

Projects ongoing:

1. Sec17/18 Studies

- His-Pull down with nanodiscs 11nm (MSP1E3D1) and 30nm (spNW30) + UT-Sec17 + His-Tev-Sec18 (Tev treat/ filter)
- Production of UT-Sec17_D1-30 (to start)

2. Proteins Nyv1 and Vam3 with single Cys in the Juxta region




- Make 4-snare complex in detergent with R0C228 and Qa-3C262/ R0C and Qa0C/ Qb3Δ/ Qc3Δ
- Cross-link assays
- Derivatization of R0C228 and Qa-3C262
- Run samples in Mono-Q FPLC (R0C228 and Qa-3C262, regular and derivatized)

3. Nanodiscs reconstitution

-Reconstitution of Empty 30nm nanodiscs

4. Making Ypt7-tm and Nyv1 with split GFP tag using pDuet system

5. 4-Snare assembly studies with Cys-Vam7 (*Qc) and Cys-sdVti1 (*sQb)

-  Ready
-  In progress
-  To start

Latest Proteins:

- MBP-R_{JxCys} (Me)
- MBP-Qa_{JxCys} (Me)
- His-Sec17 (Basic-Ser) (Bill)
- MBP-R0C (Me)
- MBP-Qa0C (Me)
- MBP-QcY42A_tmQb (Bill)
- MBP-Tev-Qb3delta (Me)
- MBP-Qa_wt (Me)
- GST-Qc3delta uncleavable (Me)
- Strep Tags (Amy): MBP-Qb_Twin Strep, MBP-Qa_Twin Strep, MBP-Strep-Qb (by Bill)
- His-Tev-Sec18 (no ATP) (Bill/ Me)
- MBP-QcSnareDomain_tmQb (Bill)
- UT-Sec17_D1-30 (Bill)

Week (4/21-25)

- 1) Cross-linking optimization: crosslinking with other versions of R_{JxCys}: R0C227 and R0C229.
- 2) Purification of His-Tev-Sec18.
- 3) Purification of MBP-QcSnareDomain_tmQb.

Crosslink without 3Rs

Glutathione Agarose 4B PROTOCOL for 100ul reaction with 40ul beads:

Will need:

1X Buffer P: 20mM HEPES-NaOH pH 7.4, 150mM NaCl, 10% glycerol, 1% B-OG

1X Buffer P + 40 mM Glutathione: 12.3 mg Glutathione in 1 ml Buffer P. Adjust pH up to 7.4 with 10N NaOH (~6 μ l)

| Q _{Agarose} + B _{Agarose} (ROC228) | | | | |
|--|-----|------|---------------|------------------------------|
| Protein amount | Box | Prep | Concentration | Volume to add in 100 μ l |
| 4 μ M GST-His-Vam7 (uncleavable) | 65 | 392 | 138 μ M | 3.2 μ l |
| 4 μ M cleaved MBP_Qa-3C262 | 342 | 526 | 15 μ M | 26.7 μ l |
| 4 μ M cleaved MBP_ROC228 | 380 | 580 | 14 μ M | 28.6 μ l |
| 4 μ M untagged Vti1 | 195 | 421 | 44 μ M | 9.2 μ l |
| 50mM DTT (1M stock) | | | | 5.0 μ l |
| 1X Buffer P + 1% β -OG | | | | 27.3 μ l |

| Q _{Agarose} + B _{Agarose} (ROC227) | | | | |
|--|-----|------|---------------|------------------------------|
| Protein amount | Box | Prep | Concentration | Volume to add in 100 μ l |
| 4 μ M GST-His-Vam7 (uncleavable) | 65 | 392 | 138 μ M | 3.2 μ l |
| 4 μ M cleaved MBP_Qa-3C262 | 342 | 526 | 15 μ M | 26.7 μ l |
| 4 μ M cleaved MBP_ROC227 | 375 | 561 | 10.2 μ M | 39.2 μ l |
| 4 μ M untagged Vti1 | 195 | 421 | 44 μ M | 9.2 μ l |
| 50mM DTT (1M stock) | | | | 5.0 μ l |
| 1X Buffer P + 1% β -OG | | | | 16.7 μ l |

