Leslie

M2D1:Evaluate mutations and site-directed mutagenesis

10/09/15

1. Prelab
   - Intro to Mod2!
2. Design mutations for Inverse Pericam
3. Set up site-directed mutagenesis reaction

Smooth transition from M1 to M2

- Wrap-up M1
  - DNA engineering summary, *in partners*
    due 10/13, revision due 10/24
    on Stellar or via email
  - DNA mini-presentation, *individual*
    due 10/17
    on MIT TechTV
  - 20.109 blog:
    before 10/25
    http://be20109f15.blogspot.com/

➢ Extra office hours this weekend:
Friday 10-12 and 2-4, Saturday 12-2, Sunday 10-4

➢ Carefully read assignment description!

Smooth transition from M1 to M2

- Good news! M2D2 is next T/F, Oct. 15/16
- Prepare M2D2: read

Proc Natl Acad Sci U S A. 2001 Mar 13;98(6):3197-202. Epub 2001 Mar 6.

Circularly permuted green fluorescent proteins engineered to sense Ca2+.

Nagai T[1], Sawano A, Park ES, Miyawaki A.

- Quiz days: M2D3 and M2D7
- Culminating assignments:
  lab report (as journal article) – 25%
  journal club presentation – 10%

Welcome to M2!

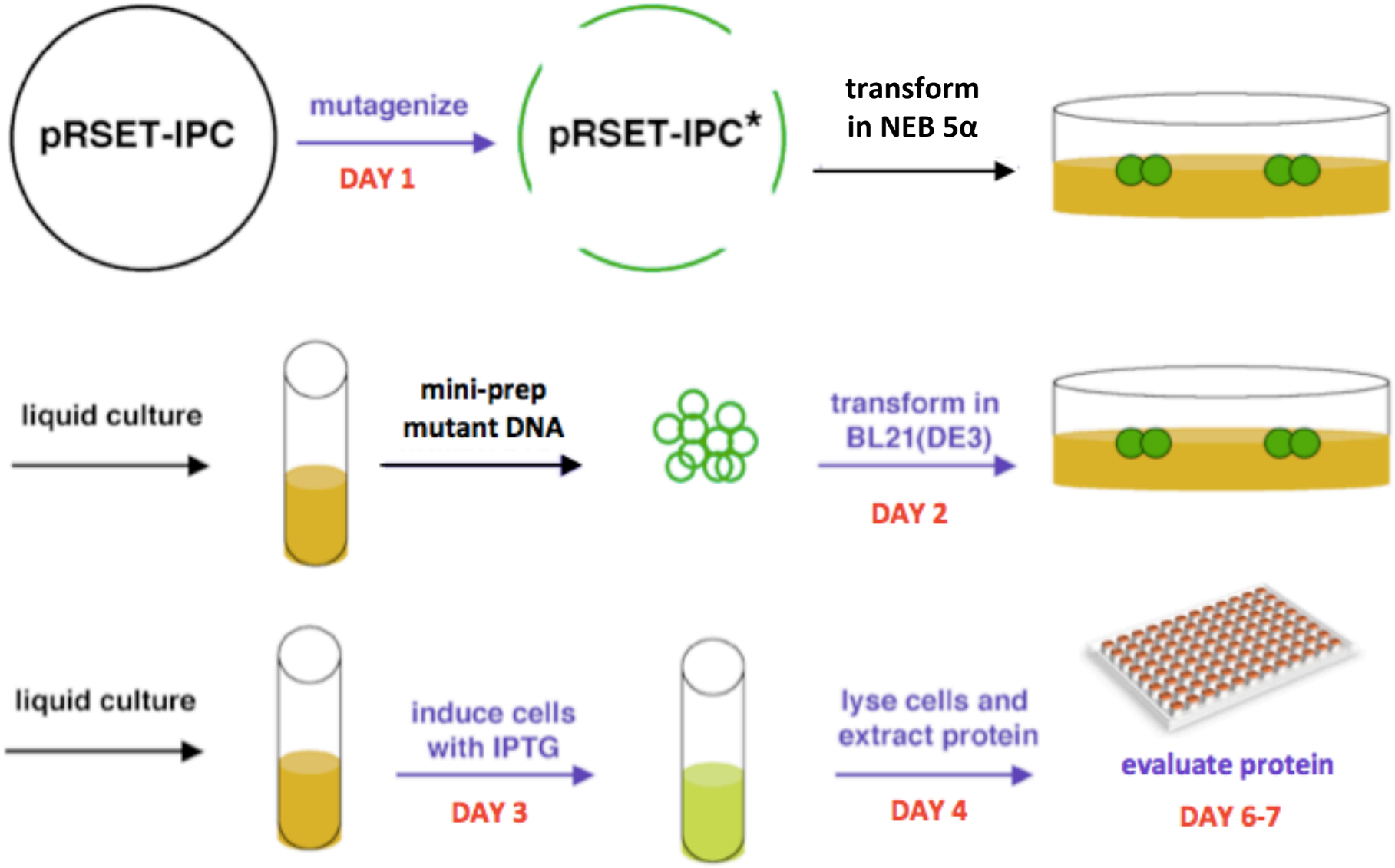
New in M2
calcium sensor, affinity and cooperativity
sequencing
protein purification
fluorescence plate reader
MATLAB coding nonlinear regression

