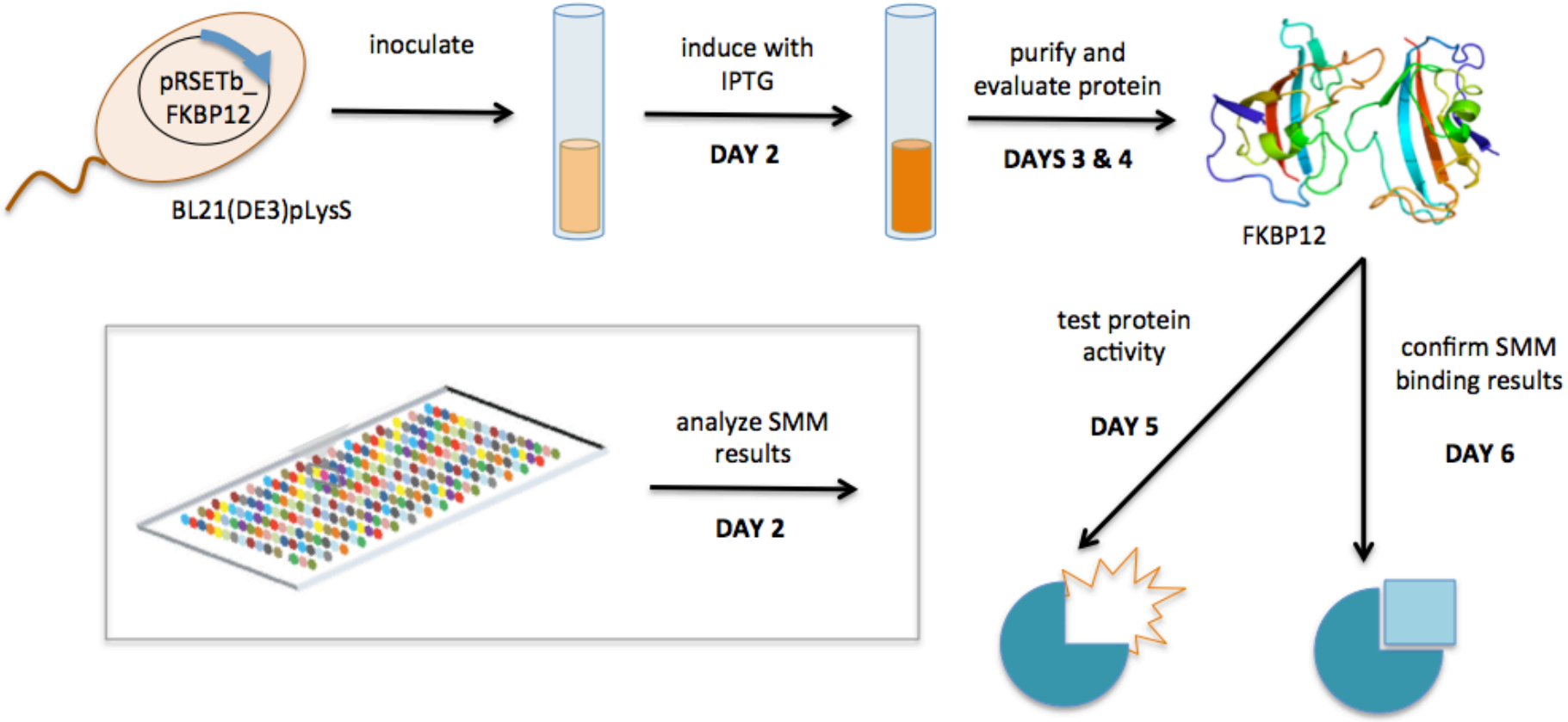
M1D1:
In silico cloning and confirmation digest of protein expression vector

1. Laboratory orientation quiz
2. Pre-lab discussion
3. Build protein expression plasmid
   - Virtually
4. Confirm protein expression plasmid
   - Actually

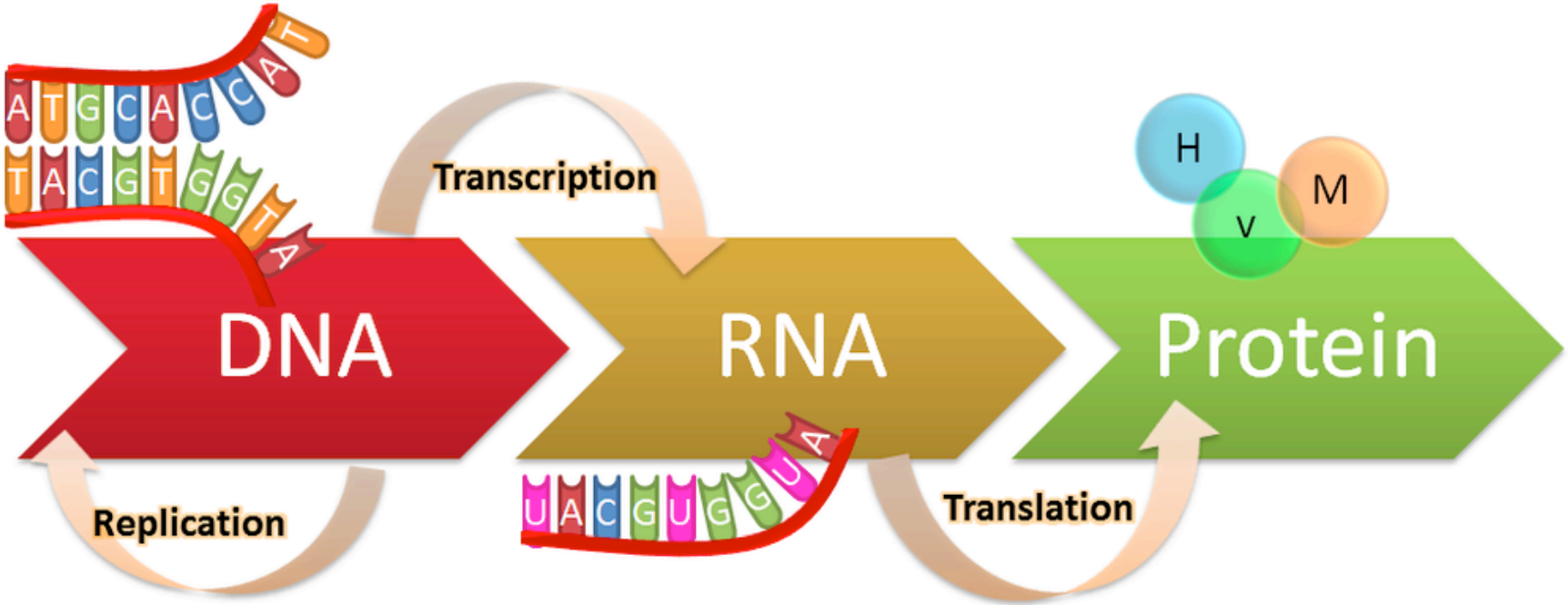
Mark your calendars!

