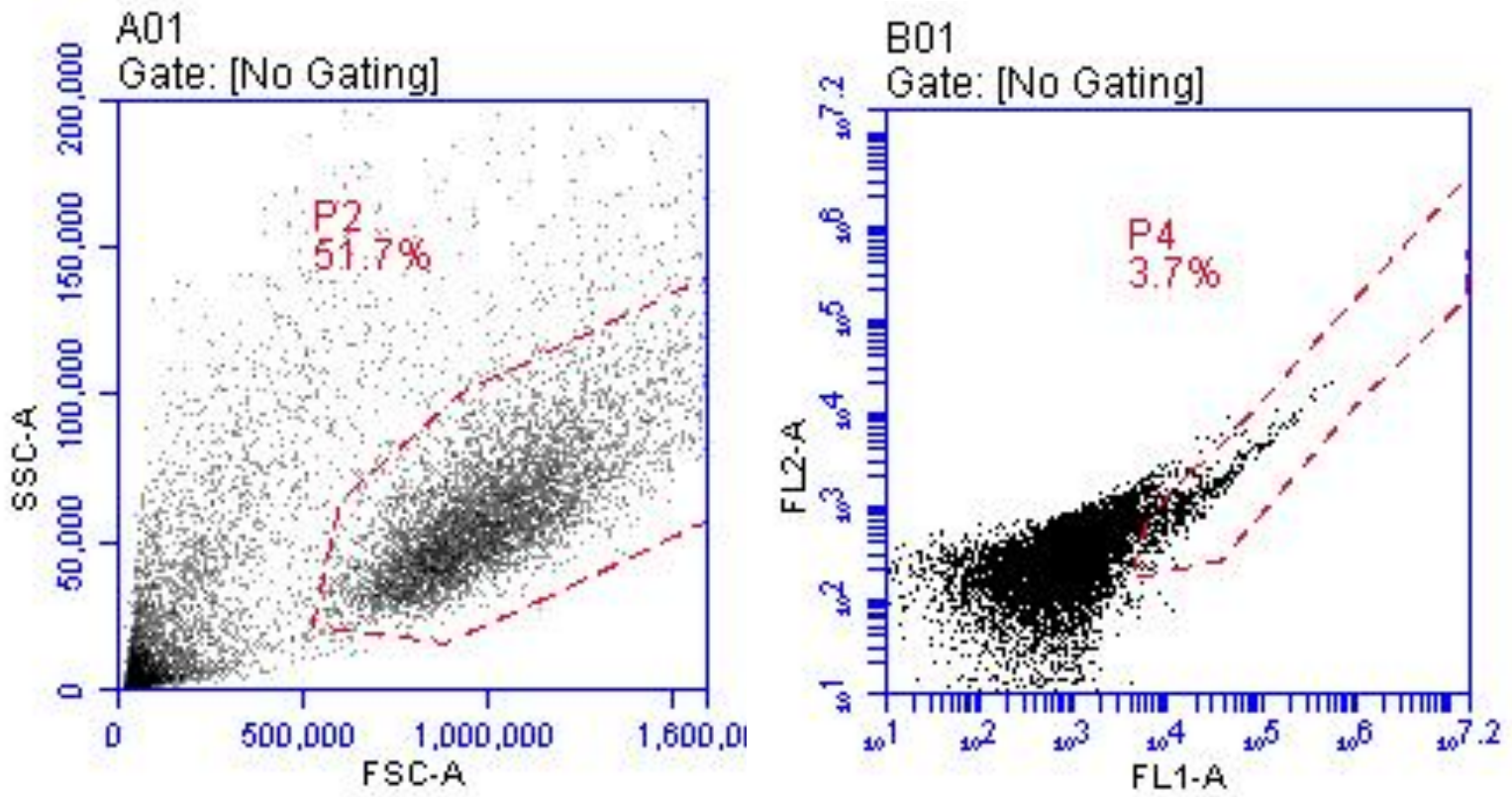
Pre-prelab: green cell population gate



The Nobel Prize in Chemistry 2015

Photo: Cancer Research UK

Tomas Lindahl

Prize share: 1/3

Photo: K. Wolf/AP Images for HHMI

Paul Modrich

Prize share: 1/3

Photo: M. Englund, UNC-School of Medicine

Aziz Sancar

Prize share: 1/3

The Nobel Prize in Chemistry 2015 was awarded jointly to Tomas Lindahl, Paul Modrich and Aziz Sancar *"for mechanistic studies of DNA repair"*.

