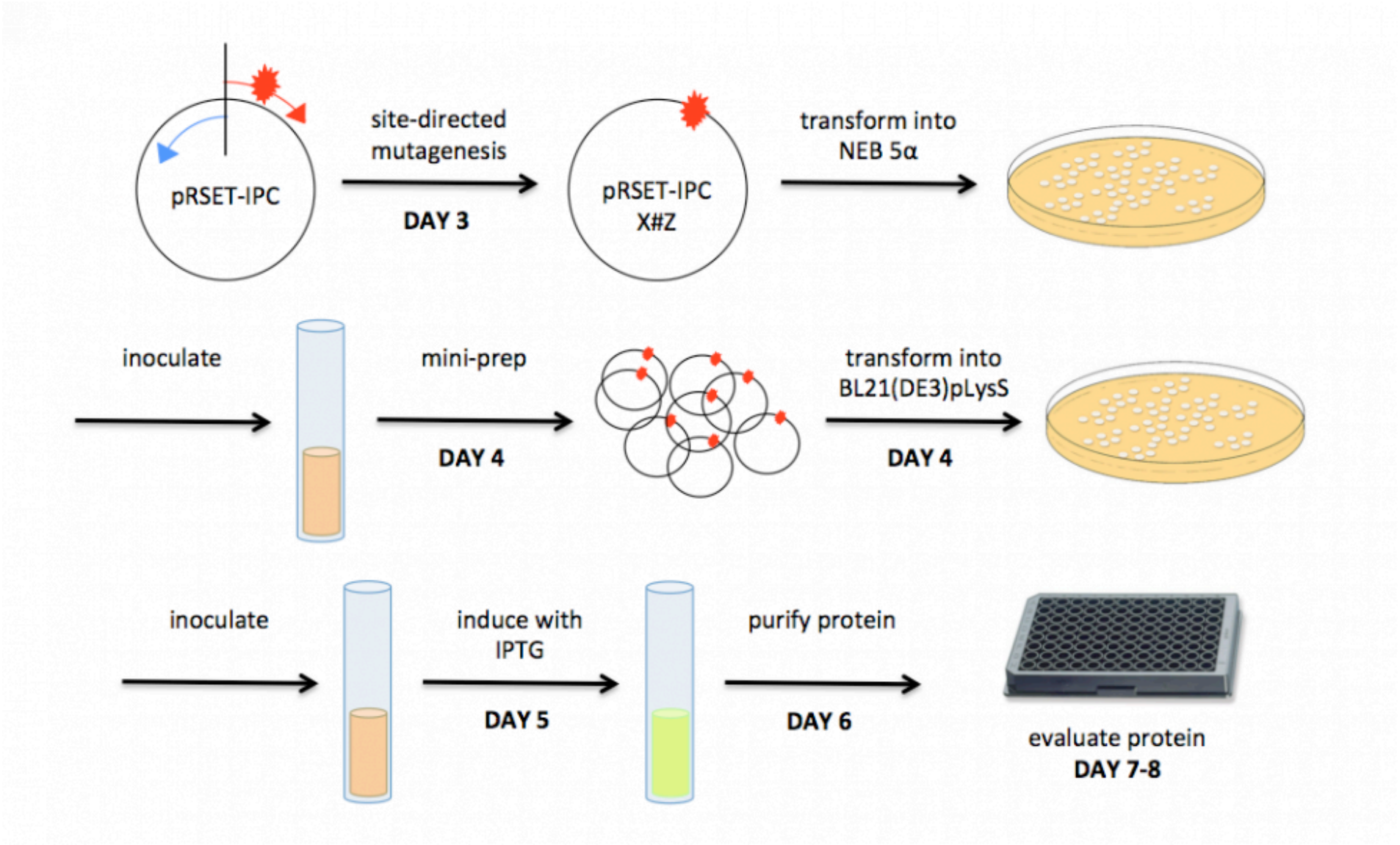
M1D2:Design site-directed mutagenesis primers

02/09/16

1. Load diagnostic digest from M1D1
2. Prelab Discussion
3. Design mutations for Inverse Pericam and create primer sequence