- 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)
  - one entry will be graded by Casper 24 hr after M1D7
- Blog (part of 5% Participation)
  - due 3/18 via Blogspot

Overview of Mod1 experiments



How are proteins made?



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

What if we want a specific protein?

- Amplification

  need to generate product for cloning

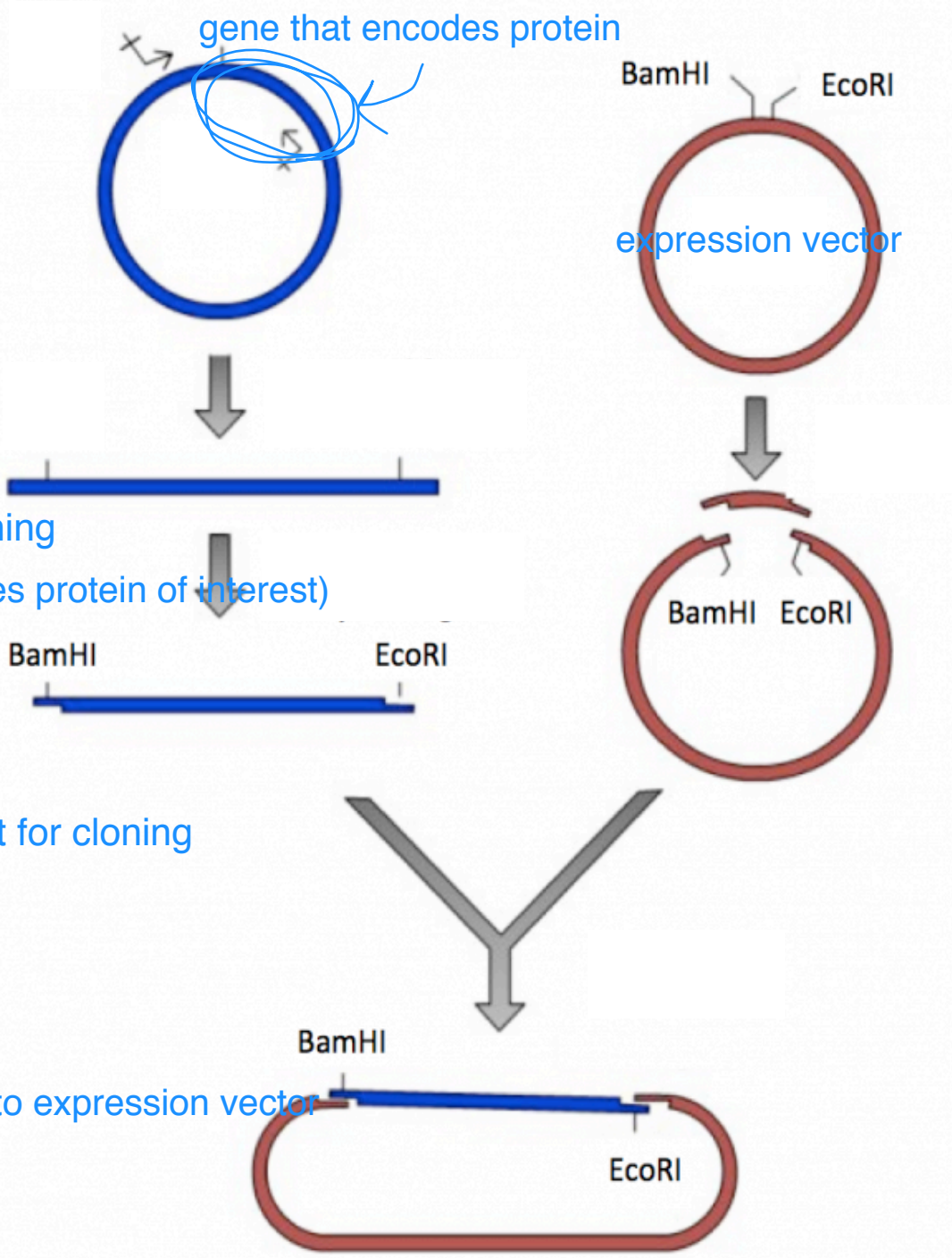
  (product = gene that encodes protein of interest)