| Q _{Agarose} + B _{Agarose} (ROC229) | | | | |
|--|-----|------|---------------|------------------------------|
| Protein amount | Box | Prep | Concentration | Volume to add in 100 μ l |
| 4 μ M GST-His-Vam7 (uncleavable) | 65 | 392 | 138 μ M | 3.2 μ l |
| 4 μ M cleaved MBP_Qa-3C262 | 342 | 526 | 15 μ M | 26.7 μ l |
| 4 μ M cleaved MBP_ROC229 | 381 | 570 | 7.8 μ M | 51.3 μ l |
| 4 μ M untagged Vti1 | 195 | 421 | 44 μ M | 9.2 μ l |
| 50mM DTT (1M stock) | | | | 5.0 μ l |
| 1X Buffer P + 1% β -OG | | | | 4.6 μ l |

1. Prepare 100ul SNARE mixes as outlined in 0.5ml tubes
2. Nutate at 4°C for 1 hour
3. While nutating, prepare GSH resin: make 1 tubes for each RXN
 Resin Binding capacity: 5 μ g of protein/ μ l of resin
 Slurry is 75%, so 55 μ l of slurry = 40 μ l of beads.
 Use wide bore tip to transfer slurry into 0.5ml epp tube
 Suspend in ~ 0.5ml **Buffer P** + 1mM DTT
 Spin 6 min @ 500xg (2300 rpm) at 4°C. Remove supe.
 Wash 3 more times in 0.5ml **Buffer P** + 1mM DTT
4. After 3rd wash, remove *all supe with gel loading tip* and add 40ul of SNARE Mix into beads.
5. Nutate at 4°C for 1 hour

6. Spin and remove unbound
7. Wash with 0.5mls with **Buffer P** 3X.
8. After 3rd wash, remove *all supe with gel loading tip*.
9. Add 40ul of **Buffer P + 40mM glutathione pH 7.4**, nutate at 4°C for 1 hour.
10. Spin, then harvest supe with gel loading tip.

- Buffer exchange sample in Column P-30 (1 column/rxn)

1. Wash column 4X with 0.5ml **Buffer P** + 5mM EDTA (Spin 4 min @1000xg).
2. Apply 75ul of elution to each column and spin
3. Save samples in 20ul aliquots

- Cross-link with BMH

1. Prepare BMH 20mM in DMSO: 2.8 mg in 500ul DMSO
2. Dilute BMH 10X in **Buffer P** + 5mM EDTA for 2mM
3. Add 4 μ l of 2mM BMH in 20 μ l Sample (final conc. 0.4mM)
4. Incubate 1h @ RT
5. Add 1ul 1M DTT to quench reaction, incubate 15 min @ RT