M2D1:
Evaluate mutations and site-directed mutagenesis

10/08/2015

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

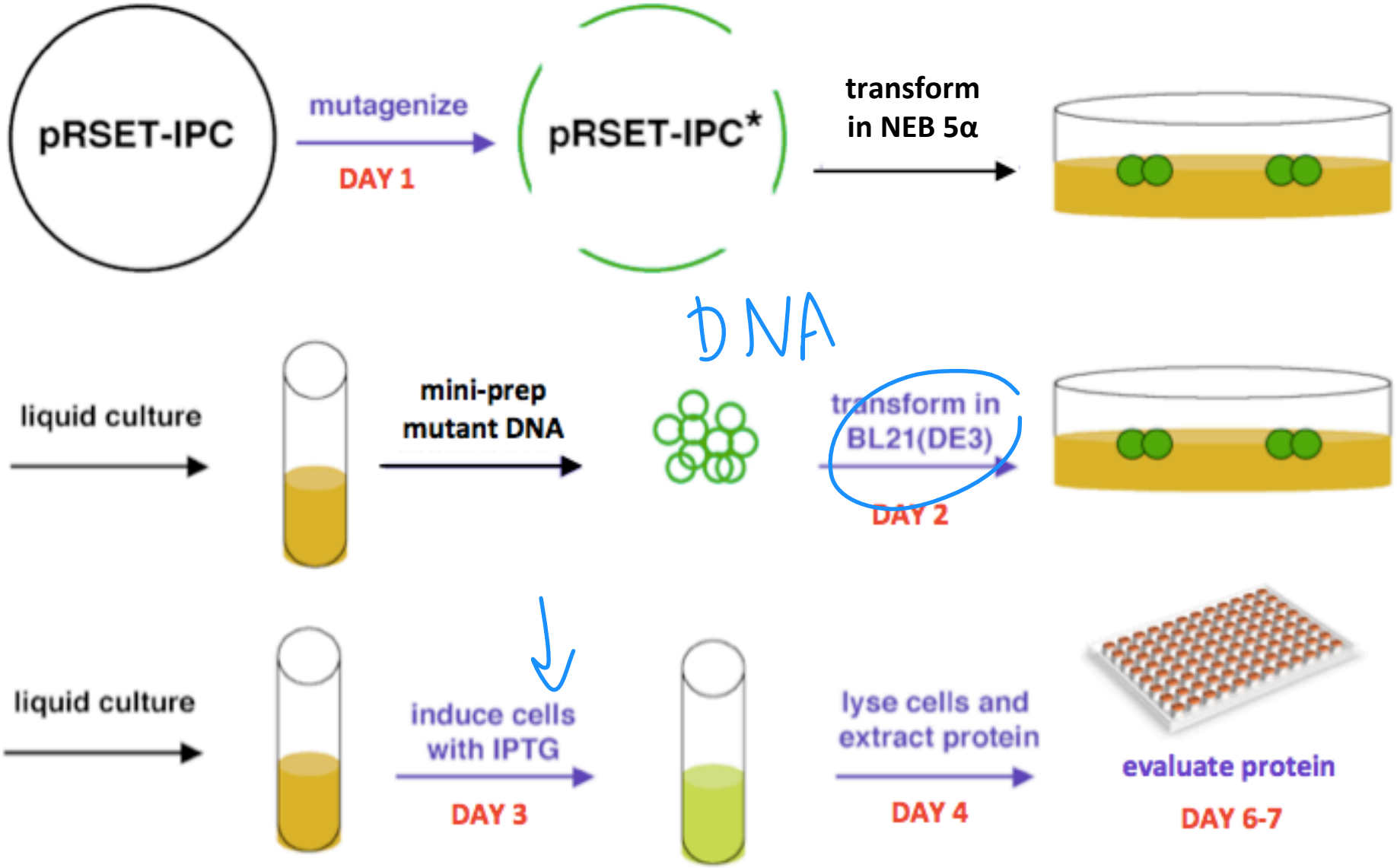
Reuse knowledge from M1
IPC based on cpEYFP
site-directed mutagenesis SDM
competent cells **x 2!**
DNA + protein engineering
restriction enzymes
SDS-PAGE gel electrophoresis
oral and written communication

