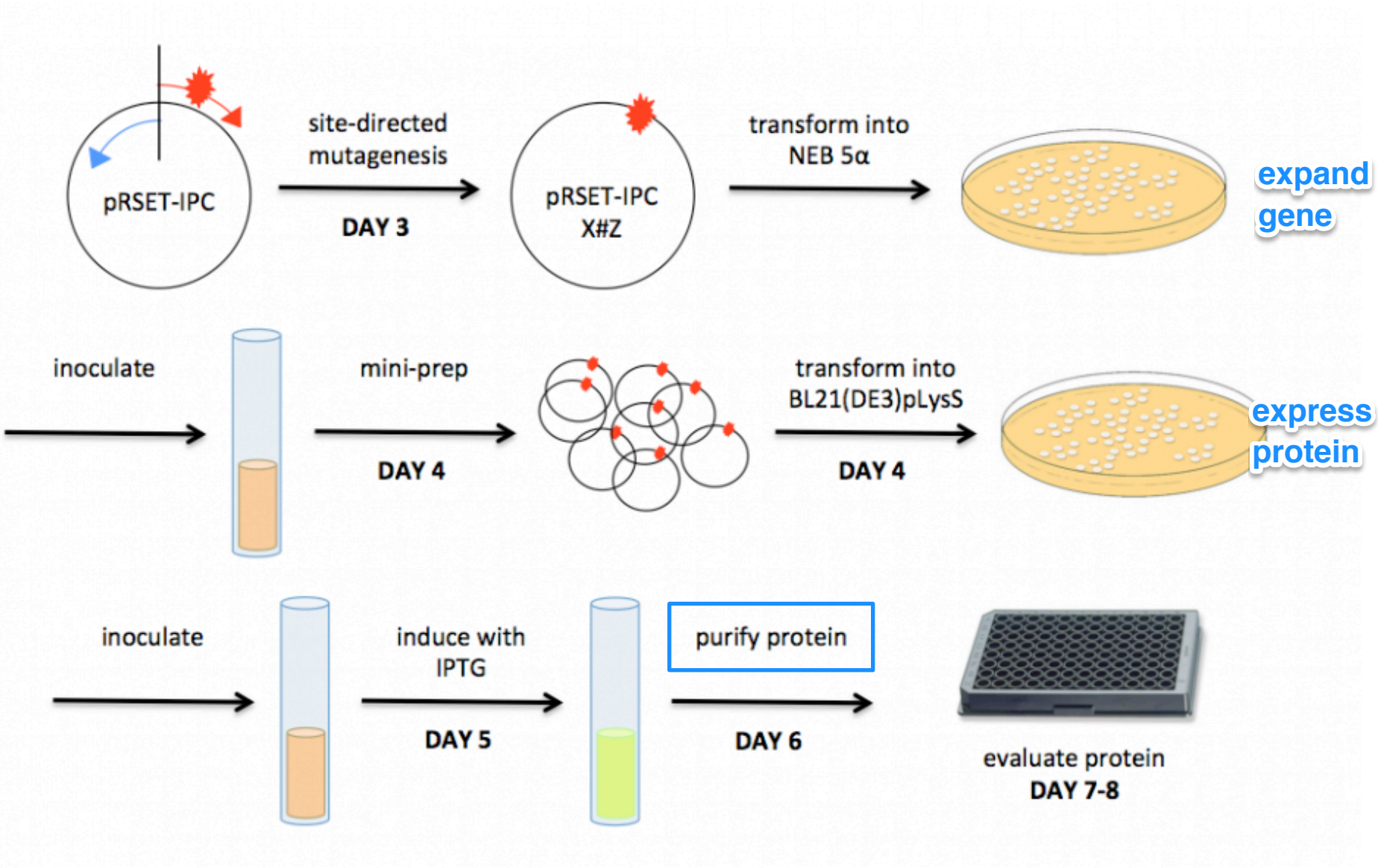
# M1D2:
# Design mutation primers

02/10/2016

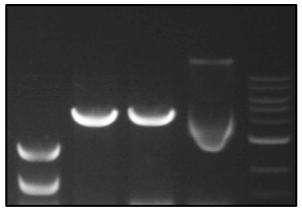
# M1 experimental overview

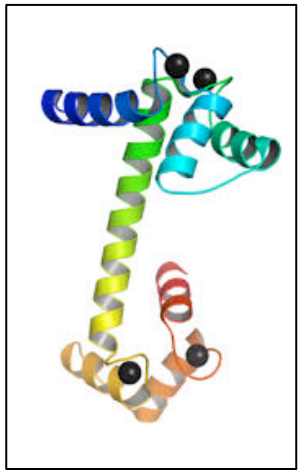


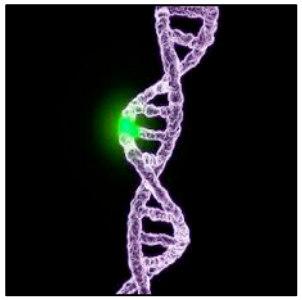
# M1 major assignments

- Protein engineering **summary** (15%)
  - in teams, on Stellar
  - draft due 03/12, final revision due 03/28 add up 1 1/3 grade (C can be become B+)
  - bullet points, .PPTX
  - Abstract + Background & Motivation + Results & Interpretation + Implications
- Protein engineering **mini-presentation** (5%)
  - individual, via MIT TechTV
  - 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 M1D7 (03/02)
- Blog: http://be20109s16.blogspot.com/ (participation: 3% total)
  - by 03/29