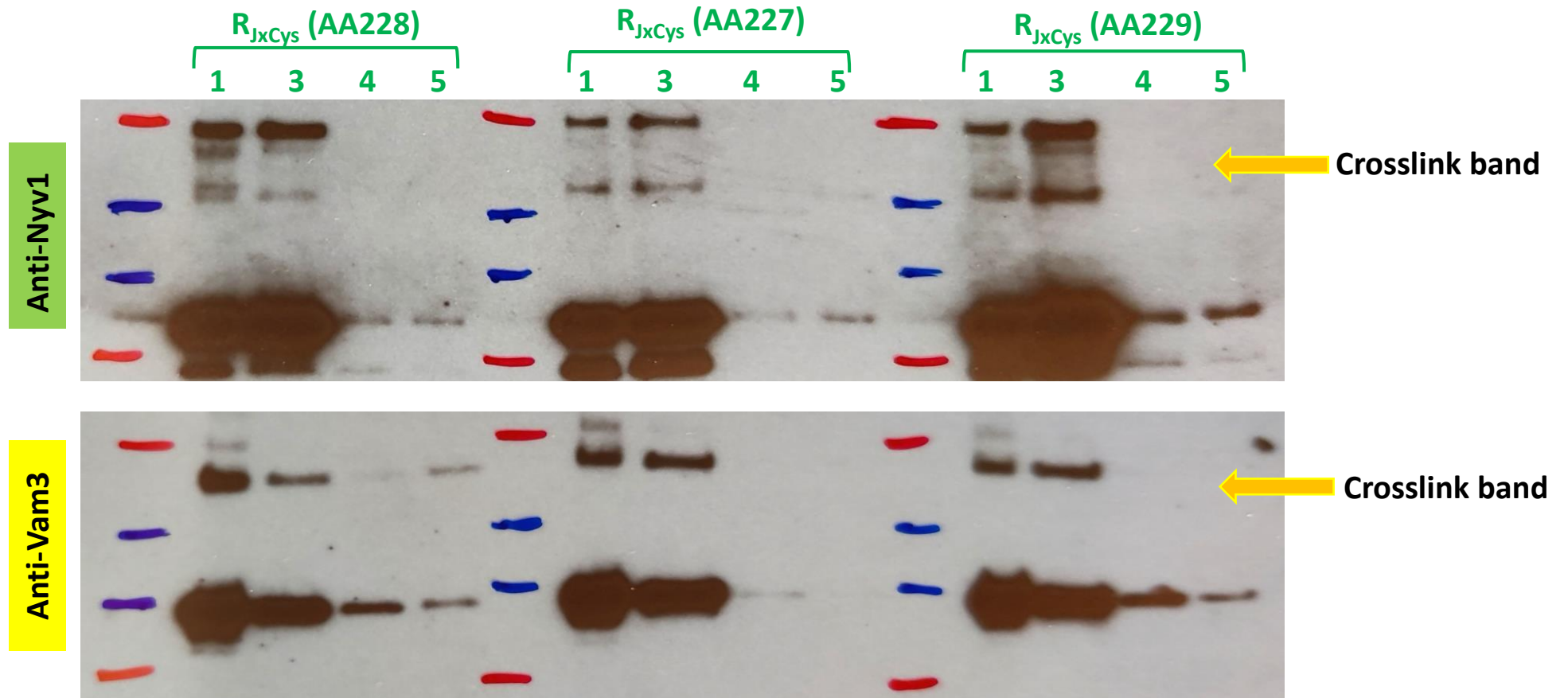
- Run samples twice for westerns anti-Nyv1 and anti-Vam3

Crosslink without 3Rs

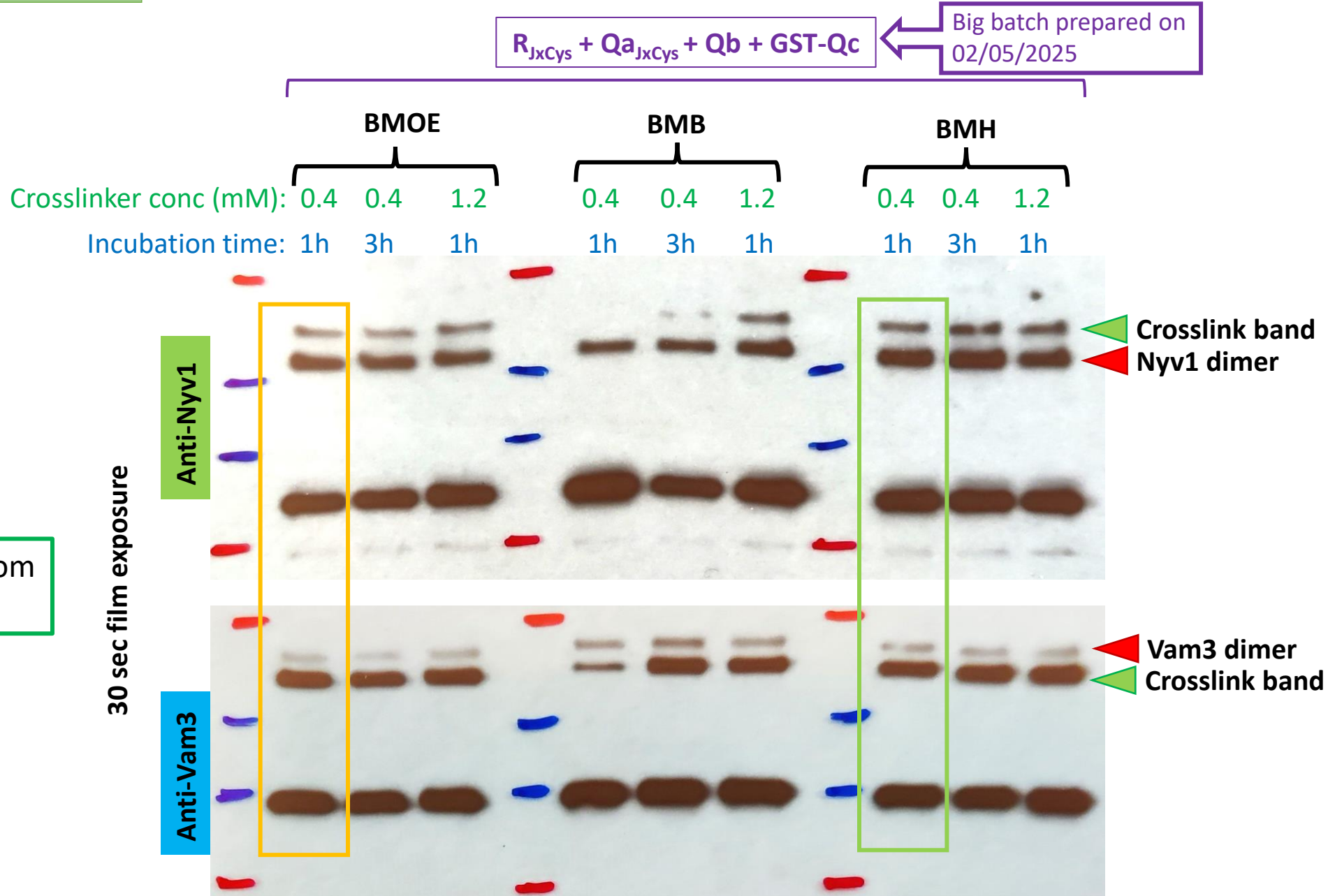
- 1) Initial mix 4-Snare (QaJxCys + RJxCys + Qb + GST-Qc)
- 2) Unbound to Glutathione resin
- 3) Elution with Glutathione
- 4) Buffer exchange (in Rb150+1% bOG+5mM EDTA)
- 5) Buffer exchange / crosslinking with BMH 1H

