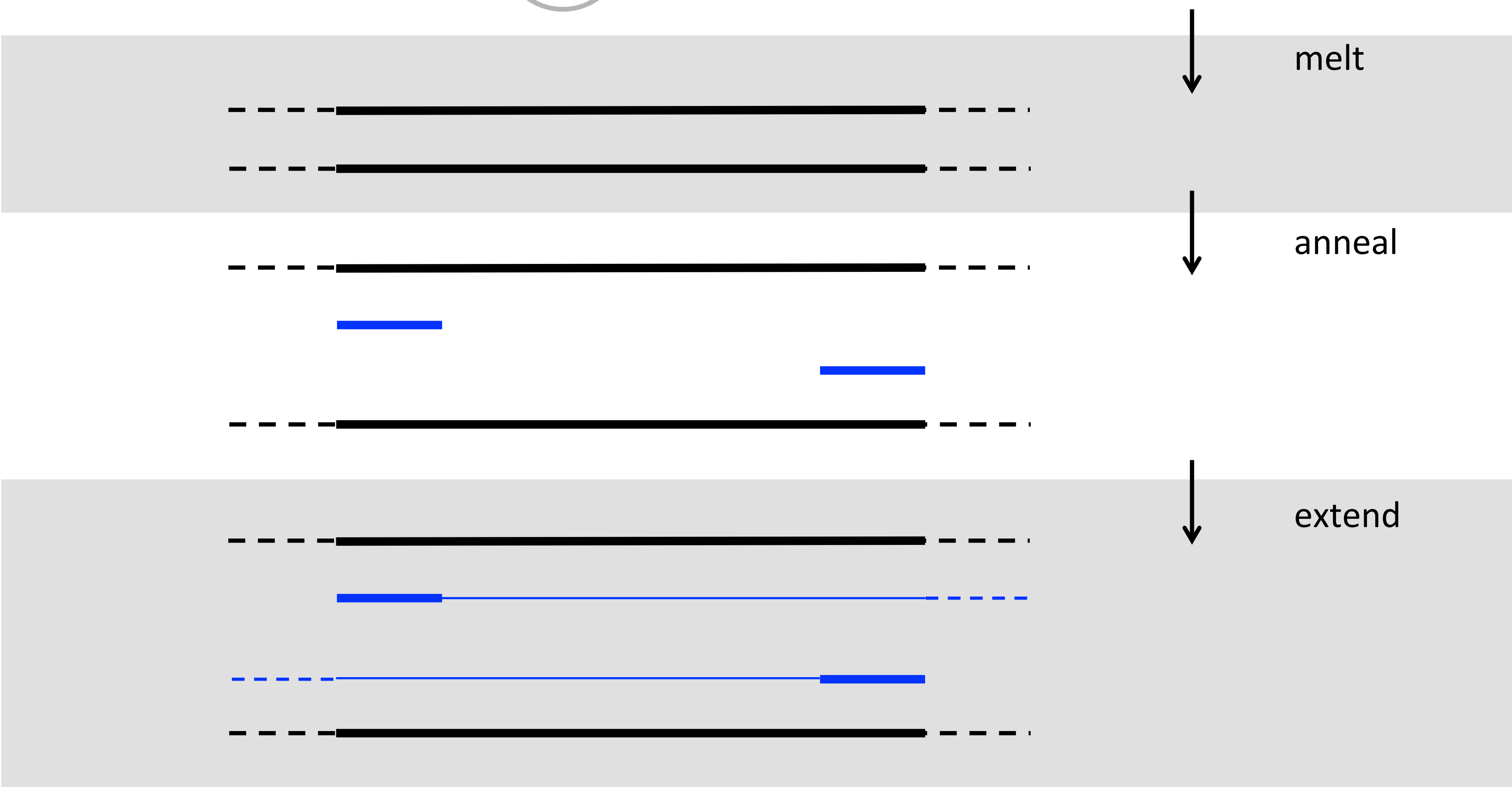
How many PCR cycles until only your amplicon?