# Today in lab

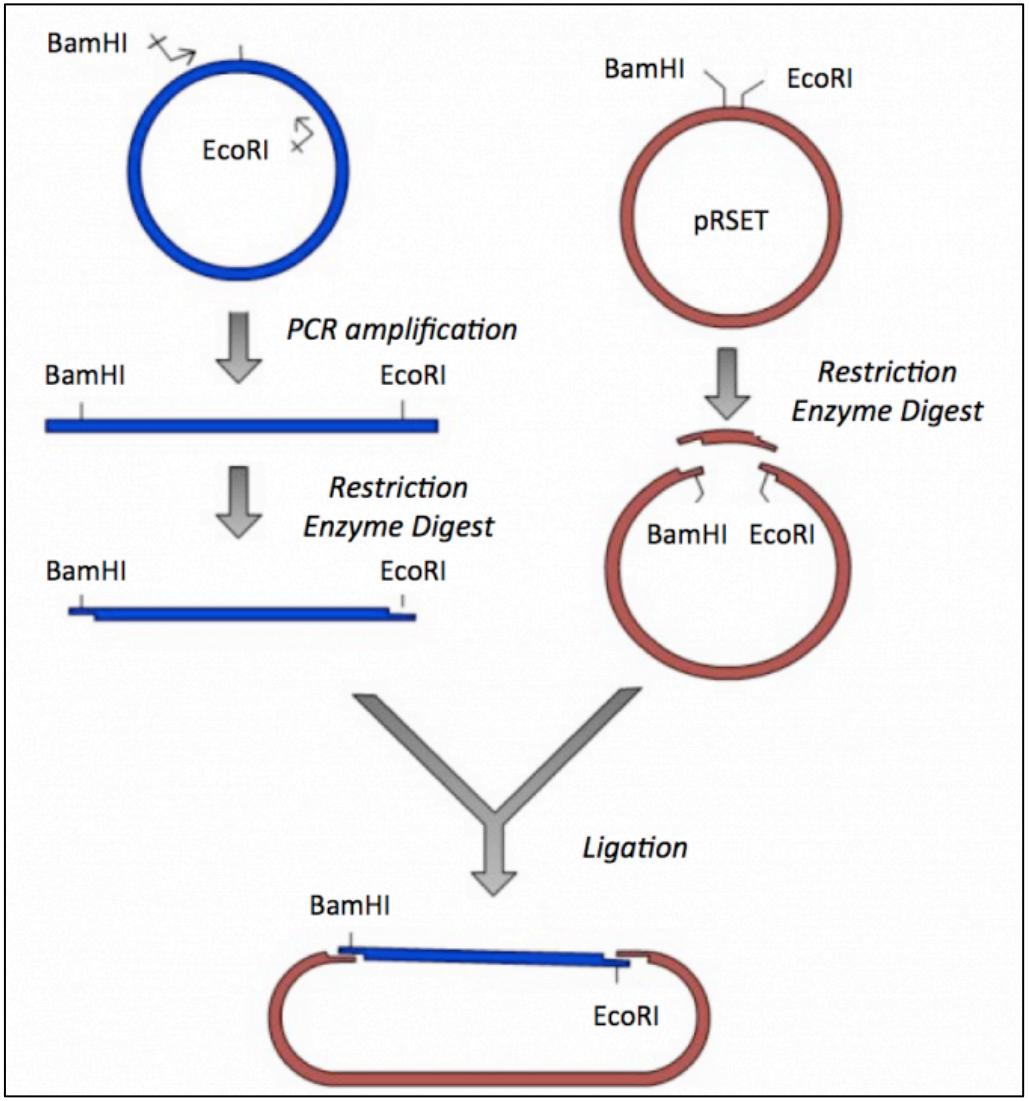




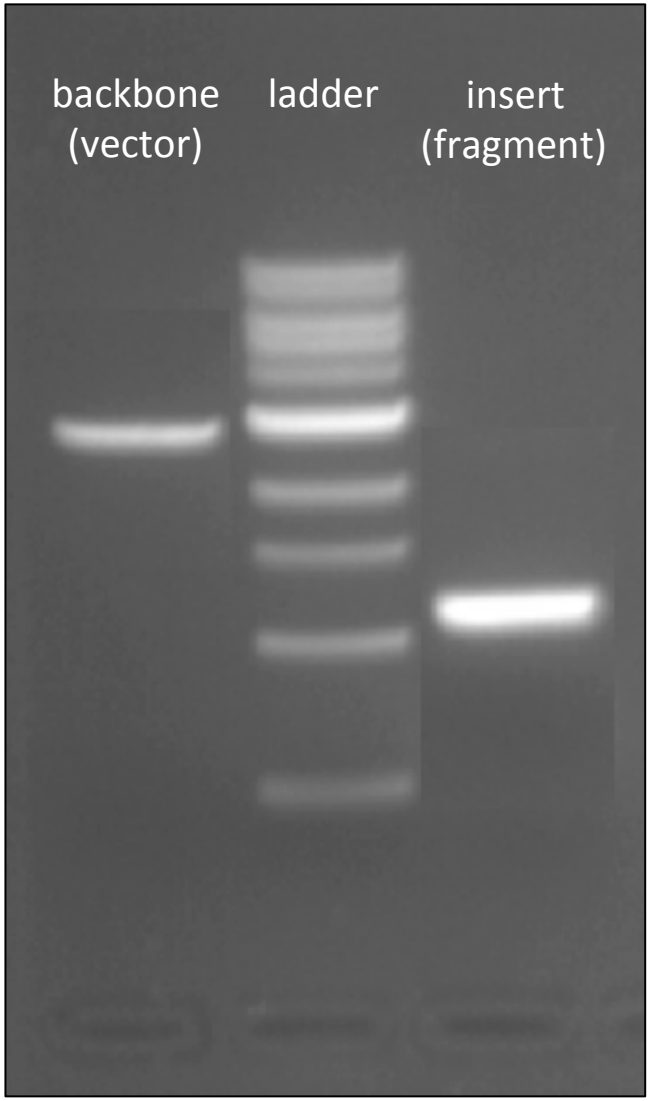


- Agarose gel electrophoresis of confirmation digests
  - pRSET-IPC cut by restriction enzymes
- Explore inverse pericam (IPC)
  - primary
  - tertiary
- <u>Submit primer sequences before leaving lab</u>
  - for site-directed mutagenesis