I am losing almost all sample in the buffer exchange step. (???)
Solve this problem!

10 min film exposure



Crosslink optimization



HIS-TEV-SEC18 purification

Things to do :

- Grow cells from constructions :

① His-TEV-SEC18 box 15 # 13 XL1-Blue (Amp, Tet)

② Parallel-His box 11 # 22 Novablue (Amp)

③ His-SEC18 (pET9) box 21 # 42 XL1-Blue (Amp, Tet)
plasmid box 29 # 36 Rosetta Blue (Amp, chloro)

- Miniprep ①

- Transform ① and ② into expression cells:

A) Rosetta Blue (1 and 2) (4/3/25)

B) XL1-Blue (only 2) (4/3/25)

C) Ros2plys5 (1, 2 and 3) (4/3/25)

D) BL21 STAR (1, 2 and 3) (4/7/25)

- Send ① for sequencing

(04/01/25) OK

- Induction test comparing ①, ② and ③ (04/10/25)
(04/11/25) coomassie

If all good...

- Production of His-TEV-SEC18 in BL21 STAR (Amp^R) 3L (04/23/25)

- TEV treatment

- Remove His-tag by filtering through 50K amicon filter

- Incubations with nanodiscs

HIS-Tev-SEC18 purification

HIS-Tev-SEC18

SOLUBLE PROTEIN PREPARATIONS (HIS-tag)

STEP 1: BL21 STAR + pParallel-His-Tev-SEC18

(4.16)

1. Grow each strain construction in 100 ml LB+ Amp (in 500 ml flask) O/N, 37°C, 200 RPM.
2. Next morning, dilute the 100 ml culture in 3 L fresh LB+ Amp (in a 6 L flask).
3. Shake at 37°C until OD₆₀₀=1 on spectrophotometer
4. Add 3 ml 1 M IPTG to each flask and shake at 37°C for another 5 hours.
5. Spin cells down at 0°C, 4K for 5 minutes.
6. Resuspend pellets in 30 ml of **Buffer A** (I. Cell suspension: 40 ml)
7. Freeze Cell suspension dropwise. Store at -80°C.

STEP 2:

(4.18)

1. Thaw cell drops and French press twice (4°C, 10⁴ psi).
2. Balance ultracentrifuge (60Ti) tubes with **Buffer A** and spin for 1h, 4°C, 50K, 60Ti.
3. Freeze Cytosol fraction dropwise, it contains the protein. II. Protein supernatant: 36 ml.
4. Resuspend pellet in 10 ml **Buffer A** using P/E homogenizer. (III. Membrane suspension: 11 ml)
5. Freeze Membrane suspension dropwise and save it too.
6. Store everything at -80°C.

