M1D1: In silico cloning & confirmation digest of protein expression vector

February 9, 2018

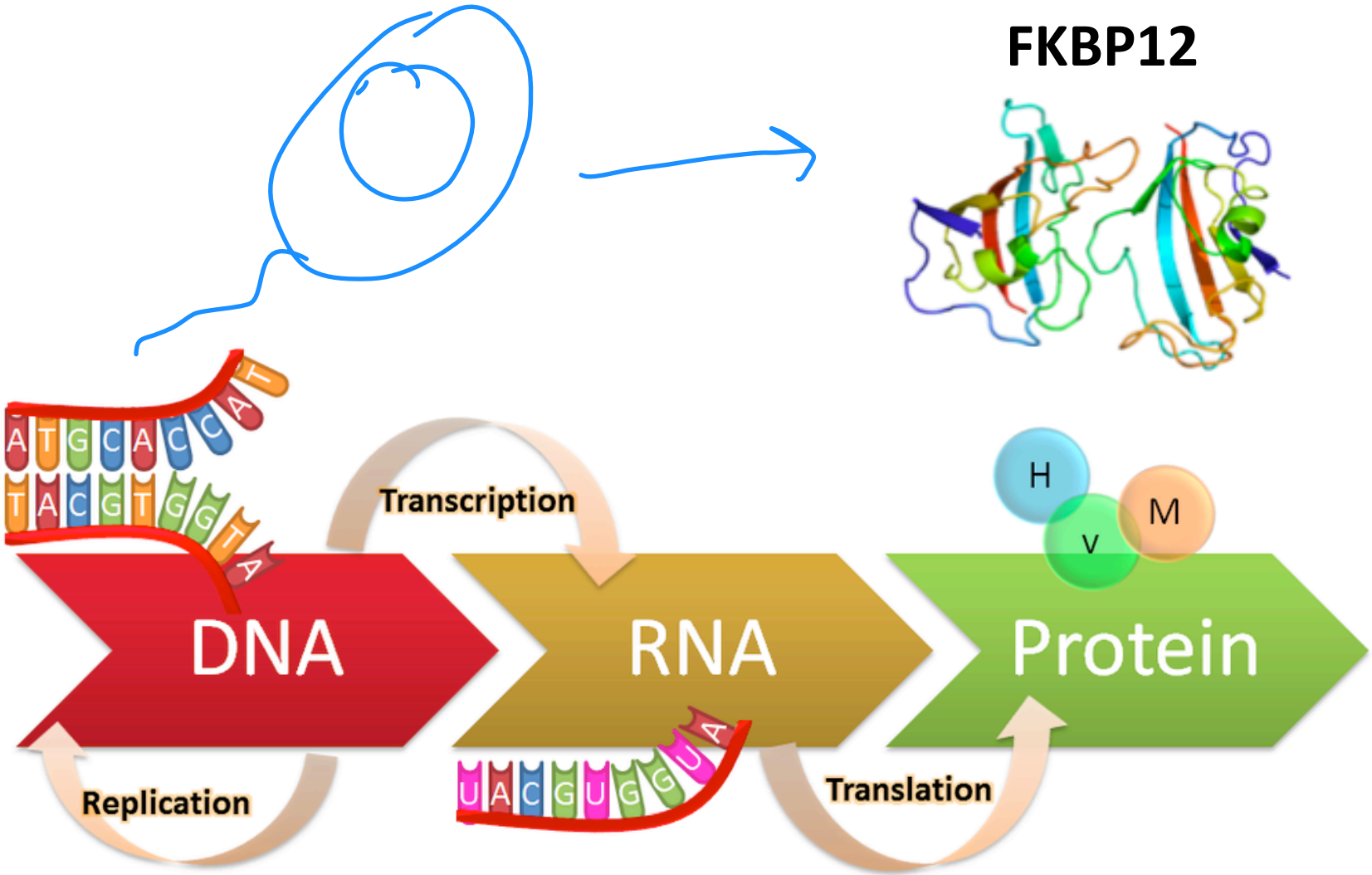
Plan for today

1. Quiz (<45 min)
2. Pre-lab discussion part 1
3. Build protein expression plasmid (virtually)
4. Pre-lab discussion part 2 (~3:45pm)
5. Confirm protein expression plasmid (actually)

Mark your calendars!

- Data summary (15%)
  - completed in teams and submitted via Stellar
  - draft due 3/12, final revision due 3/26
  - format in bullet points
- Mini-presentation (5%)
  - completed individually and submitted via Gmail
  - due 3/17
- Laboratory quizzes
  - scheduled for M1D4 and M1D7
- Notebook (part of 10% Homework and Notebook)
  - All entries viewed, one graded by Casper, due the day after M1D7
- Blog (part of 5% Participation)
  - due 3/18 via Blogspot

How can we make our protein of interest?



