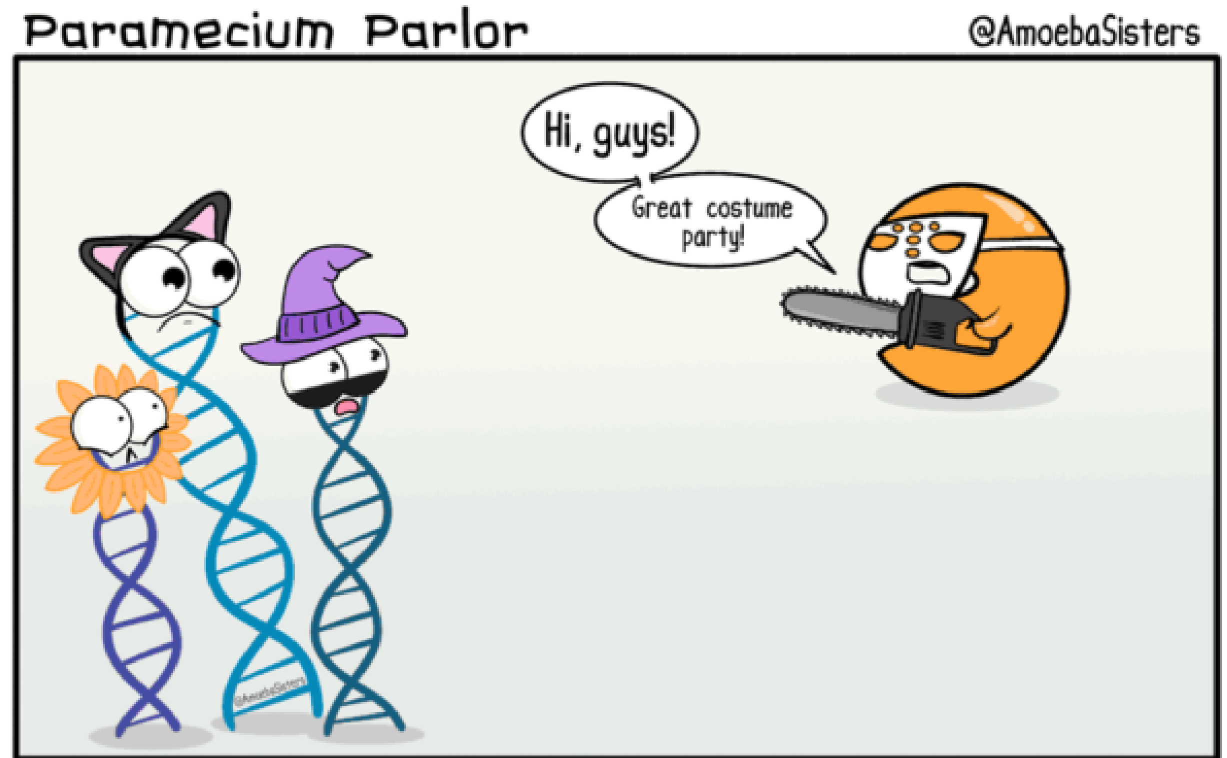
M2D1: Complete in-silico cloning of protein expression plasmid