STEP 3:

(4.23)

1. Thaw supernatant and nutate for 1h30 at 4°C with 10 ml settled Ni-NTA resin equilibrated with **Buffer A** (10:30-12:00)
2. Pour into a 2.5 cm diameter column at 4°C. Let flow through and save all. (IV. Flow-through: 36 ml)
3. Wash column with 50 ml of **Buffer B** and save all. (V. Wash: 50 ml)
4. Elute protein with 50 ml **Buffer C**. Collect 2 ml fractions.
5. Check protein concentration by Bradford. Pool: 4 to 10.
6. Total vol: 13 ml. Conc: 3.4 mg/ml. Store in box # 406, prep # 596

(40.8 μM)

| Buffer A | 100ml | Buffer B | 50ml | Buffer C | 50ml |
|------------------------|-------|-----------------------|------|------------------------|------|
| 100mM HEPES-KOH pH 7.0 | 10ml | 20mM HEPES-KOH pH 7.0 | 1ml | 20mM HEPES-KOH pH 7.0 | 1ml |
| 500mM KCl (3M) | 17ml | 400mM NaCl (4M) | 5ml | 10% glycerol | 10ml |
| 2mM bME (pure) | 14 μl | 2mM bME (pure) | 7 μl | 400mM Imidazole pH 7.0 | 25ml |
| 2mM PMSF | 2ml | 10% glycerol (50%) | 10ml | | |
| 1x PIC | 1ml | 20mM Imidazole pH 7.0 | 1ml | | |

Samples that should have the protein of interest.

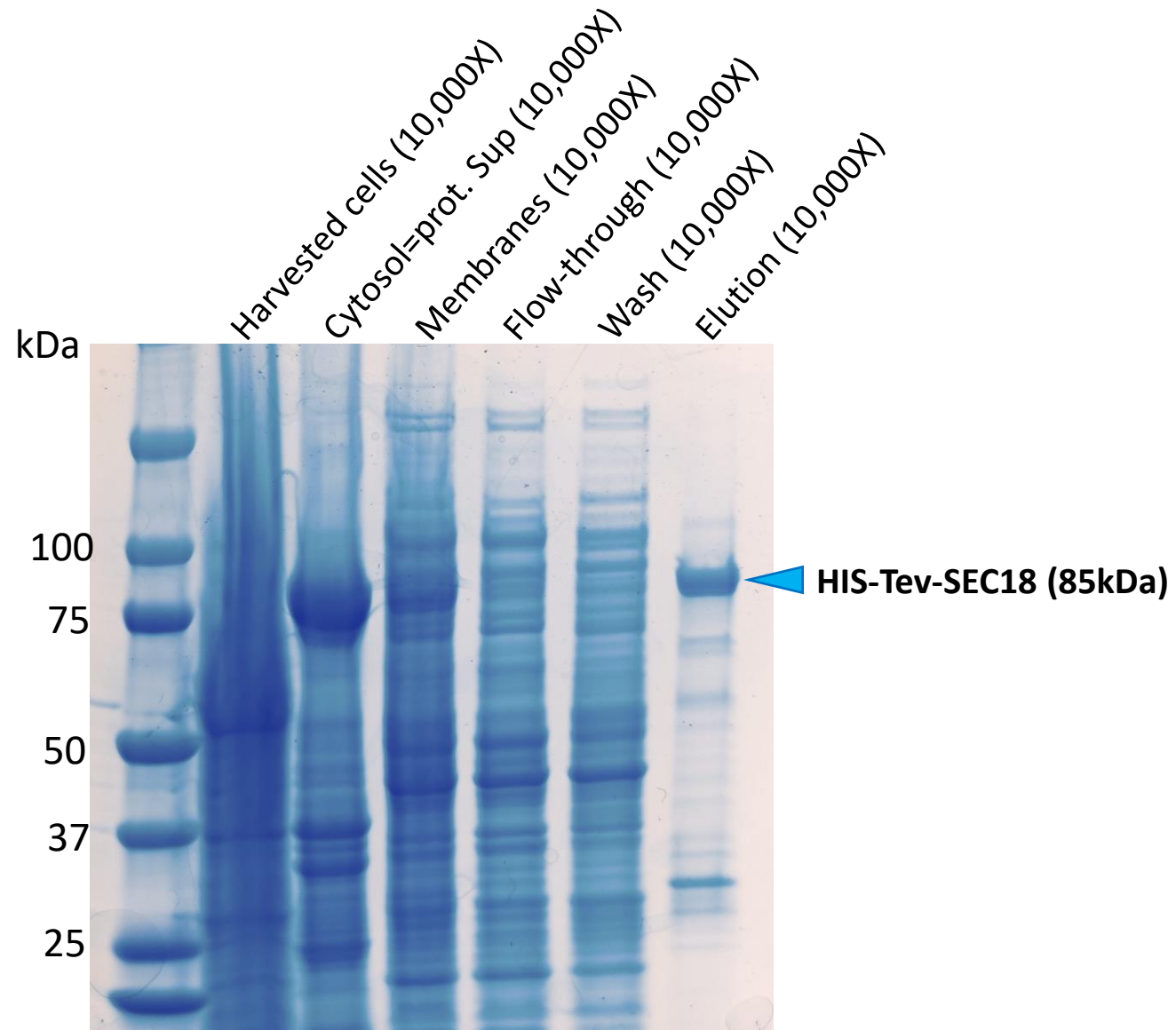
Samples that should NOT have the protein of interest.