Cycle 1

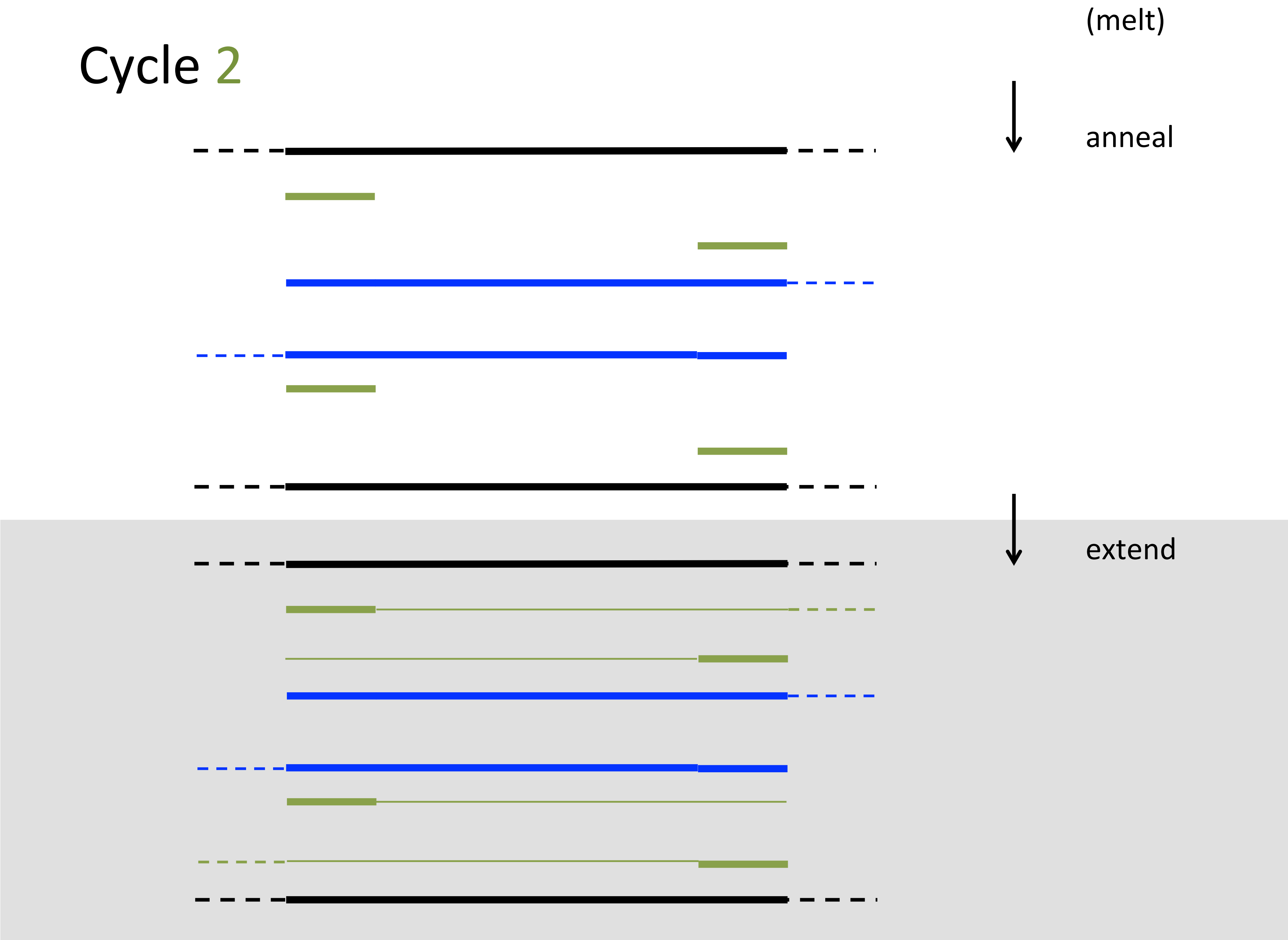


dsDNA

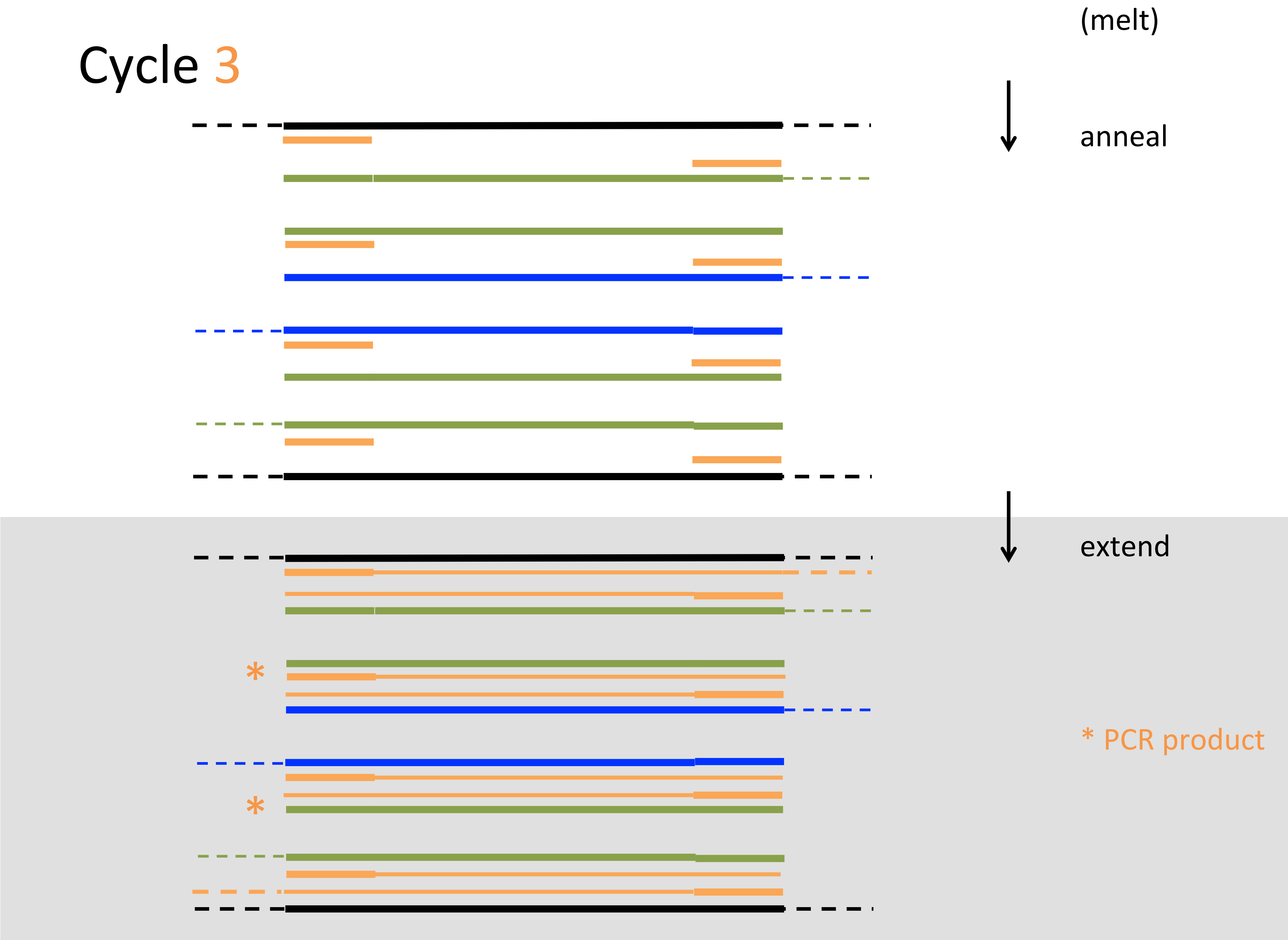
dCas9



Cycle 2

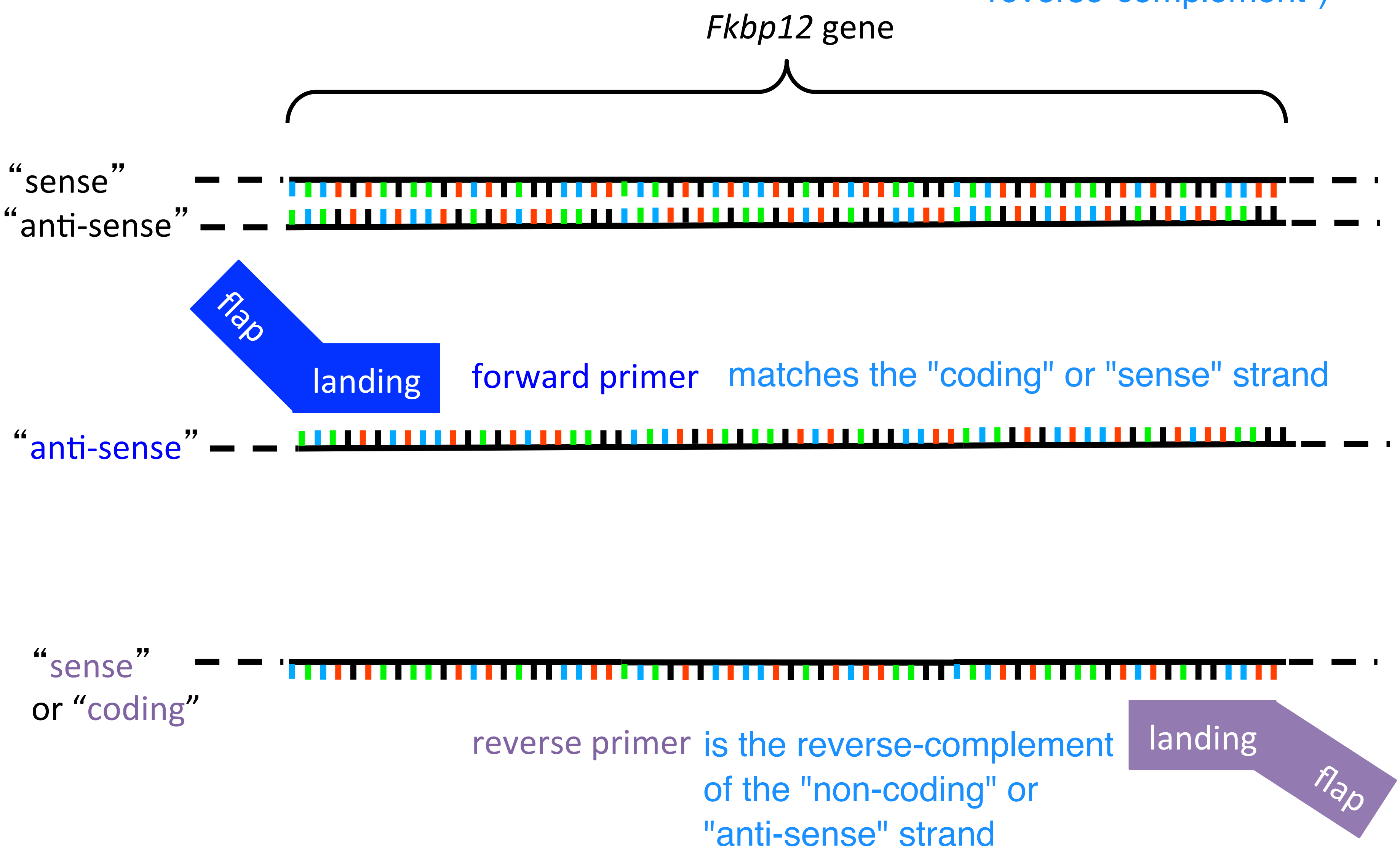


Cycle 3



How do you design primers?

always list primers' sequence 5' to 3' (even for the reverse primer, hence the terminology "reverse-complement")