1. Prelab discussion
2. Complete DNA engineering exercise
3. Set up confirmation digest of plasmid

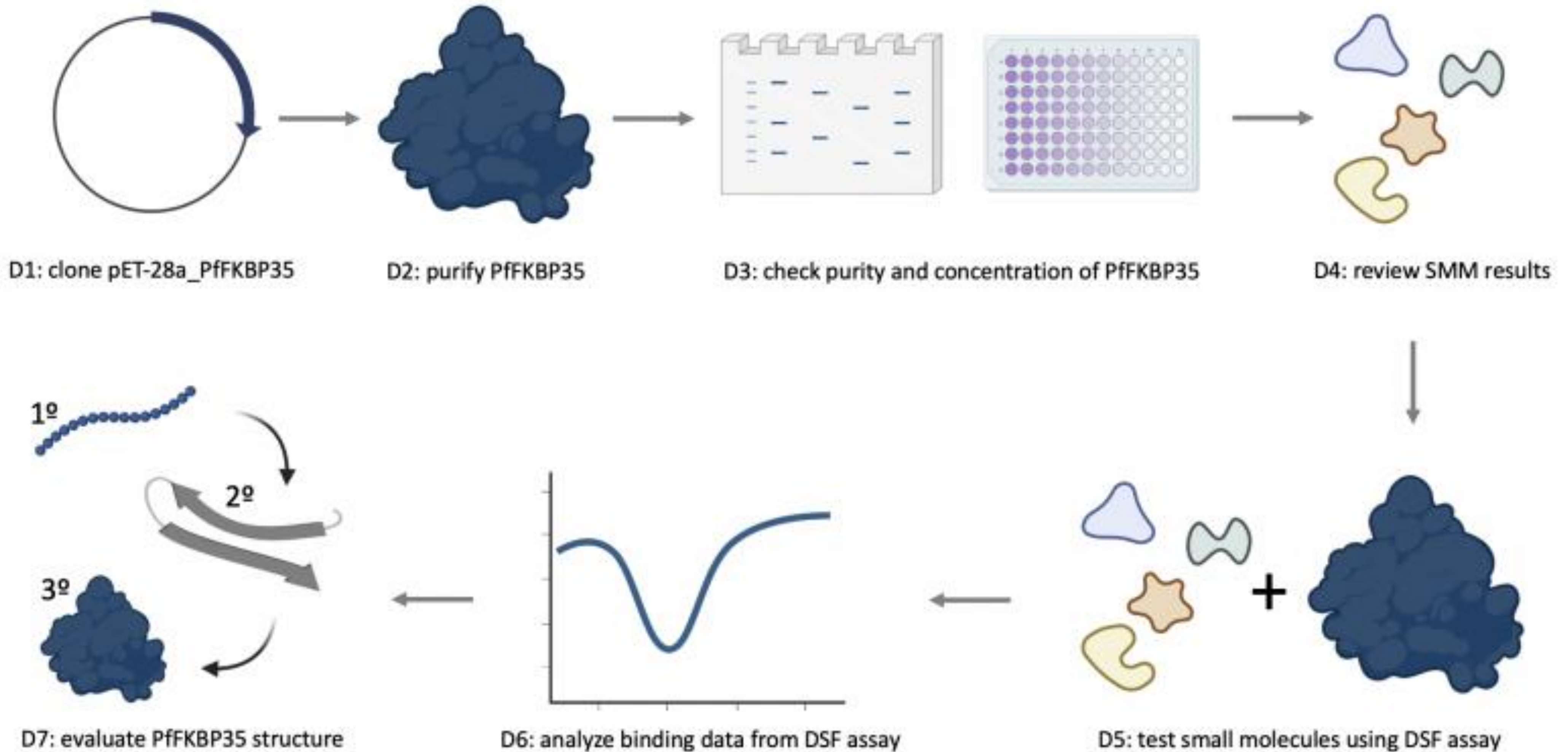


Mod 2 Major Assignments

- Journal Article presentation (15%)
  - Individual
  - Presentations on 11/1 & 11/3
- Research article (20%)
  - Individual
  - due 11/21
- Laboratory quizzes (collectively 5%)
  - M2D4 and M2D7
- Notebook (collectively 5%)
  - one entry will be graded by Alex 24 hr after M2D7
- Blog (part of 5% Participation)
  - due 11/5 & 11/22 via Slack channel

Overview of M2: drug discovery

Research goal: Test small molecules for binding to the *Plasmodium falciparum* FKBP35 protein using a functional assay.

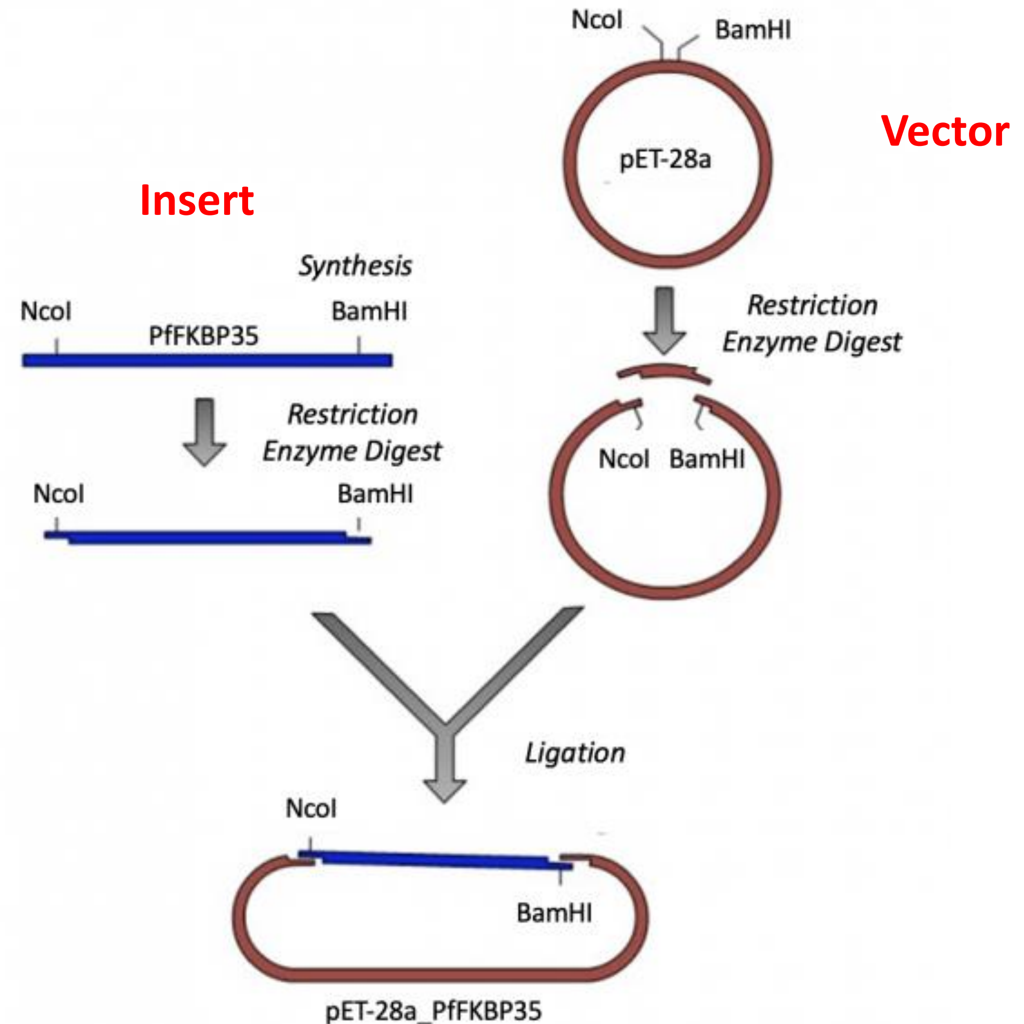


What if we want to make a specific protein?