# pRSET-IPC was constructed by ligation



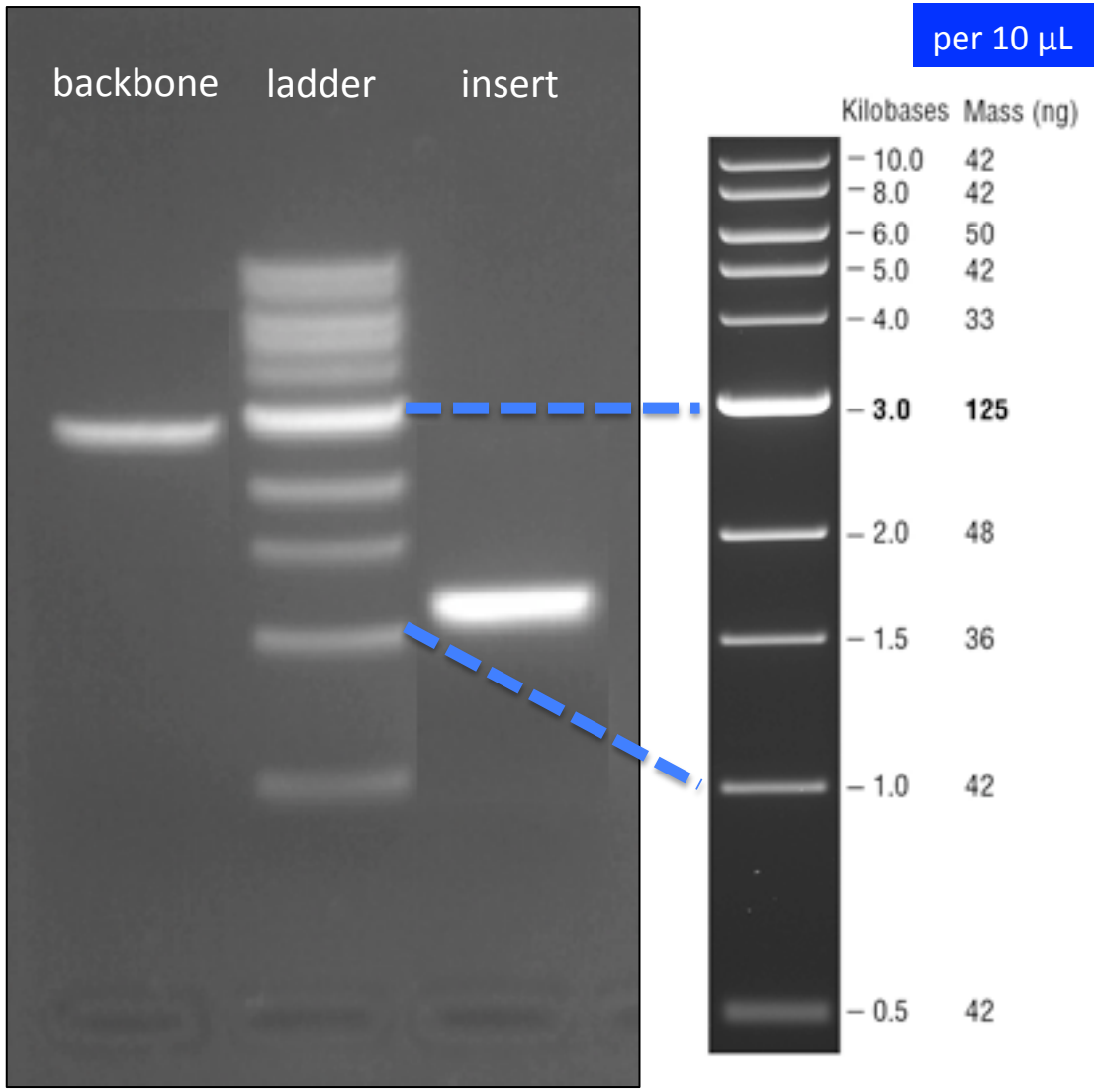

pRSET-IPC cloning strategy




recovery gel

manufacturer's recommendation

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

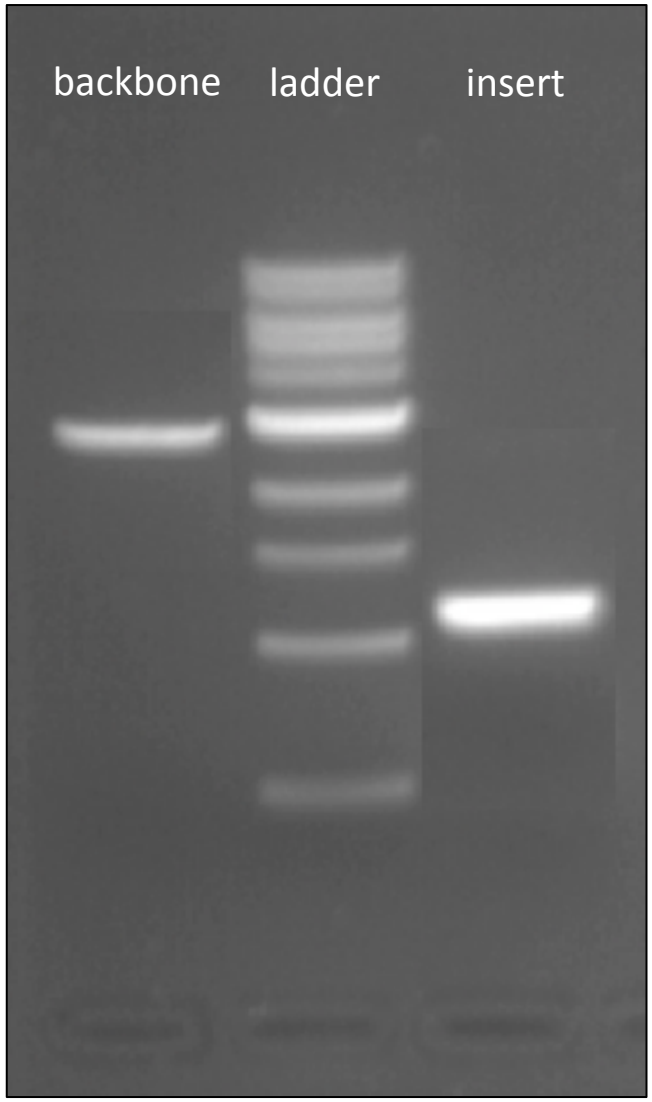



- Assuming
  - 20 µL of ladder loaded,
  - 5 µL of *BamHI-EcoRI* double digest loaded,
- amount of backbone = 200 ng
- amount of insert = 800 ng

➢ but mass of DNA ≠ molar amount of DNA

molecular weight of 1 bp = 660 Da = 660 g/mol

# Calculate the 1:4 *molar* amounts for ligation

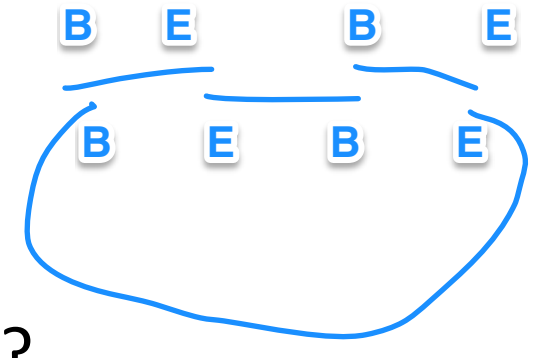



1. From recovery gel, estimate
   - backbone: 200 ng / 5 µL = 40 ng/µL
   - insert: 800 ng / 5 µL = 160 ng/µL
2. Determine volume of backbone needed
   - 50-100 ng, choose 80 ng, *i.e.* 2 µL
3. Calculate moles of backbone
   - 2887 bp * ( 660 g / (mol*bp) ) = 1.9 x $10^6$ g/mol
   - so 80 ng / (1.9 x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

# Optimal backbone-to-insert ratio

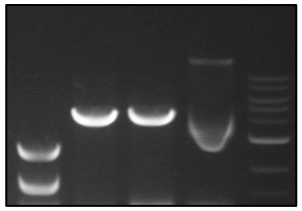
- ideally, want 1:4 backbone : insert
  - molar ratio, ***not*** mass or volume

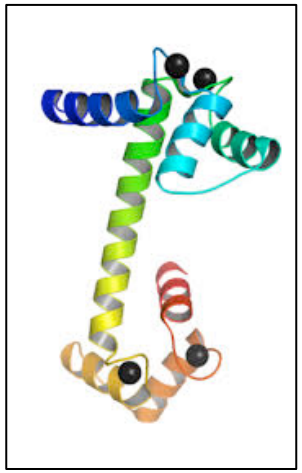
- What if too much insert?

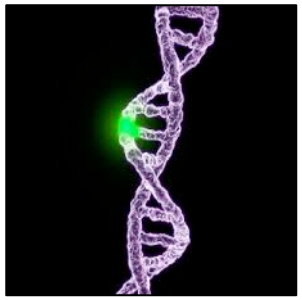



- What if too much backbone?

# Today in lab







- Agarose gel electrophoresis of confirmation digests
  - pRSET-IPC cut by restriction enzymes
- Explore inverse pericam (IPC)
  - primary: gene & protein sequence
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- Submit primer sequences before leaving lab
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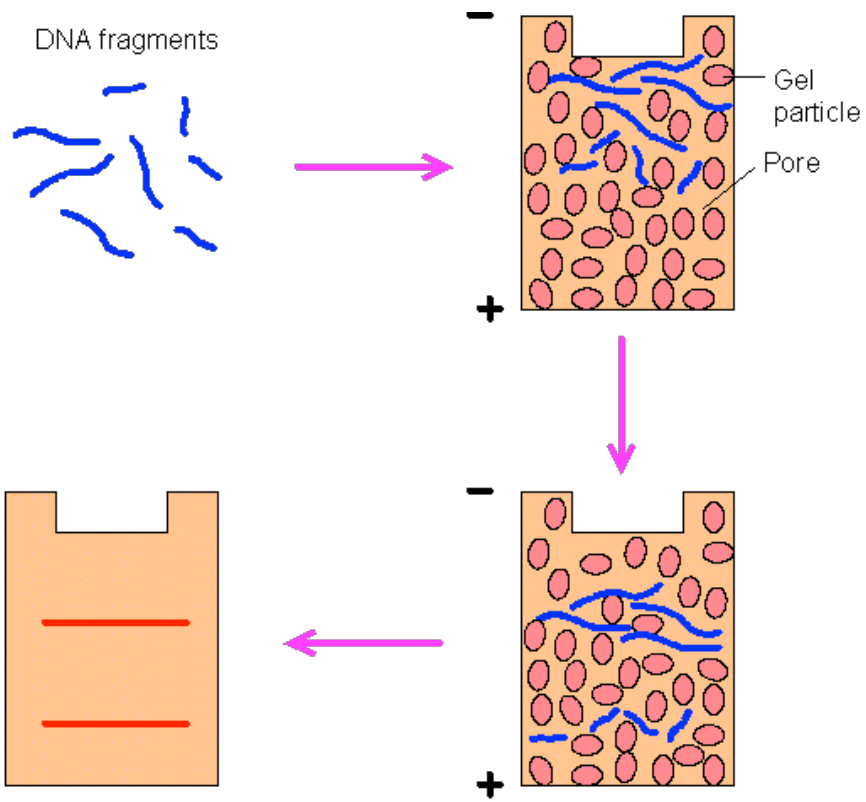
# Separate DNA by gel electrophoresis