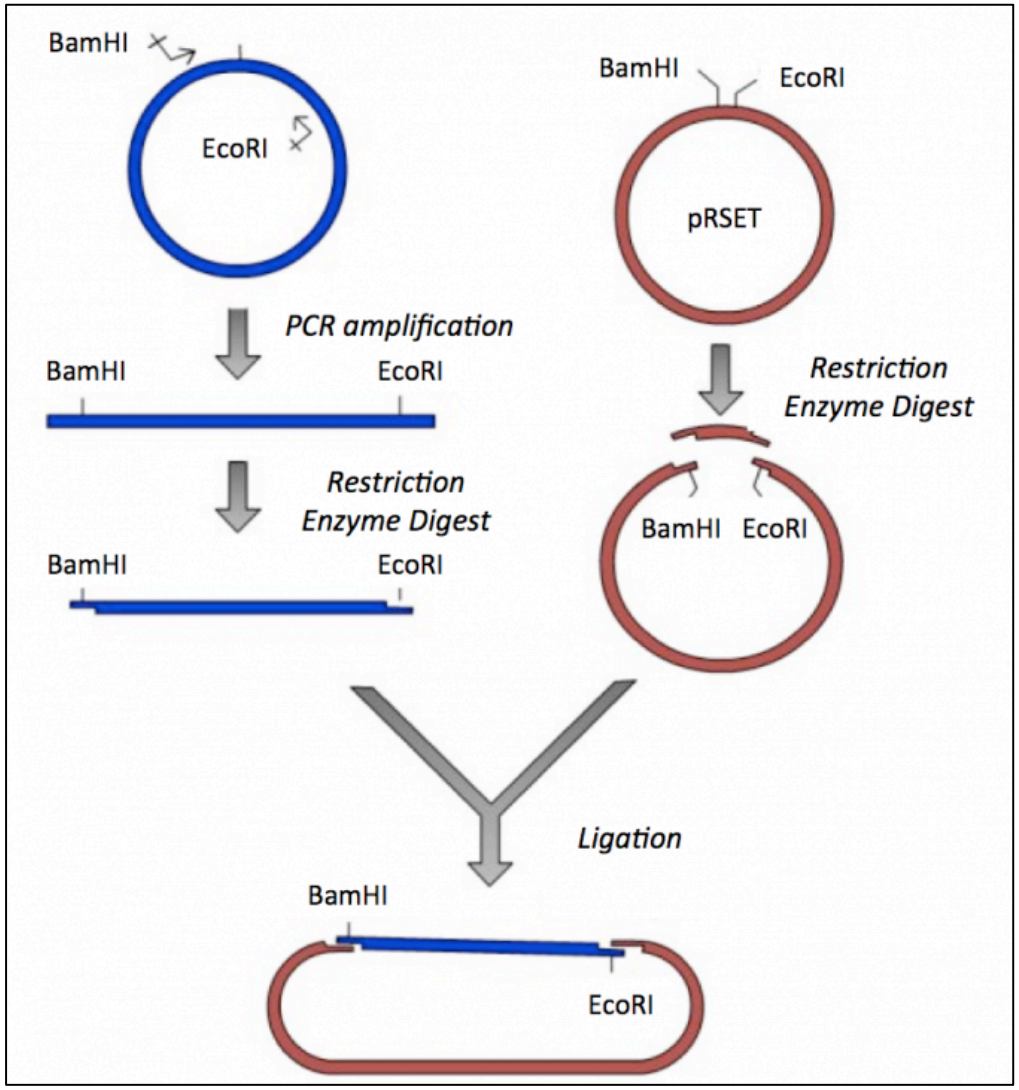
M1 experimental overview



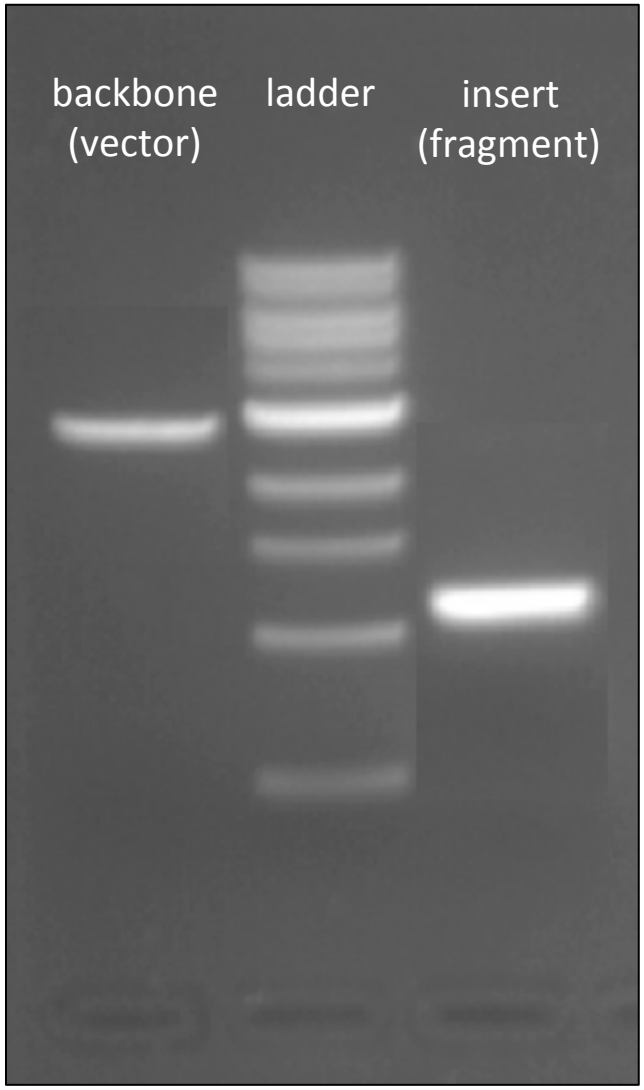
M1 major assignments

- Protein engineering **summary** (15%)
  - in teams, on Stellar
  - draft due 03/12, final revision due 03/28
  - bullet points, .PPTX
  - Abstract + Background & Motivation + Results & Interpretation + Implications
- Protein engineering **mini-presentation** (5%)
  - individual, video format
  - due 03/16
- Lab quizzes (7% total)
  - M1D4 (02/19), M1D8 (03/04)
- Notebook (3% total)
  - one day will be collected and graded by Jing on last day of module 1
- Blog: http://be20109s16.blogspot.com/ (participation: 3% total)
  - by 03/29, more info later in module