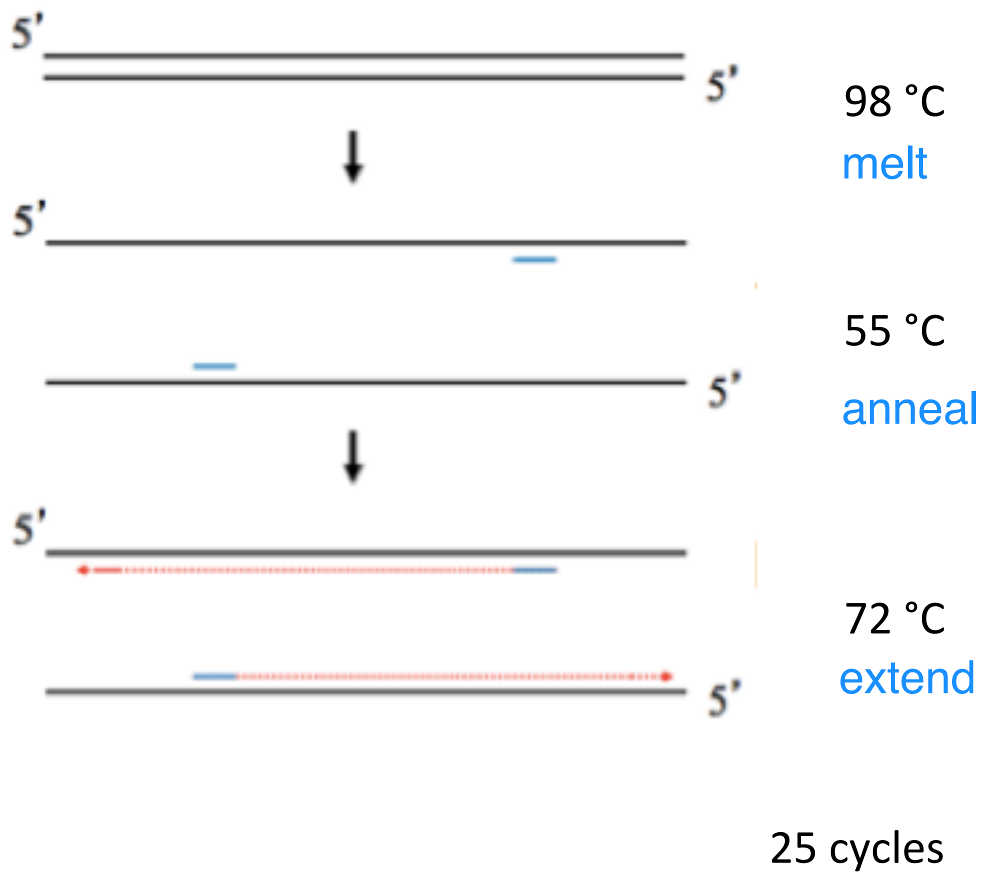
Primer design guidelines

- Length
  - 17-28 base pairs
  - long enough for specificity, short enough to anneal at T_anneal
- GC content
  - 40-60%
  - GC clamp at ends
- T_m(primer)
  - < 65 °C

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

PCR ingredients and cycling conditions

PCR ingredients
template DNA
primers
high-fidelity polymerase
Mg2+ as cofactor for enzyme
dNTPs
buffer
water