~ GFP

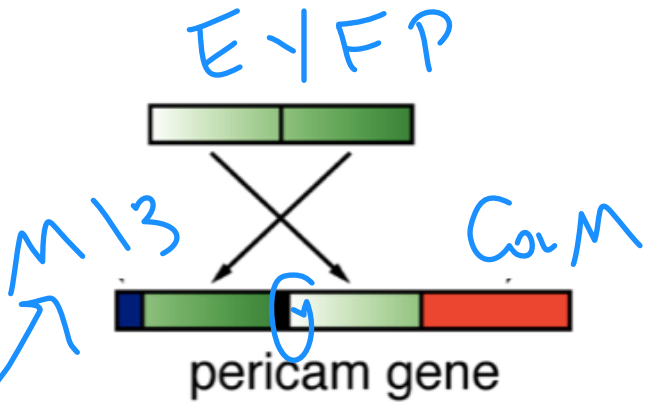
~ PCR

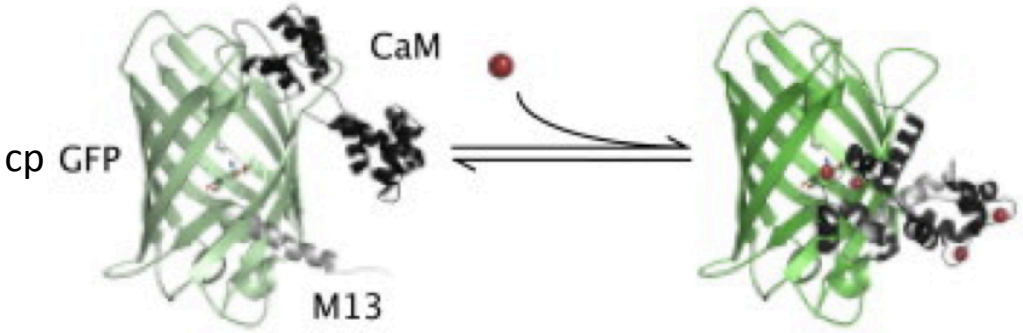
agarose

M2 experimental overview



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

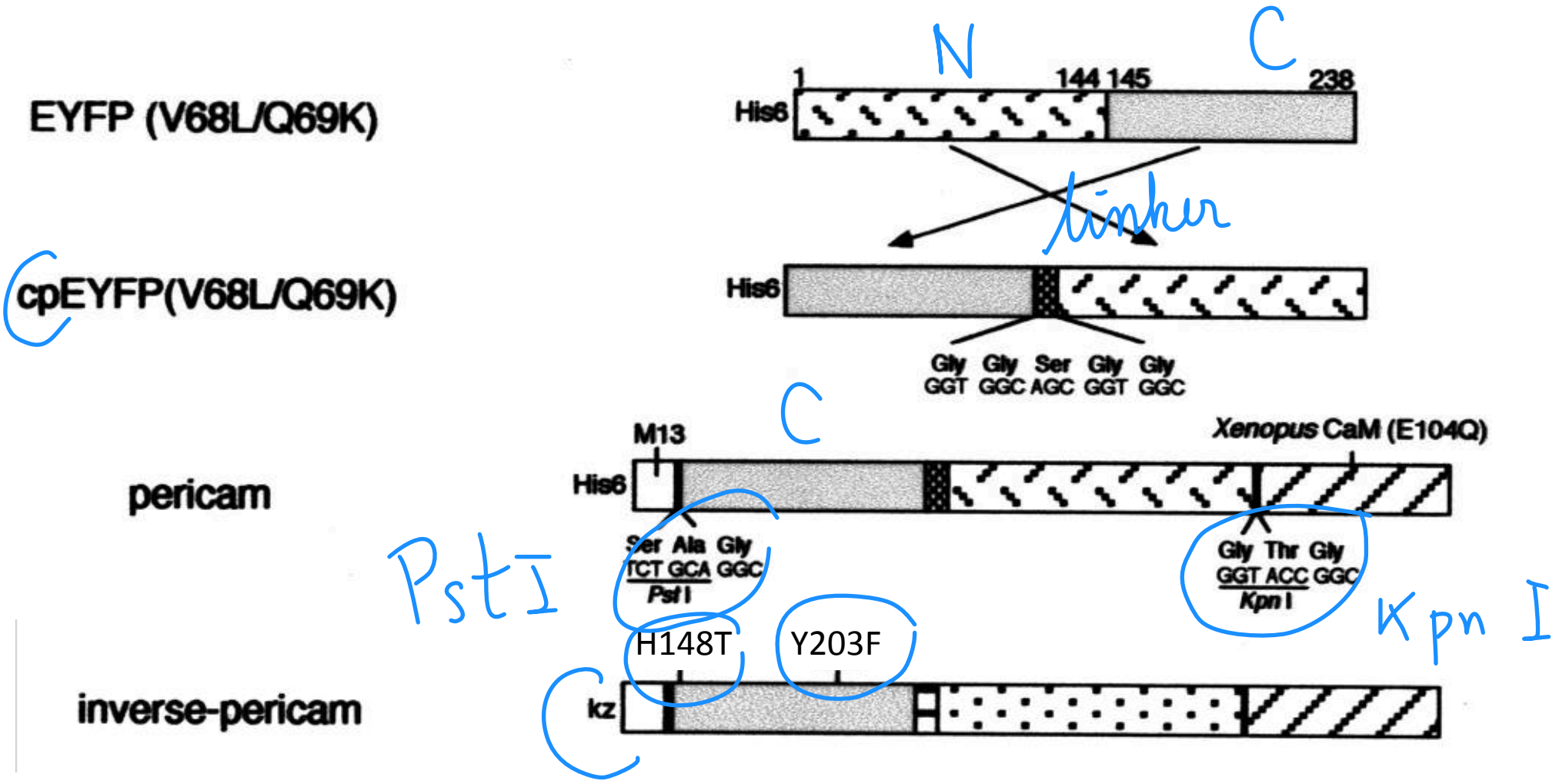




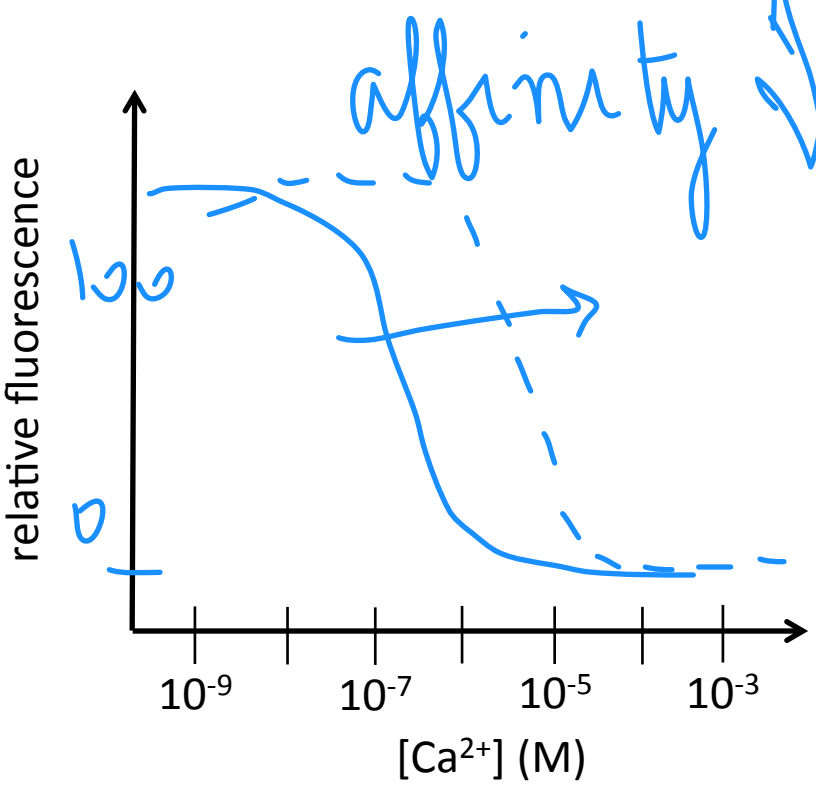
- EYFP: enhanced yellow fluorescent protein
- CaM: calmodulin (calcium-modulated protein)
- M13: CaM-binding peptide from myosin light-chain kinase