- Digestion

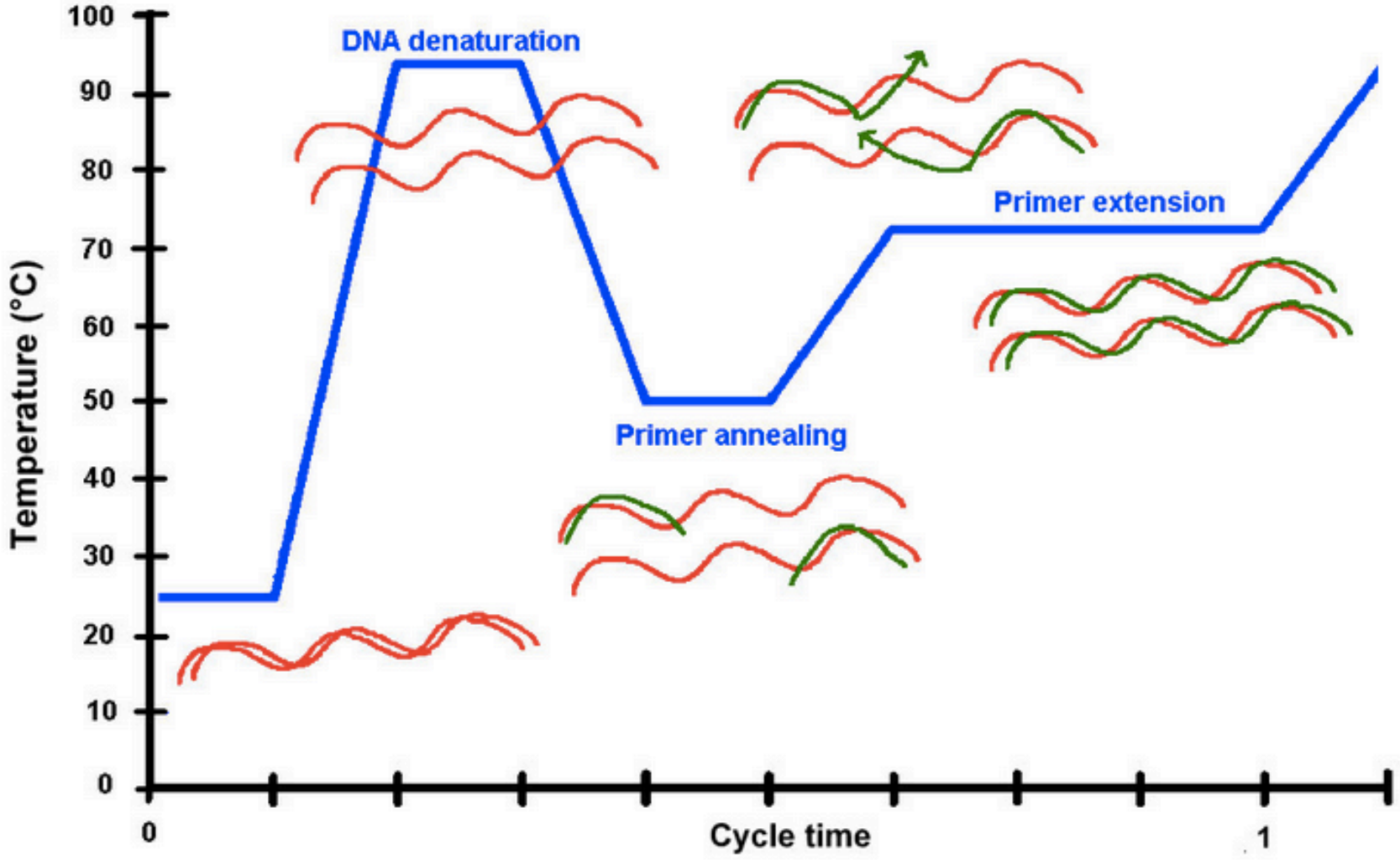
  need to prepare amplified product for cloning

- Ligation

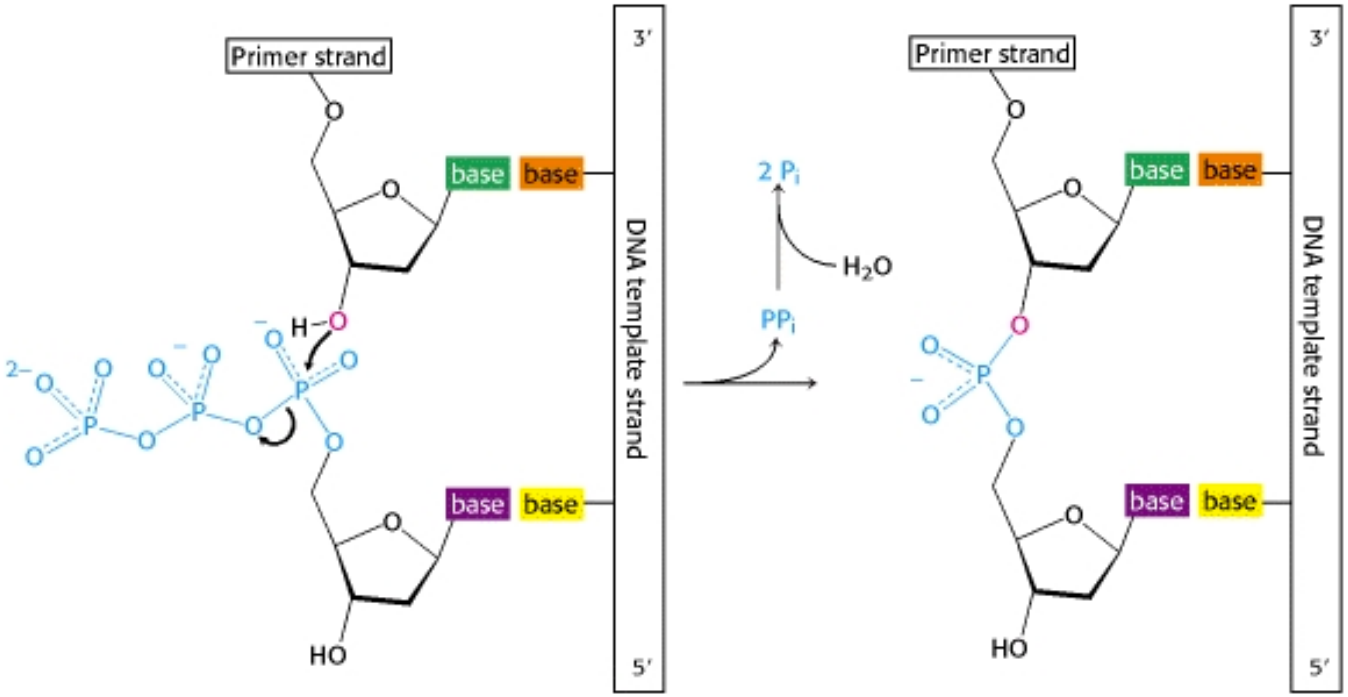
  need to insert amplified product into expression vector