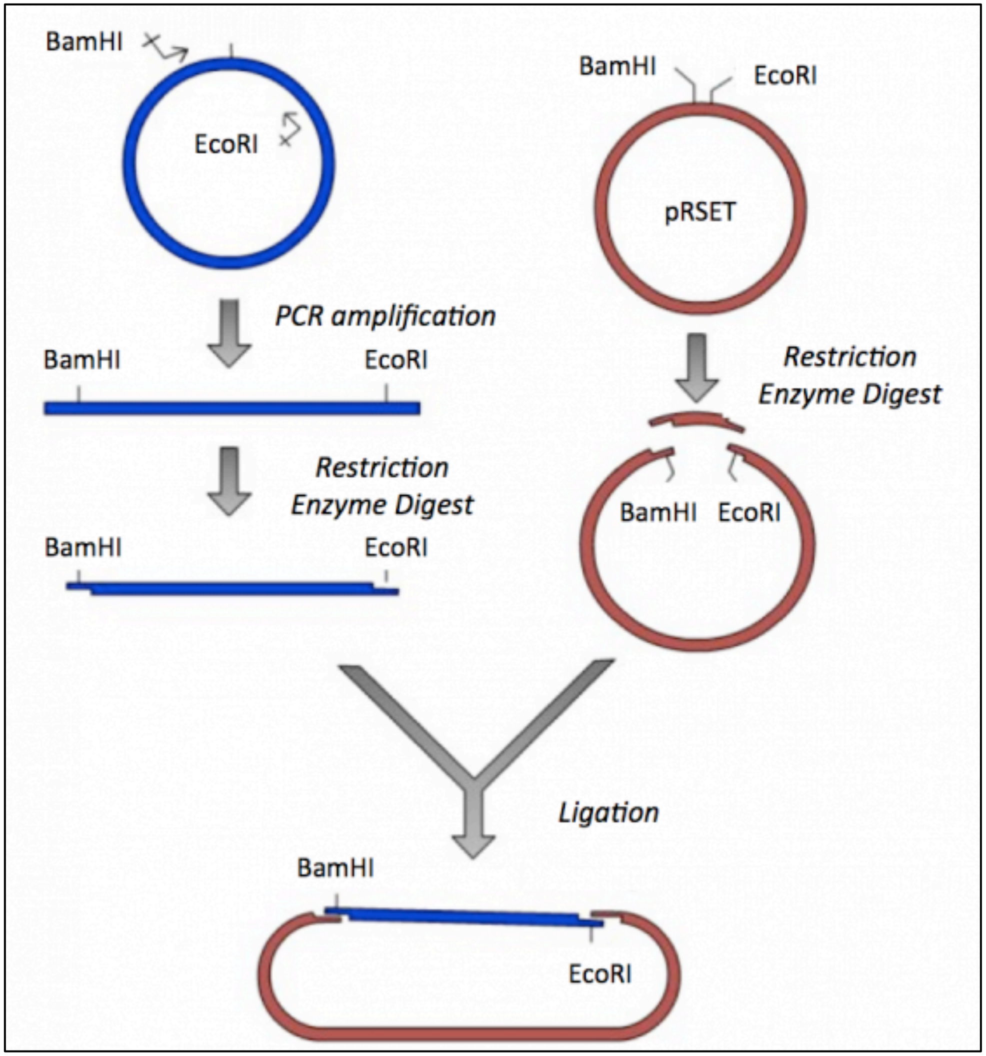


check it out !

