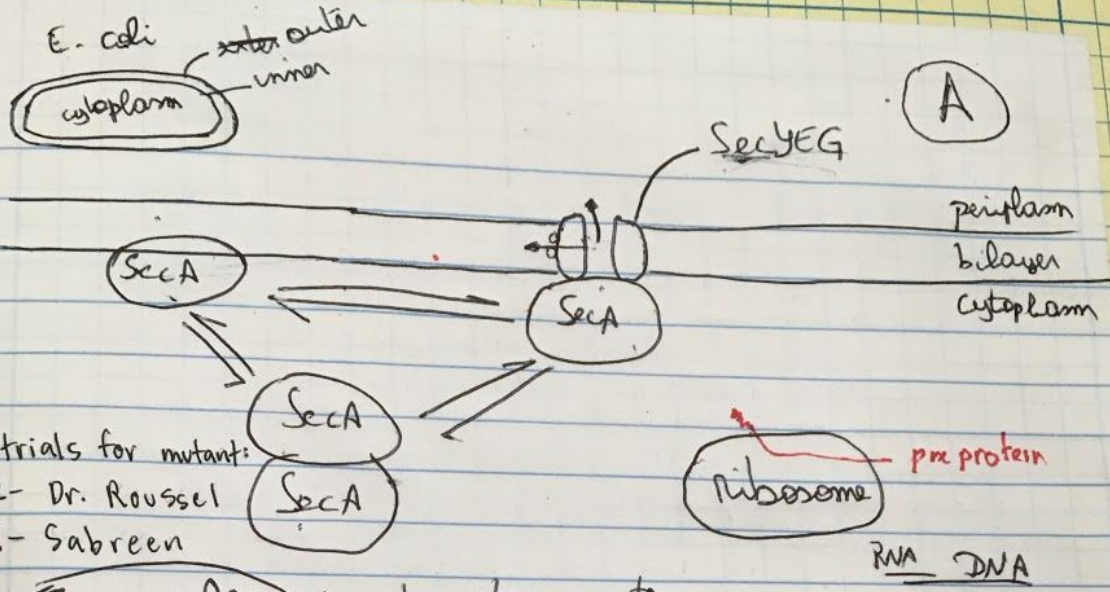




3 trials for mutant:
2- Dr. Roussel
1- Sabreen

SecA prP23 binds stronger than WT

- expression
- purification
- biophysical characterization
 - stability T_m 3PC

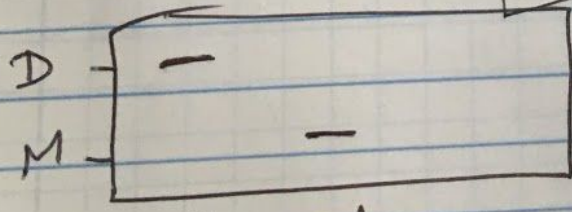
box purification

box membrane

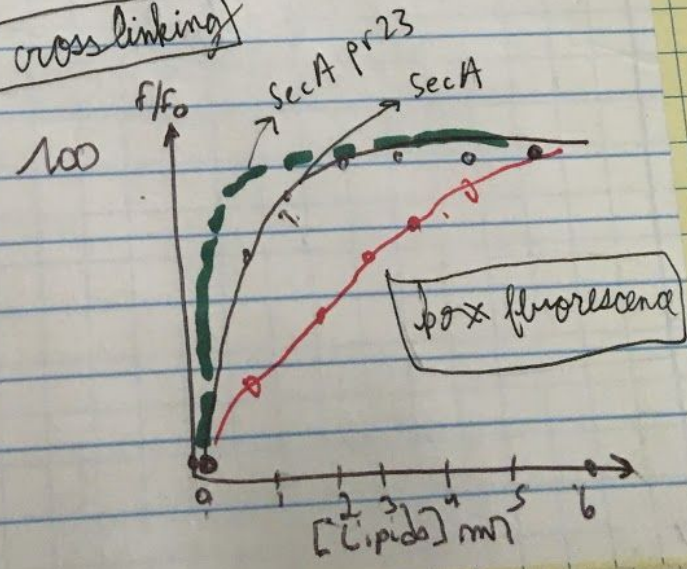
? is SecA a dimer on the membrane?

now gel electrophoresis

box cross linking



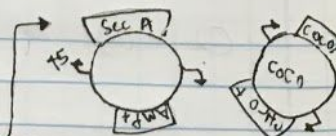
box gel electrophoresis



PURIFICATION

① EXPRESSION

↳ E. coli + DNA (plasmid)



Problem: when E. coli

divides, not all replicated bacteria

will get the SecA protein plasmid

Solution: on SecA, plasmid, also gene that confers ampicillin resistance

→ treat media w/ ampicillin to

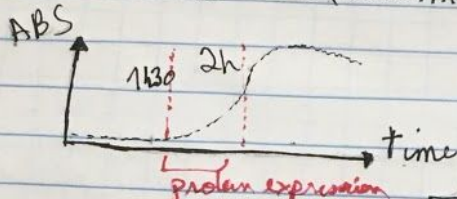
get rid of bacteria w/out plasmid

Improvement: plasmid that causes increased protein yield (coco)

→ treat bac. w/ (CH₂O) + antibiotic to get bac. w/ that plasmid as well

OW in preculture (10mL)

1 day 1.2 culture ± (800mL)