pRSET-IPC was constructed by ligation

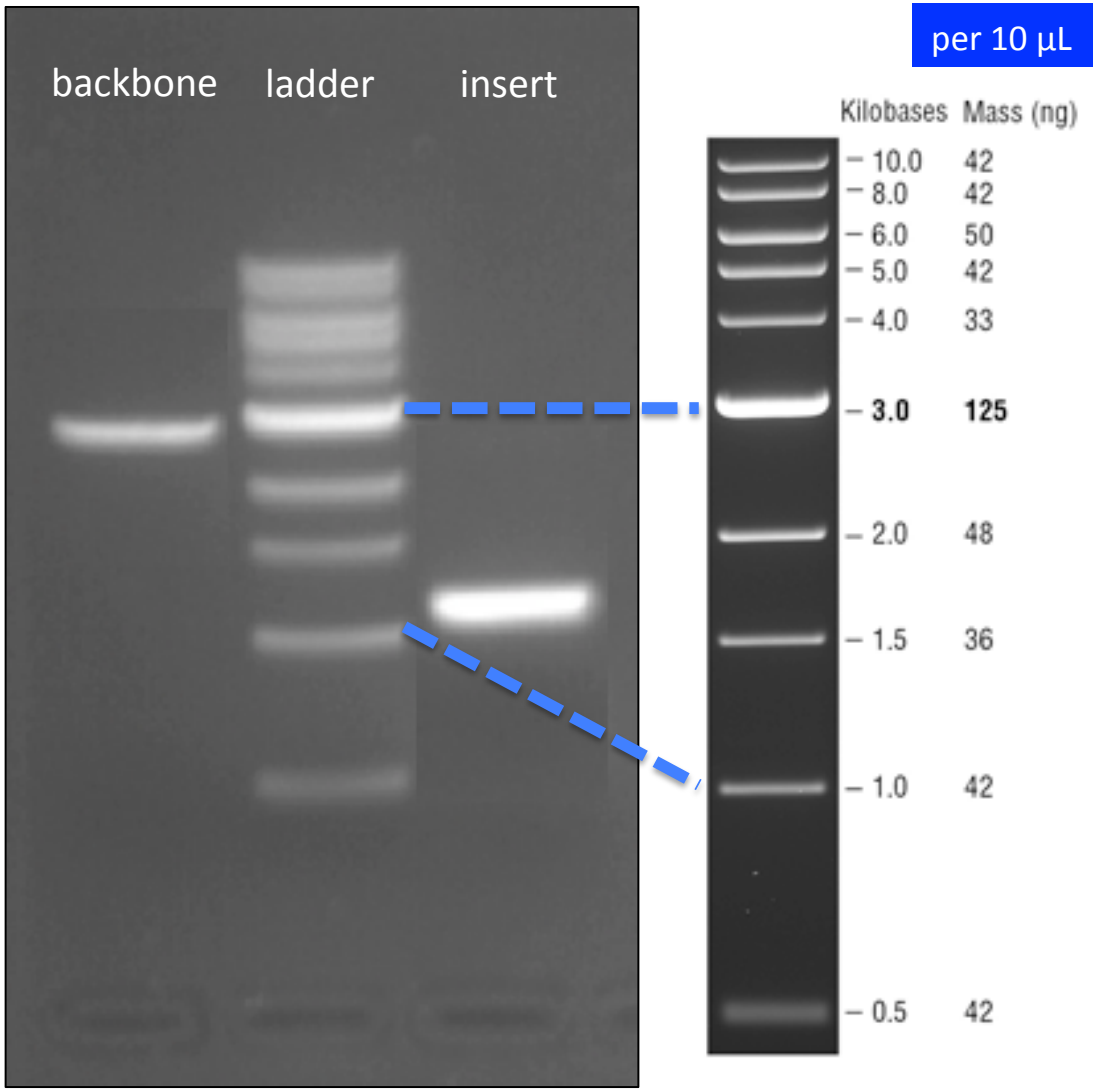


pRSET-IPC cloning strategy



recovery gel

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

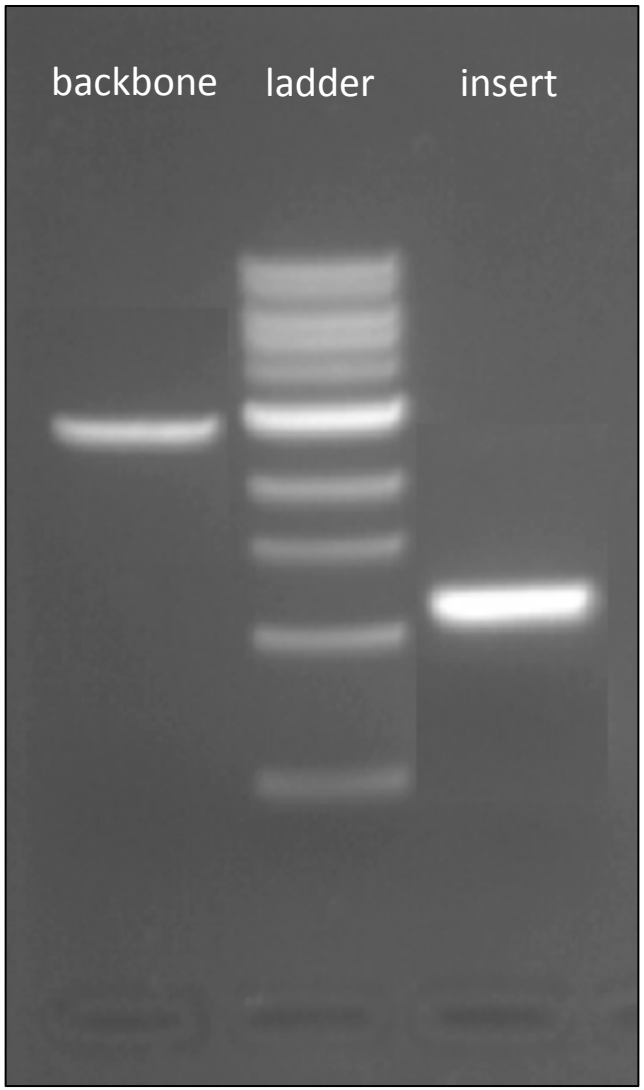


- Assuming
  - 20 µL of ladder loaded,
  - 5 µL of *BamHI-EcoRI* double digest loaded,
- amount of backbone = 200 ng
- amount of insert = 800 ng

➢ but mass of DNA ≠ molar amount of DNA

base pair=660Da=660g/mol

Calculate the 1:4 *molar* amounts for ligation



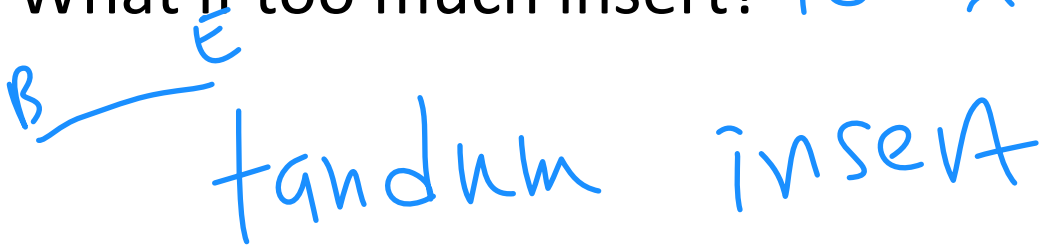
1. From recovery gel, estimate
   - backbone: 200 ng / 5 µL = 40 ng/µL
   - insert: 800 ng / 5 µL = 160 ng/µL
2. Determine volume of backbone needed
   - 50-100 ng, choose 80 ng, *i.e.* 2 µL
3. Calculate moles of backbone
   - 2887 bp * (660 g / (mol*bp)) = 1.9 x 10^6 g/mol MW bkb
   - so 80 ng / (1.9 x 10^6 g/mol) = 4.2 x10-14 mol mol bkb
4. Determine moles of insert needed (4X bkbn)
   - 4 x 4.2x10-14 ~ 1.7 x 10^{-13} mol want!
   - with 1288 bp * (660 g / (mol*bp)) = 8.5 x 10^5 g/mol MW isert
   - so use 1.7 x 10^{-13} mol * 8.5 x 10^5 g/mol ~ 143 ng
5. Calculate volume of insert needed
   - 143 ng / (160 ng/µL) = 0.9 µL

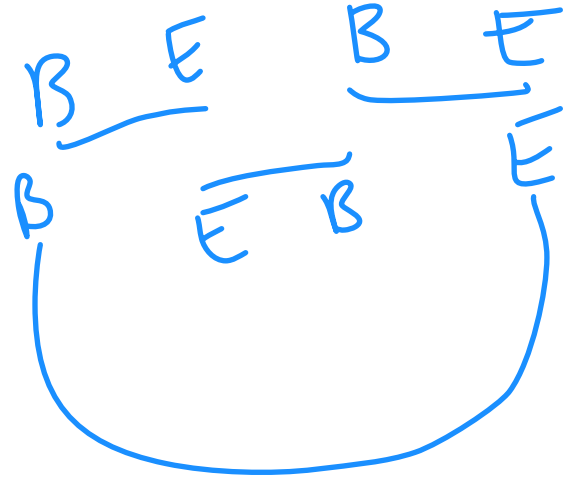
ng / (g/mol)=mol

Optimal backbone-to-insert ratio

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

- What if too much insert? 100X





- What if too much backbone? 100X

linear DNA = no amp.

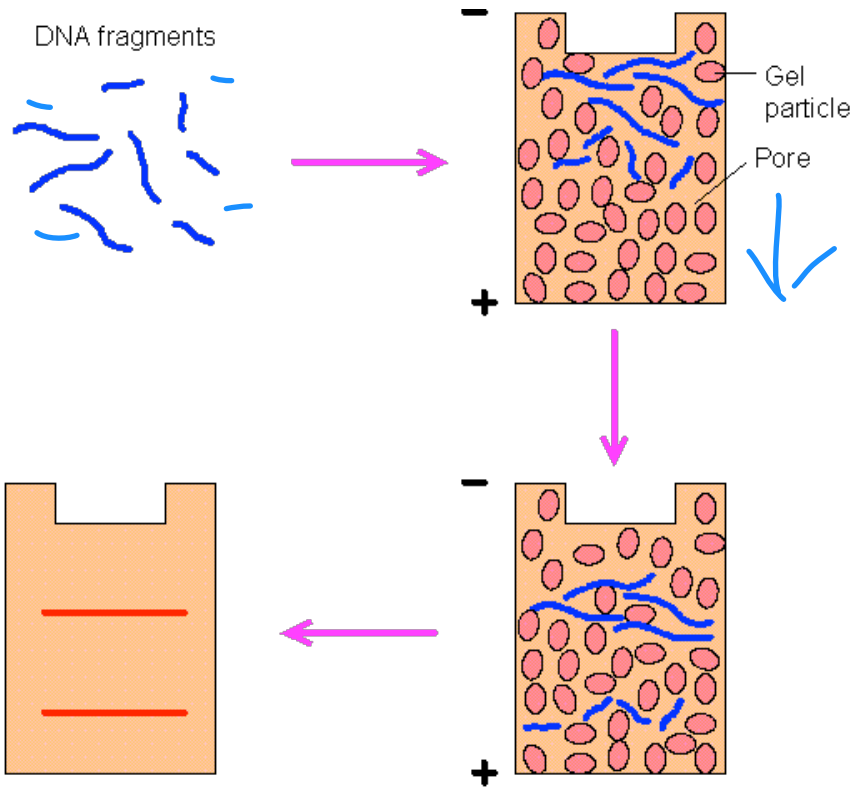
Separate DNA by gel electrophoresis

- Agarose gel electrophoresis
  – driving force:

charge DNA

  – separates DNA by:

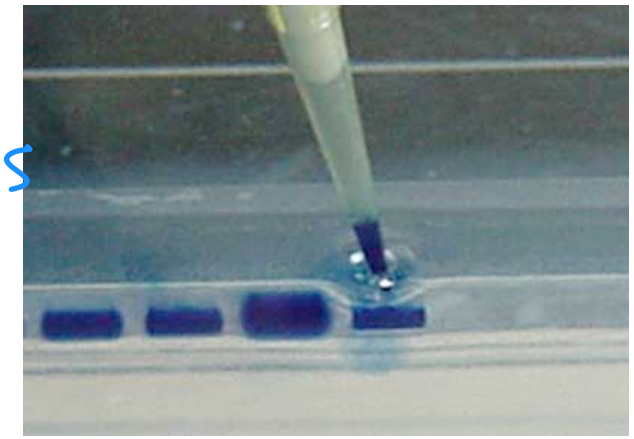
Size

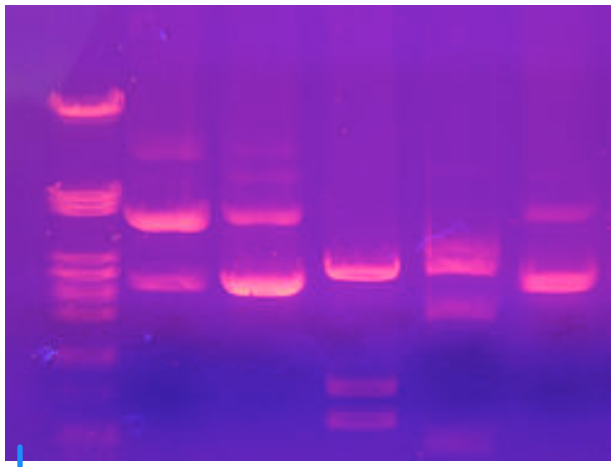


1% agarose

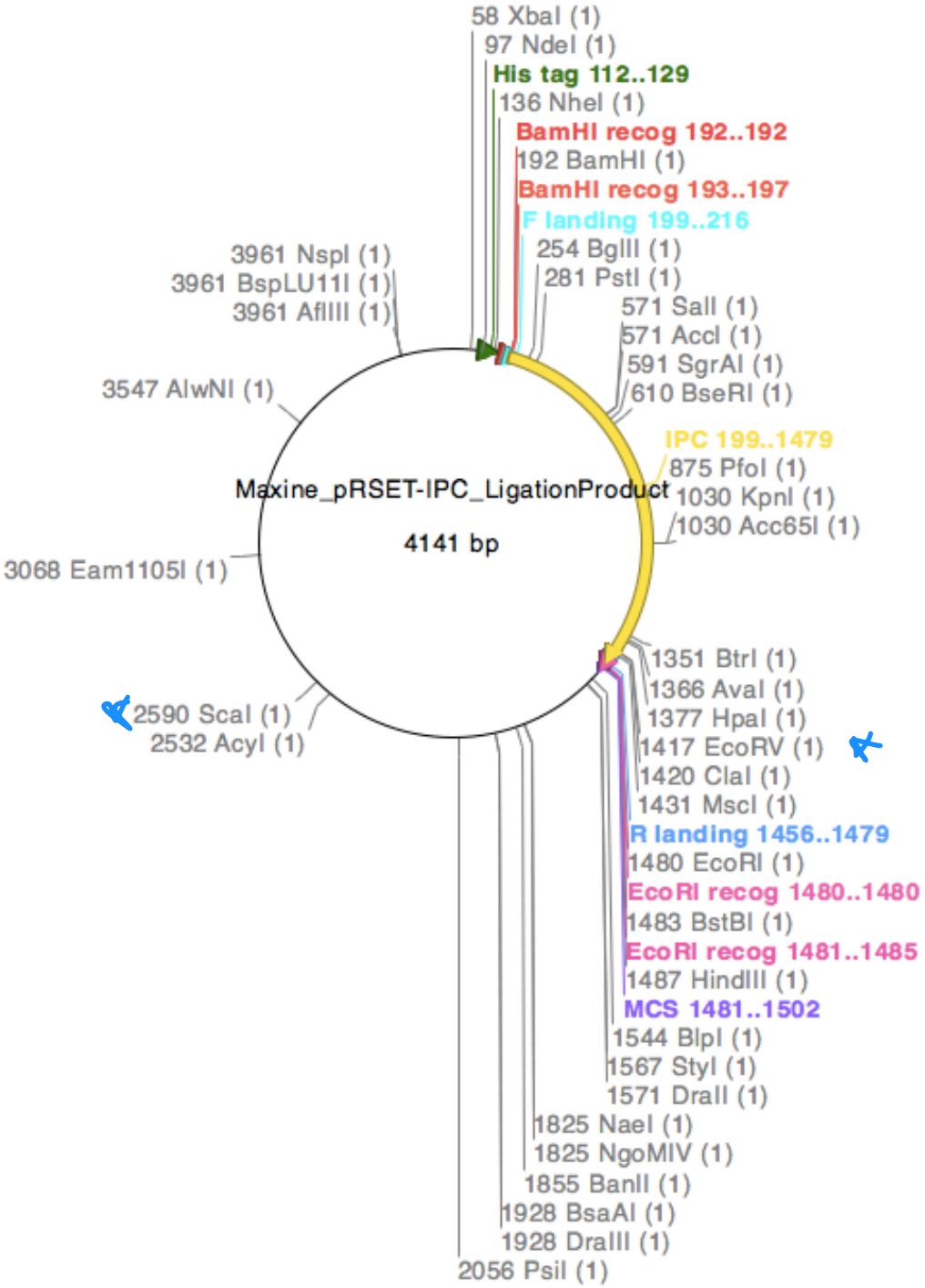
Visualize DNA

- Loading dye 6X
  - bromophenol blue 500bps
    - front of DNA
  - glycerol
    - viscosity
- DNA stain
  - Sybr-safe
    - DNA intercalator
    - visualize by UV light