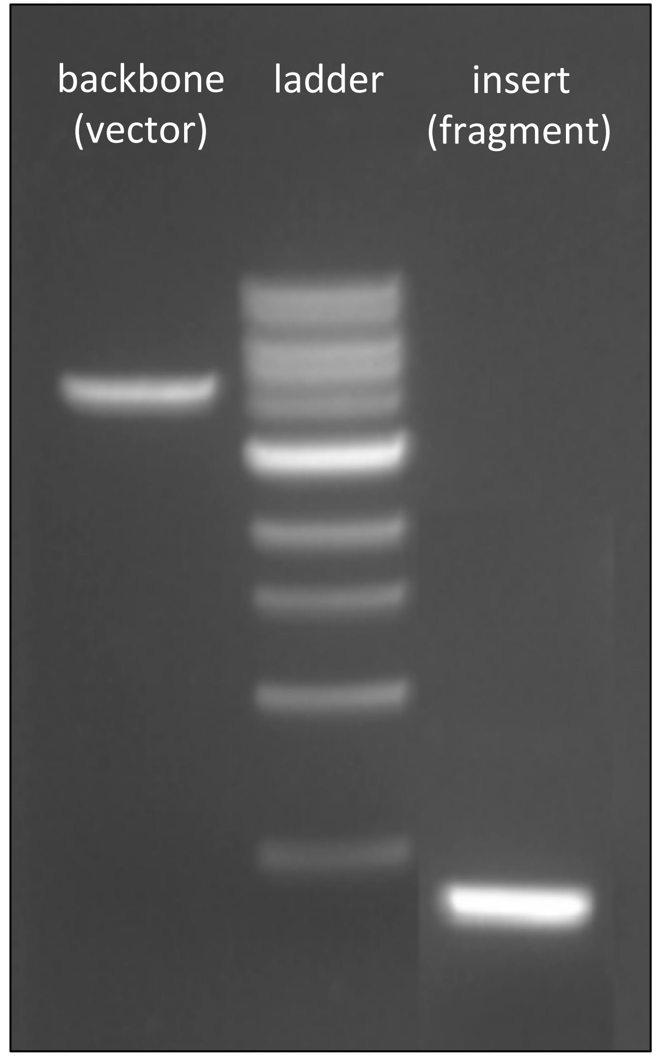
Leslie's favorite PCR animation

learn.genetics.utah.edu

pRSETb-FKBP12 was constructed by ligation

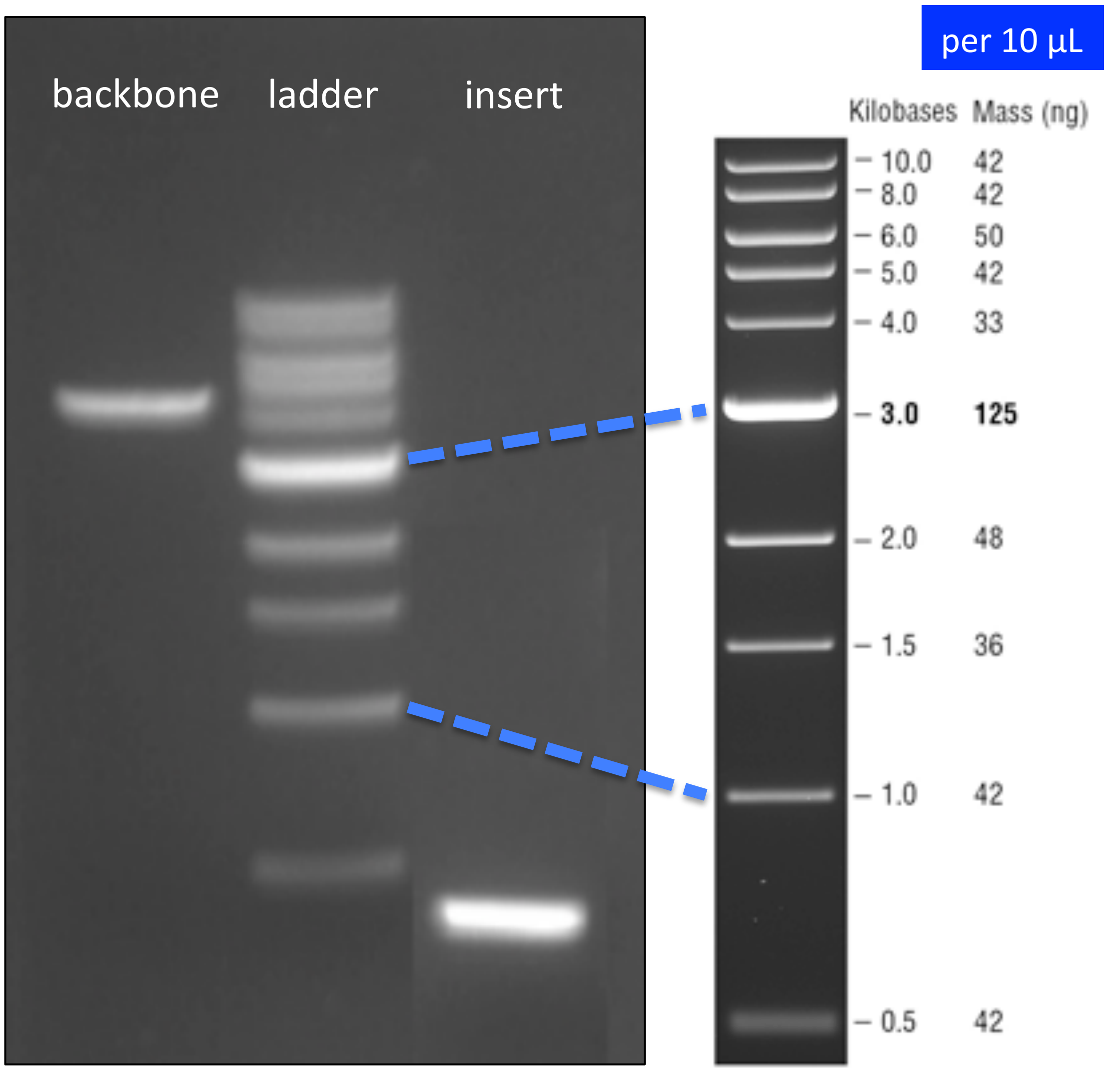


pRSETb-FKBP12 cloning strategy



recovery gel

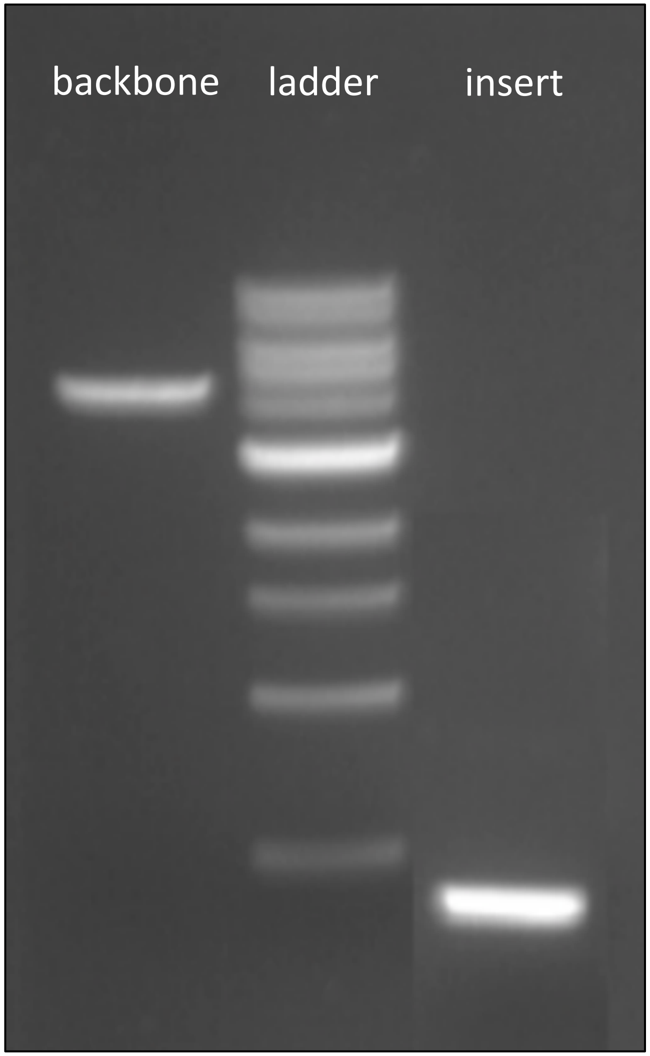
For ligation, mix 1:4 *molar* backbone : insert