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

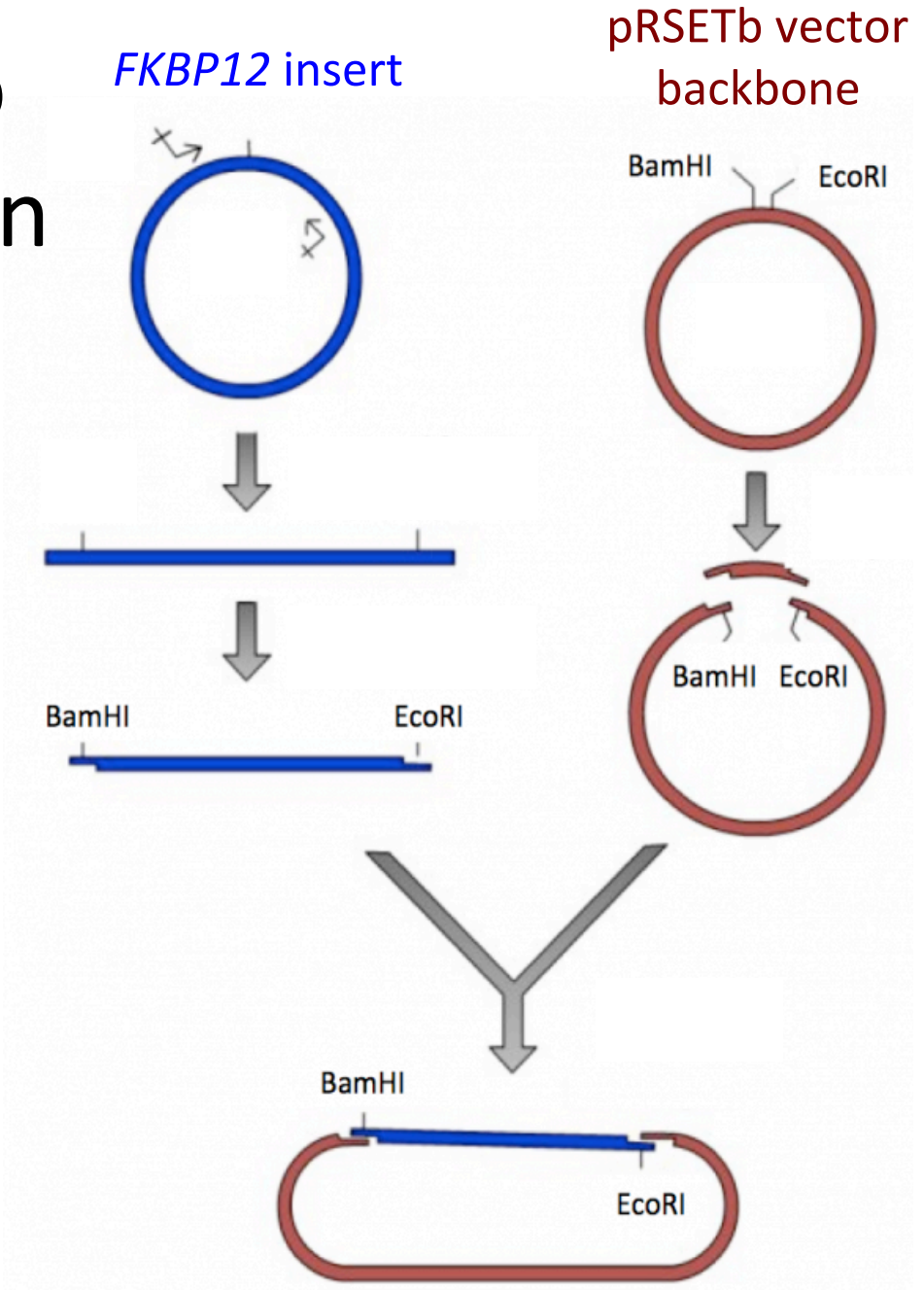
Generate plasmid to make FKBP12 protein

You will do this in silico today

1) Amplification

2) Digestion

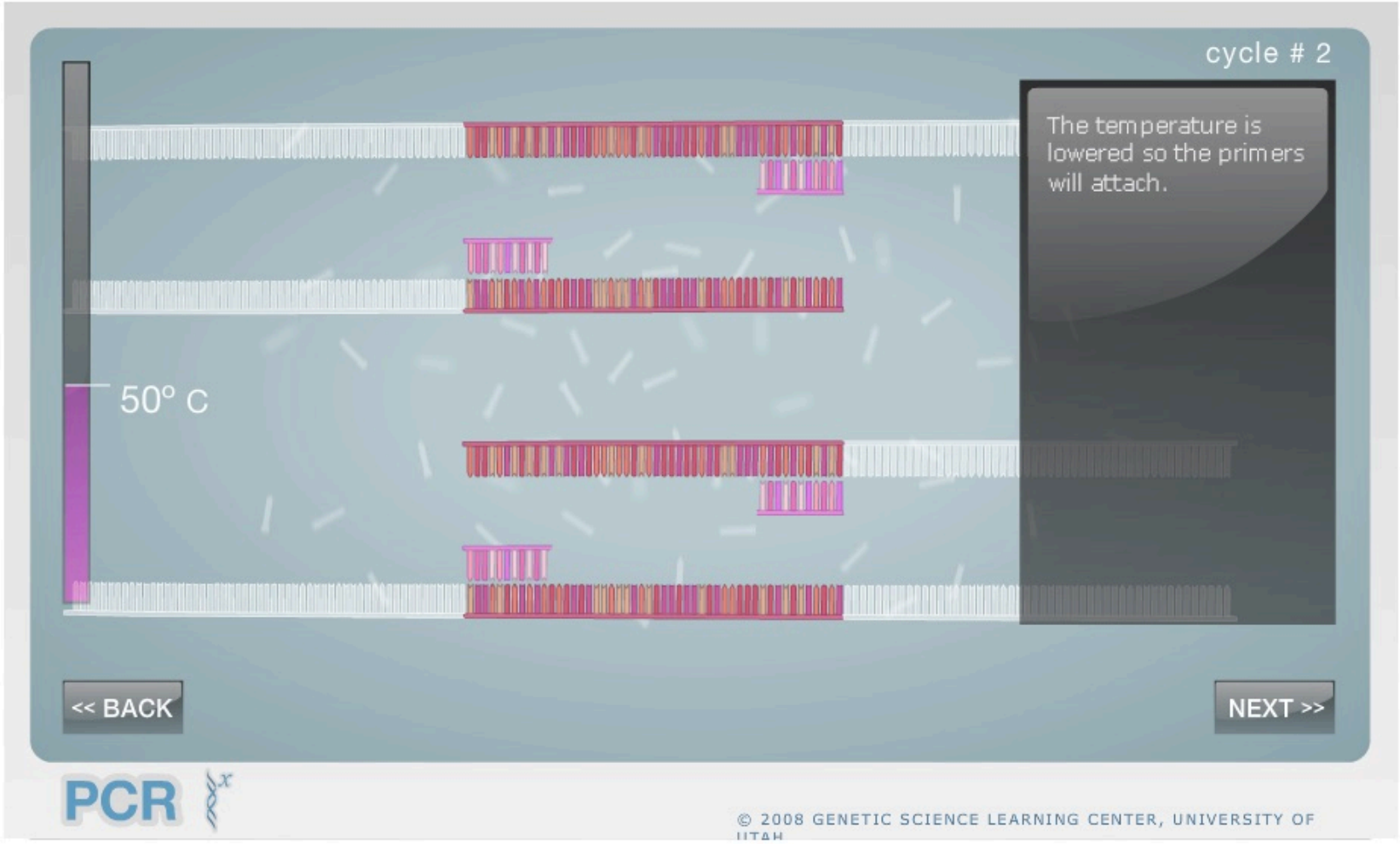
3) Ligation



1) Amplification by polymerase chain reaction (PCR)