- Safety : wear nitrile gloves

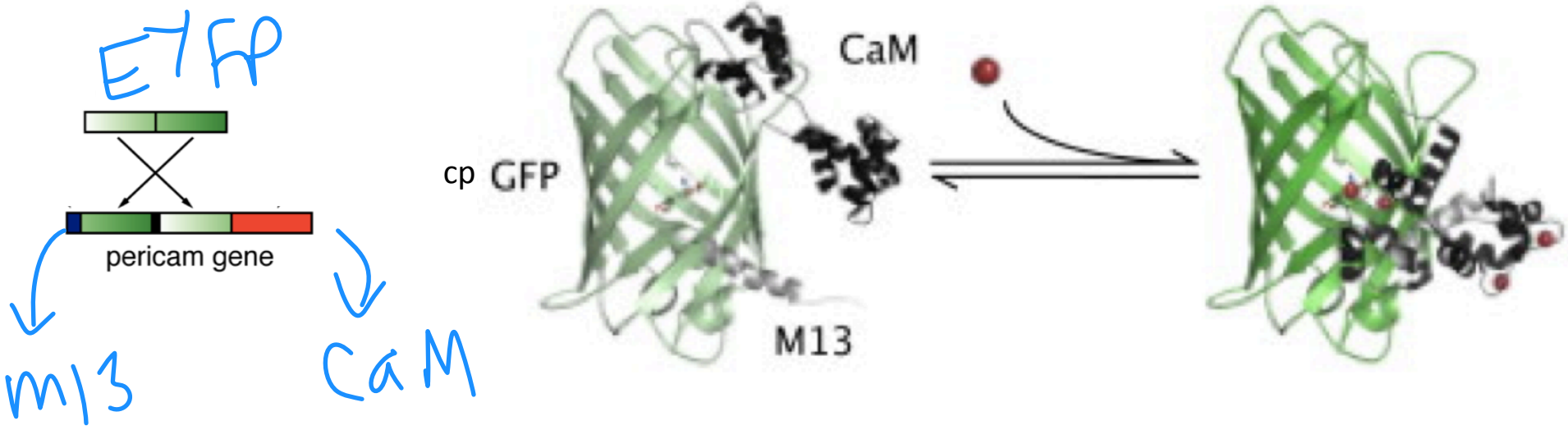


-2968
-1173

Confirmation digest

- *ScaI* (2590) in backbone
- *EcoRV* (1417) in insert
- NEB buffer 3.1

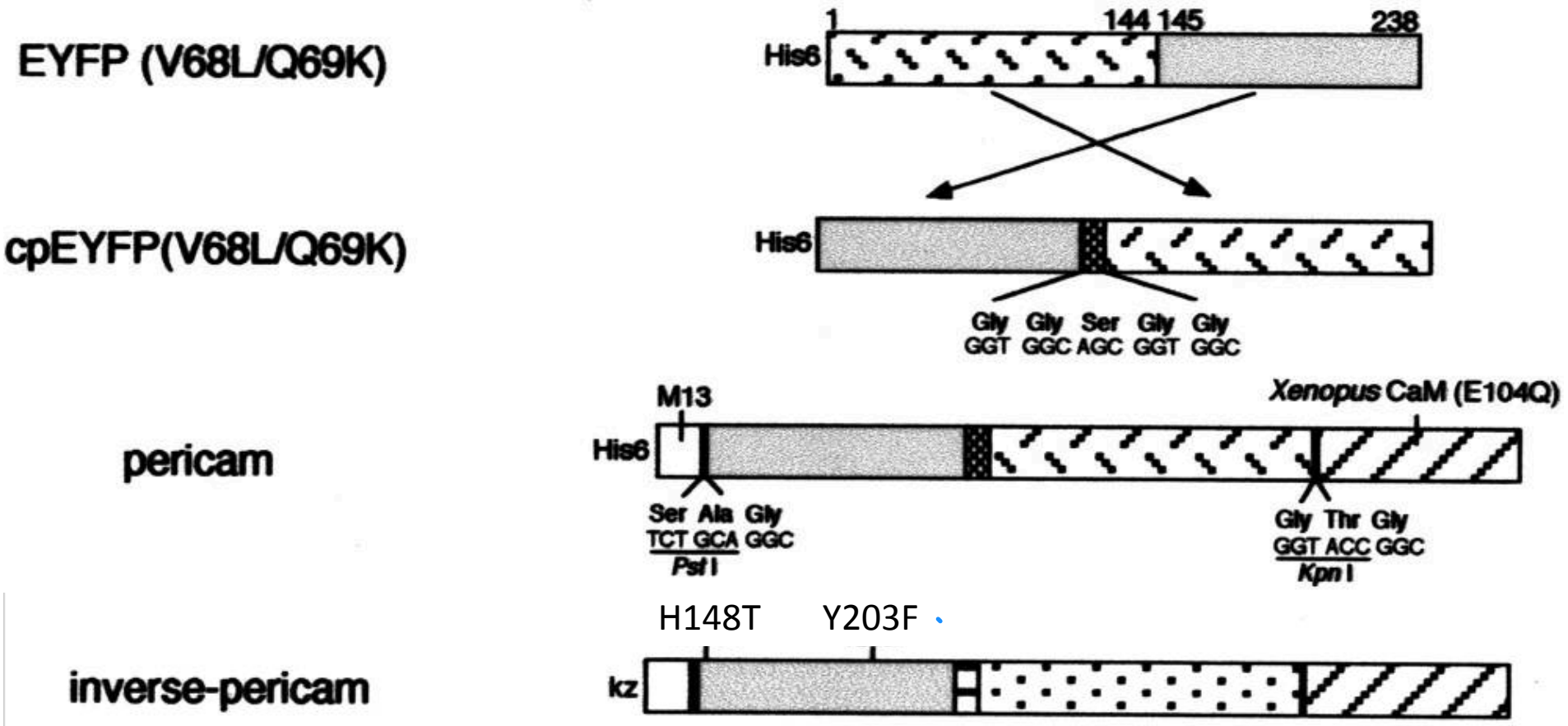
Pericam (and GCaMP family) is a GECI: genetically engineered calcium indicator