+ CENTRIFUGE

→ PELLET = bacteria containing SecA

② PURIFICATION / ISOLATION

②.1 LYSIS

CHEMICALLY

THERMALLY

MECHANICALLY



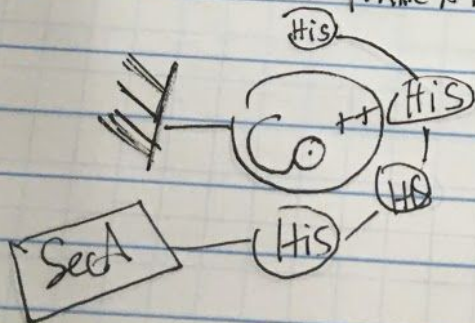
↳ Mix: MEMBRANE + CYTOPLASM / PROTEIN

Day 2

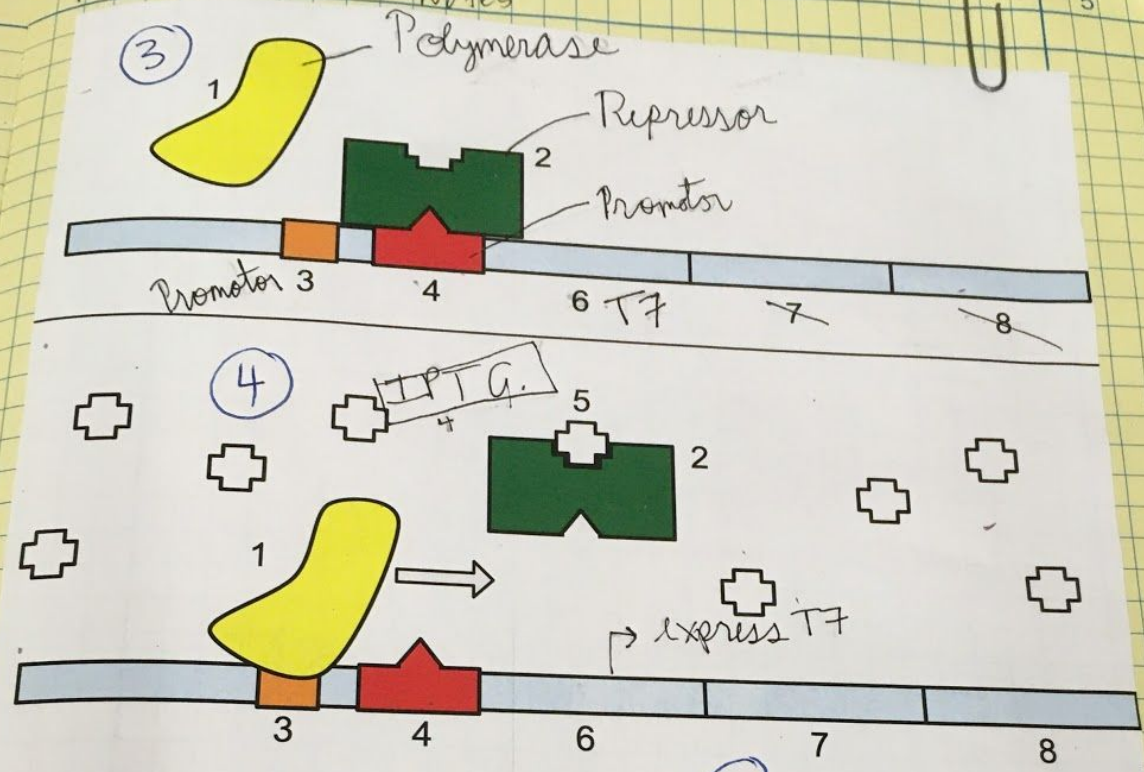
②.2 PURIFICATION

↳ COBALT PURIFICATION

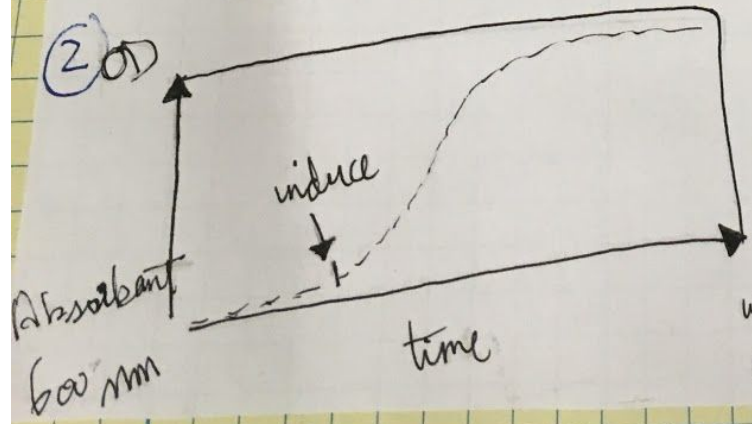
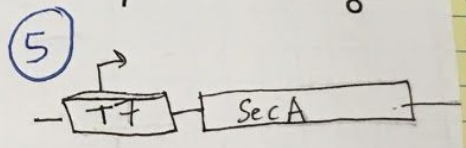
SecA History
Histidine residue



Future Research: in vitro, Sec A is shown to not bind to the membrane - possibly due to lack of space b/c of existing proteins on membrane? → remove proteins and see if SecA binds



① E. coli BL21 Sec A-His tag



- ① current culture: E. coli of strain BL21, plasmid containing Sec A and His tag (for purification, see p. 4)
- ② measure optical density over time by exciting bacteria w/ 600 nm laser-induced protein expression (induce IPTG) at indicated time

③ site-bacterial operon; ^{lac} repressor bound to promoter preventing expression of T7; active inhibition of polymerase

well (appears less yellow)

* Cold Spring Harbor Protocols

cshprotocols.cshlp.org

doi:10.1101/pdb.rec9077

Cold Spring Harb Protoc 2006. 2006: pdb.rec9077-

Recipe

Coomassie Brilliant Blue solution

Dissolve 0.25 g of Coomassie Brilliant Blue R-250 in 90 ml of methanol:H₂O (1:1, v/v) and 10 ml of glacial acetic acid. Filter the solution through a Whatman No. 1 filter to remove any particulate matter. Store at room temperature.