Leslie's favorite PCR animation

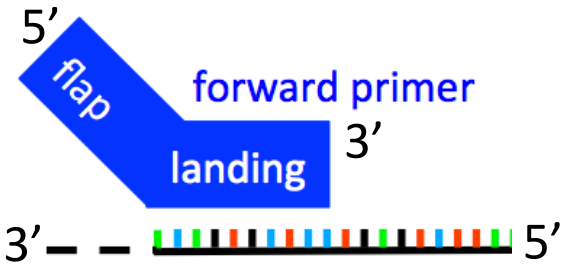
http://learn.genetics.utah.edu/content/labs/pcr/



1) Amplification—Guidelines for primer design

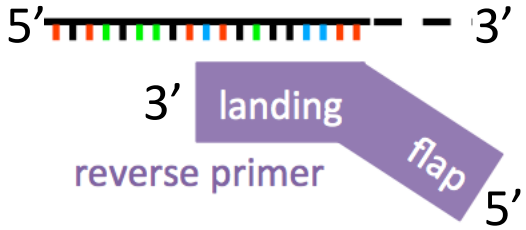
- **Landing sequence**: match to FKBP12 gene
- **Flap sequence**: endonuclease recognition sequence, junk DNA

- Length (landing sequence): 17-28 bp



- GC content: 40-60 %

- Tm : < 65 °C

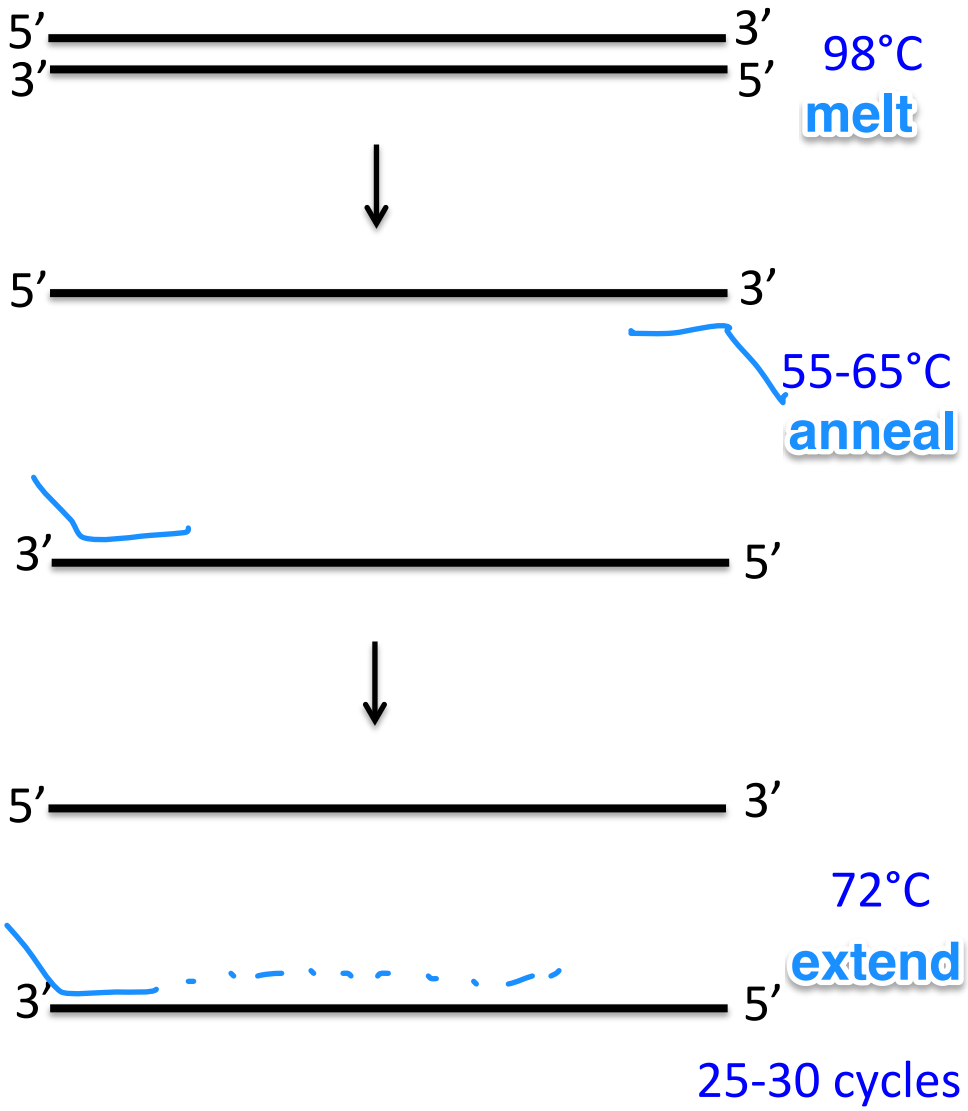


- Avoid secondary structure and repeat sequences

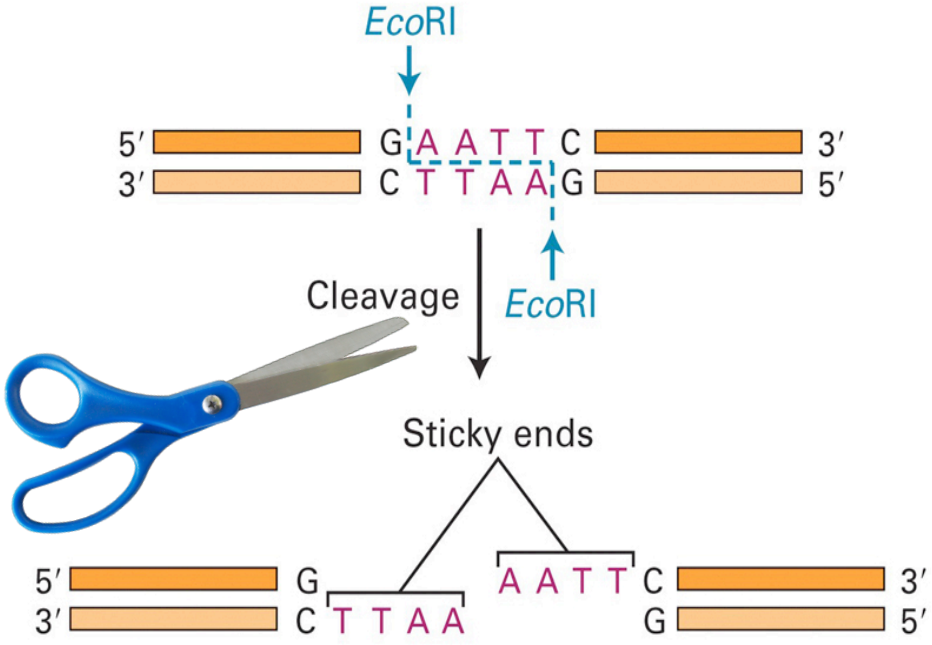