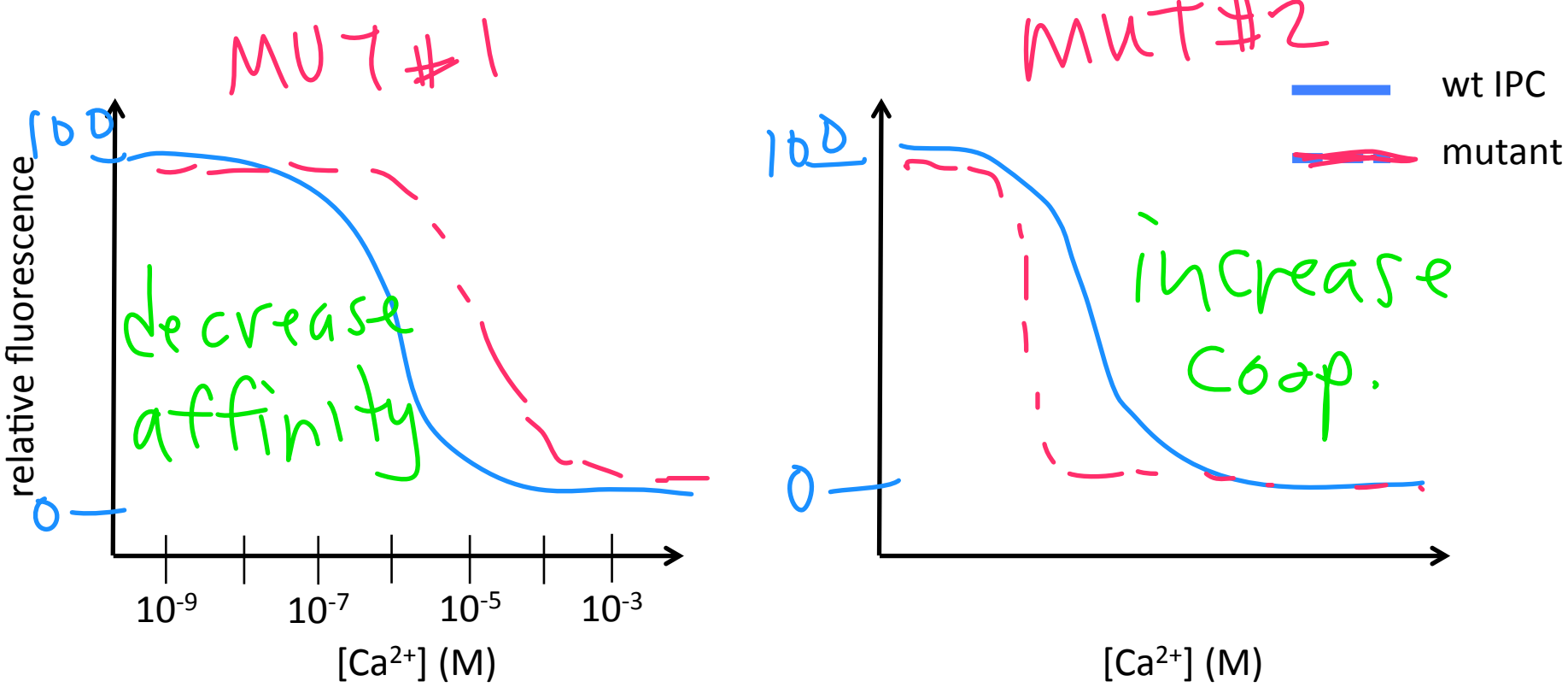
- EYFP: enhanced yellow fluorescent protein
- CaM: calmodulin (calcium-modulated protein)
- M13: CaM-binding peptide from myosin light-chain kinase