- Agarose gel electrophoresis
  – driving force:

    charge

  – separates DNA by:

    size



# Visualize DNA

- Loading dye

  **bromophenol blue: ~500 bp**

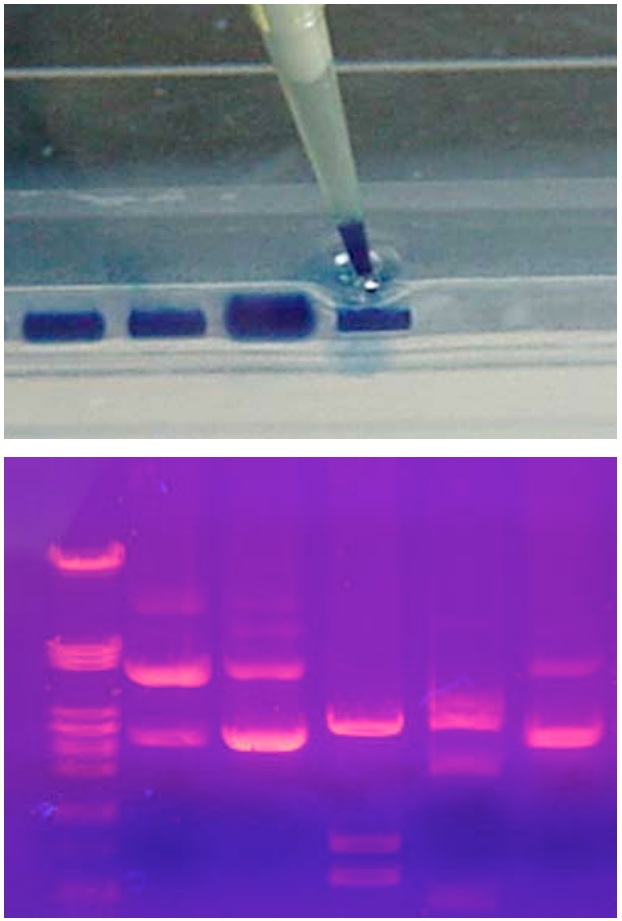
  **glycerol: density, viscosity**

- DNA stain

  **SYBR-Safe ~ SYBR-Green ~ EtBr**

  **intercalating DNA dye**

  **UV-blue excitable fluorophore**

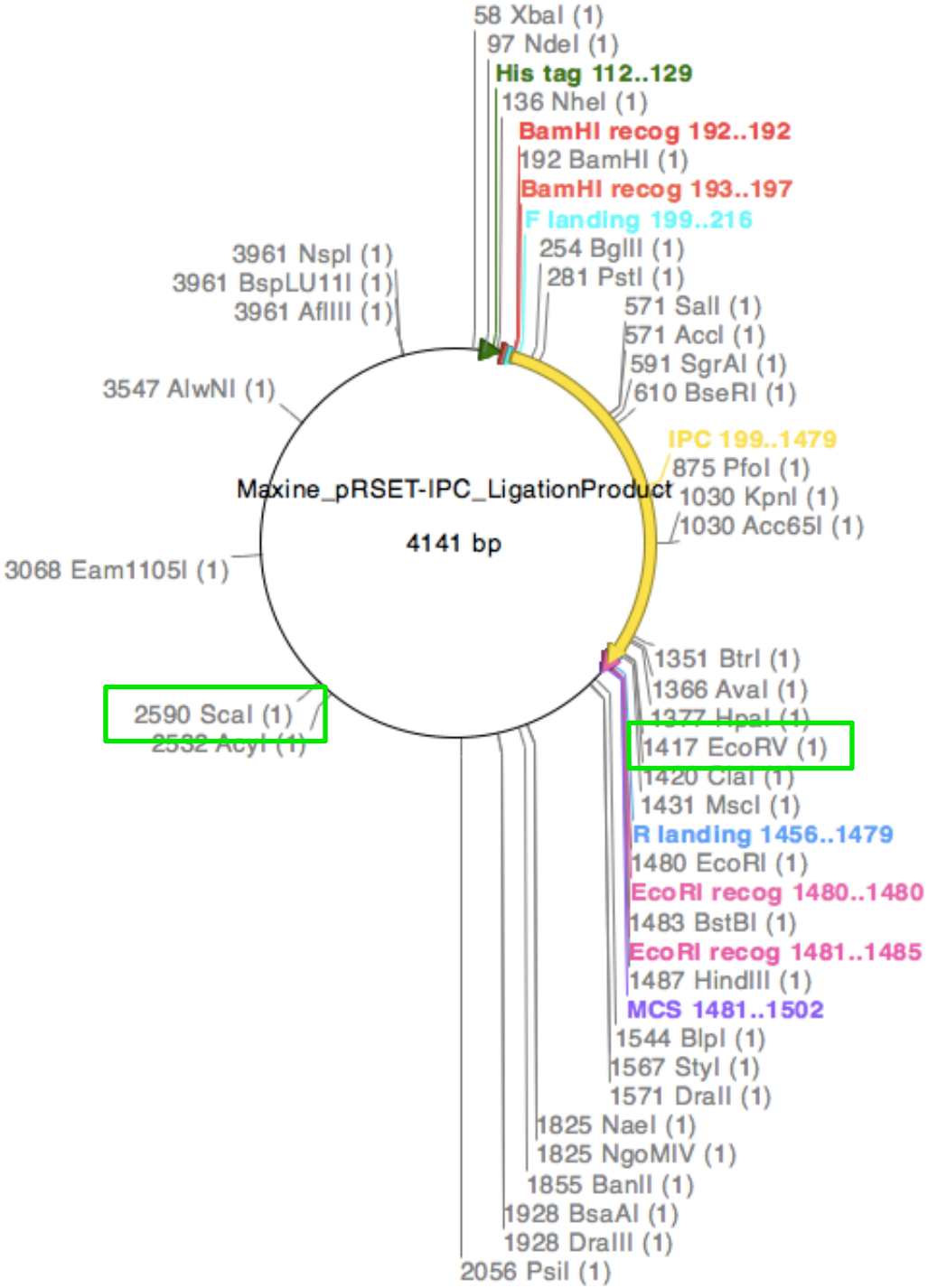


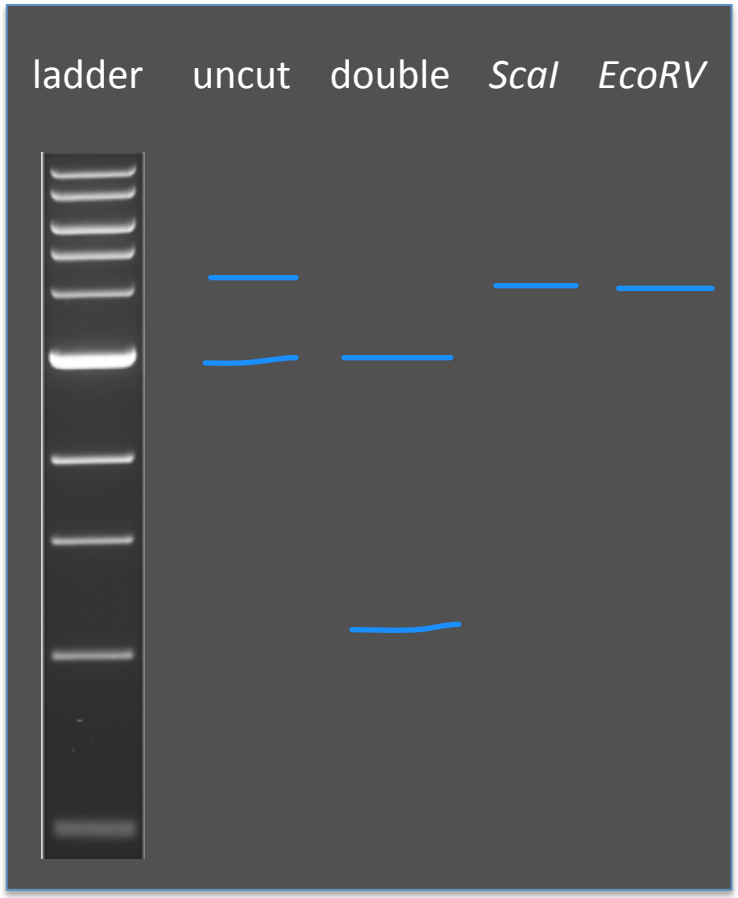
➢ Safety : wear nitrile gloves

expect fragments at 2590 - 1417 ~ 1173 bp
and 4141 - 1173 = 2968 bp

# Confirmation digest

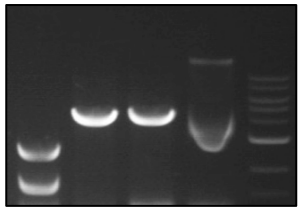
- *ScaI* (2590) in backbone
- *EcoRV* (1417) in insert
- NEB buffer 3.1