- Chemically synthesize protein by successively linking each amino acid
  - Complicated, **have to make each protein,** expensive
- Synthesize RNA encoding the protein (1 step back in the central dogma)
  - RNA degrades easily
  - Amplification: 1 RNA -> Many Proteins
- Create DNA encoding the protein (2 steps back in the central dogma)
  - Plasmids are highly stable, easily transformed into bacteria
  - Amplification Cascade: 1 DNA -> Many RNA -> Many Proteins

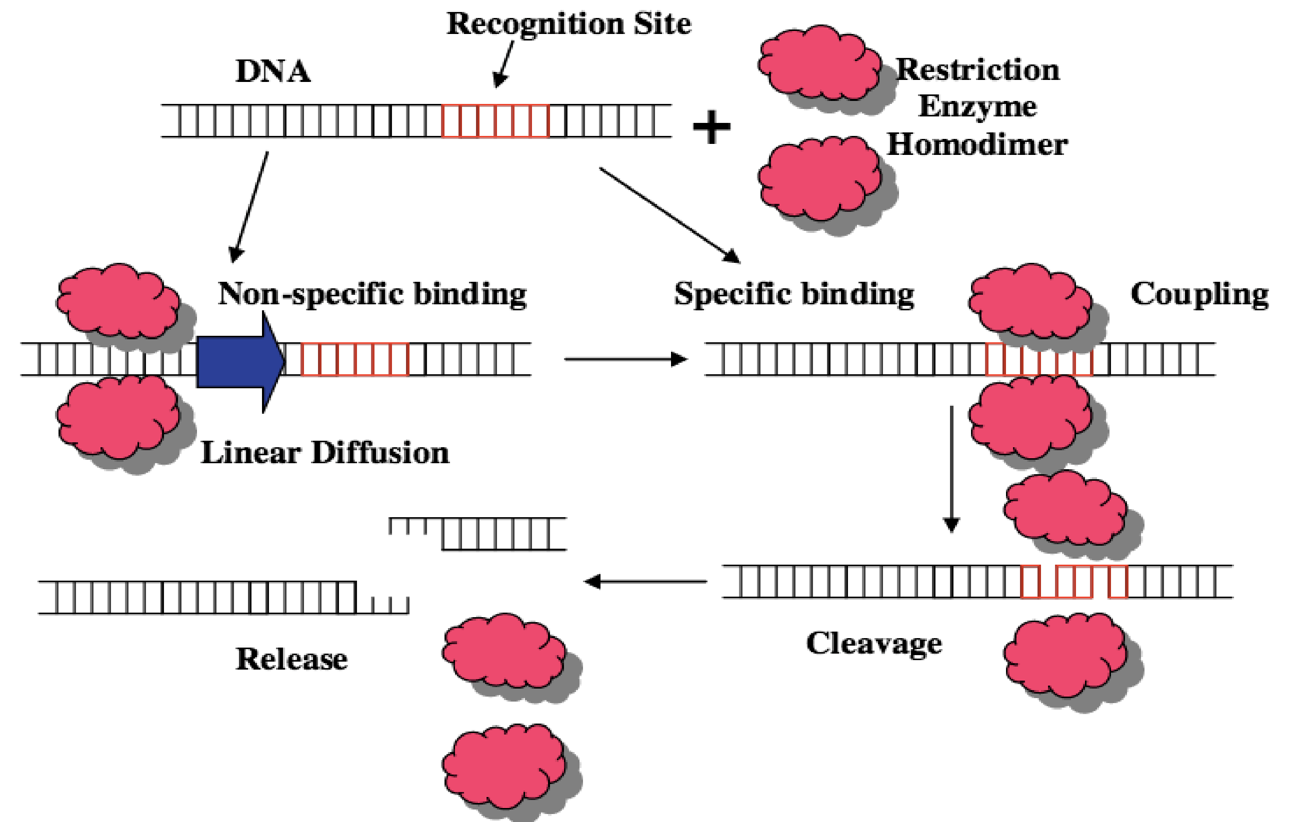
What if we want to make a specific protein?

- Who are the players?
  - Insert   GOI - **FKBP35**
  - Vector   Other essential stuff – Ab resistance genes, promoters, ORIs…
- What is the process?
  - Digestion   Cut DNA with Restriction Enzymes
  - Ligation   Staples the insert DNA into the open plasmid