Amplification: PCR cycling



Amplification: DNA polymerase

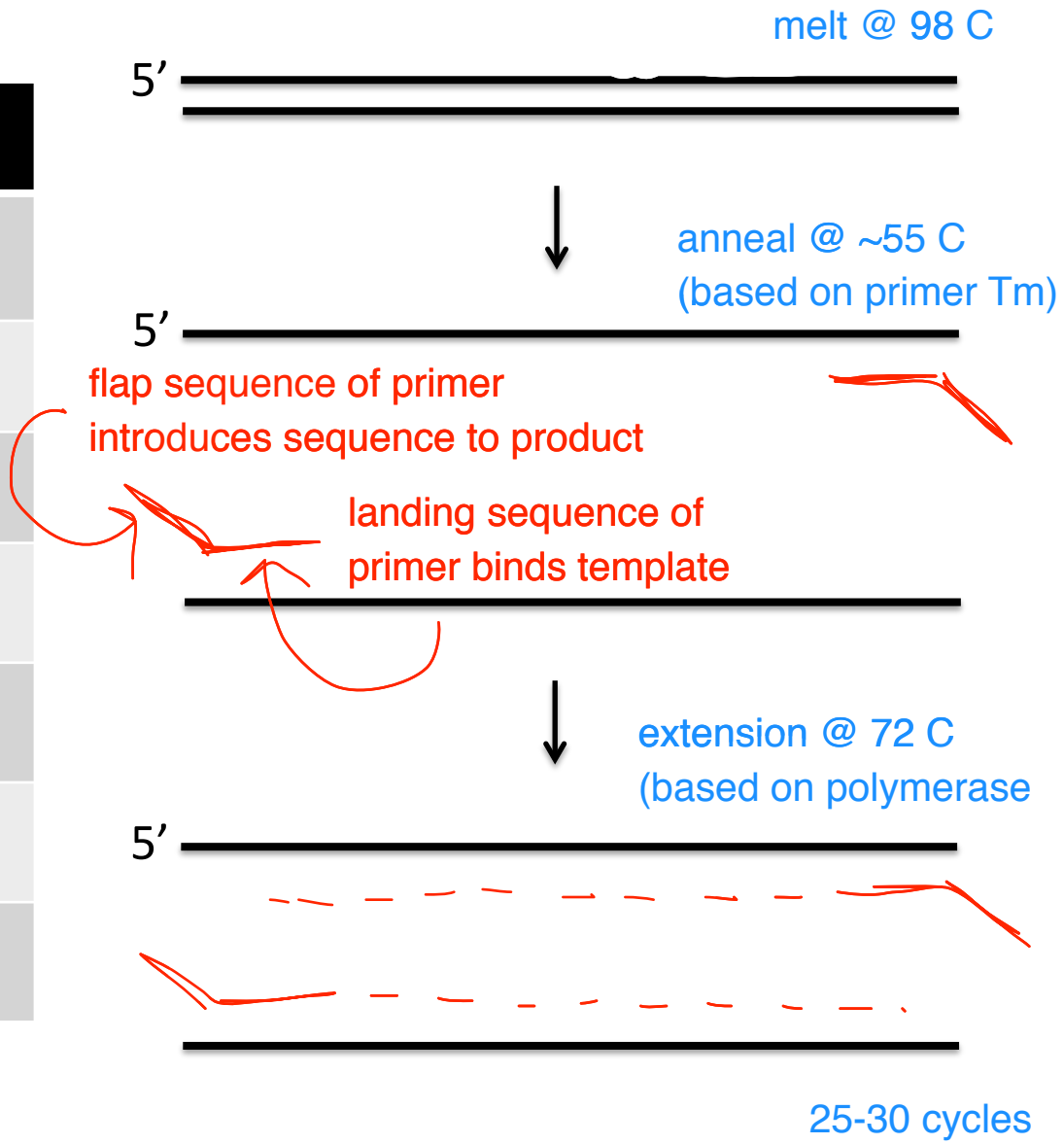


- Catalyzes formation of polynucleotide chains
- Requires a primer base-paired to template

http://www.ncbi.nlm.nih.gov/books/NBK22374/

PCR reagents and conditions

Reagents



A closer look at primer design

- Length: 17-28 bp

  length increases specificity (reduces chance of nonspecific binding / amplification