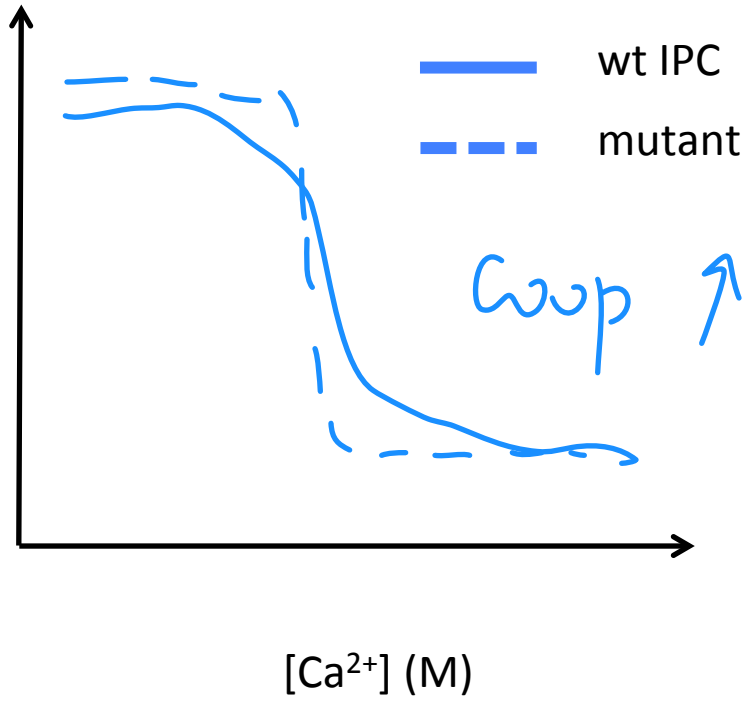
* Roger Tsien won the 2008 Nobel Prize for engineering novel forms of GFP