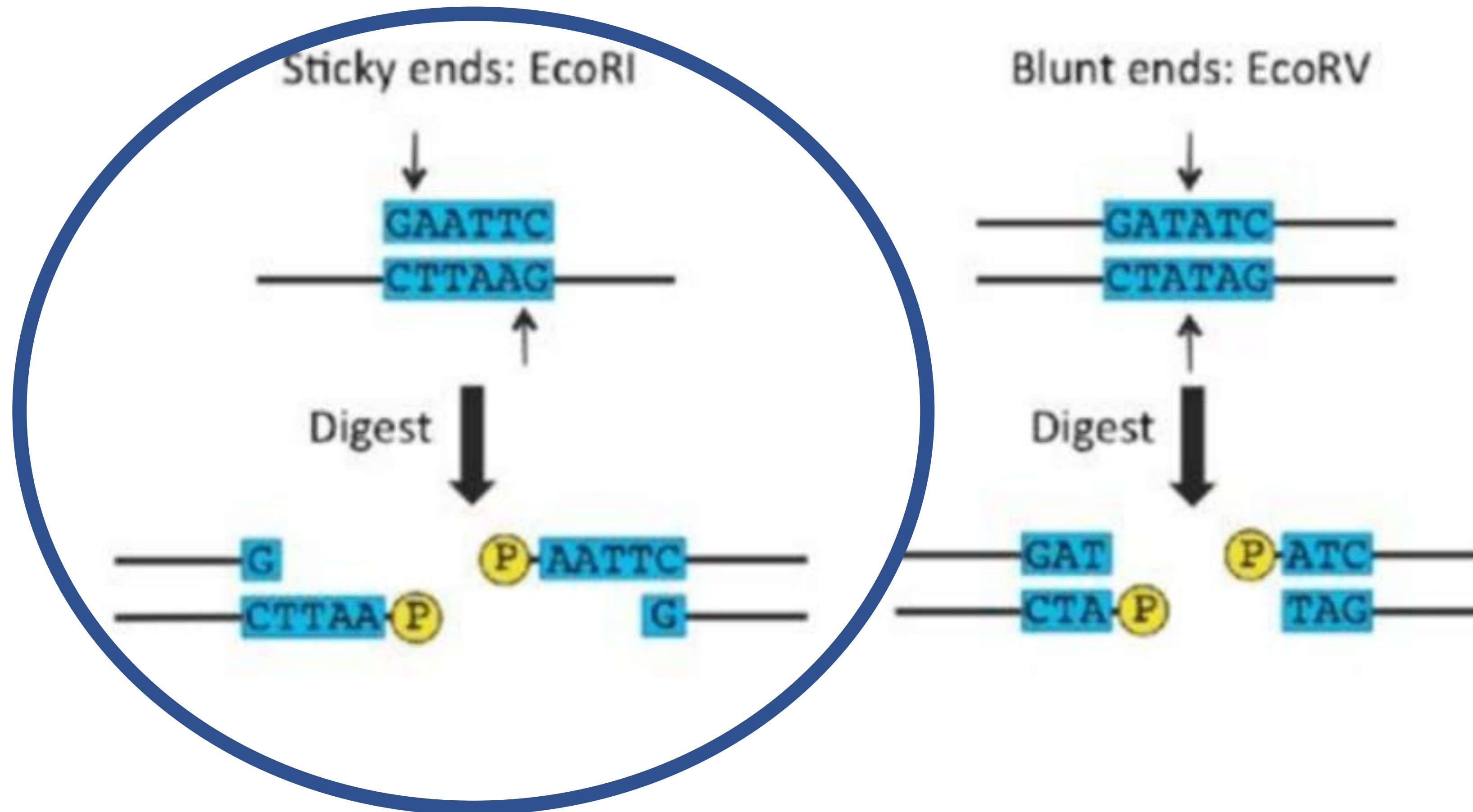


Digestion: restriction enzymes

- Function as homodimers
- Each dimer contains active site that cleaves backbone at site of palindromic recognition sequence
- Results in cleavage of both strands



In our cloning strategy, we used enzymes that created *sticky ends*



Digest reagents and conditions

Reagents

1) Restriction Enzymes
2) DNA Template
    1) Insert
    2) Vector
3) Buffer/H20

Conditions

- Temperature:

37C

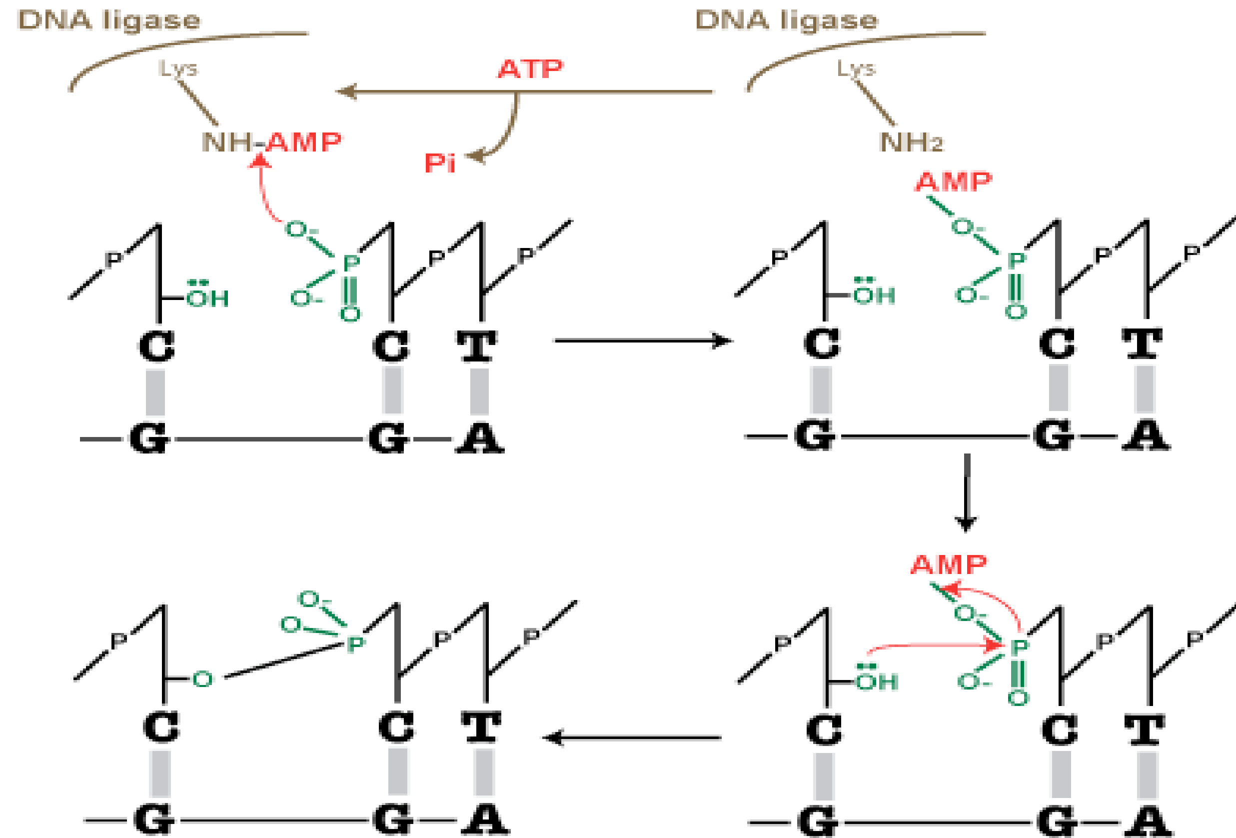
- Time:

1 hr – ON

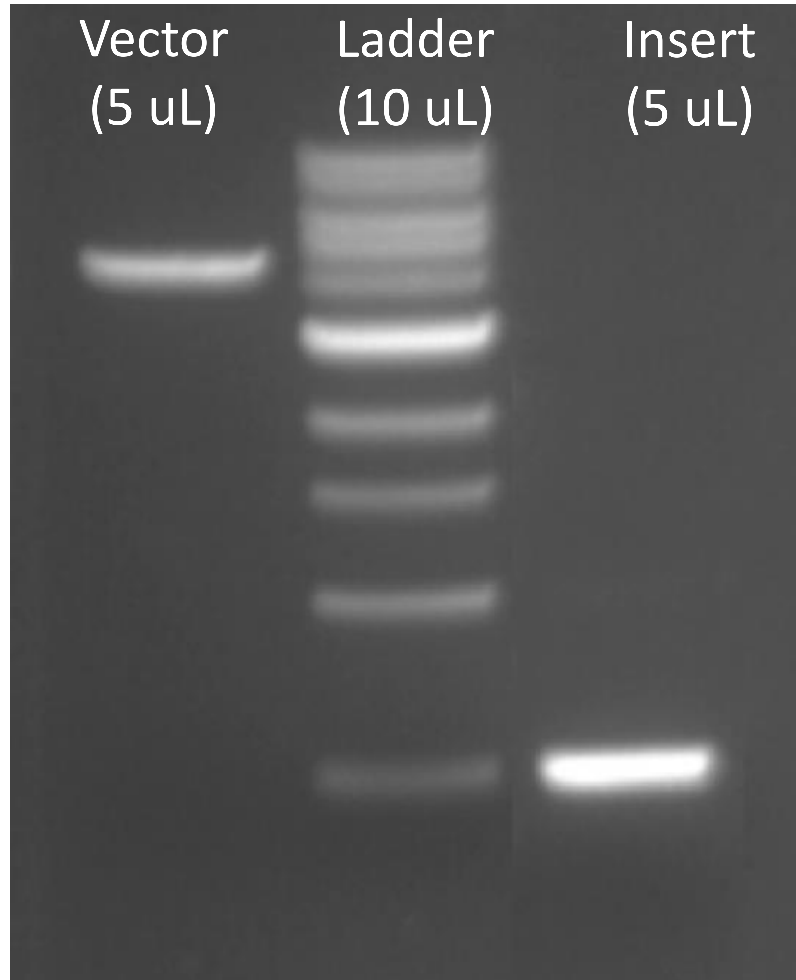
Be wary of Star Activity

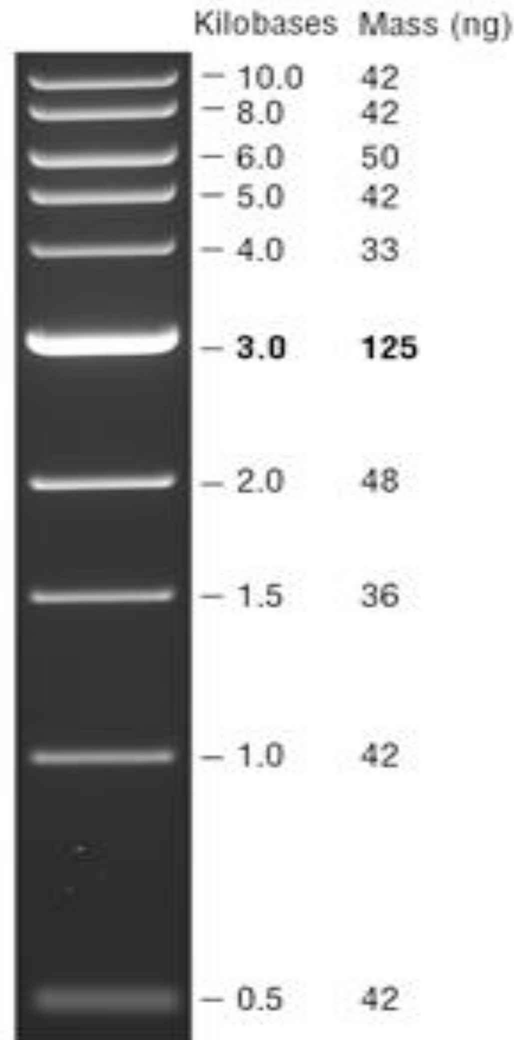
Ligation: T4 DNA ligase *T4 is a bacteriophage*

- Functions as a carrier for AMP leaving group
- Forms covalent phosphodiester bond between 3' OH acceptor and 5' phosphate donor
- Requires ATP



Ligation conditions





- Ideally, want 3:1 molar ratio of insert:backbone
- Calculate molar amounts from measured concentrations and known sizes of DNA molecules

Pro tips for ligation calculations

1. Determine volume of vector
    - Use backbone concentration = 50 ng/uL
    - Want 50 – 100 ng
2. Calculate moles of vector
    - Vector = (you will discover this in the exercise) bp, MW bp = 660 g/mol
3. Calculate moles of insert
    - Insert = (you will discover this in the exercise) bp, **3**:1 ratio of insert:vector
4. Calculate volume of insert
    - Use insert concentration = 25 ng/uL

How do we confirm the cloning product?