19.09.25 - Purification of WT-SeeA by IMA

I FT S W

Imidazole

60 kDa

Not

Elution

- take out imidazole from protein Sec A
- measure protein concentration
- see whether or not protein is active (on W-3-19)

PURIFICATION

① Expression:

- x overnight culture
- x culture
- x induction
- x expression
- x centrifugation

→ bacteria express Sec A,
centrifuge pellet, etc.
(Ref: page 6)

② Purification

- x Solubilisation
- x lysis
- x loading / binding
- x Washing
- x Elution

→ isolate Sec A:
- lyse chemically,
mechanically, thermally
- cobalt purification
(Ref: page 8)

- SDS-PAGE

50 mM HEPES pH 7.5

400 mM imidazole

1 M NaCl

50 mM KCl

→ two main methods:
dialysis (next page)
and size exclusion / desalting
(next page)

Note: use size exclusion/
dialysis for lab purposes

x Remove imidazole

Dialysis - can use any volume of protein and
elute it

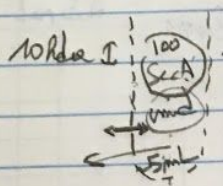
- must repeat 3 times for 2 hours
each for a total of 6 hours

Size exclusion / desalting - very fast - 15 minutes
- can only use 2.5 mL
of the sample

Method 1

Dialysis

membrane



50 mM Hepes
1 mM $MgCl_2$
50 mM KCl

2 liter

$\frac{5}{2000} \rightarrow$ dilution factor ~ 400 times

~ 1 mM imidazole

3x 2h ~ 6 hours

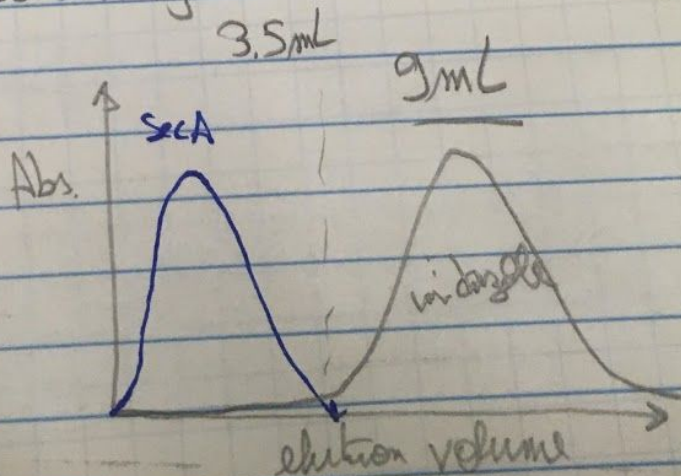
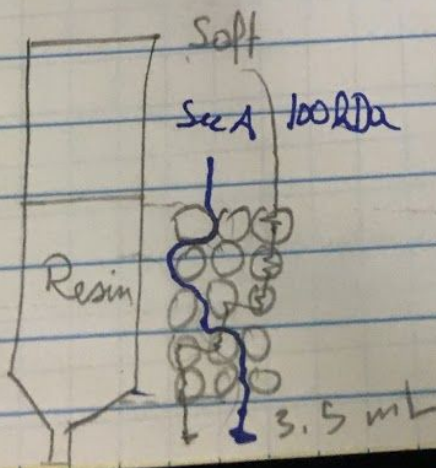
Overall Process: Cut off a piece of a porous membrane to be. Pores are the size of 10 kilo Daltons - SecA is 100 kDa and imidazole is smaller so only imidazole can diffuse through the membrane. A buffer without imidazole we used before (2L of 50 mM Hepes, 1 mM $MgCl_2$, and 50 mM of KCl) surrounds the membrane, and the dialysis bag holds 5 mL of material. The respective volumes can be used to find the molarity of imidazole after it diffuses into the surrounding buffer, which is about 1 mM imidazole. The buffer is left soaking the membrane, and replaced every 2 hours 3 times (the whole process takes 6 hours).

Method 2

Size Exclusion

$\rightarrow$ desalting

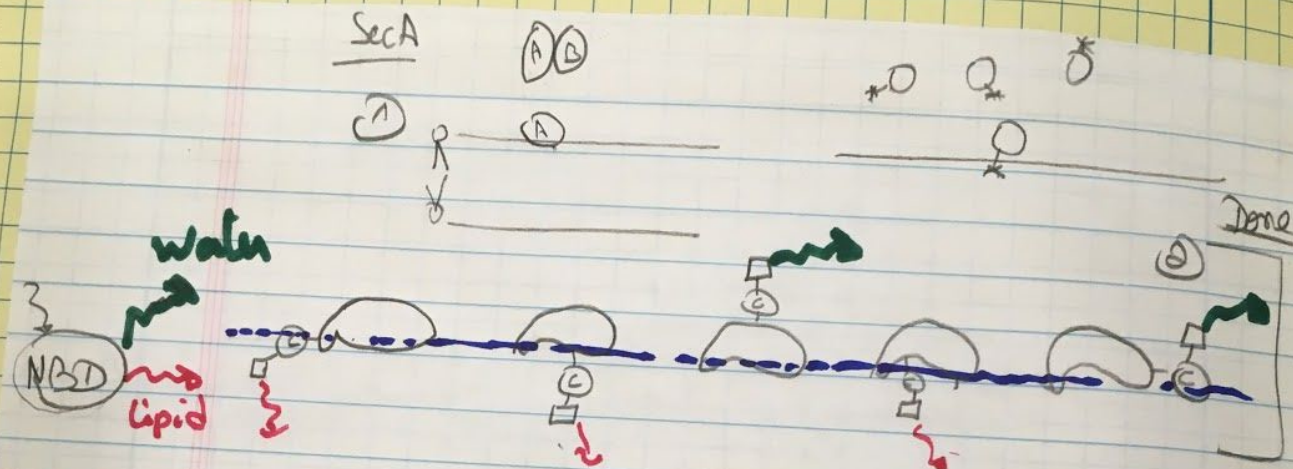
15 minutes



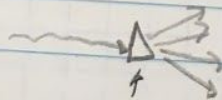
11-7-19 Background: Notes

Objective: What is the specific structure of SecA when it binds to the membrane? We know it is a monomer, but want to know about 3-dimensional structure

Diagrams:



③ Structure → CD circular dichroism → 2nd α -helices
* crystal preparation + x-ray diffraction



cons. x salt
- detergent

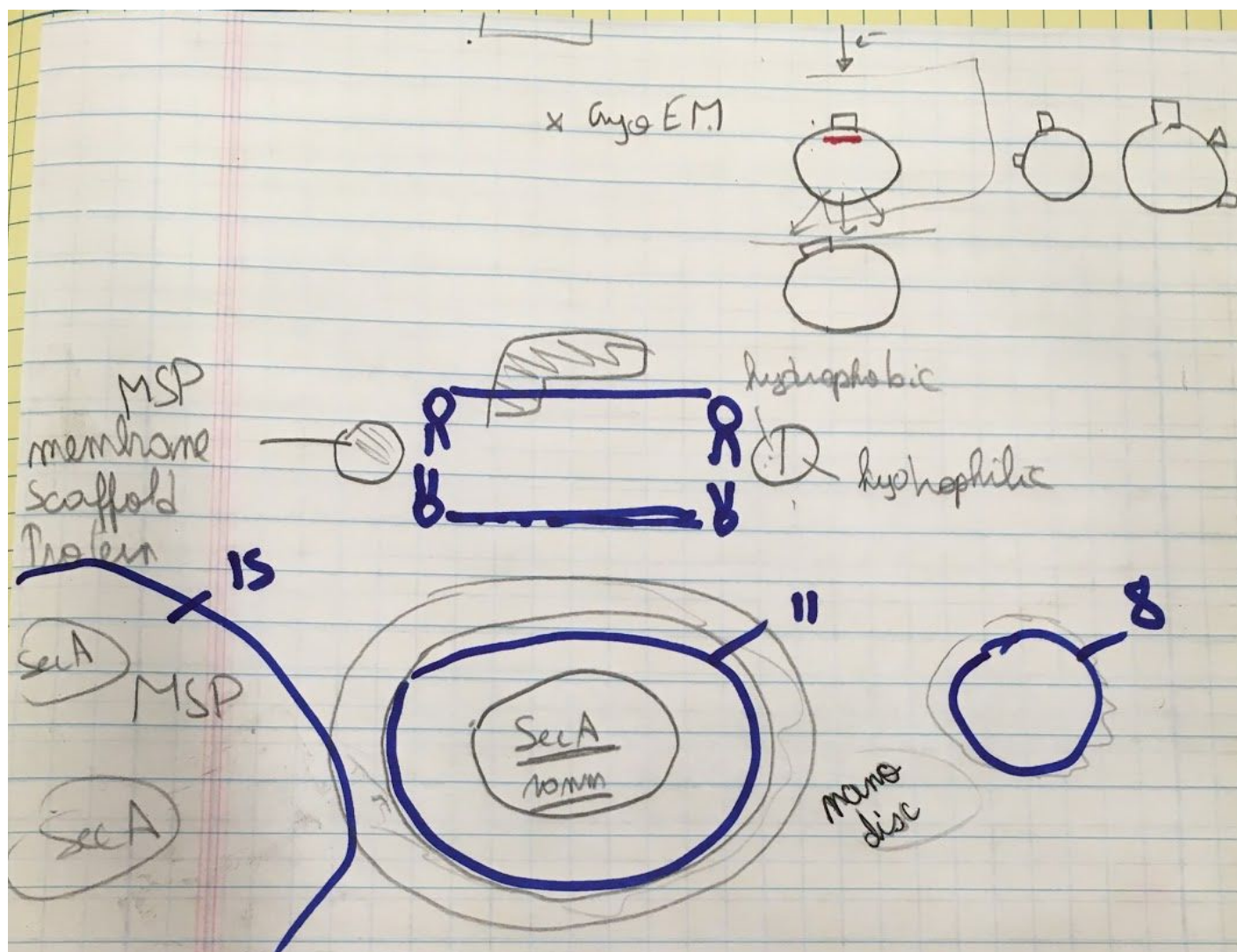
Circular Dichroism: uses circularly polarized light to look at the structure of optically active (asymmetric) chiral media. Difference in absorption of left and right circularly polarized light can be measured/quantified.

Vibrational (IR) CD - used to study structure of small organic molecules/proteins/DNA

* UV CD - used to determine aspects of protein secondary structure

UV/Vis CD - looks at charge transfer transitions in metal-protein complexes

* peptide bonds in proteins are optically active (ellipticity changes based on local conformation of molecule). Analyzed using far-UV (190-250 nm); ordered α -helices, β -sheets, β -turn, & random coils have unique spectra. Note: only relative pos. shown, not exact loc. → best to see changes in comp. (i.e. structural changes upon ligand binding, extent of denaturation).



Crystallography: highly ordered lattice of tightly packed molecules is rotated through an X-ray beam, and resolution of resulting image calculated from diffraction patterns made by deflected photons, which are turned into "maps" of electron density, which + prot. seq. = model. Model represents how specific chemical building blocks of proteins fold into sheets & helices, and how side chains of amino acids are positioned. The smaller and more rigid protein, $\rightarrow$ more amenable to crystallography (b/c big, floppy, objects tend not to form crystals).

Cryo-EM: proteins + macromolecular complexes are flash-frozen in thin layer of water (not much thicker than protein itself), then irradiated w/ low energy e^- to make 2D images of individual particles on detector. Repeated thousands/100s of thousands of times $\rightarrow$ computationally sorted $\rightarrow$ reconstructed to create 3D map $\rightarrow$ protein sequence fit to map to create a model. Smaller object $\rightarrow$ noisy image, so cryo-EM works best for larger structures.