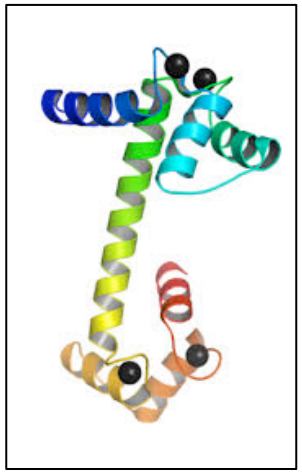


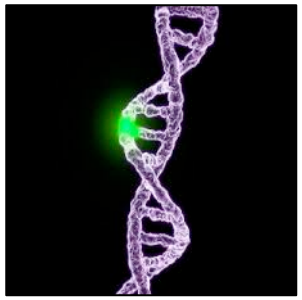




supercoiled, nicked

# Today in lab







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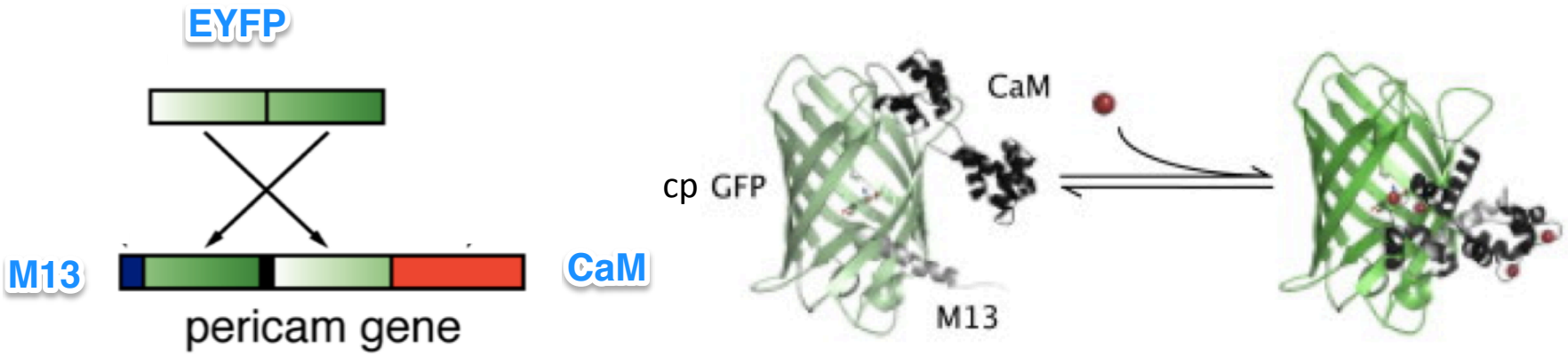
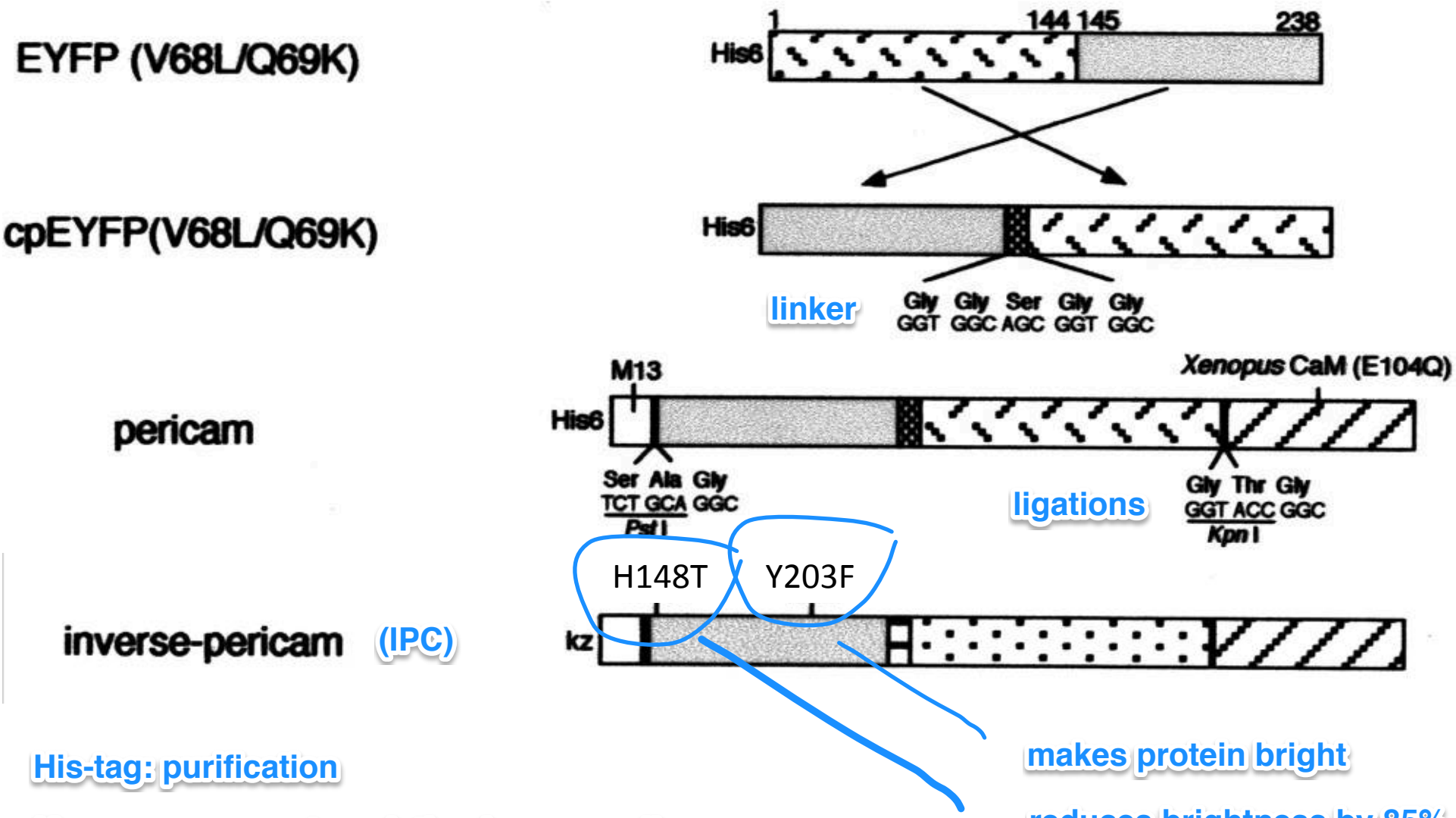
# Pericam (and GCaMP family) is a GECI: genetically engineered calcium indicator