- Transformation

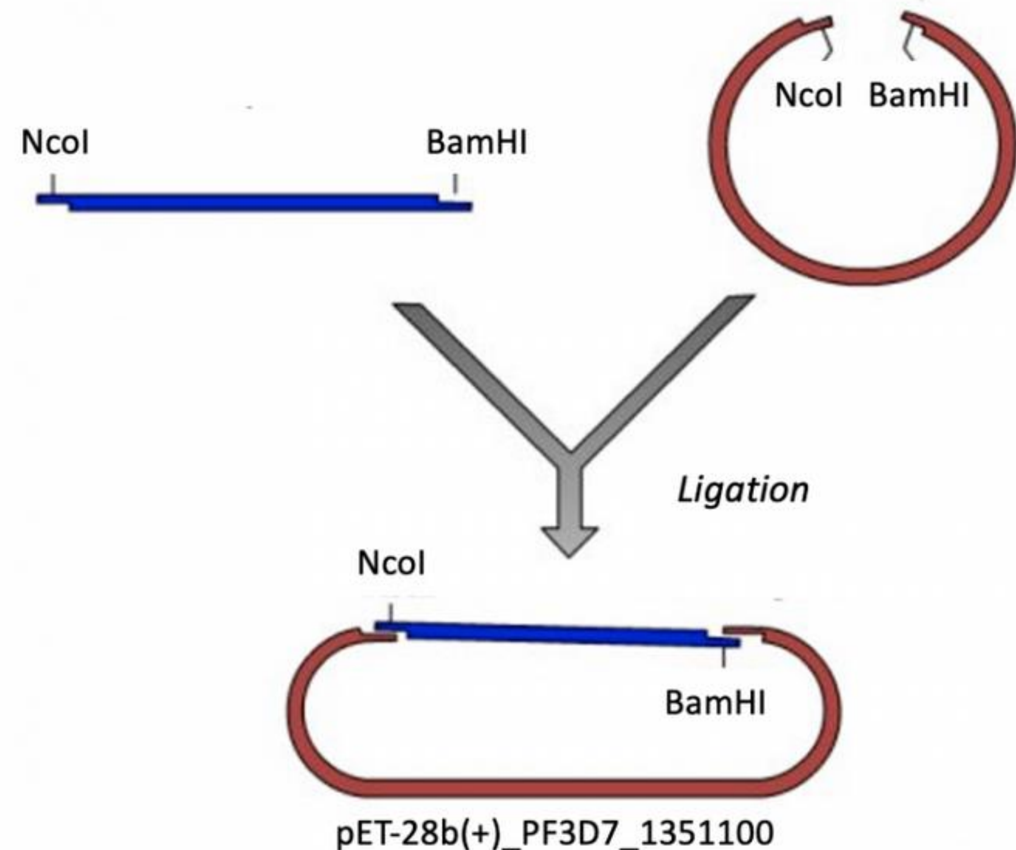
Allow E.coli to take up our new plasmid
Allow that E.coli to amplify our plasmid as it replicates