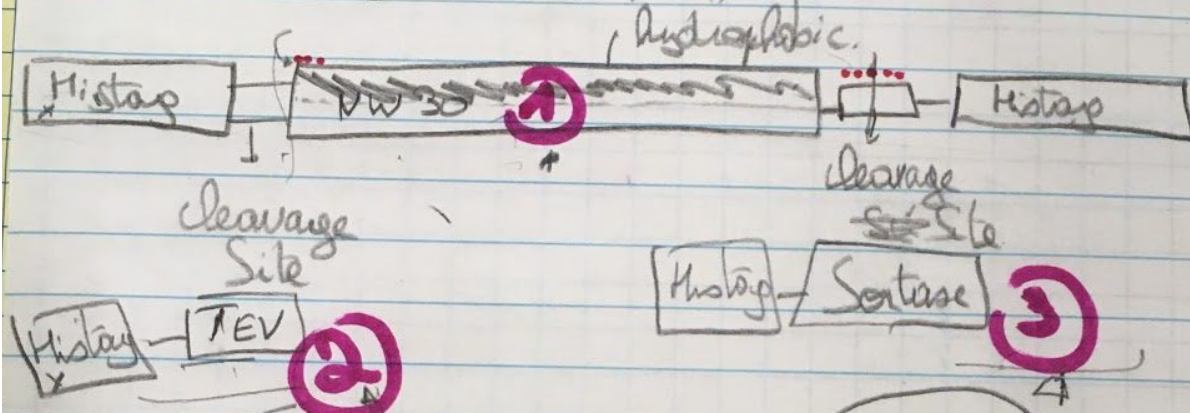
MSP = NW nanodisc width

NW30 $\rightarrow$ 15 nm

NW11 $\rightarrow$ 11 nm

NW9 $\rightarrow$ 8.5 nm

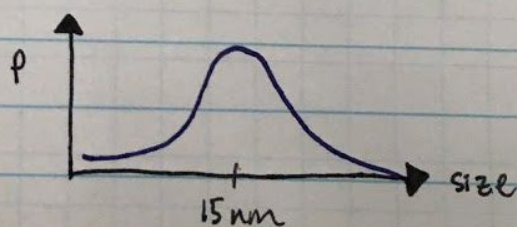
④ Sec A



- ① Purification
- ② release imidazole
- ③ remove imidazole
- ④ introduce TEV
- ⑤ load onto Talon
- ⑥ recover NW30 (does not bind)



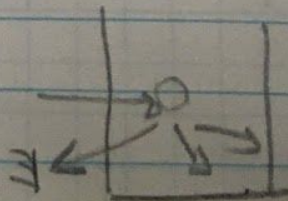
Objective:



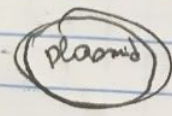
⑦ TEV

⑧ SORTASE

⑨ Dynamic light scattering

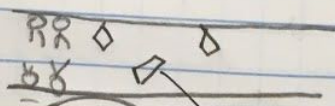


3°C

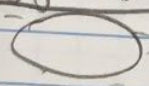


same"

30min 4°C

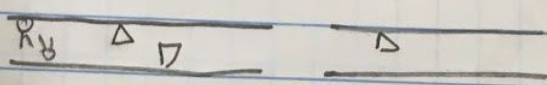


fluidity of lipids ↓

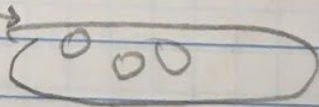
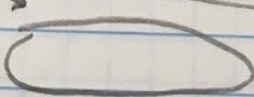
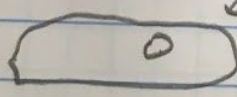
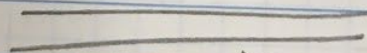


uses cholesterol to keep membrane fluid at this temp

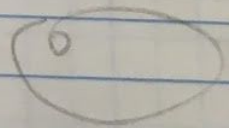
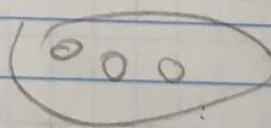
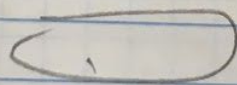
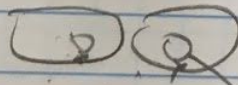
45sec 42°C



1min 4°C



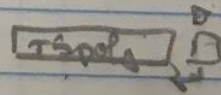
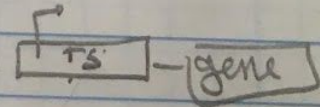
Shower 3°C in LB



low copy plasmid (not for our use)

introduce Ampicillin

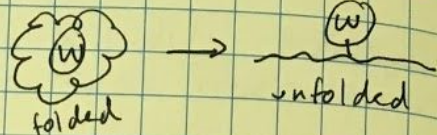
select for plasmid (has resistance to Amp^r will be selected for if cells are plated w/ amp^r)



Background: Fluorescence Microscopy

Sec A Structure:

- dimer
- folded protein when stable
- consists of alpha helices and beta-pleated sheets
- contains tryptophan that releases light when excited

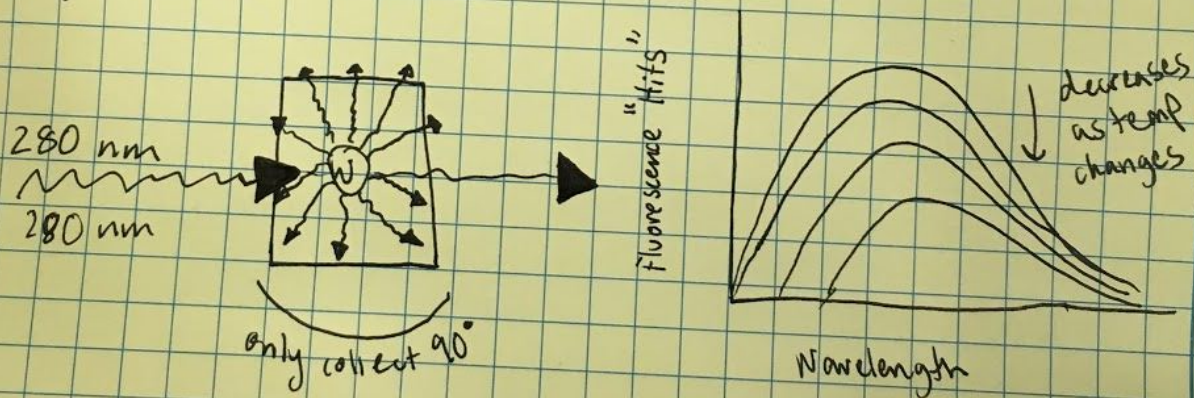


Fluorescence:

- laser excites electrons to go to a higher energy level
- electrons release light when they fall back to ground level
- emitted light is of a different wavelength based on compound hit by laser (different molecules absorb different amts of light)
- emitted light measured and plotted

Main Idea: At which temperature does SecA unfold? We know that mutant Y1345 binds unusually well to the membrane. Is there a difference b/w the temperature at which WT and Y1345 SecA unfolds? → Does the addition of a salt (K6lu, NaCl) change this temperature? What does this imply about mutant Y1345 SecA structure compared to WT SecA?