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



Protein engineering: modulate binding affinity and/or cooperativity





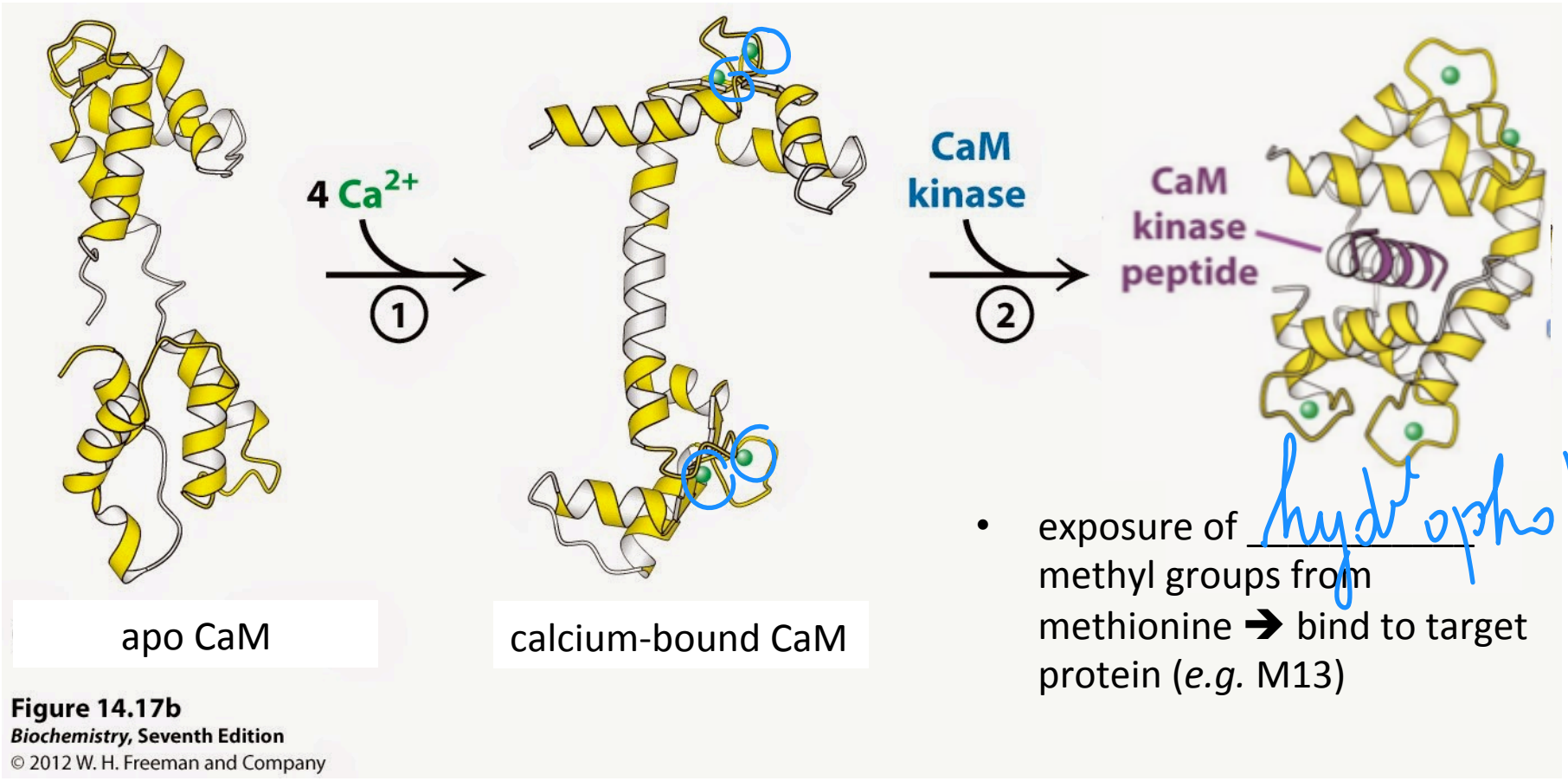
$[Ca^{2+}]_{cytosol}$ ~ 10 - 100 nM

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

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

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

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

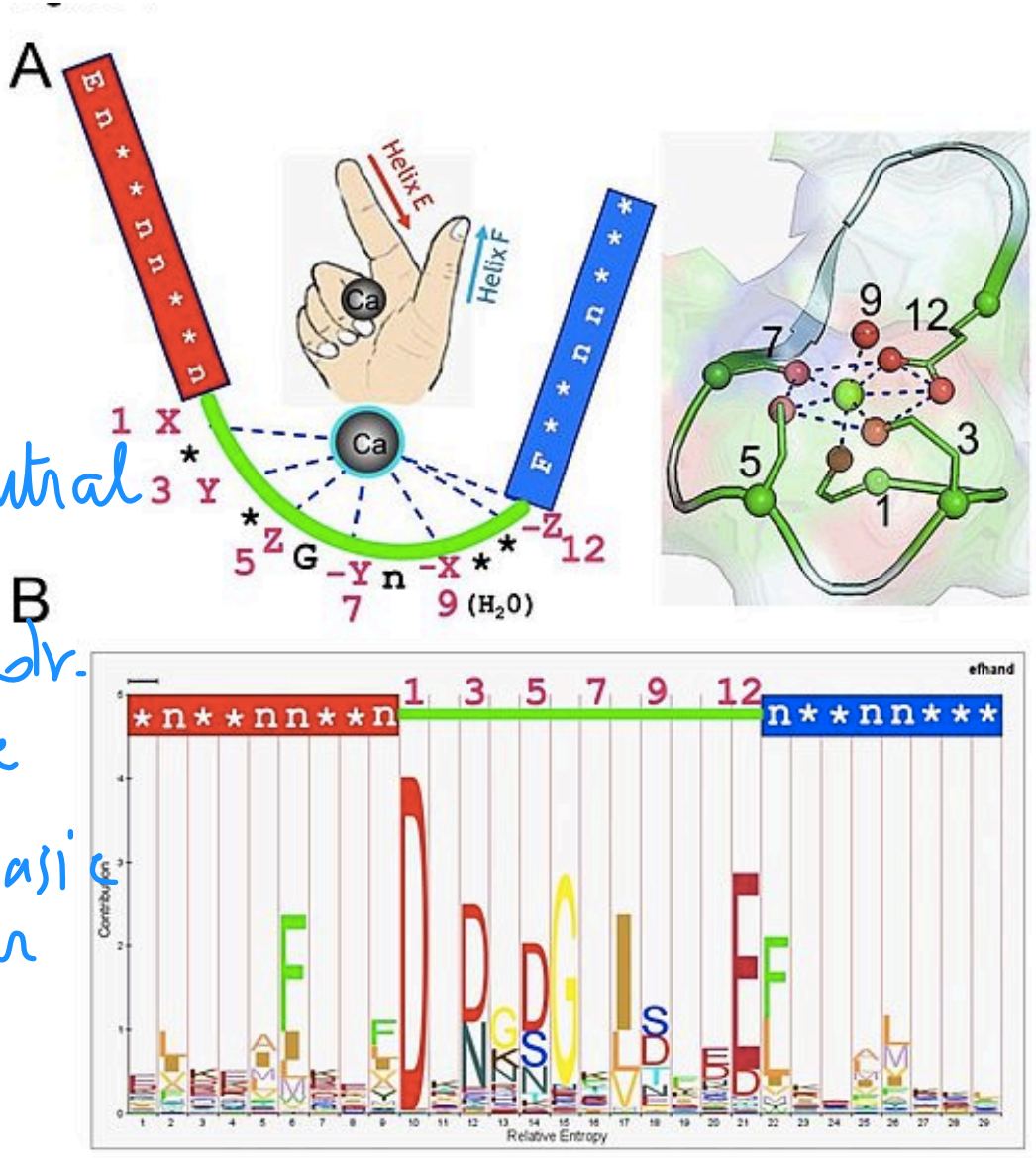


- exposure of _____ methyl groups from methionine ➔ bind to target protein (*e.g.* M13)

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

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

- Binding pocket residues
  - charge vs. neutral
  - H-bond
  - size, steric hindr.
  - loss. of selective
  - pH: acidic vs basic
  - nonpolar / polar
- Interface with M13