- GC content: 40-60 %

  GC base pairing provides better binding to template - 3 H bonds

- Tm : < 65 °C

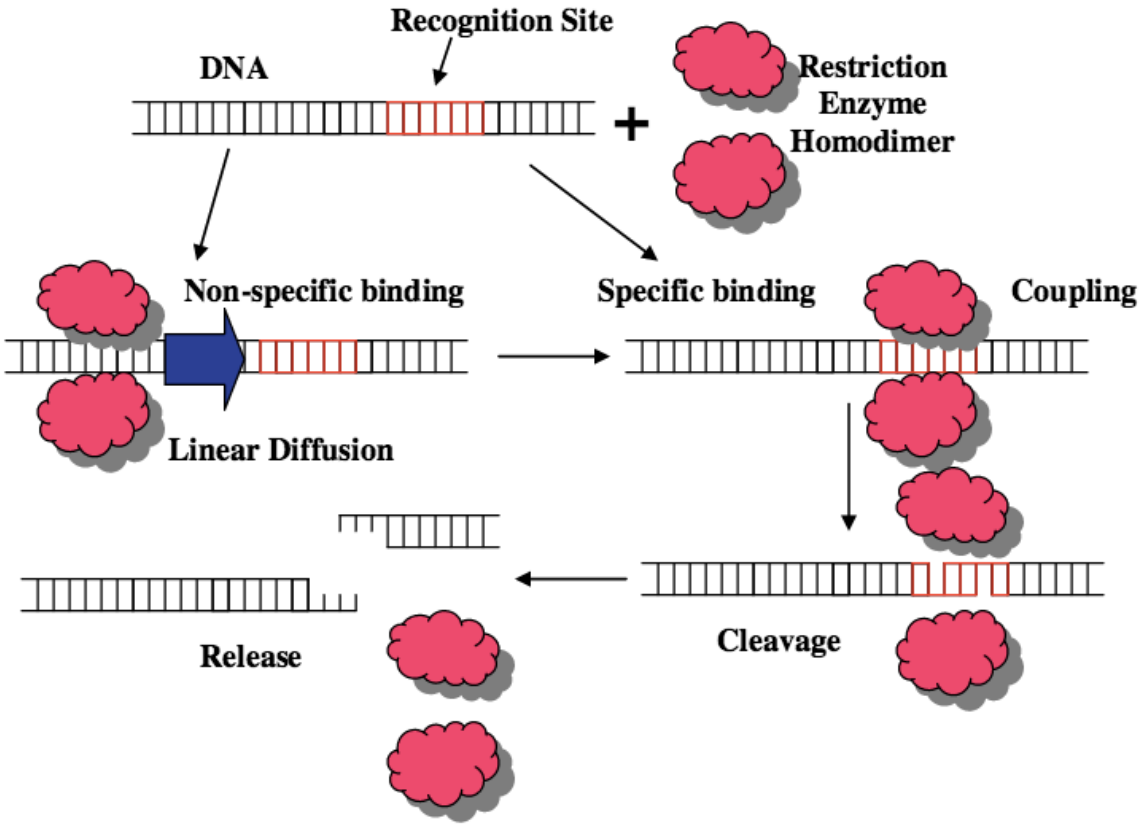
- Avoid secondary structure and repeat sequences

  secondary structures prevent primer binding to template:
  - hairpins
  - primer dimers

  repeat sequences promote slippage of polymerase and mispriming

Digestion: restriction enzymes

- Function as homodimers
  - Each dimer cleaves backbone at site of palindromic recognition sequence



Digest reagents and conditions

Reagents
DNA substrate

- Temperature

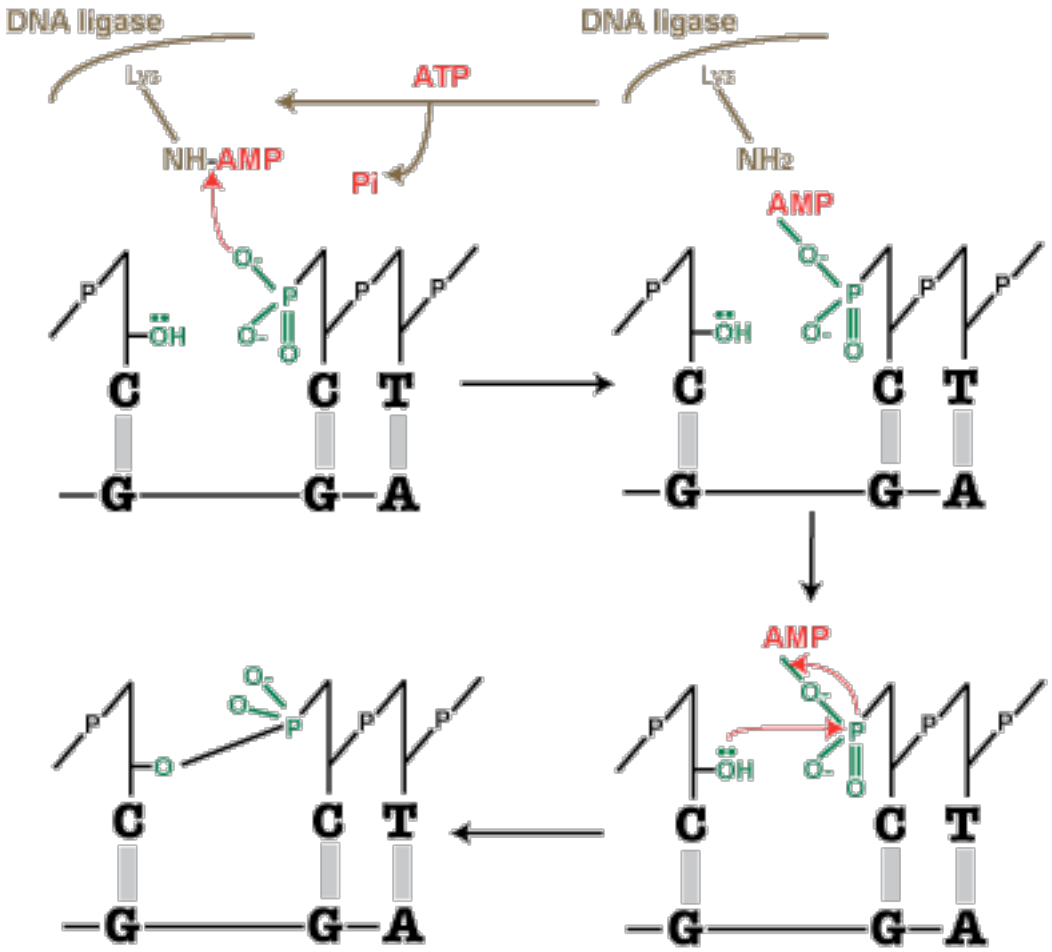
  typically 25 or 37 C, follow manufacturer protocol