(e.g. hairpins, primer dimers, ATATAT)

1) Amplification—PCR reagents & conditions

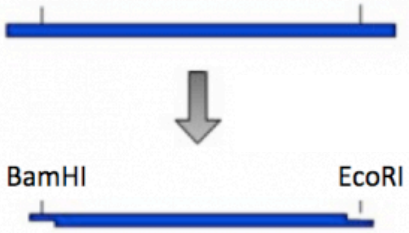
Reagents
dNTPs
DNA polymerase
primers
template
buffer



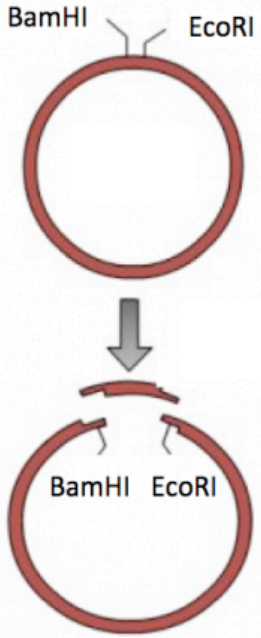
2) Digestion—Create compatible ends on insert fragment and backbone



FKBP12 insert

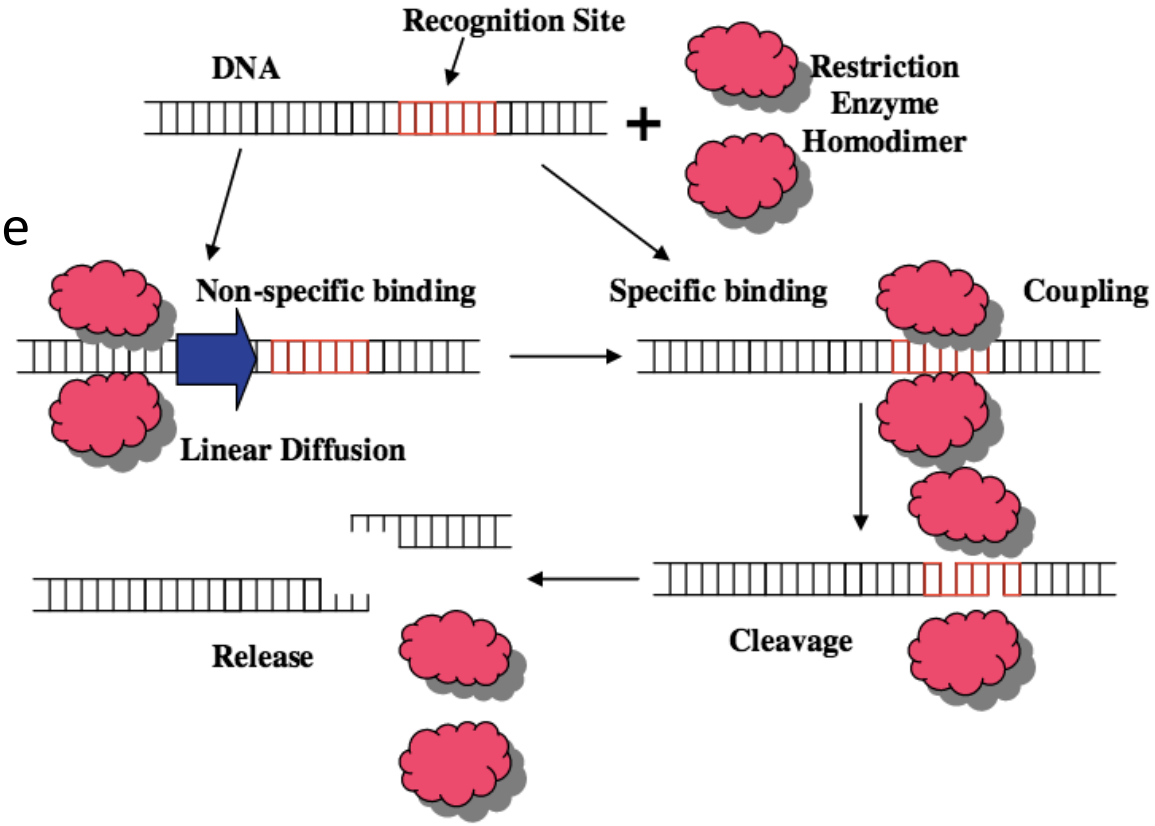


pRSETb vector backbone



2) Digestion—Restriction enzymes

- Many function as homodimers
  - Cleaves backbone at site of palindromic recognition sequence