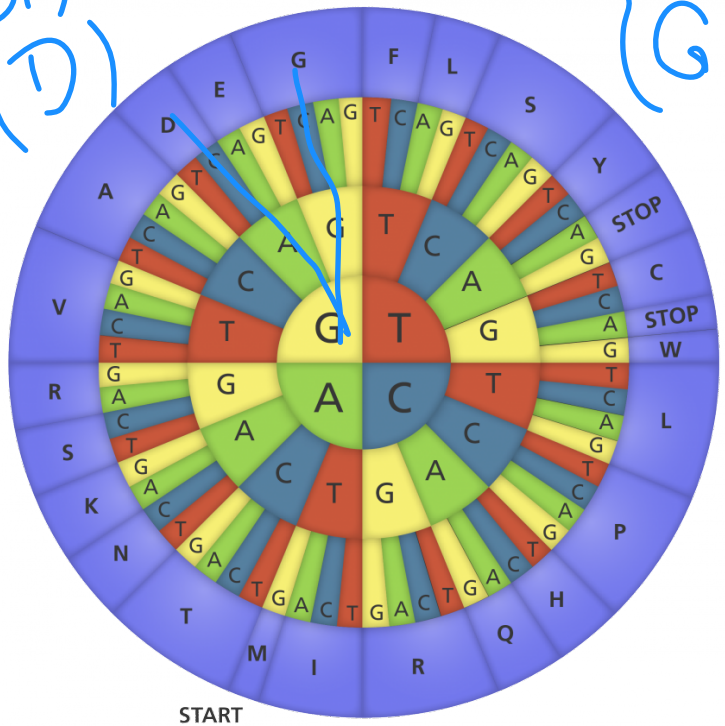


from “viral calciomics: interplays between Ca2+ and virus”. Zhou Y, Frey TK, Yang JJ. Cell Calcium. 2009 Jul;46(1):1-17.

Which residues might you try to alter?

GAC → GGC

(D) (G)



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

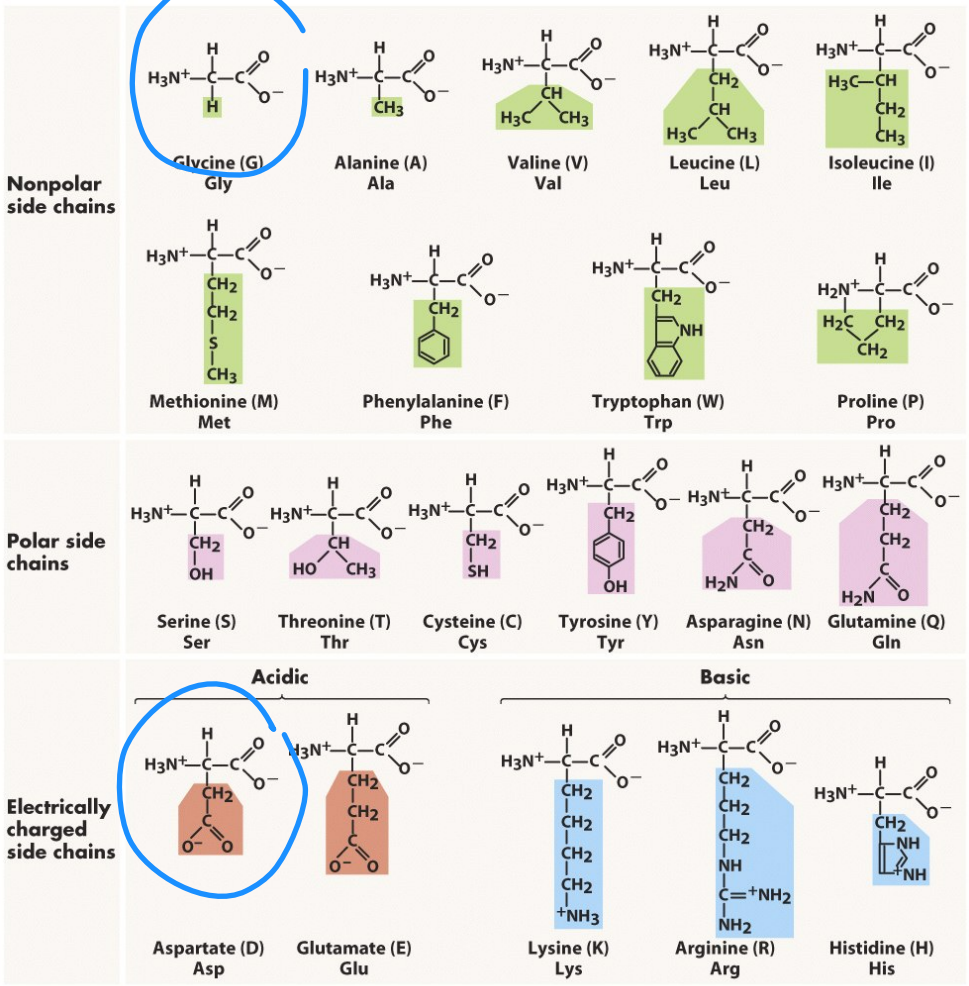
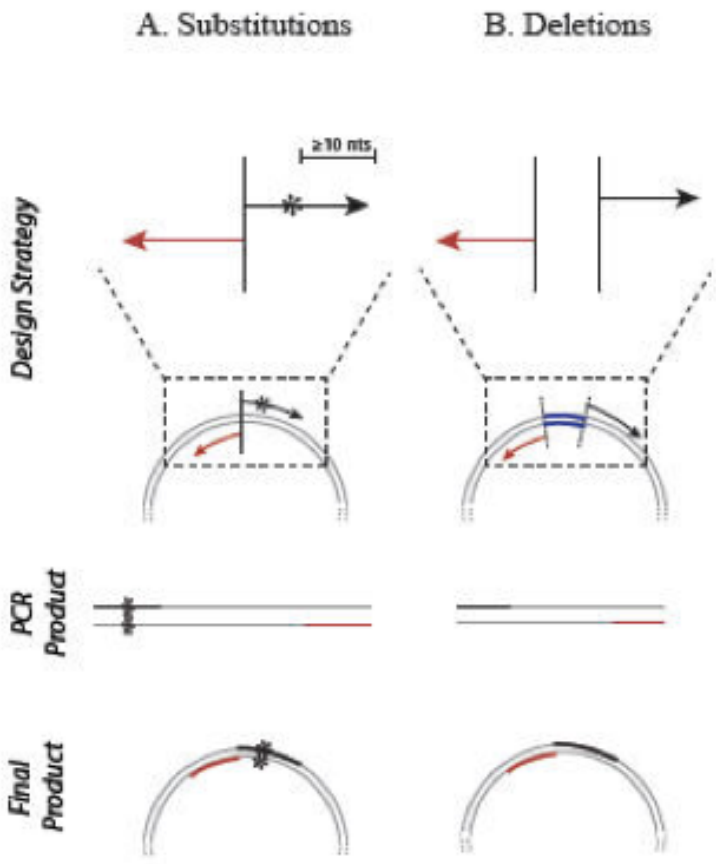