| | | | | | | | | | | | |
|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| 0.541 | 0.570 | 0.582 | 0.736 | 0.798 | 0.865 | 0.837 | 0.769 | 0.687 | 0.644 | 0.589 | 0.423 |
| 0.395 | 0.425 | 0.436 | 0.590 | 0.653 | 0.719 | 0.691 | 0.623 | 0.541 | 0.498 | 0.444 | 0.277 |
| 0.392 | 0.366 | 0.358 | 0.333 | 0.300 | 0.292 | 0.289 | 0.267 | 0.267 | 0.257 | 0.256 | 0.227 |
| 0.246 | 0.220 | 0.213 | 0.187 | 0.154 | 0.146 | 0.143 | 0.121 | 0.121 | 0.111 | 0.110 | 0.081 |
| 0.233 | 0.146 | | | | | | | | | | |
| 0.087 | 0.000 | | | | | | | | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |

HIS-Tev-SEC18

F: 4-10 13mls

HIS-Tev-SEC18 purification



HIS-Tev-SEC18 purification

[illegible]

MBP-QcSnareDomain_tmQb purification

MEMBRANE PROTEIN PREPARATIONS (MBP-tag)

STEP 1: B21 STAR- MBP-QcSD-tm (04/22)

1. Grow each strain construction in 100 ml LB+ Amp (in 500 ml flask) O/N, 37°C, 200 RPM.
2. Next morning, dilute the 100 ml culture in 3 L fresh LB+ Amp (in a 6 L flask).
3. Shake at 37°C until OD₆₀₀=1.0 (cuvette spectrophotometer)
4. Add 3 mls 1 M IPTG to each flask and shake at 37°C for extra 5 hours. (10:30-3:30)
5. Spin cells down at 0°C, 4K for 5 minutes.
6. Resuspend pellets in 40 ml of **Buffer 1**. (I. Cell suspension: 60 ml).
7. Freeze dropwise. Store at -80°C.

STEP 2: (04/23)

1. Thaw cell drops (save 2 for gel) and French press twice (4°C, 10⁴ psi).
2. Balance ultracentrifuge (60Ti) tubes with **Buffer 1** and spin for 45 min, 4°C, 50K, 60Ti.
3. Suspend membrane protein pellet in 23 ml **Buffer 2** using P/E homogenizer.
4. Nutate membranes at 4°C, 60 min. (11:15-12:15)
5. Save cytosol supernatant dropwise. Store at -80°C. (II. Cytosol suspension: 60 ml).
6. Spin membranes for 1 h, 4°C, 50K, 60Ti. (12:20-1:20)
7. III. Protein suspension: 23 ml. Freeze protein supernatant dropwise. Store at -80°C.
8. Resuspend membrane pellet in 10 ml **Buffer 1** using P/E homogenizer.
9. IV. Membrane suspension: 19 ml. Freeze dropwise and save it too.

STEP 3: One day before step 4, wash the Amylose resin very well at least 3x with water and 2x with **Buffer 2** (-PMSF/-PIC). Leave the equilibrated resin in the fridge.