2) Digestion—Reagents and conditions

Reagents
restriction enzymes
DNA
Buffer

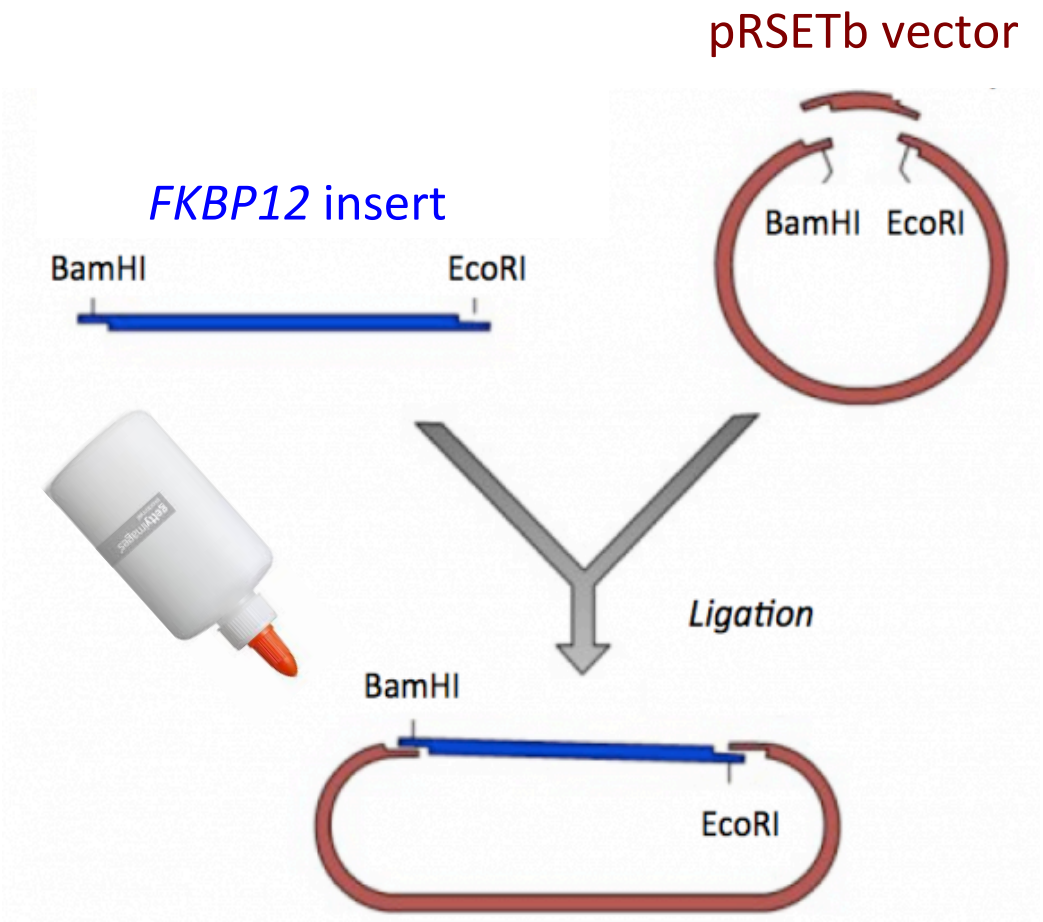
- Temperature

  25 or 37C

- Time

  1hr- overnight

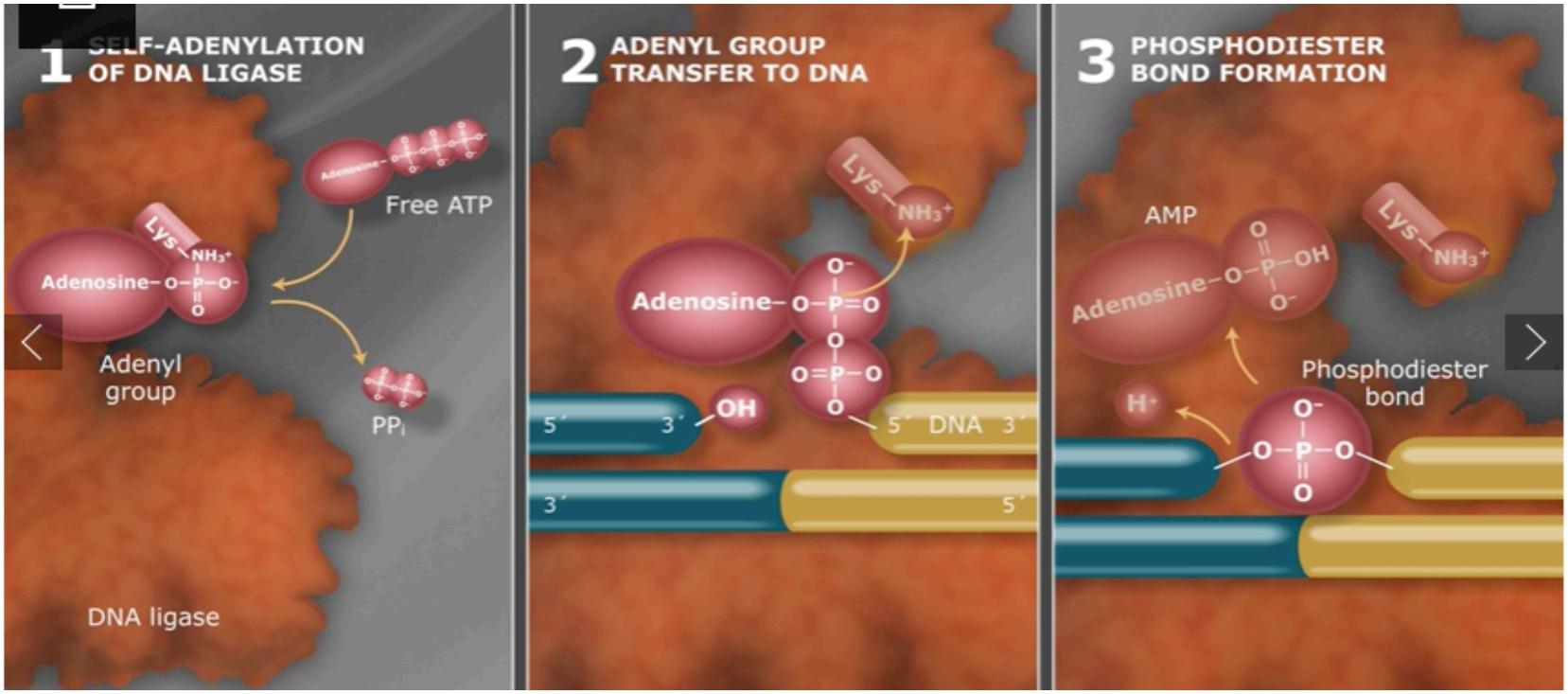
3) Ligation—Create plasmid pRSETb_FKBP12