* Roger Tsien won the 2008 Nobel Prize for engineering novel forms of GFP

Inverse pericam (IPC) is dimmer with Ca^{2+}



Protein engineering:
modulate binding affinity and/or cooperativity



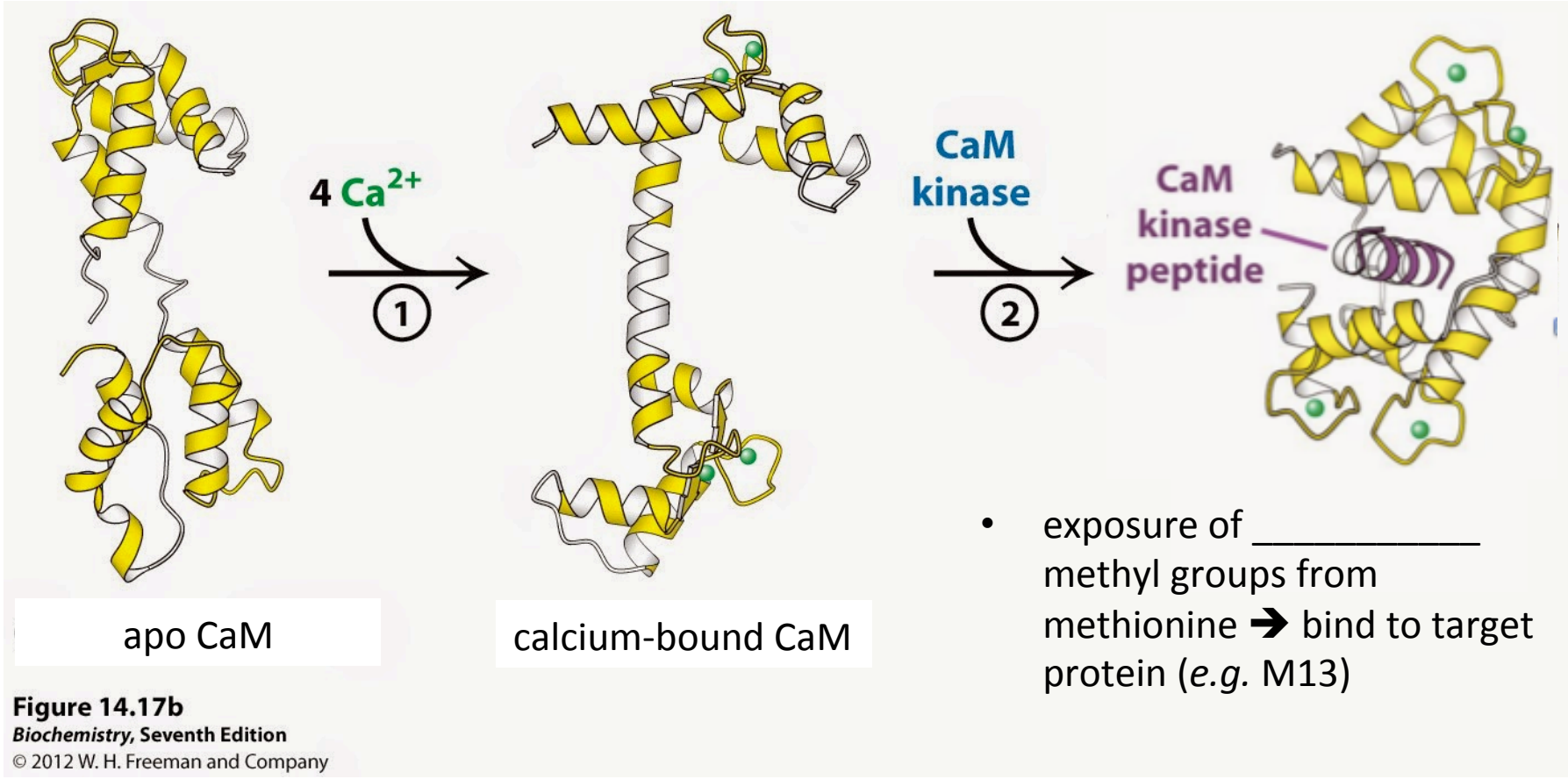
$[Ca^{2+}]_{cytosol}$ ~ 10-100 nM

$[Ca^{2+}]_{ER\ /\ mitochondria}$ ~ 20 nM

$[Ca^{2+}]_{extracellular}$ ~ 1mM

CaM interacts with Ca^{2+} and with target kinase

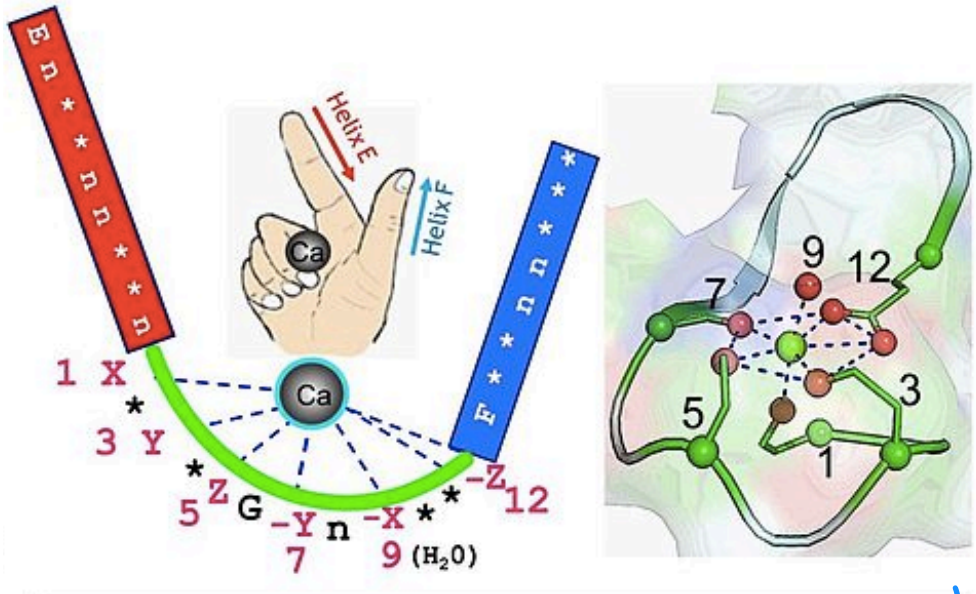
- 4 EF hands: 2 at N-terminal + 2 at C-terminal
- EF hand domain = helix-loop-helix
- loop = Ca^{2+} binding pocket, offers electro neg environment



- exposure of _____ methyl groups from methionine ➔ bind to target protein (*e.g.* M13)

Figure 14.17b
***Biochemistry*, Seventh Edition**

Mutate CaM Ca^{2+}-binding EF hand domain



- Binding pocket residues

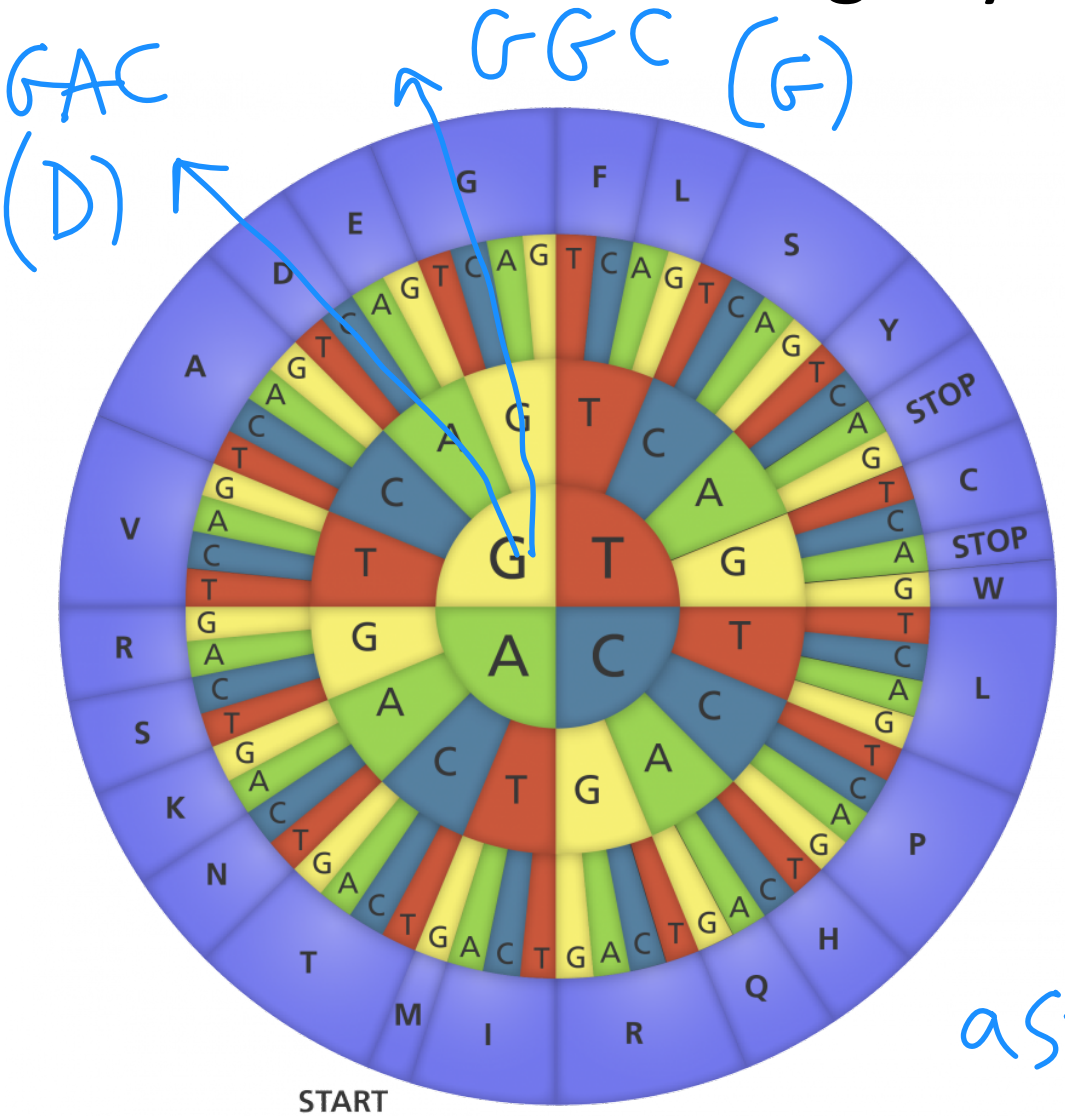
polarity
charge
hydrophobicity

topology / steric hinderance
pH

- Interface with M13

from “viral calciomics: interplays between Ca2+ and virus”. Zhou Y, Frey TK, Yang JJ. Cell Calcium. 2009 Jul;46(1):1-17.

Which residues might you try to alter?