Reuse knowledge from M1
IPC based on cpEYFP
site-directed mutagenesis SDM
competent cells **x 2!**
DNA + protein engineering
restriction enzymes
SDS-PAGE gel electrophoresis
oral and written communication

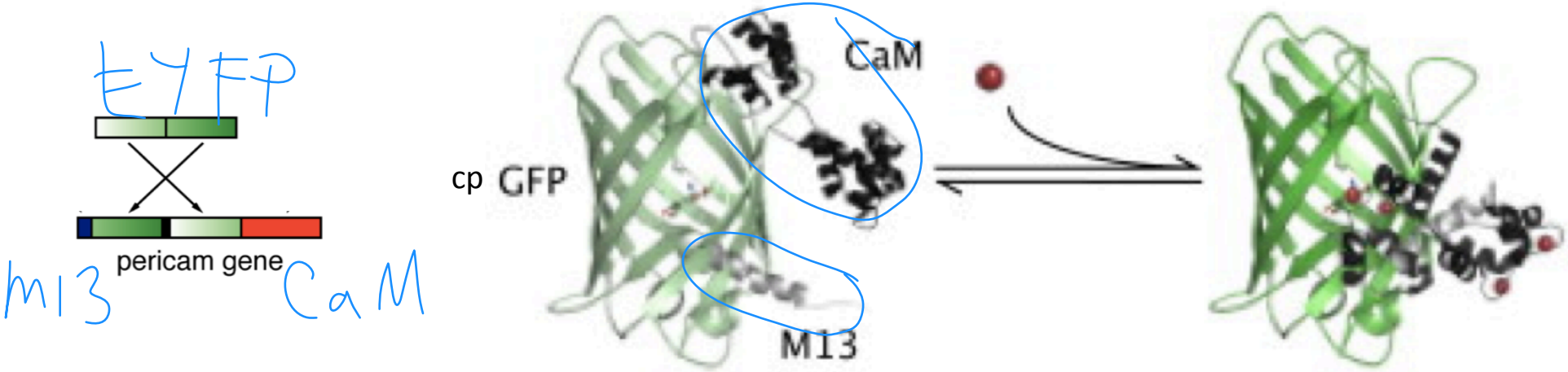
~EGFP

~PCR

M2 experimental overview



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

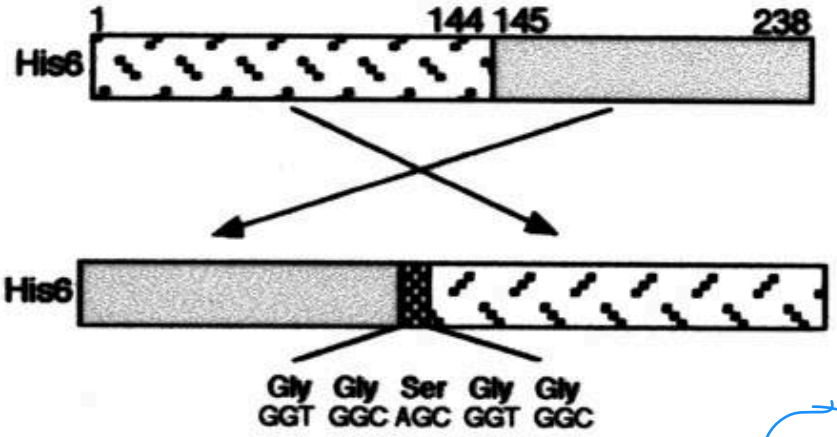
Inverse pericam (IPC) is dimmer with Ca^{2+}

normal

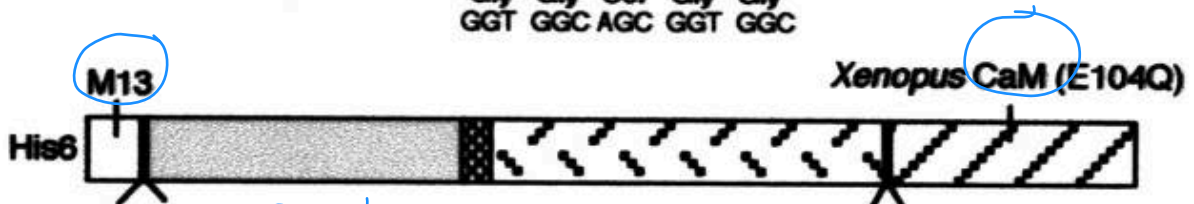
EYFP (V68L/Q69K)

circular permutated

cpEYFP(V68L/Q69K)