- 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

Calculate the 1:4 *molar* amounts for ligation

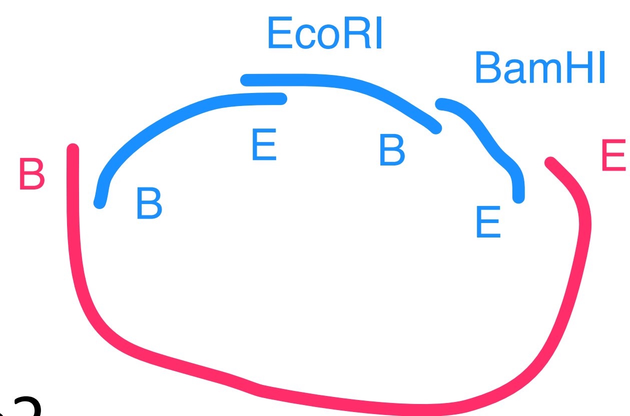


1. From recovery gel, estimate
   - backbone: 100 ng / 5 µL = 20 ng/µL
   - insert: 200 ng / 5 µL = 40 ng/µL
2. Determine volume of backbone needed
   - 50-100 ng, choose 60 ng, *i.e.* 3 µL
3. Calculate moles of backbone
   - 2776 bp * (660 g / (mol*bp)) = 1.83 x 10^6 g/mol
   - so 60 ng / (1.83 x 10^6 g/mol) = 3.27 x 10^{-14} mol
4. Determine moles of insert needed (4X bkbn)
   - 4 x 3.27 x 10^{-14} ~ 1.31 x 10^{-13} mol
   - with 408 bp * (660 g / (mol*bp)) = 2.69 x 10^5 g/mol
   - so use 1.31 x 10^{-13} mol * 2.69 x 10^5 g/mol ~ 35.3 ng
5. Calculate volume of insert needed
   - 35.3 ng / (40 ng/µL) ~ 1 µL

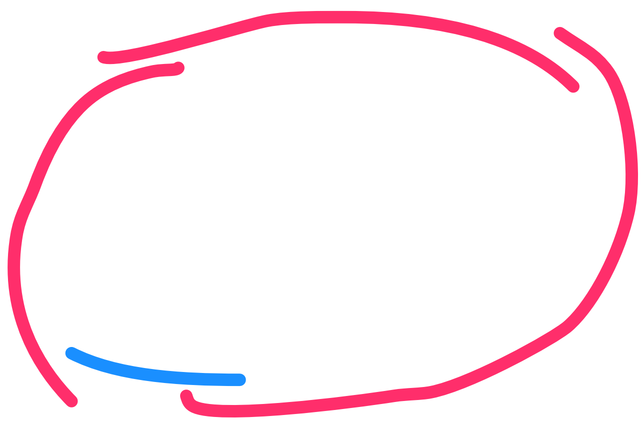
Optimal backbone-to-insert ratio

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

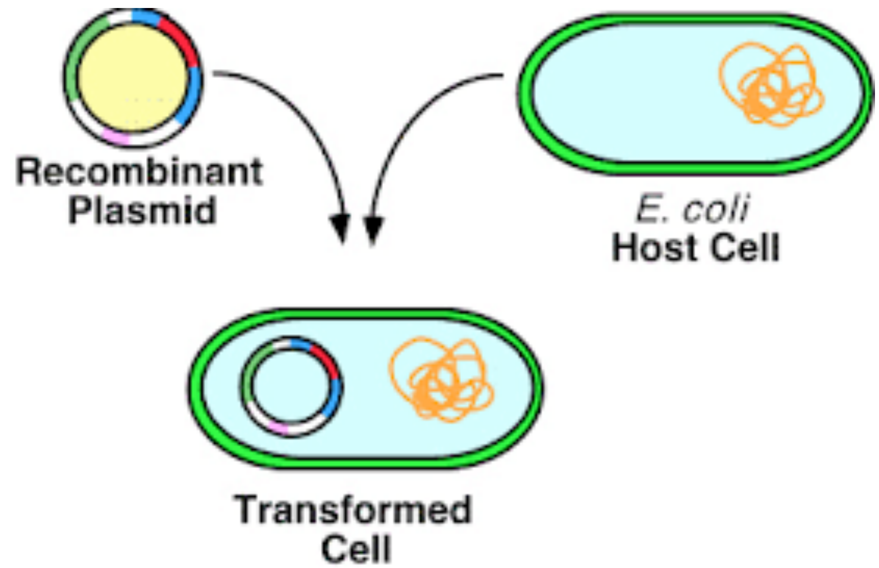
- What if too much insert?



- What if too much backbone?



How do we confirm our product?