- Time

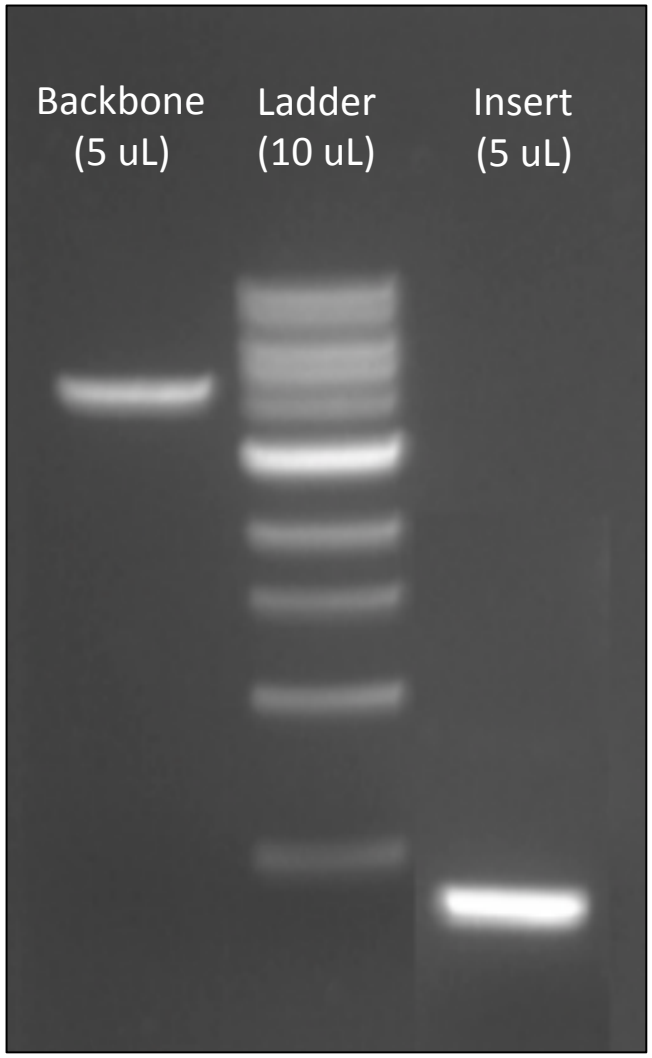
  typically 1 hr or overnight
  -shorter incubation times preferred for enzymes with star activity

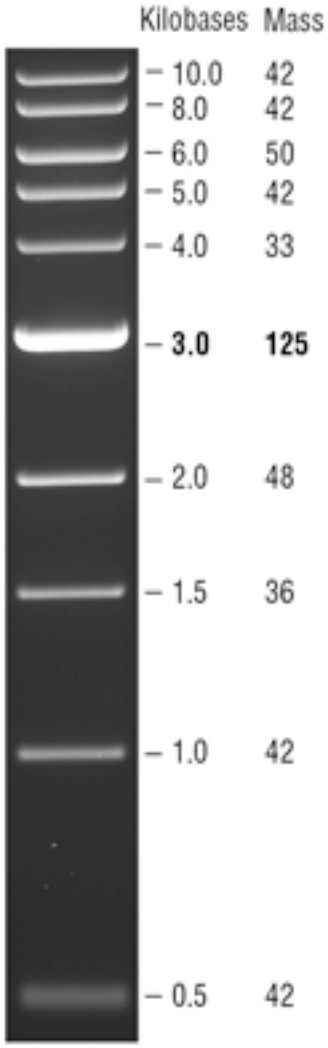
Ligation: T4 DNA ligase

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



Ligation conditions





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

- First, estimate concentrations

insert =

200 ng / 5 uL
OR 40 ng / uL

backbone =

100 ng / 5 uL
OR 20 ng / uL

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

How do we confirm the product?

- Transformation

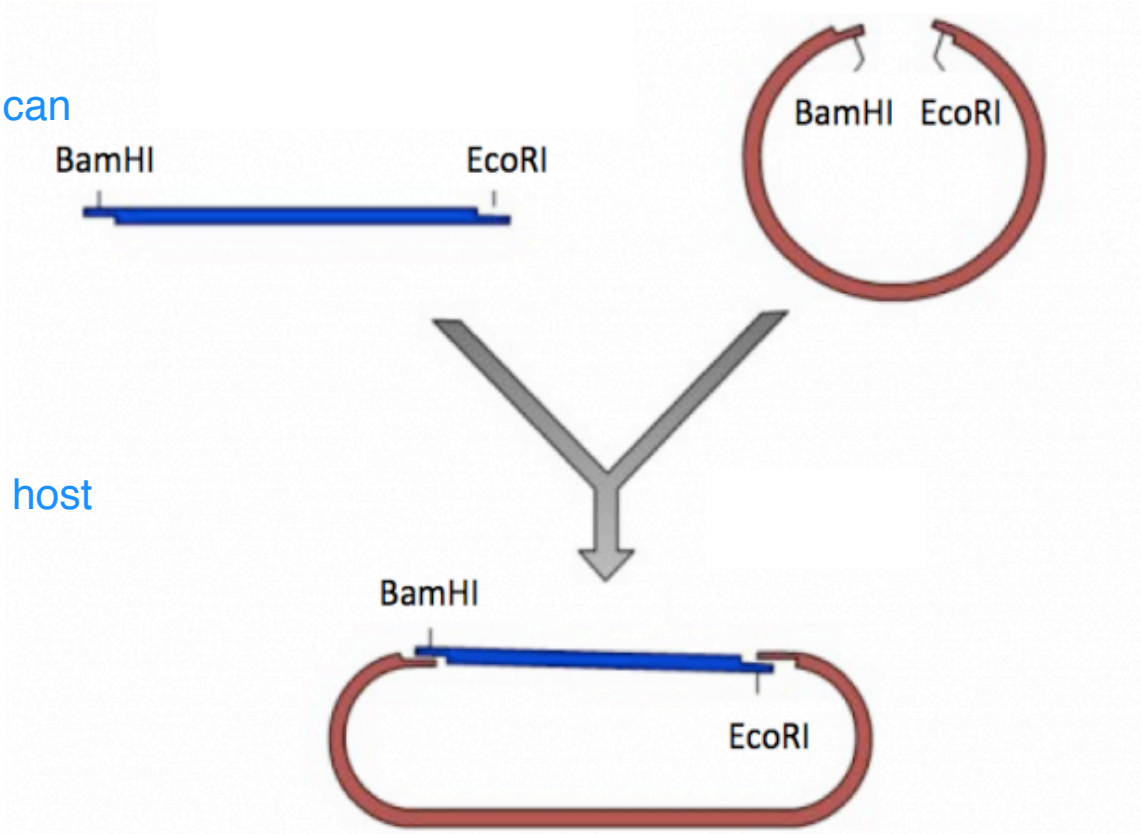
  need to move into host that can replicate, generate enough product to test