pericam

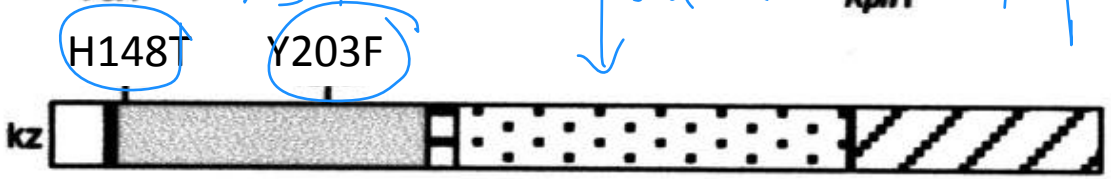


Pst1

same

Kpn1

inverse-pericam



directed evolution or rational design

Protein engineering: modulate binding affinity and/or cooperativity

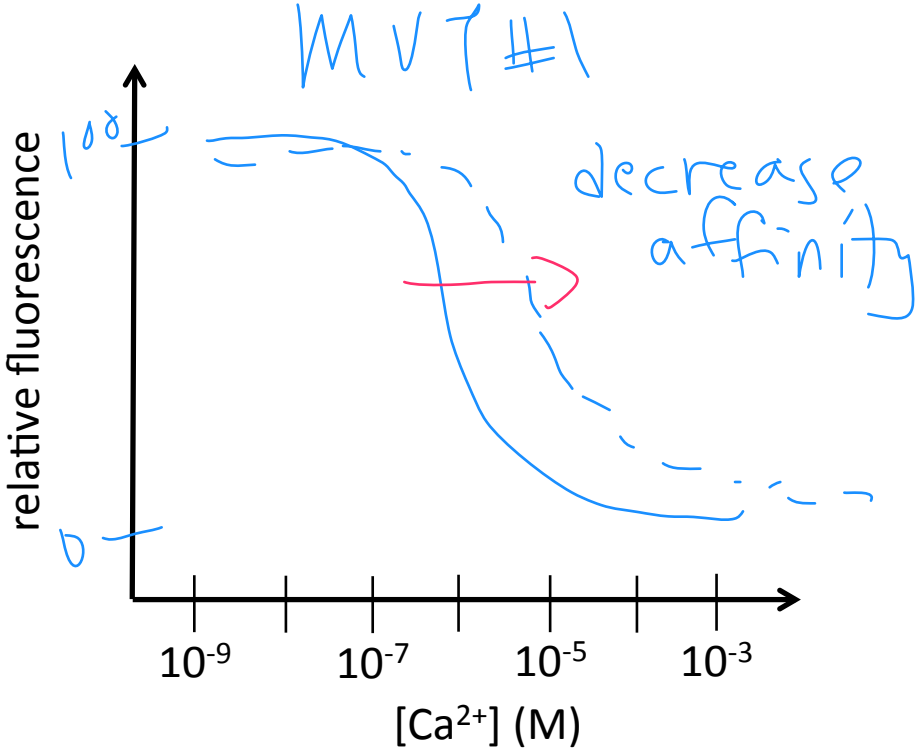
Inverse Pericam (IPC)