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

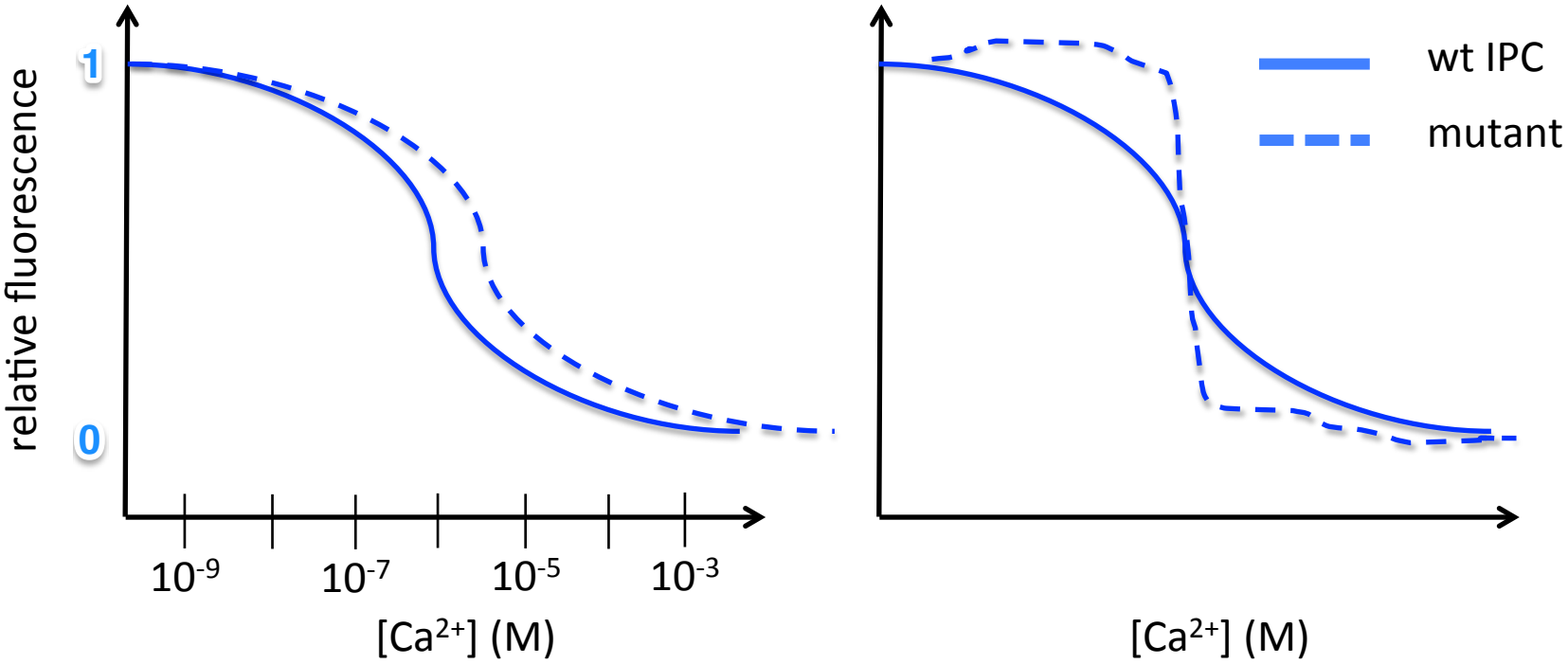
* Roger Tsien won the 2008 Nobel Prize for engineering novel forms of GFP