- Purification

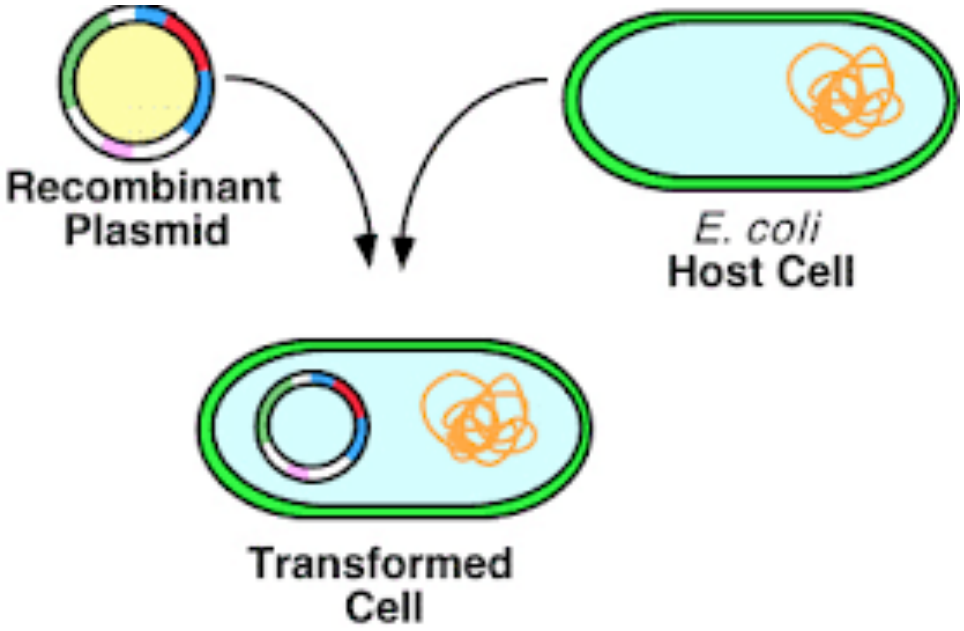
  need to retrieve product from host

- Digestion

  need to confirm that cloning product is indeed the intended insert / vector



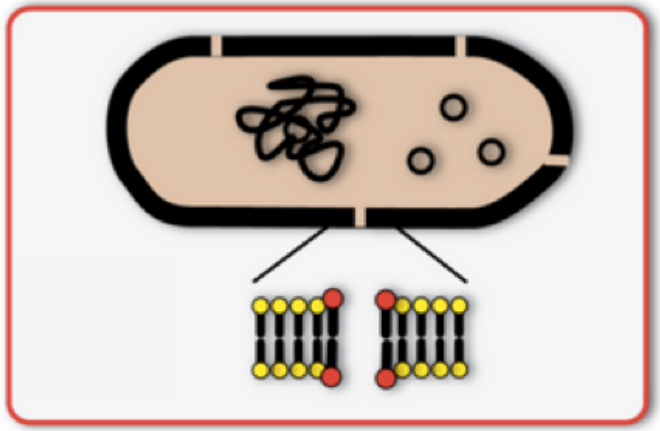
Transformation

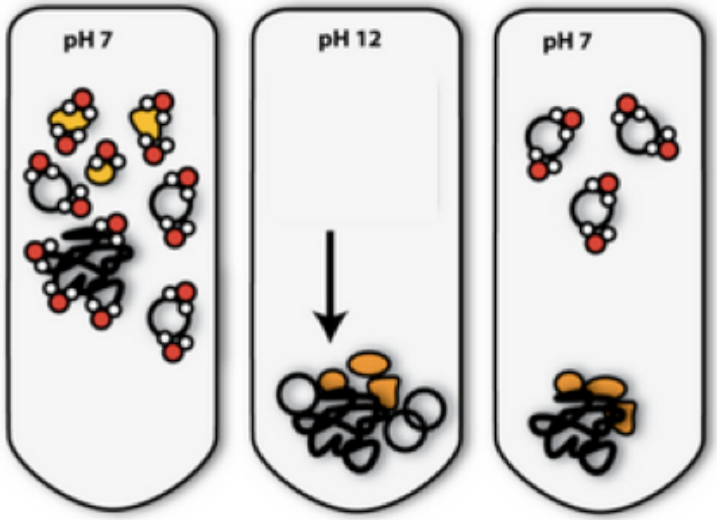


1. Incubation
2. Heat shock
   - DNA taken in by competent cells
3. Recovery
4. Selection

cells prepared such that membranes can be made permeable via electroporation or heat shock