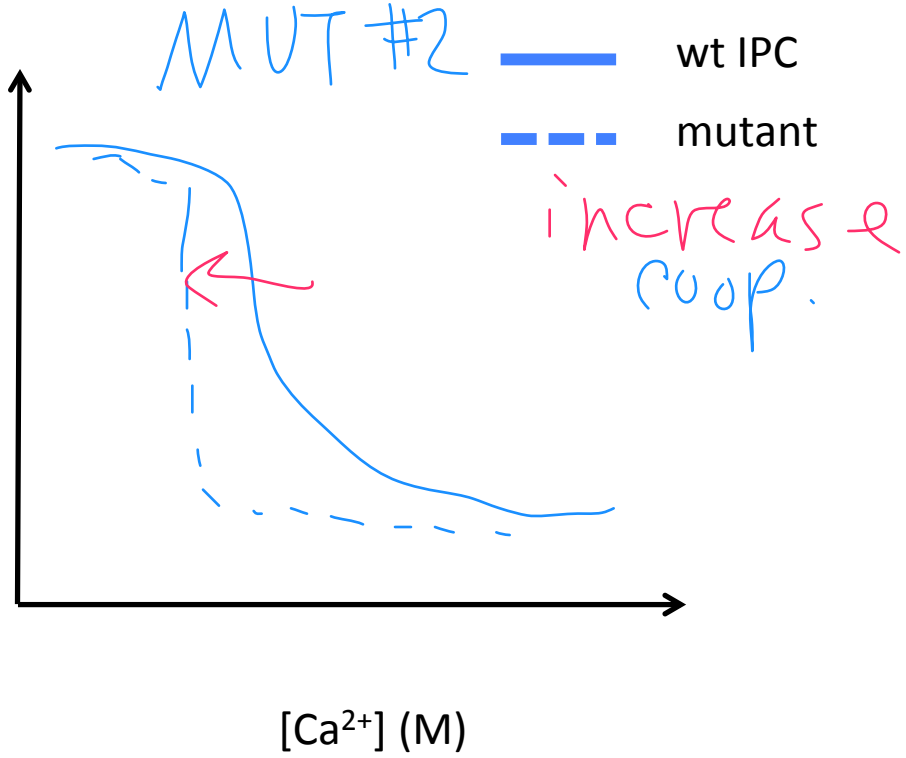
[IPC] = concentration

[Ca+] = increase

MUT #1

MUT #2





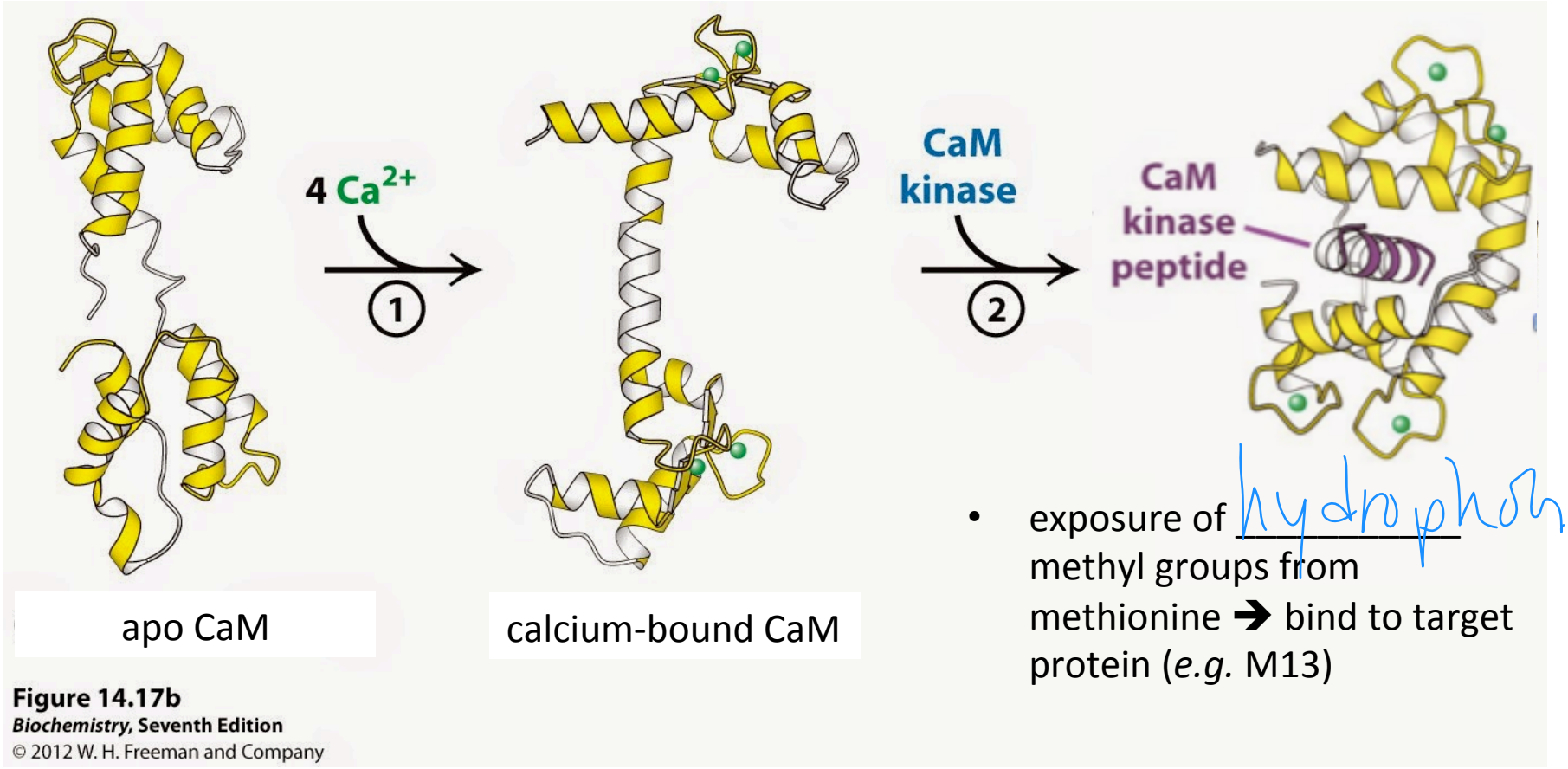
$[Ca^{2+}]_{cytosol}$ ~ 10-100 nM

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

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

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

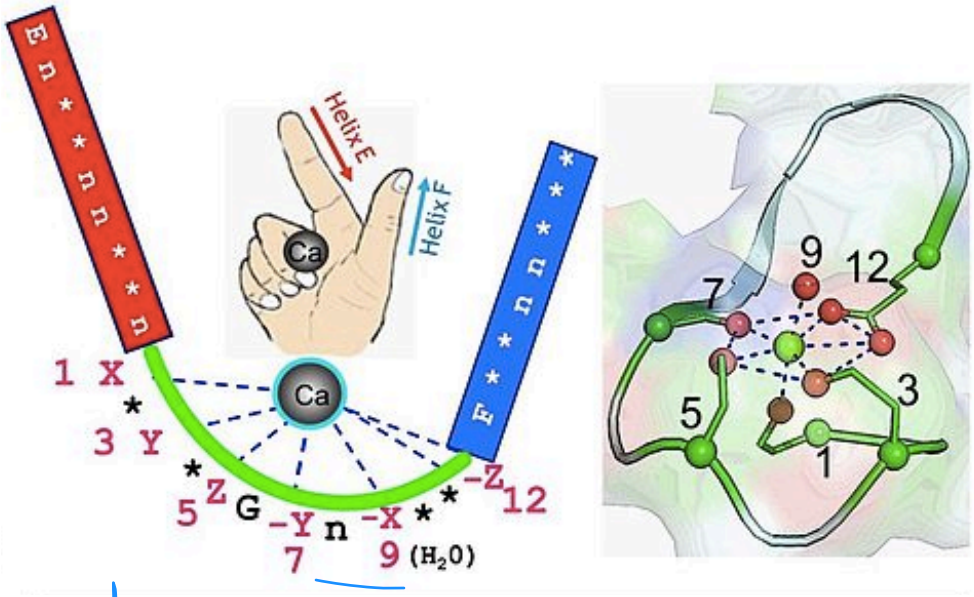
- 4 EF hands: 2 at N-terminal + 2 at C-terminal