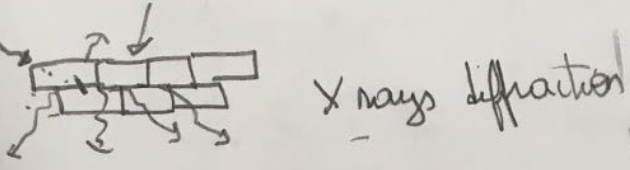
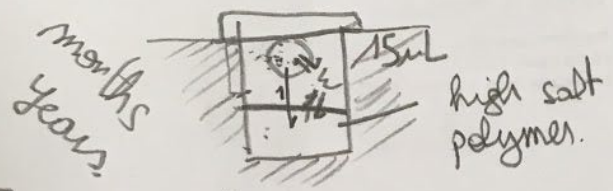
Diagram:



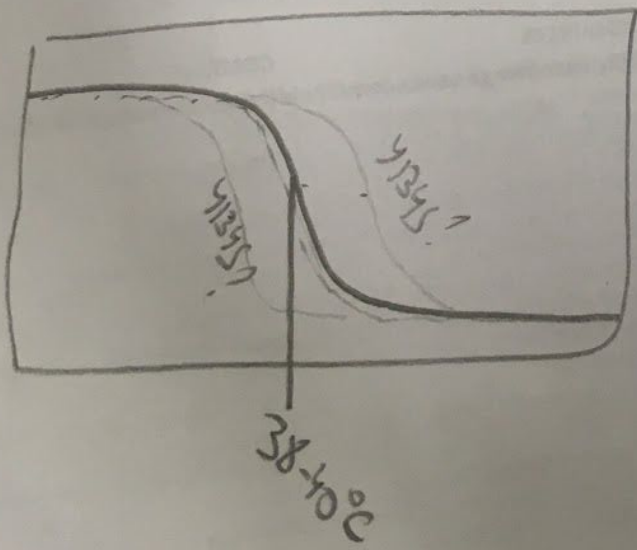
- ideally, temp would be increased by small increments from 20°C to 60°C, and long periods of wait time (max 10 mins) in between to have most accurate results
- also ideally: express, purify, etc. for 3 different strains of WT and mutant SecA to ensure integrity of results (not necessary in this case - can just plot 3 curves for each line (samples from the same stock))

CRYSTALLIZATION

High concentration 10-20 mg/mL

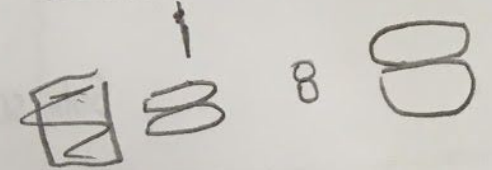
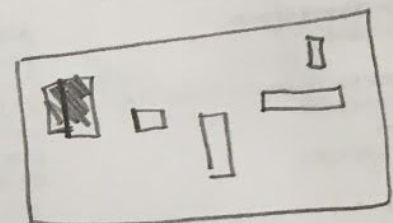
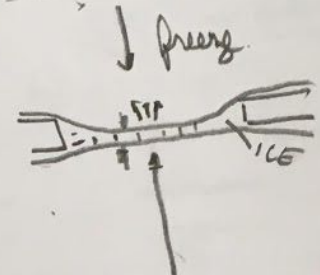
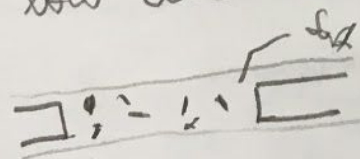


months to years
3D structure



CRYO-ELECTRON MICROSCOPY

low concentration <1 mg/mL



3D structure

Q2.4
overhang
4.6 kb
ground

Bacteria with plasmid DNA

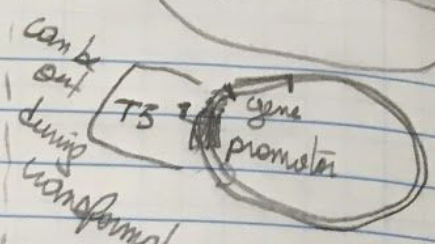
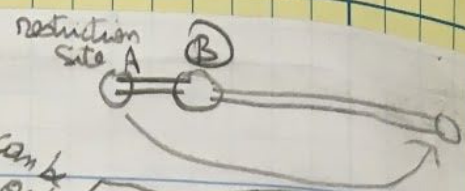
Plasmid

Transform in fresh BL21

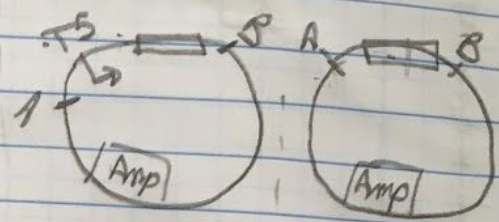
Bacteria with plasmid expression
one copy