pRSETb_FKBP12 plasmid

3) Ligation—Use T4 DNA Ligase to insert *FKBP12* gene into expression vector (pRSETb)

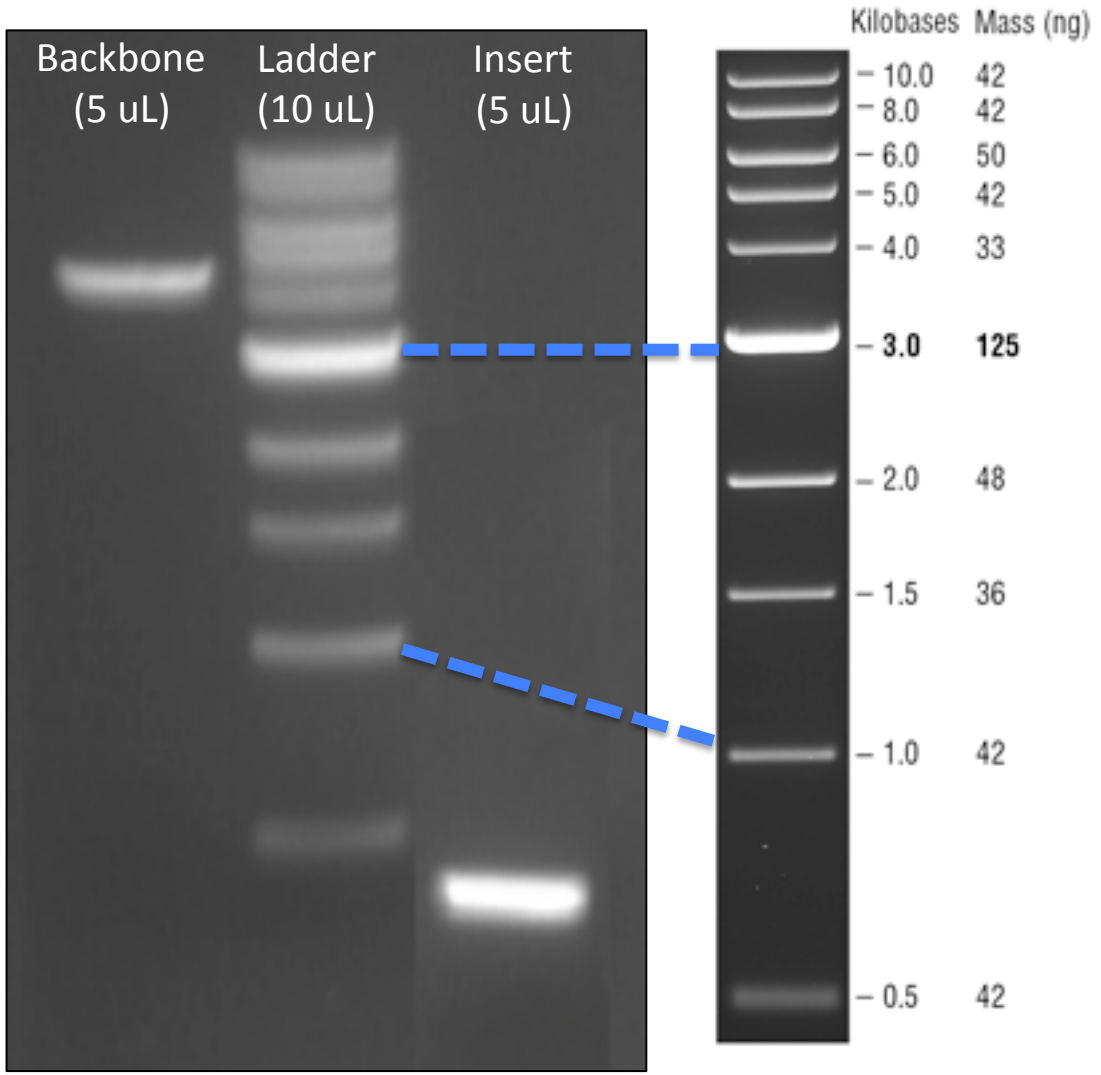
- Requires ATP
- Forms covalent phosphodiester bond between 3’ OH acceptor and 5’ phosphate donor



https://www.neb.com/products/m0202-t4-dna-ligase#Product%20Information

3) Ligation conditions—Part 2c of lab

- Calculate volumes of insert and backbone needed for ligation
- Use recovery gel to estimate insert and backbone concentrations