- EF hand domain = helix-loop-helix
- loop = Ca^{2+} binding pocket, offers electronegative environment



- 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



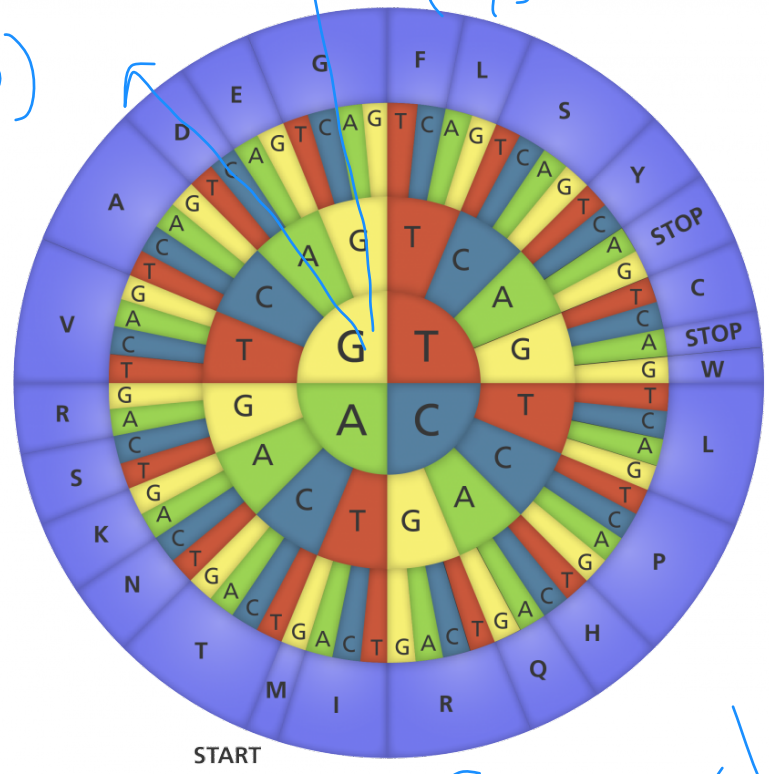
- Binding pocket residues
  - charge vs neutral
  - polar vs. nonpolar
  - size = steric hinderance
  - acidic vs. basic
- Interface with M13

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?

GAC → GGC (G)

(D)