Figure 3-5 Biological Science, 2/e

Site-directed mutagenesis (SDM)

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



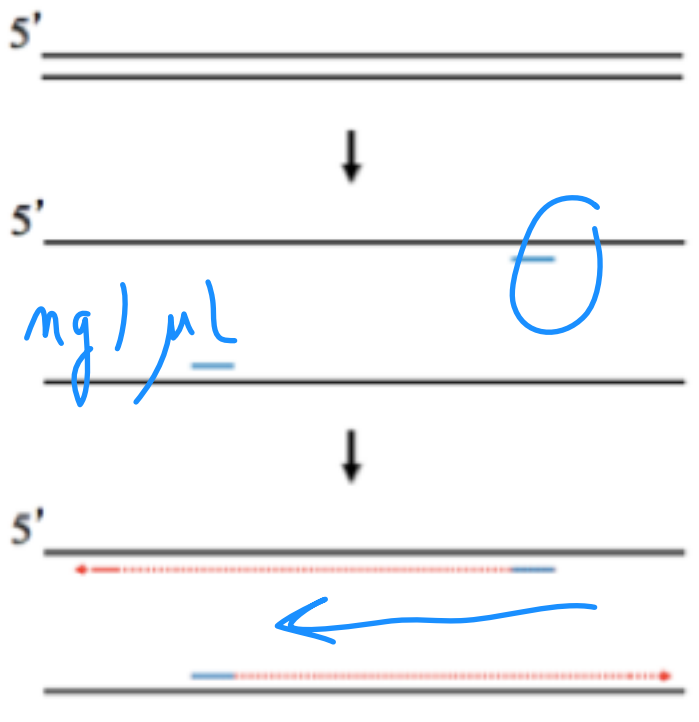
~PCR

pRSET-IPC

SDM ingredients and cycling conditions

SDM ingredients
primers
dNTPs
template DNA 25 ng / µL
polymerase HF
Mg 2+
buffer
water