- Purification

Break the plasmids out of bacterial cells
Mini/Maxiprep

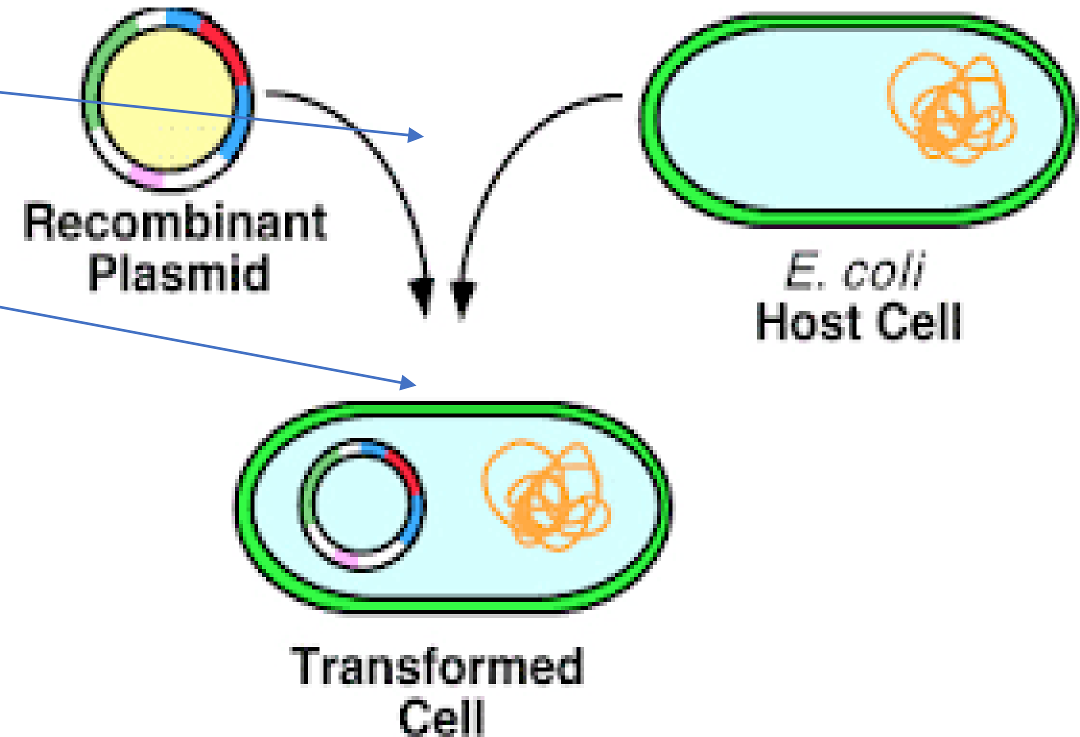
- Digestion

Use REs to cut new plasmids in a stereotyped way



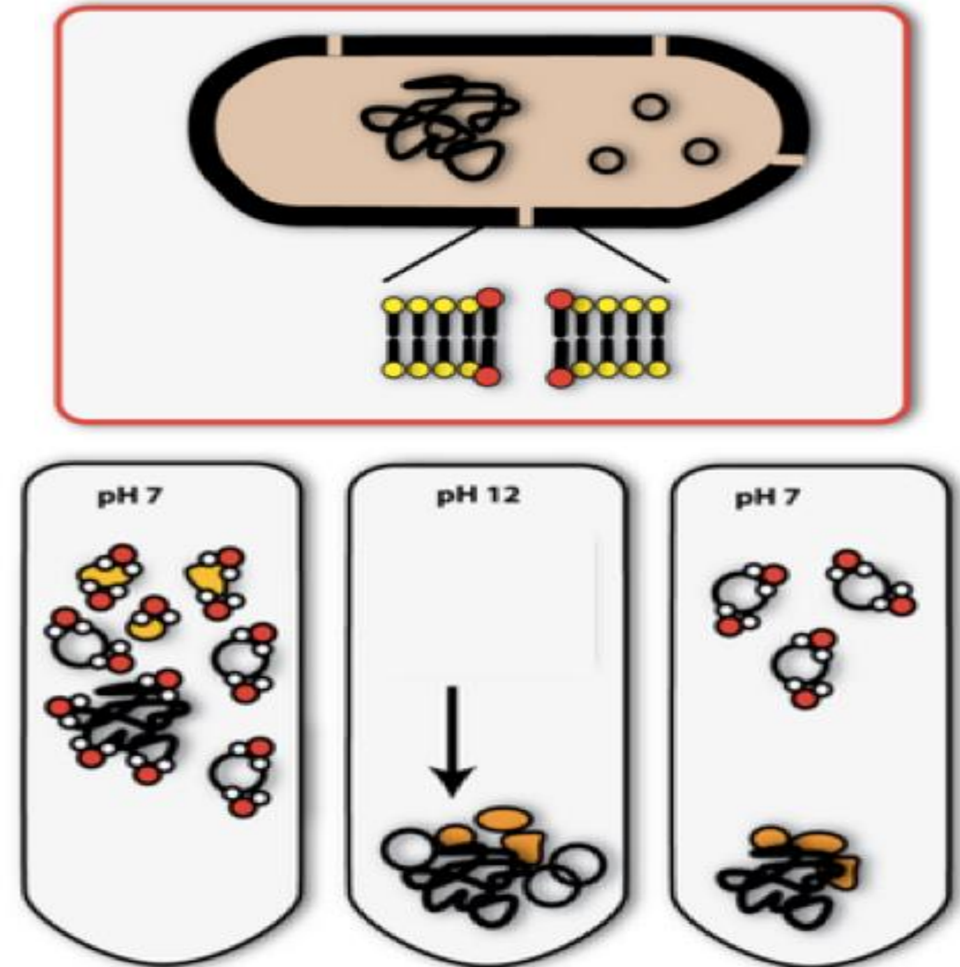
Transform plasmid into bacteria for amplification

1. Incubation
2. Heat shock @ 42C
3. Recovery
4. Antibiotic selection



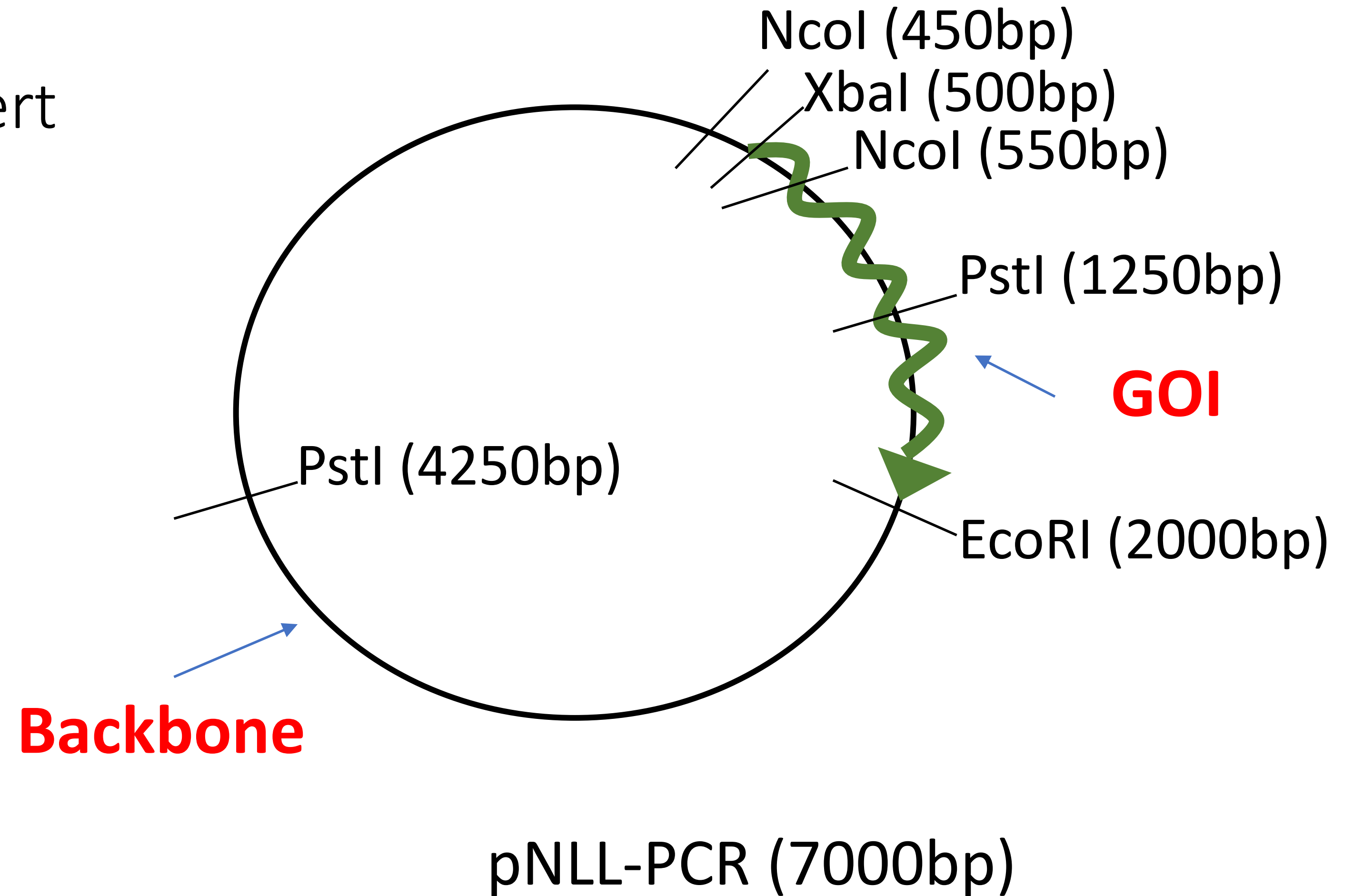
Purify amplified plasmid for confirmation w/ miniprep