Difference charged vs uncharged big vs small

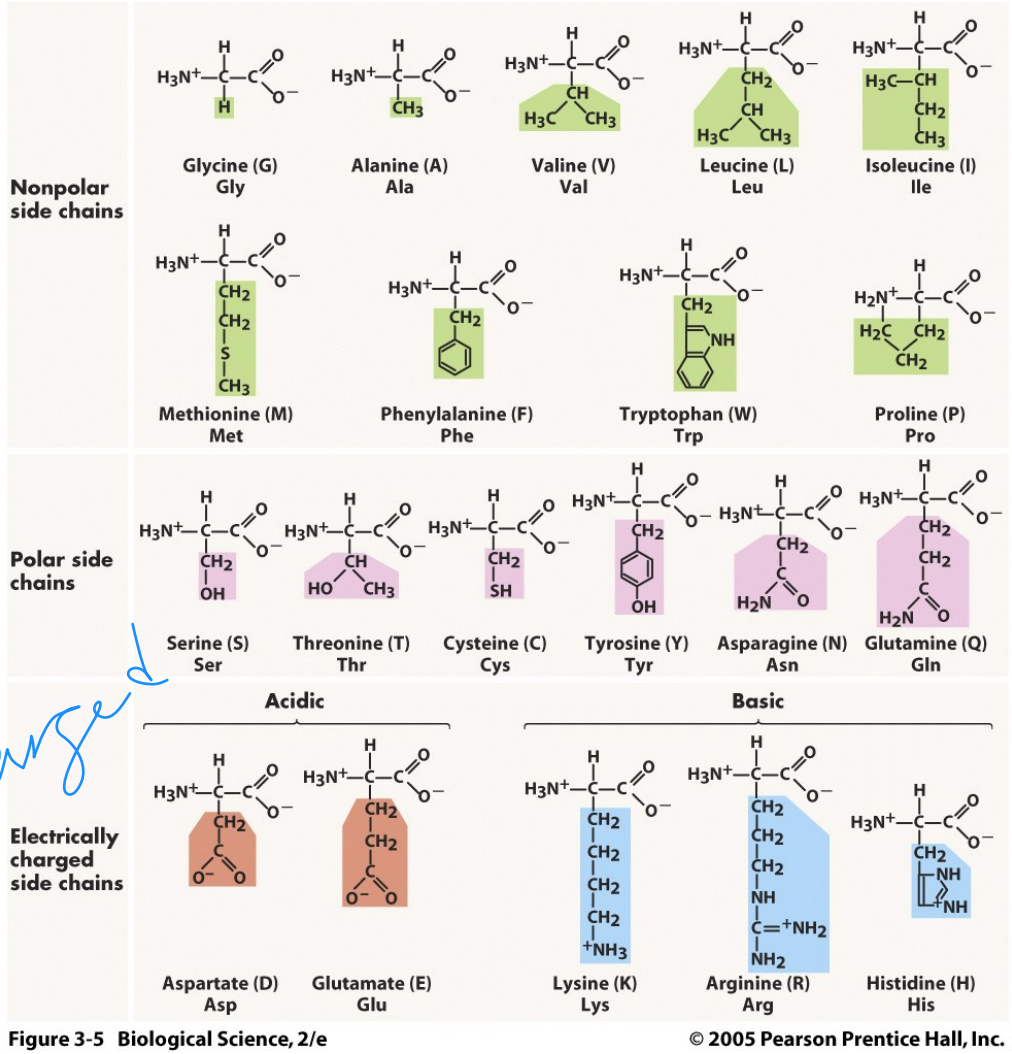
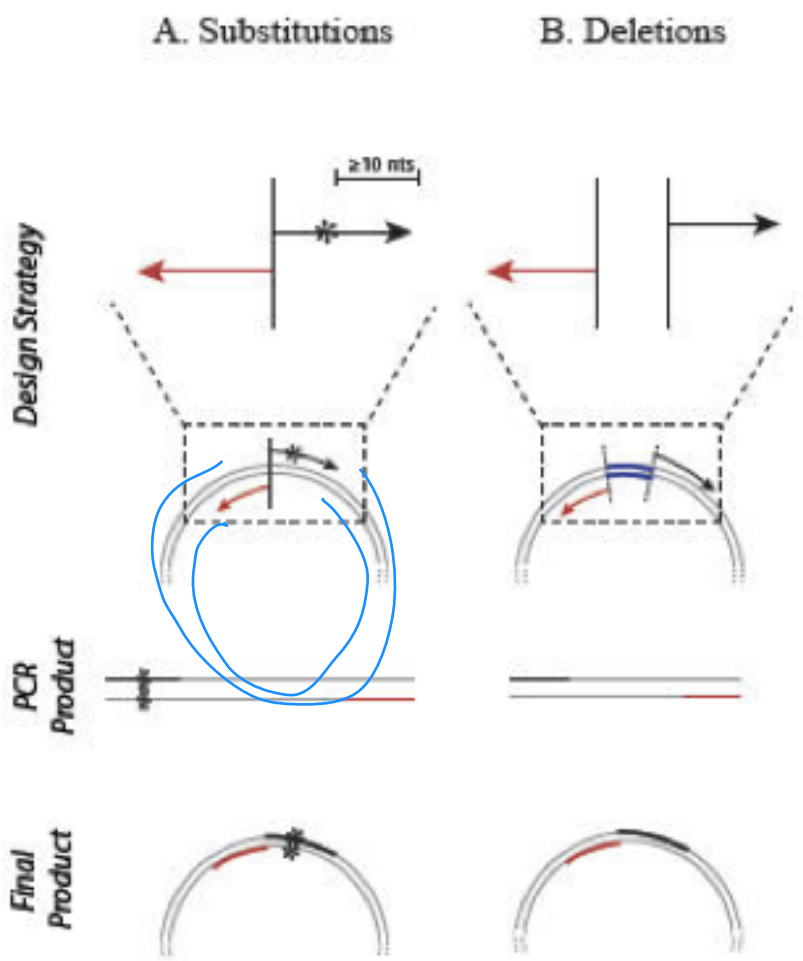