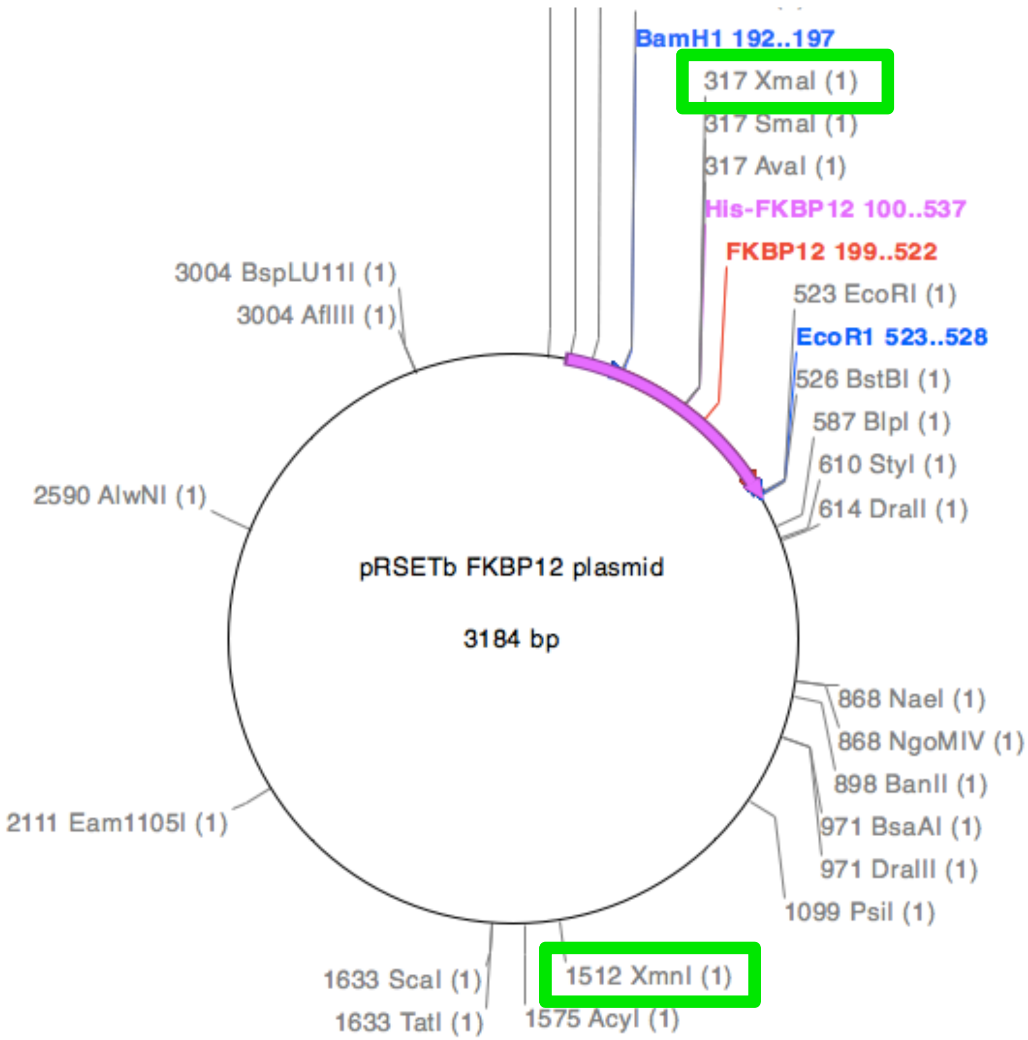


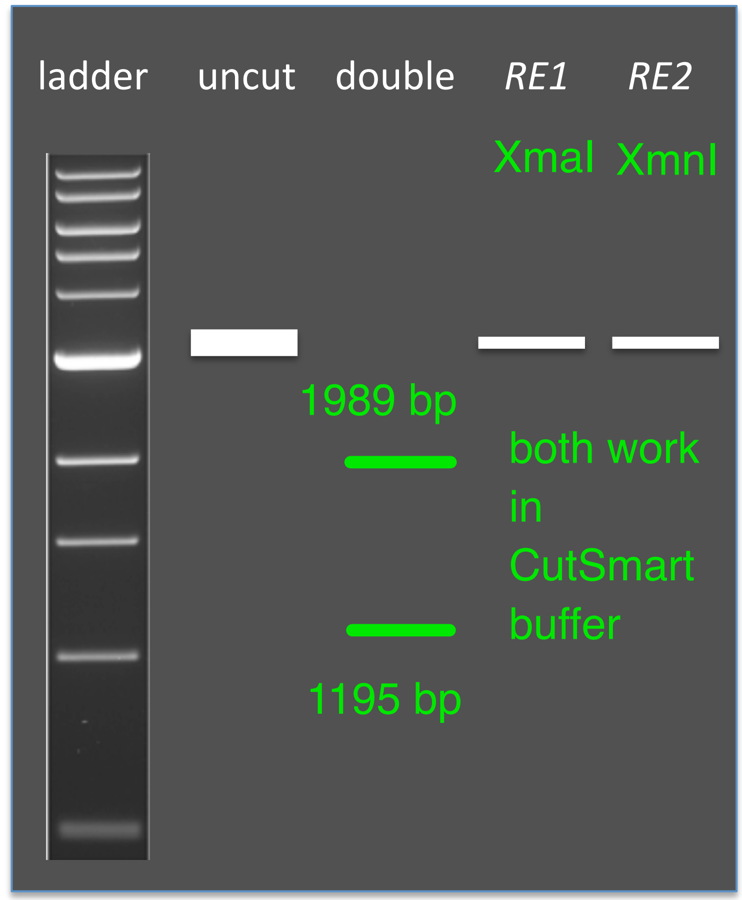
1. Transformation
   - of "competent cells"
   - incubation
   - heat shock
   - recovery
   - selection by antibiotics resistance pRSETb contains an ampicillin resistance cassette
2. Purification (mini-prep)
   - separate plasmid from host (chromosomal) DNA
3. Digestion
   - again
   - by *different*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

Confirmation digest

Goal: 1 cut only in backbone + 1 cut only in insert

- Are fragments easily distinguished on an agarose gel? > 500 bp
- Do you have access to the enzymes?
- Are the two enzymes compatible? work well in same buffer





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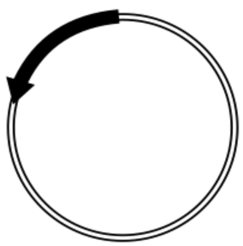

- "Clone" FKBP12 protein into pRSETb vector
- Induce FKBP12 protein expression in BL21(DE3)pLysS *E. coli* bacteria

- Make sure to keep notes in Benchling