start circular



melt
98 °C

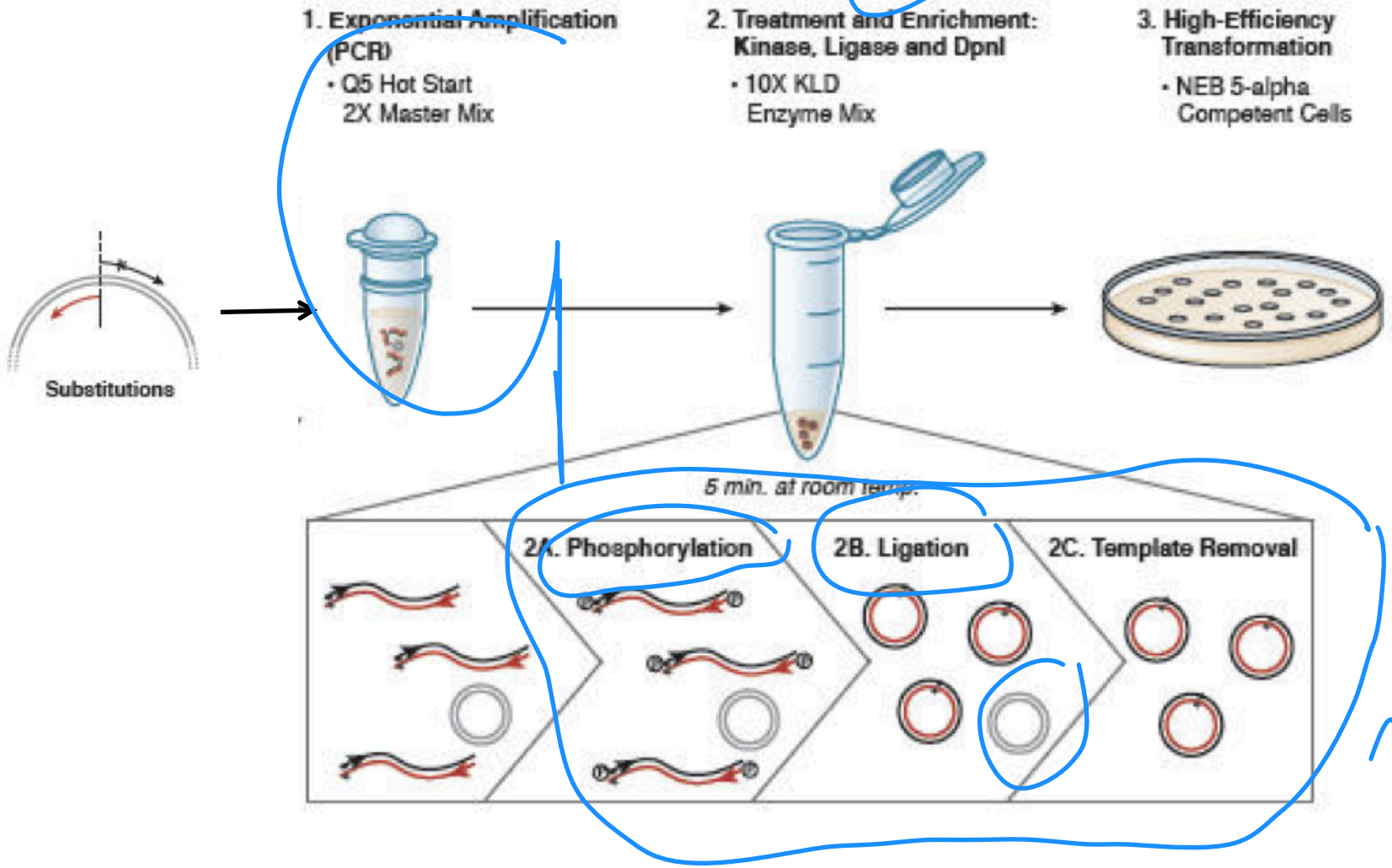
anneal
55 °C

extend
72 °C

25 cycles

end linear

SDM steps with NEB Q5 kit

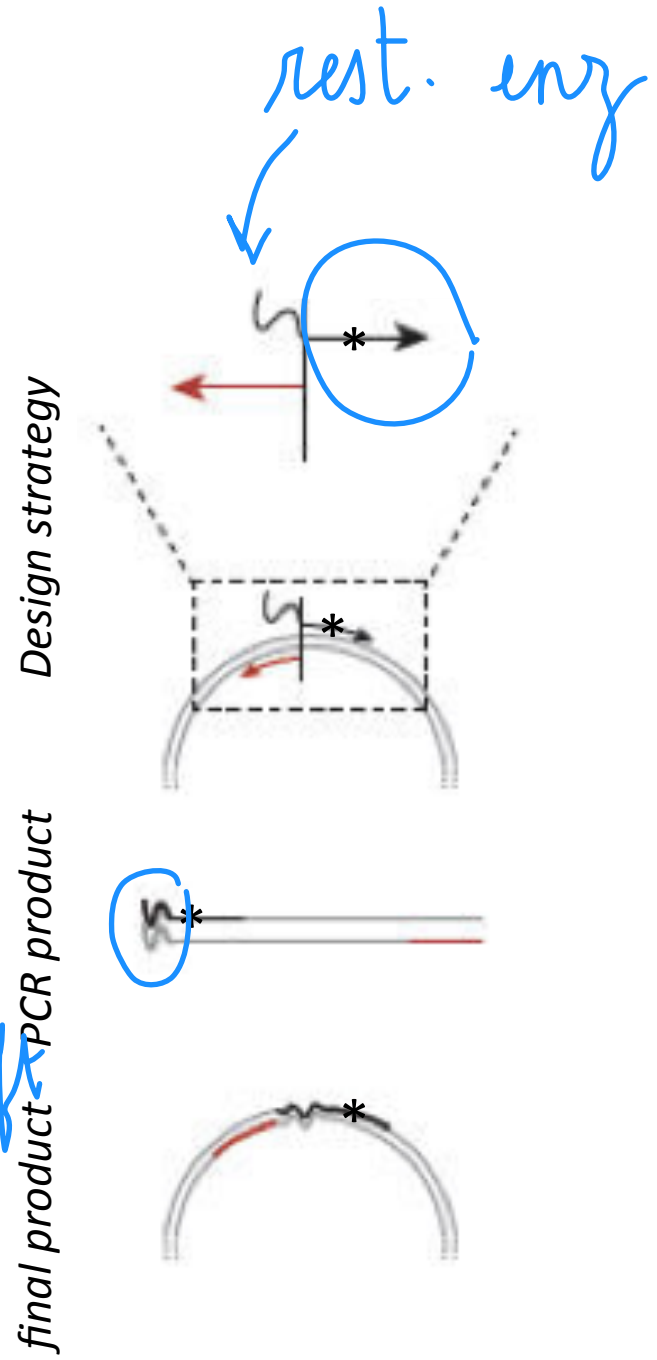


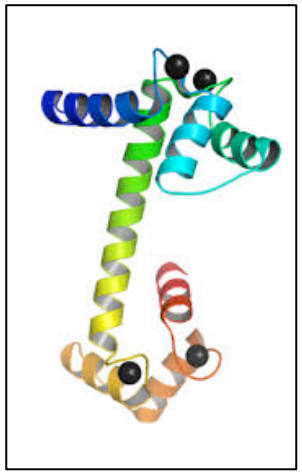
DpnI digests methyl DNA

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 otherwise frame shift
  - how can it be useful?

- diagnostic digest
- more DNA insertions





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