Figure 3-5 Biological Science, 2/e

Site-directed mutagenesis (SDM)

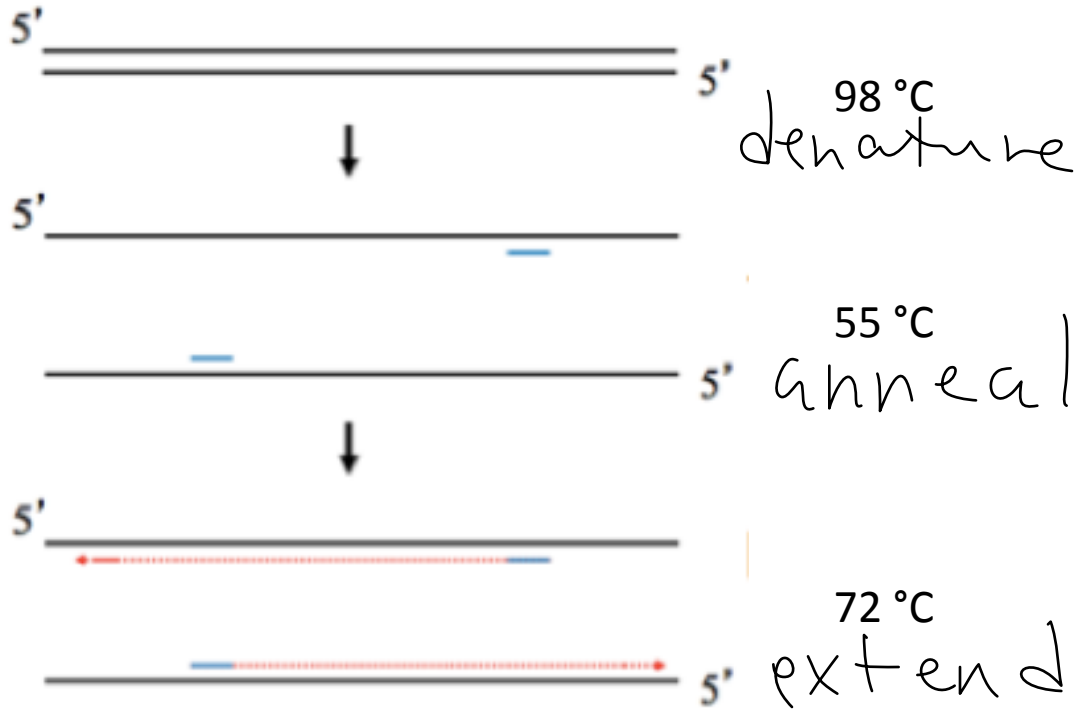
- Create specific, targeted changes in double-stranded plasmid DNA
  - mutation
  - deletion
  - insertion
- Primers contain the desired mutation
- Using NEBα Q5 SDM kit
  - back-to-back primers
  - forward primer imposes mutation



SDM ingredients and cycling conditions

pRSET-IPC (circular)

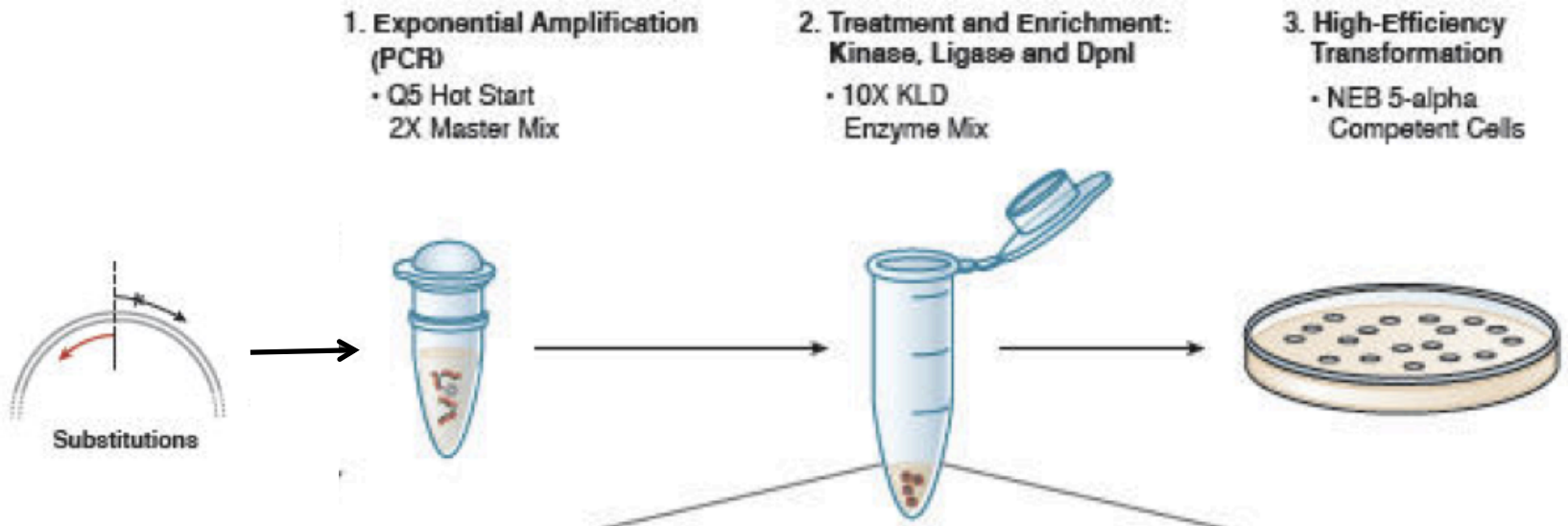
SDM ingredients
primers
buffer
template DNA
dNTPs
Mg^{++}
HF polymerase
H_2O