- Ideally, want 4:1 **molar** ratio of insert:backbone

- First, estimate concentrations

  insert =

  200 ng/ 5uL

  backbone =

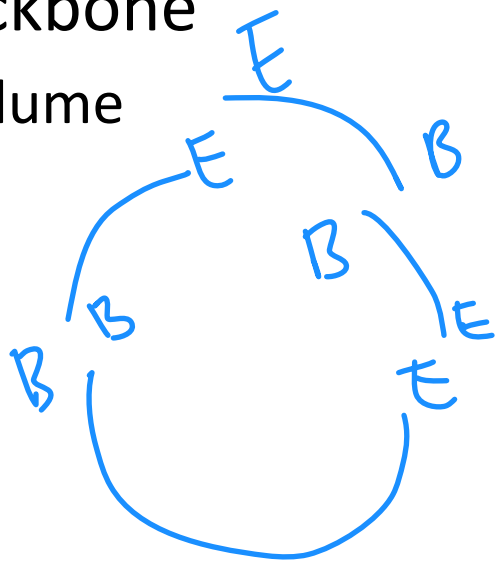
  100 ng/ 5uL

3) Ligation—Calculations

1. Determine volume of backbone
   - Use backbone concentration estimate from gel
   - Want 50 – 100 ng
2. Calculate moles of backbone
   - Vector = 2776 bp, MW bp = 660 g/mol
3. Calculate moles of insert
   - Insert = 480 bp, 4:1 ratio of insert:backbone
4. Calculate volume of insert
   - Use insert concentration estimate from gel

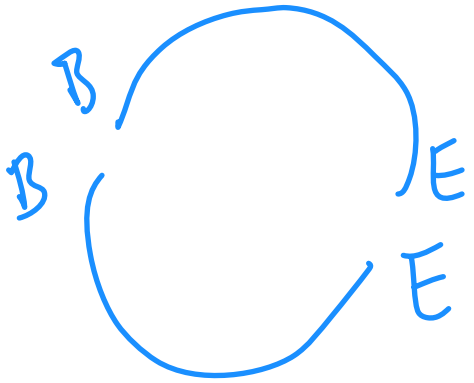
3) Ligation—Backbone-to-insert ratio

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



- What if too much insert?

- What if too much backbone?



What should go in your notebook?

Laboratory notebook entry component:	Score:		
	Complete	Partial	Incomplete
Date of experiment (include Module#/Day#)	1	0.5	0
Title for experiment	1	0.5	0
Hypothesis or goal / purpose	1	0.5	0
Protocols (link to appropriate wiki sections)	1	0.5	0
Notes on protocol changes / clarifications	1	0.5	0
Observations (qualitative / raw data)	1	0.5	0
Data analysis (calculations / graphs / tables)	1	0.5	0
Summary and interpretation of data	1	0.5	0
Information is clear	1	0.5	0
All days represented	1	0.5	0
OVERALL /10			

Due 10pm the day after each module

http://engineerbiology.org/wiki/20.109(S18):_Assignments

How should you format your notebook?

Insert H B I U S A x_2 x^2 More

M1D1: In silico cloning and confirmation digest of protein expression vector

THURSDAY, 2/8

Hypothesis or goal:
What are you testing and what do you expect of your results?

Protocols: [include link to wiki]
Part 2: Construct pRSETb_FKBP12 in silico

- Include all work / notes / images / sequences generated.
- Be sure to note any interesting observations or protocol changes!

Part 3: Confirmation digest

- Include completed table with volumes.
- Include calculations.
- Be sure to note any interesting observations or protocol changes!

Summary and interpretations:
What, if any, conclusions can be made and what does this prepare you to do next?

How should you organize your notebook?

- Entitle your project “20.109(S18)_YourName”
  - Make each module a new folder
  - Make each day a new entry within module folder
- Share the project with Josephine and Casper
  - Right-click and choose ‘settings’
  - Add collaborators by email

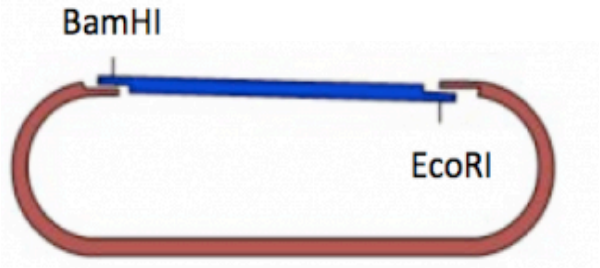
Today in lab…

- Virtual cloning exercise to build pRSETb_FKBP12 expression plasmid
- Actual confirmation digest of pRSETb_FKBP12: prepare eppendorf tubes with plasmid, enzyme and buffer and bring to front bench

For next time…

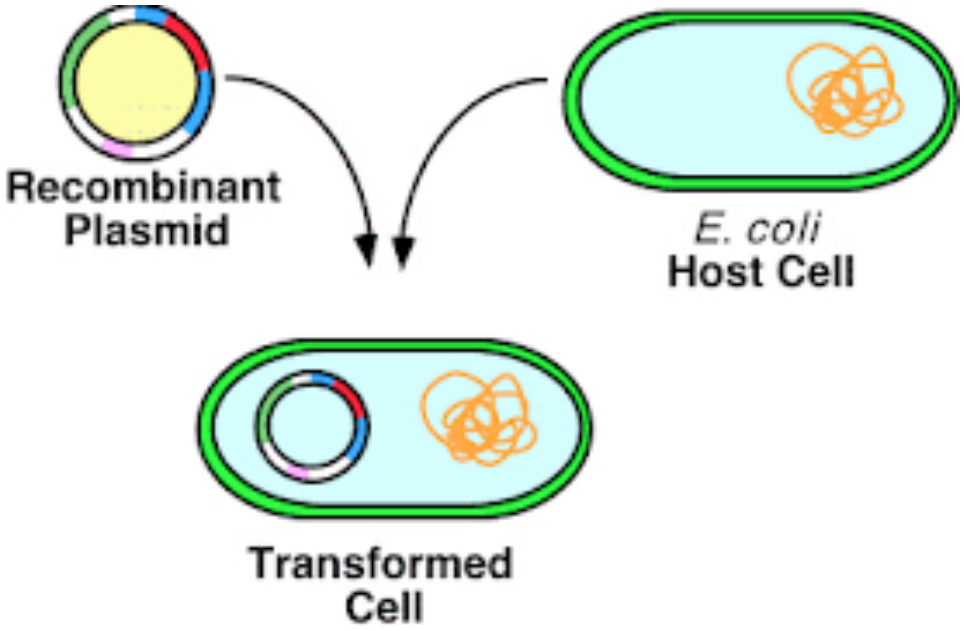
- Prepare a template for Benchling entries
- See wiki for homework details

How do we confirm the plasmid product?