1. Resuspend cells
2. Alkaline Lysis (denatures all DNA)
3. Neutralization (renatures plasmid DNA first)
4. Wash
5. Resuspend or elute DNA



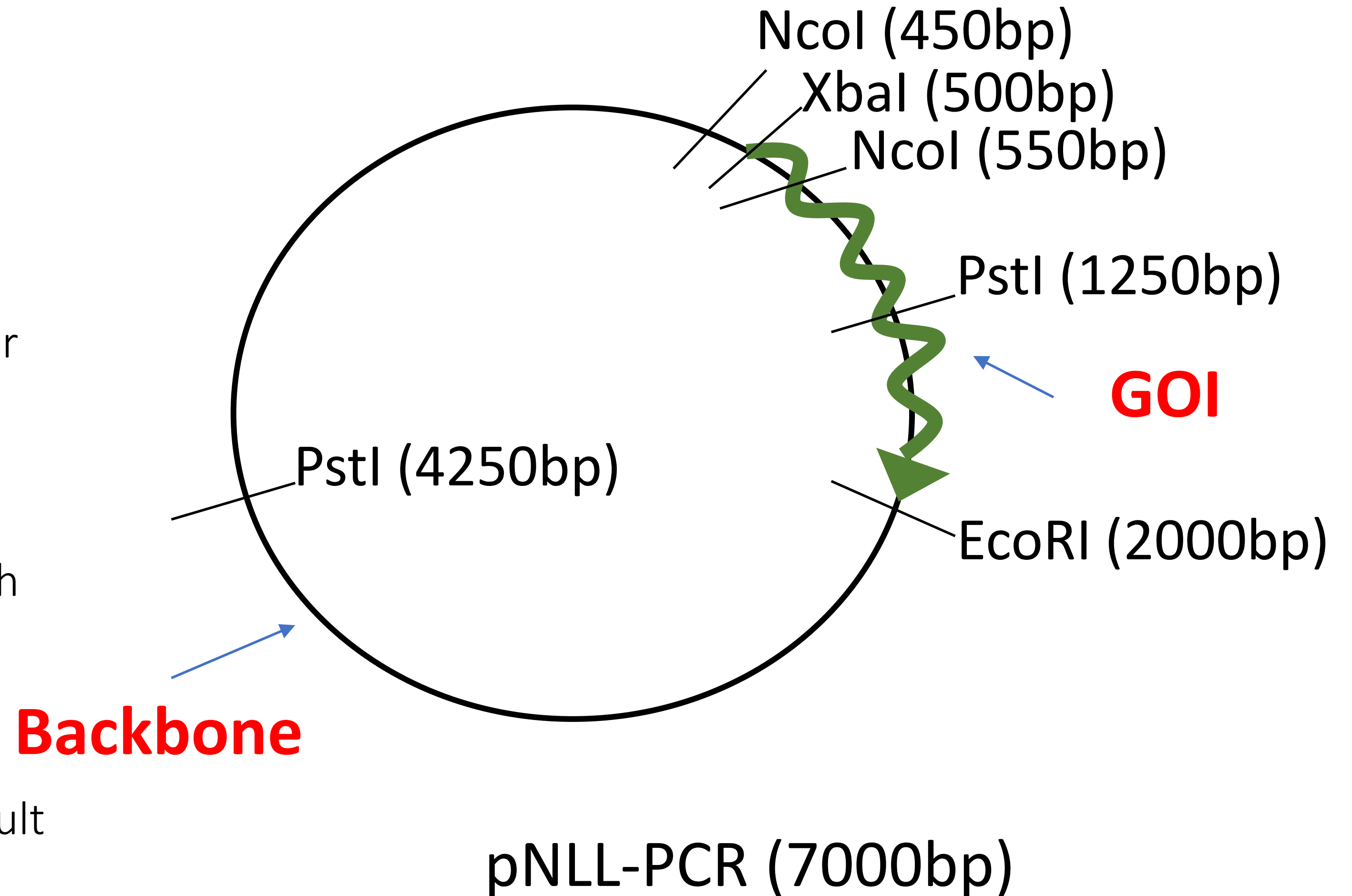
Confirmation digest follows plasmid purification

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



Confirmation digest follows plasmid purification

- Ideally, will cut once in insert and once in vector
  - XbaI and EcoRI?
    - Can't distinguish between chaining of multiple inserts per backbone
  - PstI?
    - Wouldn't be able to distinguish band sizes on a gel
  - NcoI?
    - Size difference would be difficult to see



For today…

- In silico cloning of your plasmid
- Set up restriction enzyme digest
    - Begin by 4:30pm

For M2D2…

- Sign up for your article for the Journal Article presentation on the wiki
    - Each article can only be covered by ONE person per section!

- Read your journal article, chose the figures you want to be the focus of your story, and answer the questions on the wiki