Purification



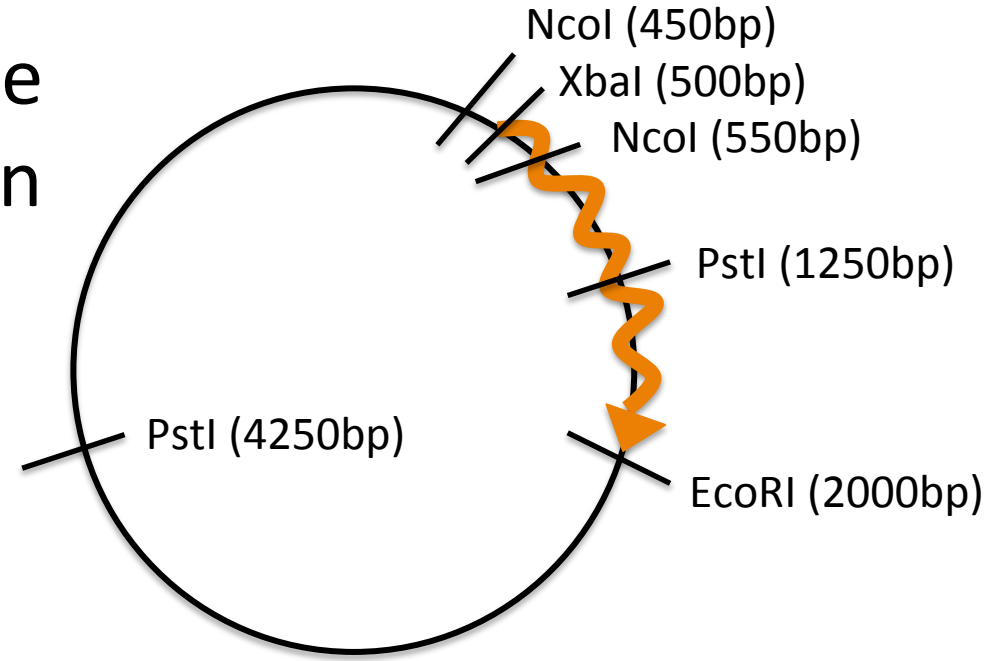


1. Resuspend cells
2. Lysis
3. Neutralization
   - Separates chromosomal DNA from plasmid DNA
4. Wash
5. Resuspend or elute DNA

neutralization step allow shorter fragments of DNA (plasmid) to reanneal and go into solution while longer fragments (chromosome) remain denatured and in the precipitate

Digestion, again

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



make sure sizes can be distinguished with gel electrophoresis and that fragments are within 500 - 5000 base pairs

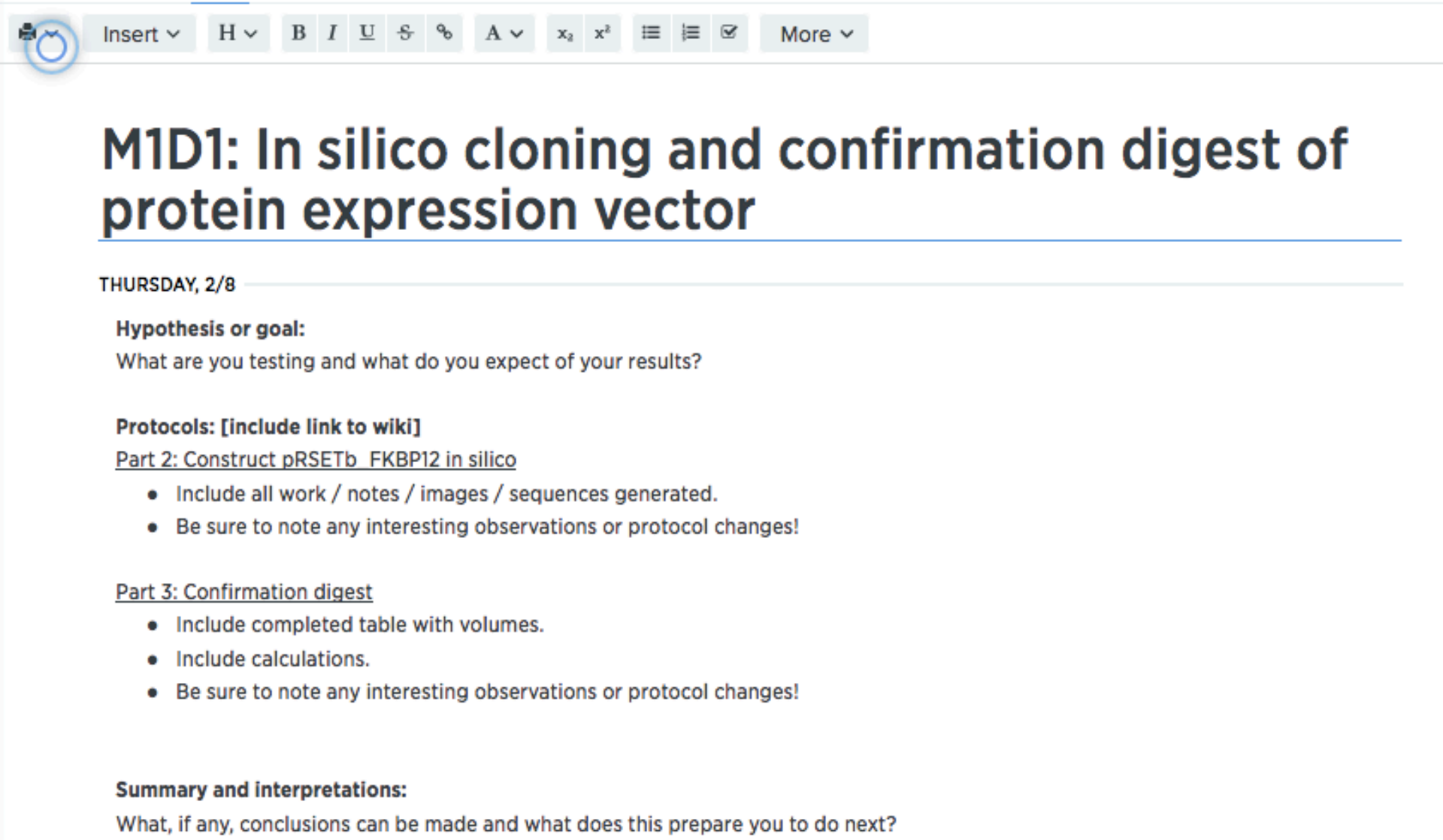
pNLL-PCR (6000bp)

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

How should you format your notebook?



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 Noreen and Casper
  - Right-click and choose ‘settings’
  - Add collaborators by email

Today in lab…

- Virtual cloning exercise to build pRSETb_FKBP12 expression plasmid
- Confirmation digest of pRSETb_FKBP12

For next time…

- Prepare a template for Benchling entries