25 cycles

linear

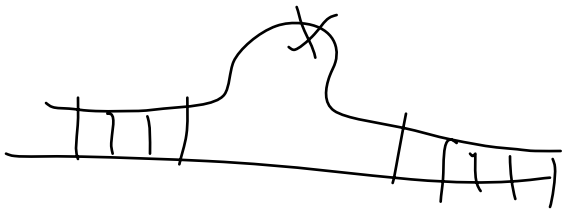
SDM steps with NEB Q5 kit



Dpn I digests meth DNA

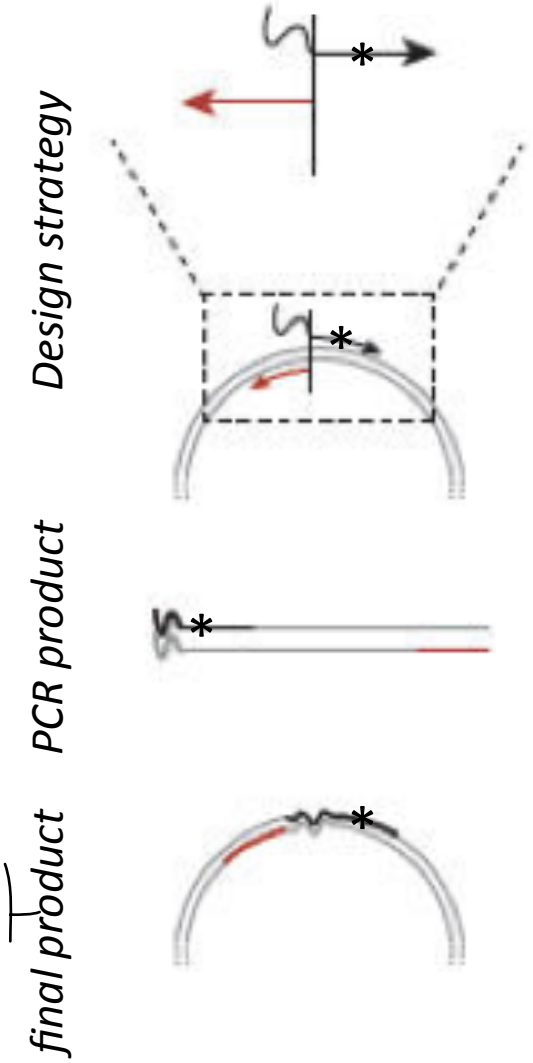
necessary ligation

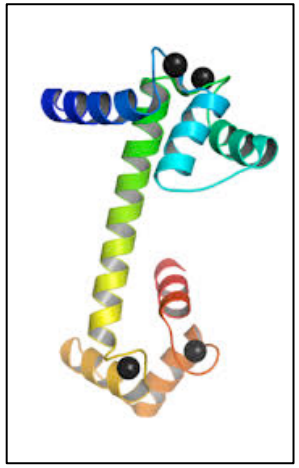
Methylated

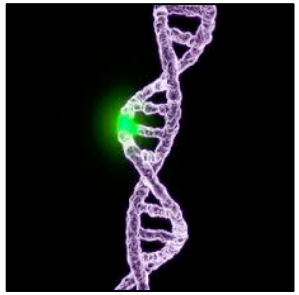


Primer design guidelines

- substitution
  - mutation location middle
  - length 25-40
  - G/C content > 40 %
  - start and end with at least one G/C
  - melting temperature > 78 °C
- + insertion
  - recognition site for endonuclease
  - # bp is a multiple of 3 frameshift
  - how can it be useful?
    - RE - diagnostic digest
    - insert more DNA







Today in lab

- Explore inverse pericam (IPC)
  - primary: gene & protein sequence
  - tertiary: 3D structure from Protein Data Bank (PDB)
- Pick 1 of 9 suggested mutations <u>before 4:30pm</u>
  - choose mutation site of interest
  - understand (forward and reverse) primer design
- Set up SDM reaction