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



mutant shows lower affinity  mutant shows higher cooperativity

# Protein engineering:
# modulate binding affinity and/or cooperativity



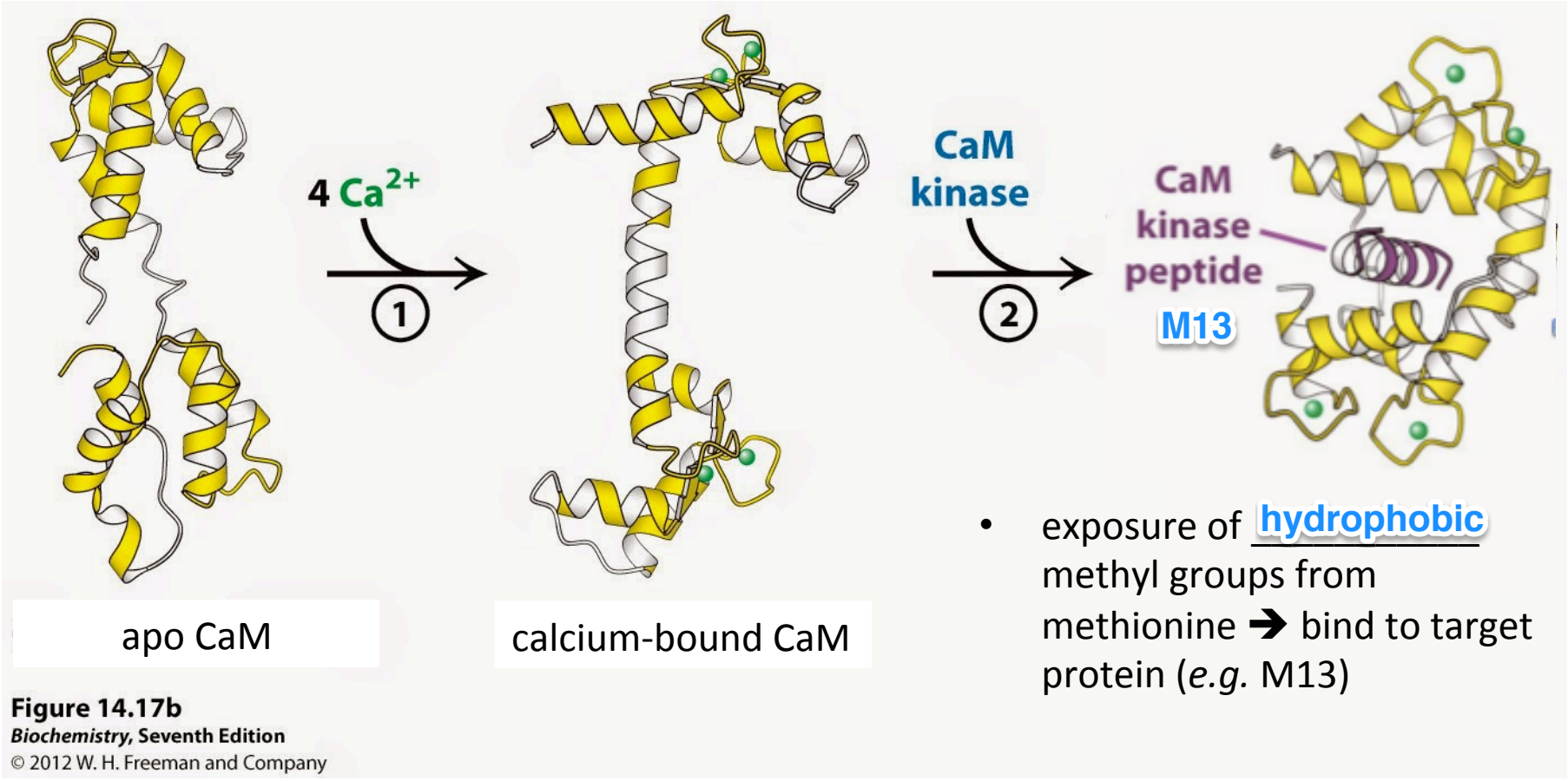

$[Ca^{2+}]_{cytosol}$ ~ 10-100 nM

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

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

# 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 negative environment

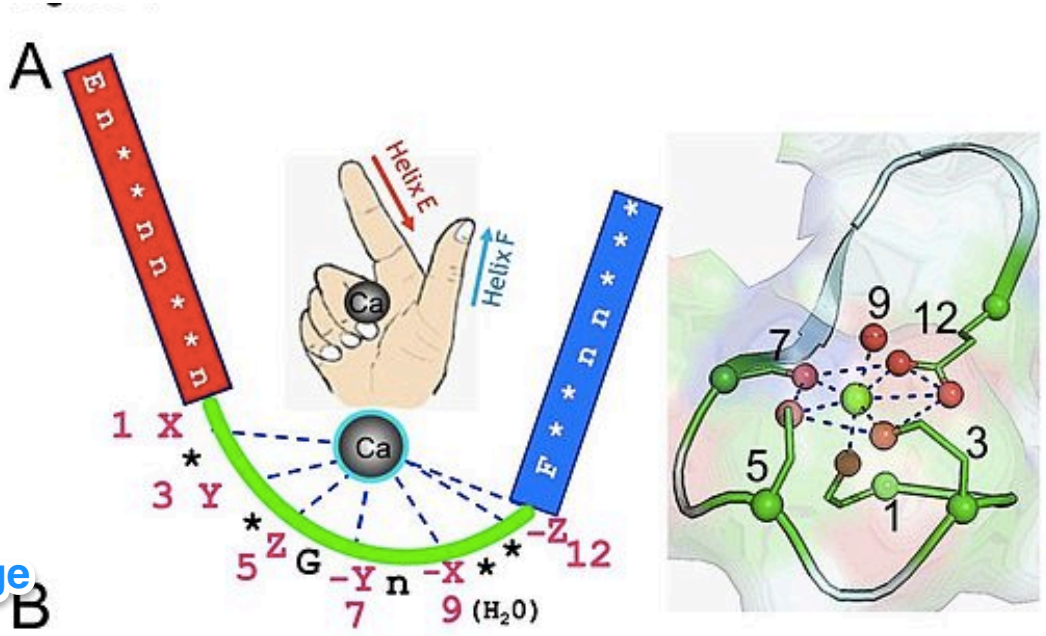


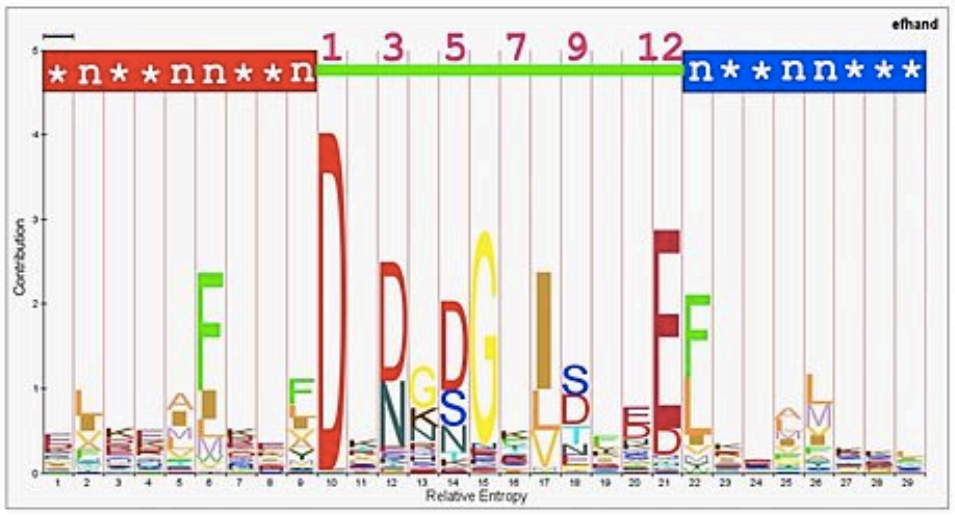

- exposure of hydrophobic methyl groups from methionine ➔ bind to target protein (*e.g.* M13)

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

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

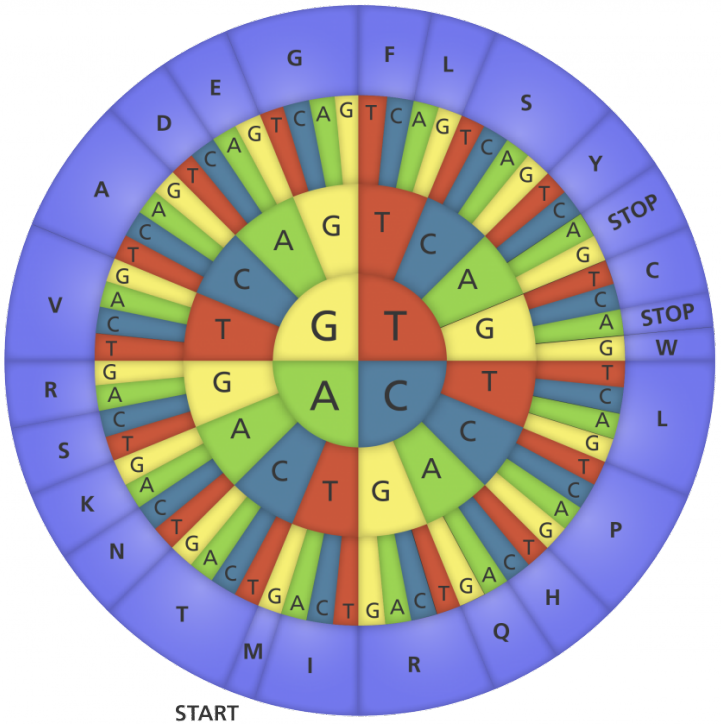
- Binding pocket residues
  - charged vs. neutral
  - negative vs. positive charge
  - polar vs. nonpolar
  - size: topology, steric hindrance, orientation
  - local pH
- Interface with M13




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?




**Amino acid code**

| | | | |
|---|---|---|---|
| A – Alanine | G – Glycine | M – Methionine | S – Serine |
| C – Cysteine | H – Histidine | N – Asparagine | T – Threonine |
| D – Aspartic acid | I – Isoleucine | P – Proline | V – Valine |
| E – Glutamic acid | K – Lysine | Q – Glutamine | W – Tryptophan |
| F – Phenylalanine | L – Leucine | R – Arginine | Y – Tyrosine |