CULTURE

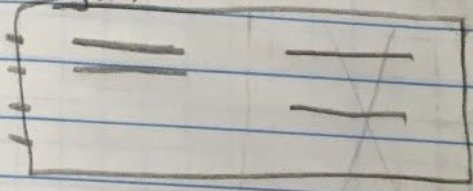
PURIFICATION



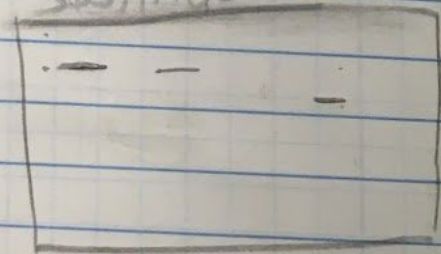
Recombination



A
Amp^r



B
SDS/PAGE



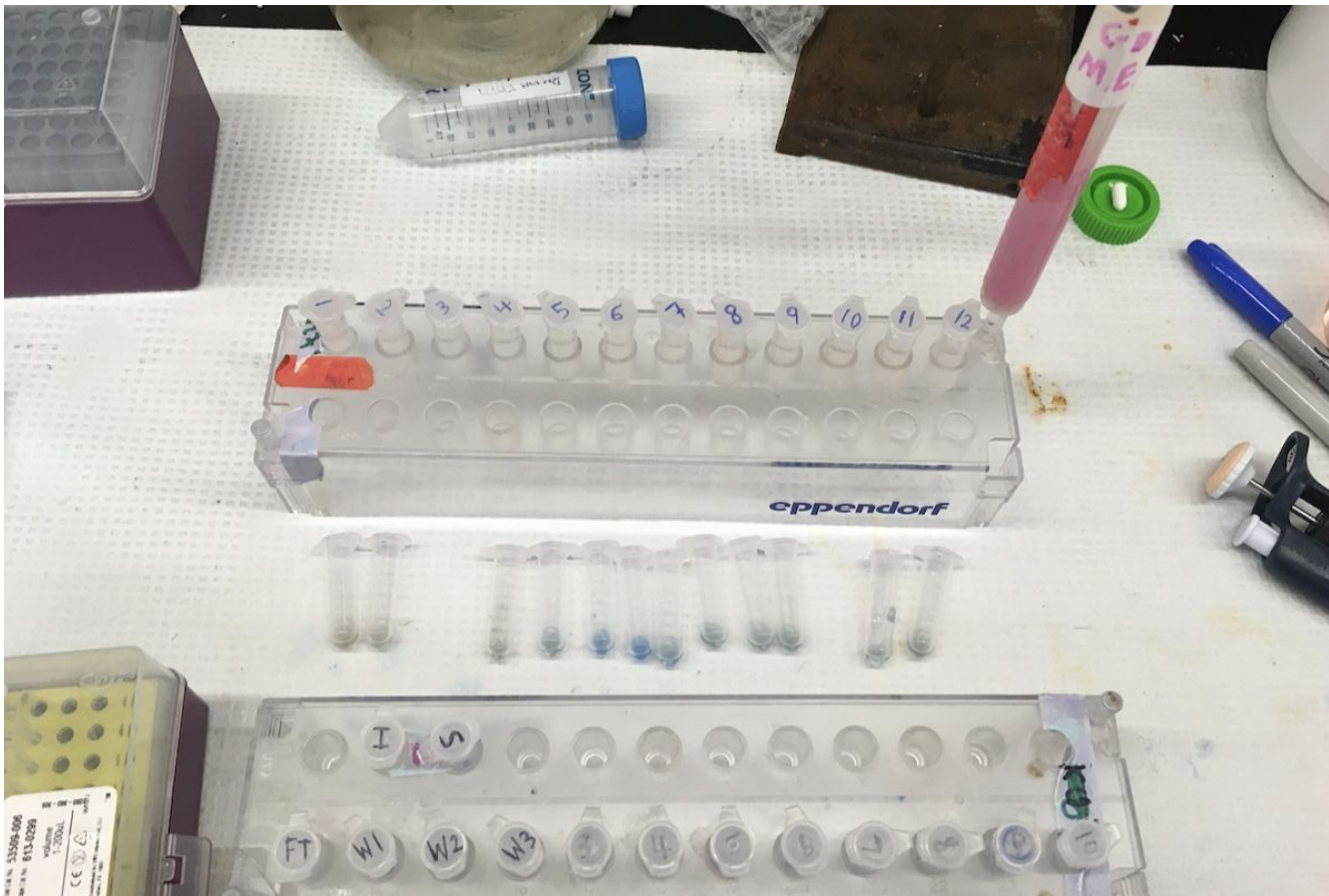
1	2	3	4	5
✓	✓	x	x	x

SEQUENCING

BL21
protein expression



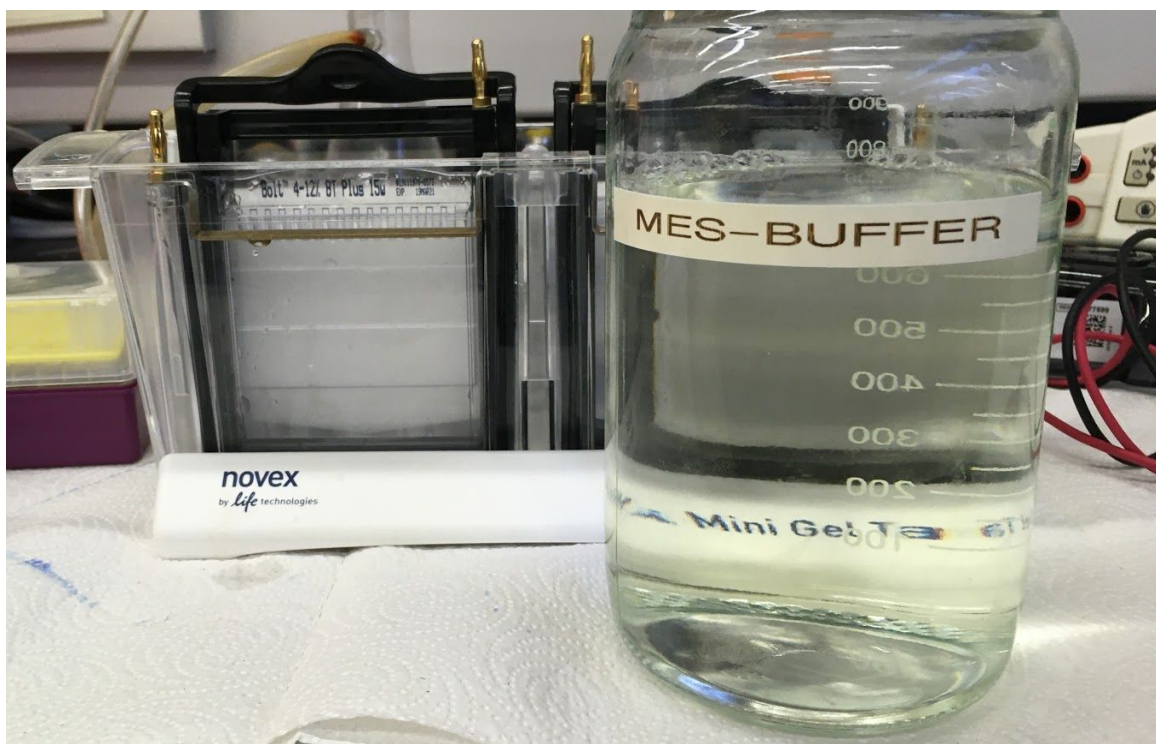
BSA Protein Concentration Analysis. Left to Right: BSA Standards (0, 0.5, 1, 2, 3, 4), Protein Samples (A, B, C)