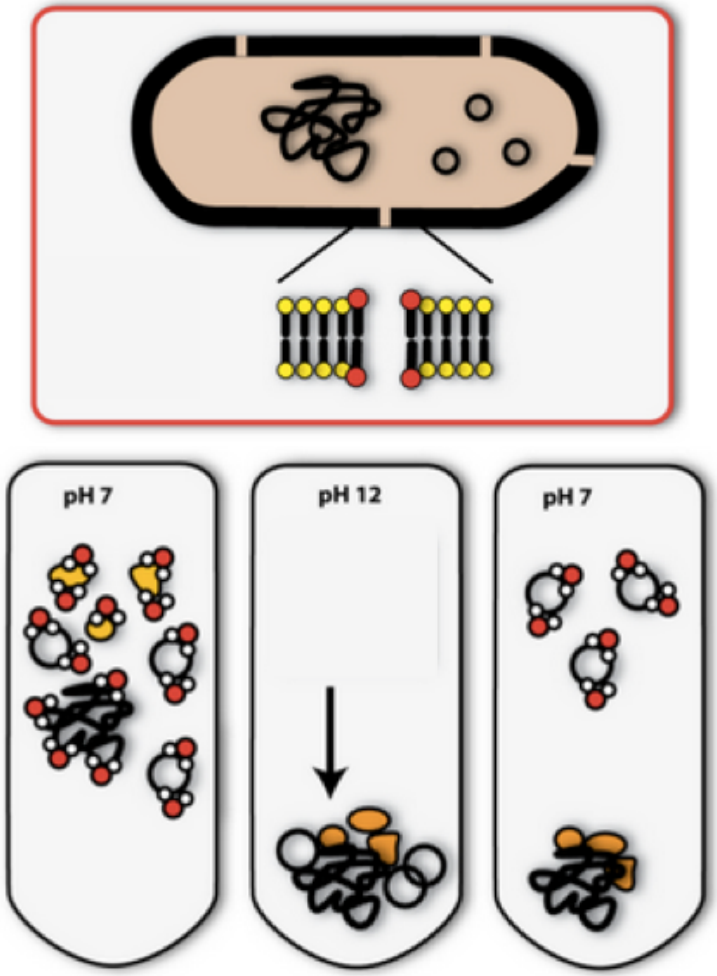
- Amplify plasmid
  - Transform into bacteria
- Purification
  - Separate plasmid from chromosomal DNA
- Digestion
  - Confirm the plasmid contains expected fragments

Transformation



1. Incubation
2. Heat shock (or electroporation)
   - DNA taken in by competent cells
3. Recovery
4. Selection

Purification of plasmid DNA a.k.a. “Mini-prep”

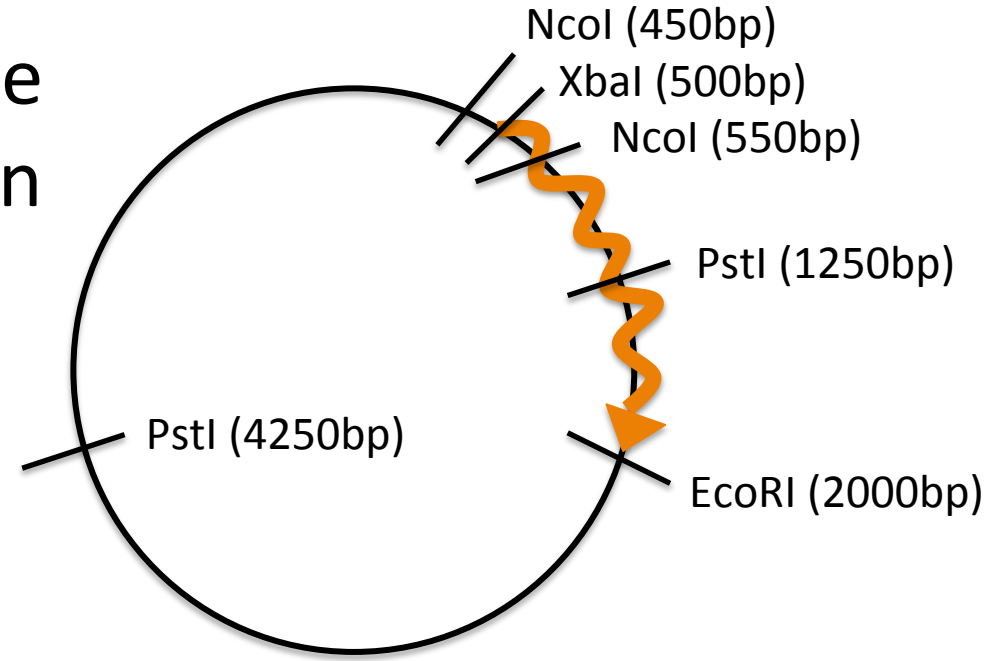


1. Resuspend cells
2. Lysis
3. Neutralization
   - Separates chromosomal DNA from plasmid DNA
4. Wash
5. Resuspend or elute DNA

Digestion to confirm plasmid

This is what you will do in lab today

- Confirmation digests
- Ideally, will cut once in insert and once in vector
  - XbaI and EcoRI?
  - PstI?
  - NcoI?



Don't cut with same enzymes used to make the vector
Ideally want 2 fragments of different lengths
Fragments should be > 200 bp