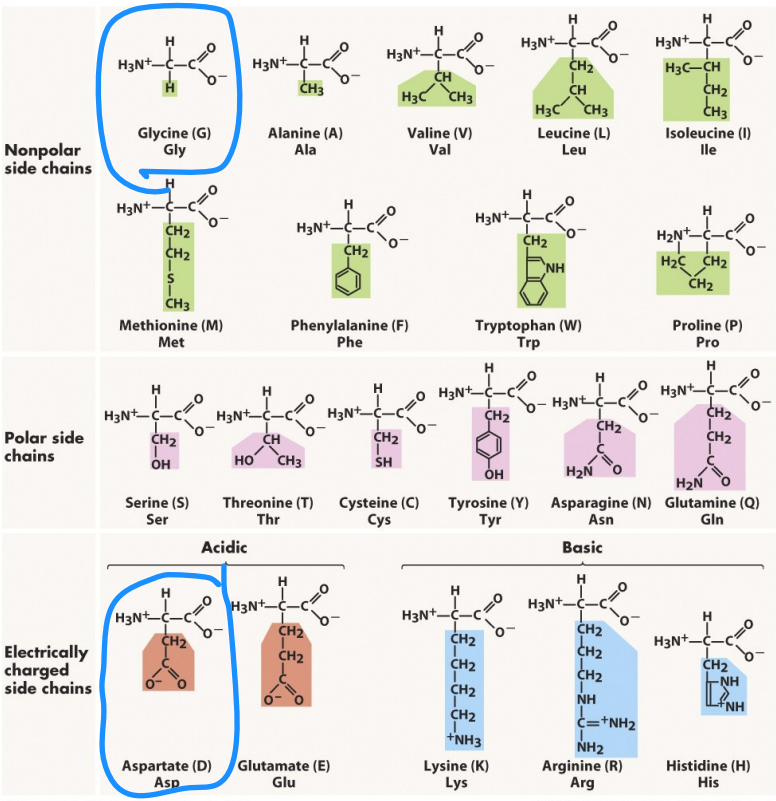


Figure 3-5 Biological Science, 2/e

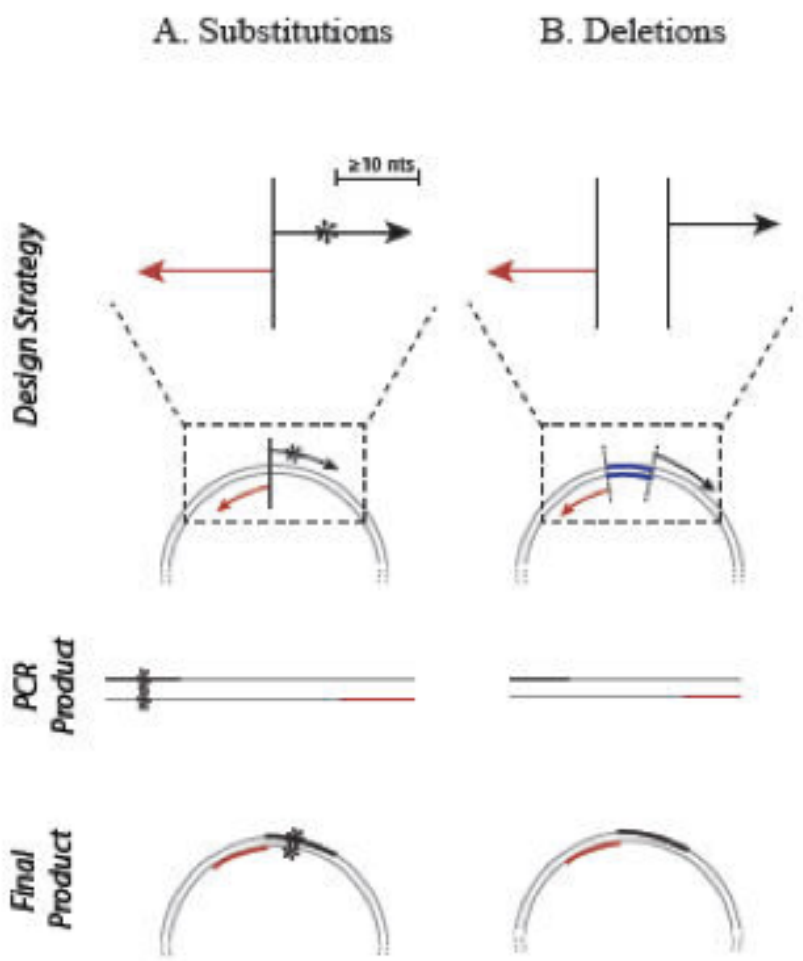
aspartate → glycine

big difference

size + charge

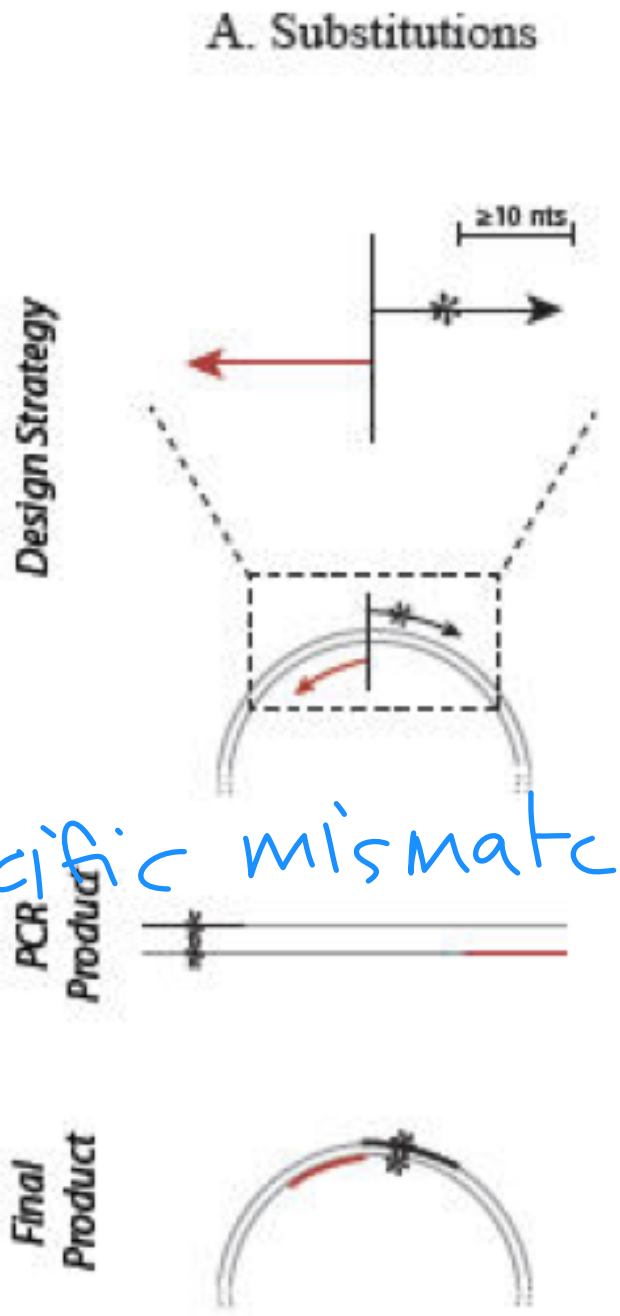
Site-directed mutagenesis (SDM)

- Create specific, targeted changes in double-stranded plasmid DNA
  - substitution
  - deletion
  - insertions
- Primers contain the desired mutation
- Using NEBα Q5 SDM kit
  - back-to-back primers
  - forward primer imposes mutation



Primer design guidelines

- substitution
  - mutation location: middle
  - Length: 25-45 bps
  - G/C content: >40 %.
  - start and end with at least one G/C
  - melting temperature > 78 °C
- PCR amplification *vs.* mutagenesis primers
  - sequence match: perfect vs specific mismatch
  - T_m: 60 C vs 80 C
  - length: 17-27 bps vs 25-45 bps



Today in lab:

1. Load diagnostic digest from M1D1
2. Image agarose gel
3. Analyze features of Inverse Pericam protein
4. Create primer sequence (start this no later than 4:30pm)