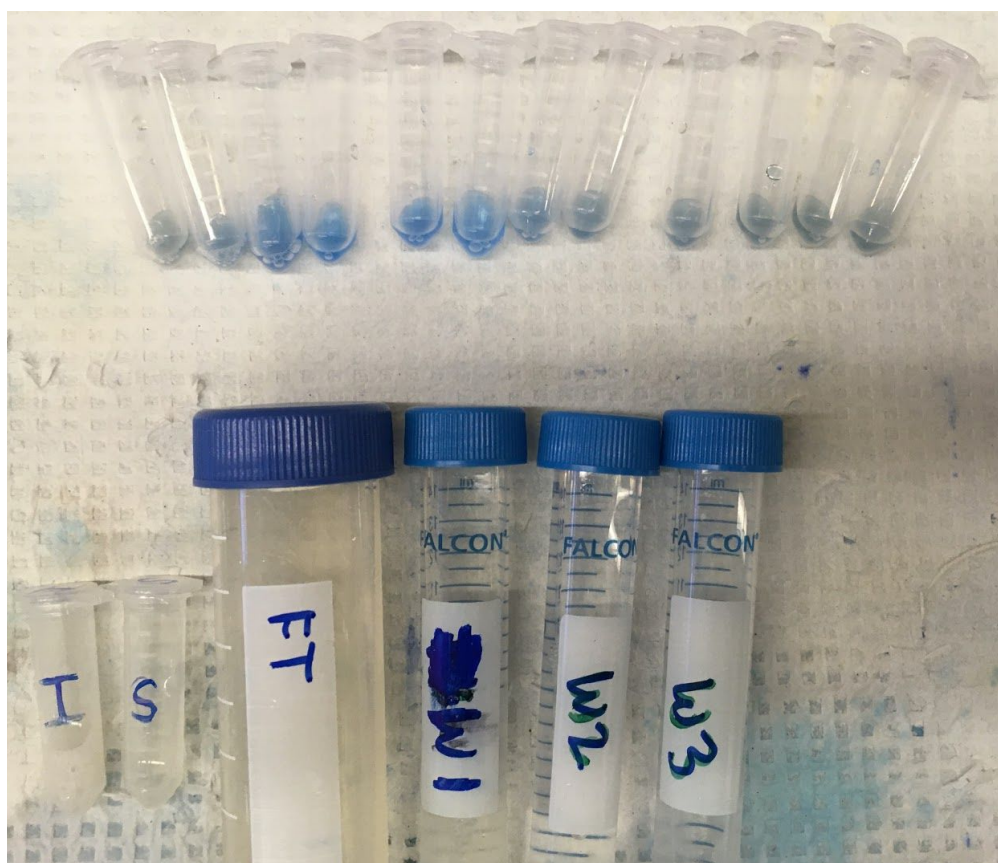
Top: Protein fractions after IMAC purification

Middle: Bradford's assay of protein fractions (middle 8 were selected to run in gel)

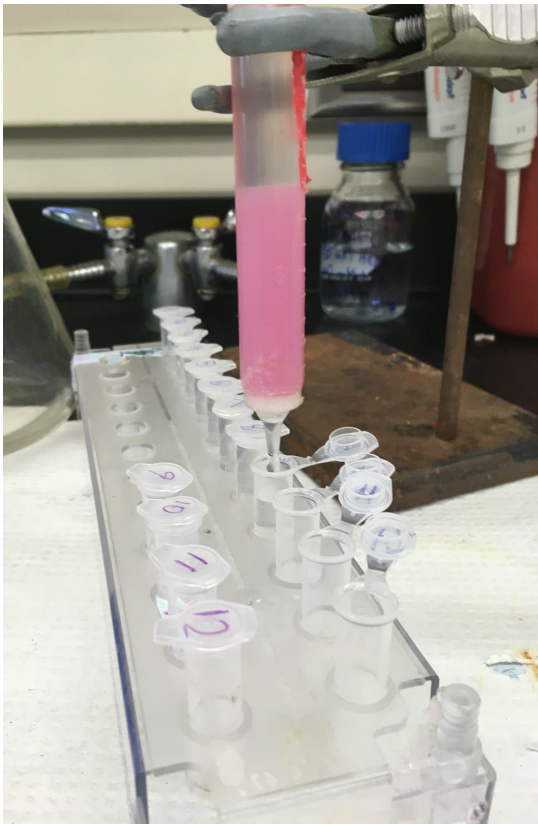
Bottom: SDS-PAGE fractions (I, S, FT, W1, W2, W3, Imidazole)



Mes Buffer and gel electrophoresis equipment



Bradford's assay of protein fractions after IMAC purification
Source material for SDS-PAGE fractions (excluding imidazole)



Left: IMAC purification (collecting final protein fractions)

Right: IMAC purification equipment (with mechanical loading tool)



IMAC purification equipment (mechanical loading tool): currently set to 0.8 mL/min