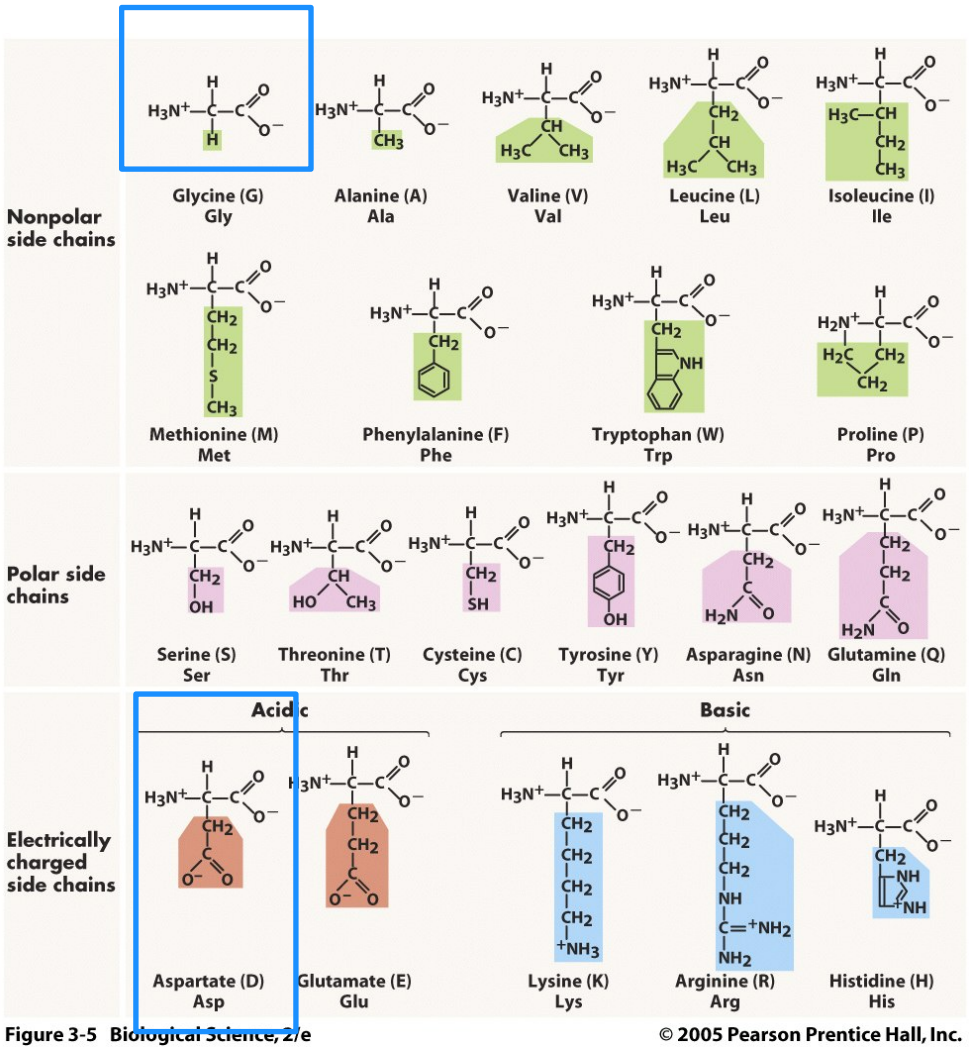


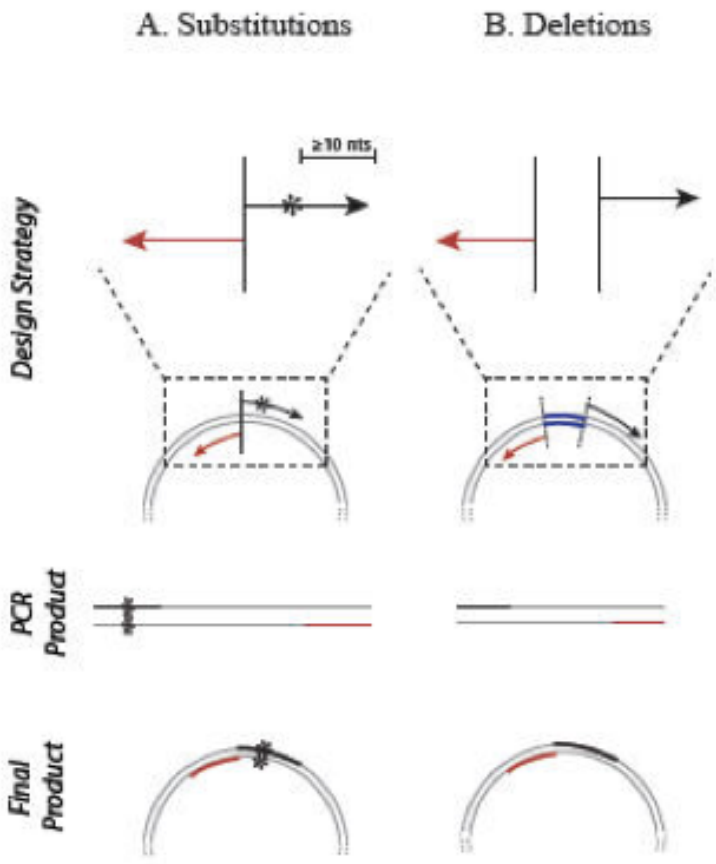

Figure 3-5 Biological Science, 2/e



**GAC (D = Asp) ----> GGC (G = Gly)**

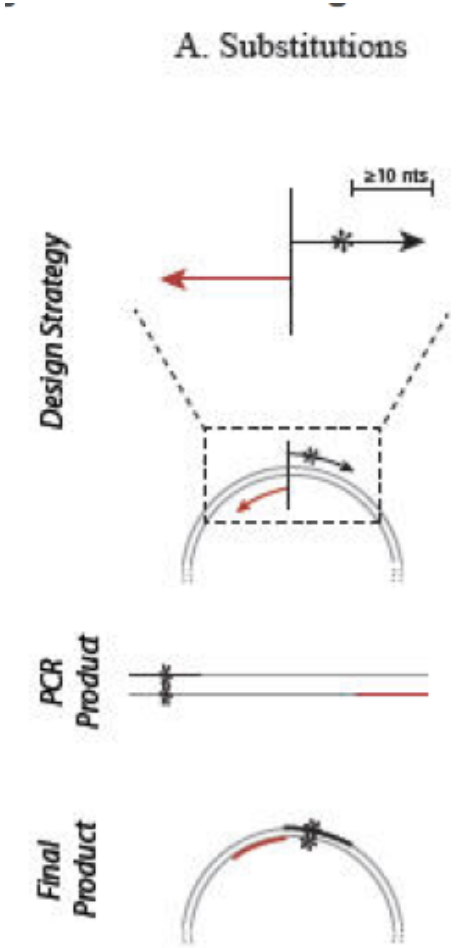
# Site-directed mutagenesis (SDM)

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



# Primer design guidelines

- substitution
  - mutation location: toward middle of F-primer
  - length: 25-45 bp
  - G/C content: > 40%
  - start and end with at least one G/C
  - melting temperature > 78 °C
- amplification *vs.* mutagenesis primers
  - sequence match:
  - $T_m$: higher for mutageneis
  - length: longer for mutagenesis



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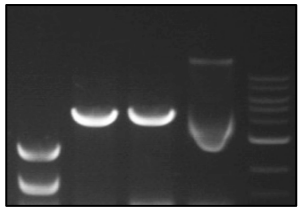

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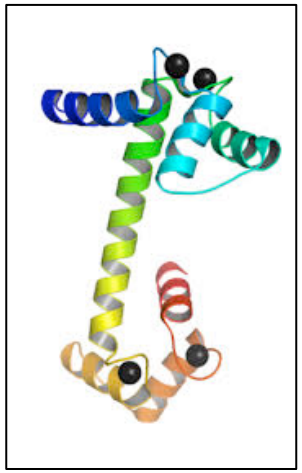

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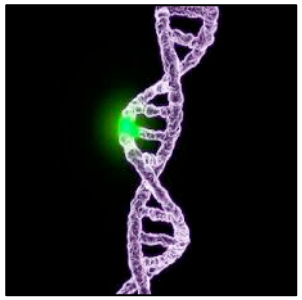

- Submit primer sequences before leaving lab 4:30pm
  - choose mutation site of interest
  - understand (forward and reverse) primer design

- For M1D3: read Nagai *et al.*
  - be ready to present your part

# Gel loading order

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | |
| Top 1 | | ladder | uncut | double | enzyme 1 | enzyme 2 | | | ladder | uncut | double | enzyme 1 | enzyme 2 | |
| Bottom 1 | | ladder | uncut | double | enzyme 1 | enzyme 2 | | | ladder | uncut | double | enzyme 1 | enzyme 2 | |
| | | | | | | | | | | | | | | |
| Top 2 | | ladder | uncut | double | enzyme 1 | enzyme 2 | | | | | | | | |
| Bottom 2 | | | | | | | | | | | | | | |

# M1D3:
# Site-directed mutagenesis

02/12/2016

# SDM ingredients and cycling conditions

| SDM ingredients |
| --- |
| |
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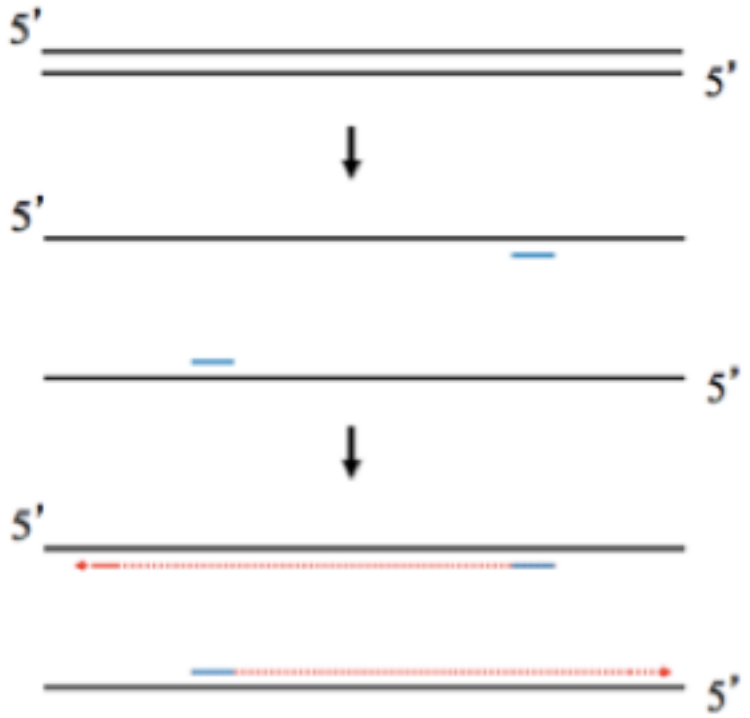


98 °C

55 °C

72 °C

25 cycles

# SDM steps with NEB Q5 kit