STEP 4:

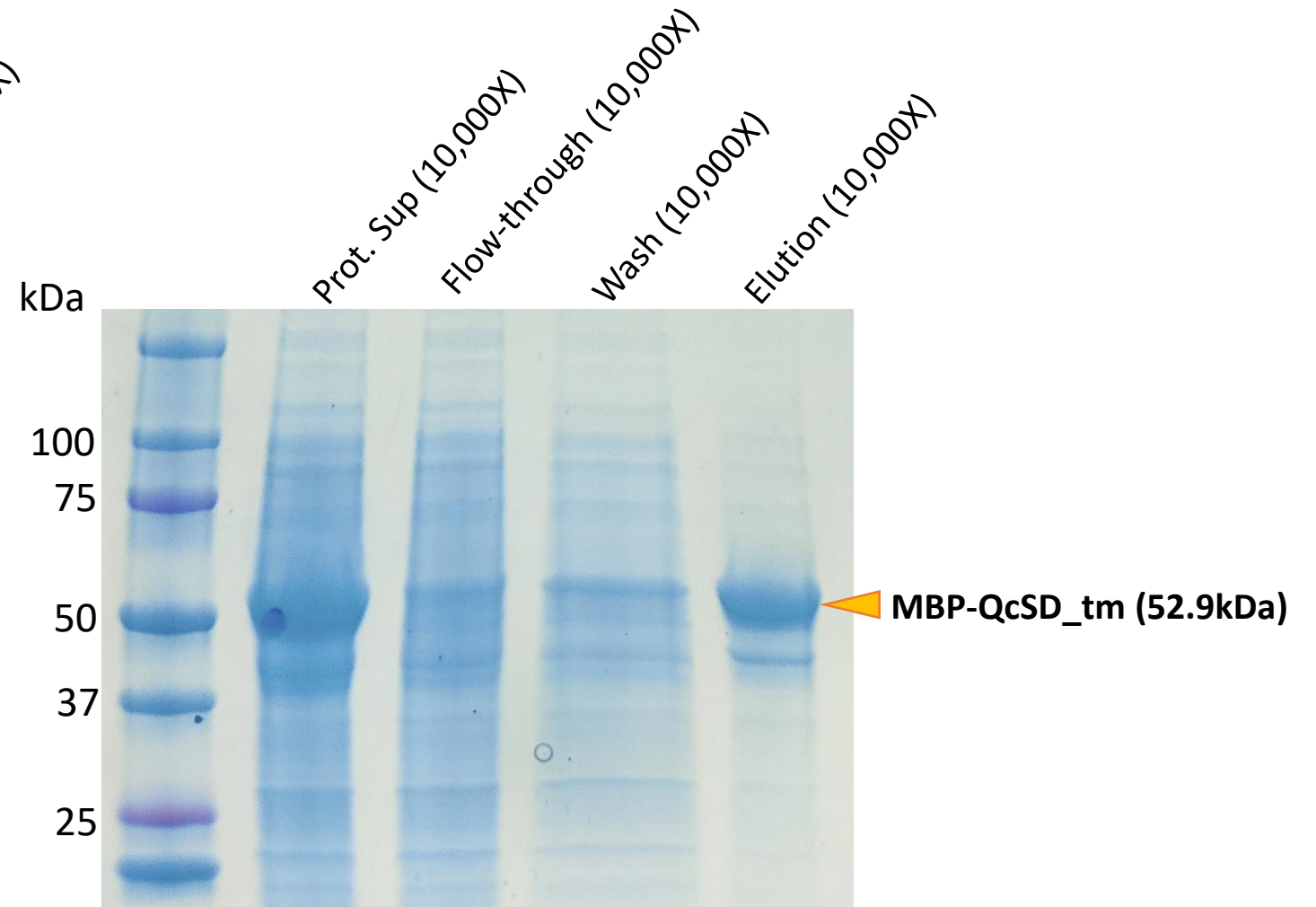
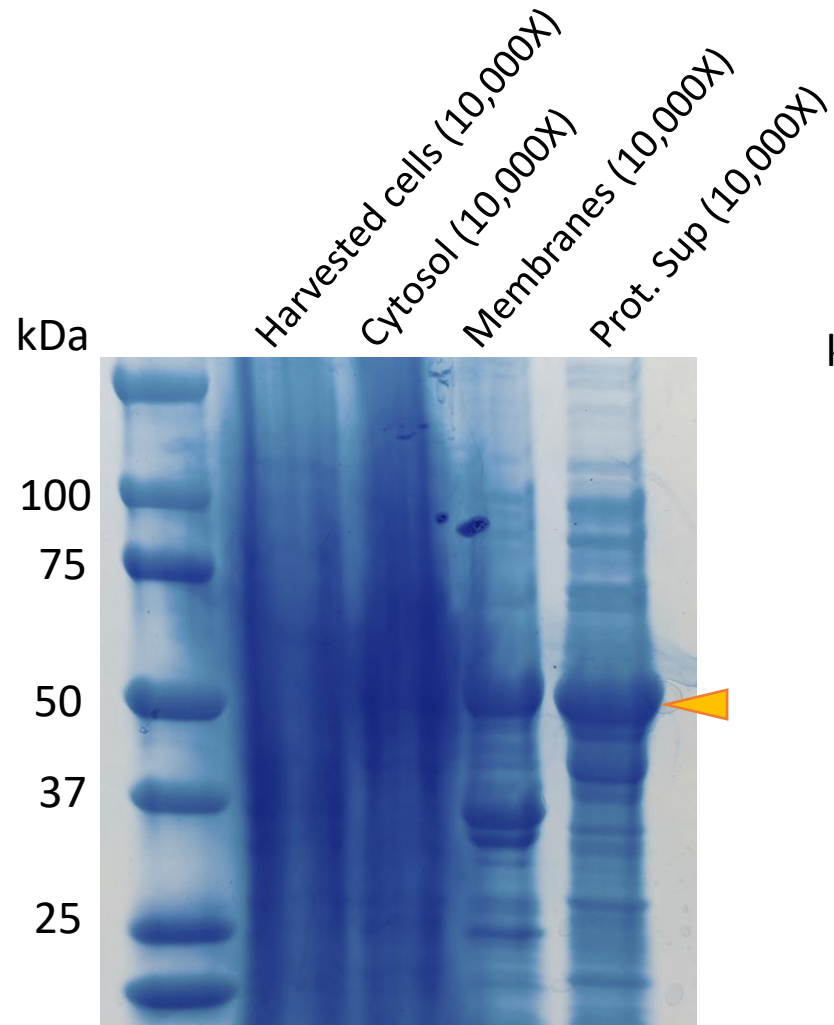
1. Thaw protein supernatant drops (save 2 for gel) and nutate for 2h at 4°C with 15 ml settled Amylose resin equilibrated with **Buffer 2**. (09:05-11:05)
2. Pour into a 2.5 cm diameter column at 4°C. Let flow-through and save all. (V. Flow-through: 23 ml).
3. Wash column with 100 ml of **Buffer 3** and save all. (VI. Wash: 100 ml).
4. Elute protein with 40 ml **Buffer 3** + 10 mM Maltose. Collect 2 ml fractions.
5. Check protein concentration by Bradford. Pool: 1 to 14.
6. Total vol: 76 ml. Conc: 2.95 mg/ml. Store in box # 407, prep # 597. (55.7 μM) 52.9 kDa
7. Do not dismantle column or discard anything until the SDS-PAGE gel confirms we have the protein recovered and pure in a good concentration.

| Buffer 1 | Buffer 2 | Buffer 3 |
|---------------------------------------|--------------------------------|--------------------------|
| - 20 mM Tris-HCl pH8 (1M) <u>5</u> ml | - 20 mM Tris-HCl pH8 | - 20 mM HEPES-NaOH pH7.4 |
| - 200 mM NaCl (4M) <u>12.5</u> ml | - 200 mM NaCl | - 200 mM NaCl |
| - 1 mM EDTA (0.5M) <u>1</u> ml | - 1 mM EDTA | - 10% Glycerol |
| - 1mM DTT (1M) <u>500</u> μl | - 1 mM DTT | - 1% (34mM) βOG |
| - 1 mM PMSF (in ethanol) <u>1</u> ml | - 1% Triton X100 | - 1mM DTT |
| - 1X PIC (100X stock) <u>500</u> μl | - 0.2 mM PMSF (in isopropanol) | - 10mM Maltose |
| | - 1X PIC (100X stock) | |

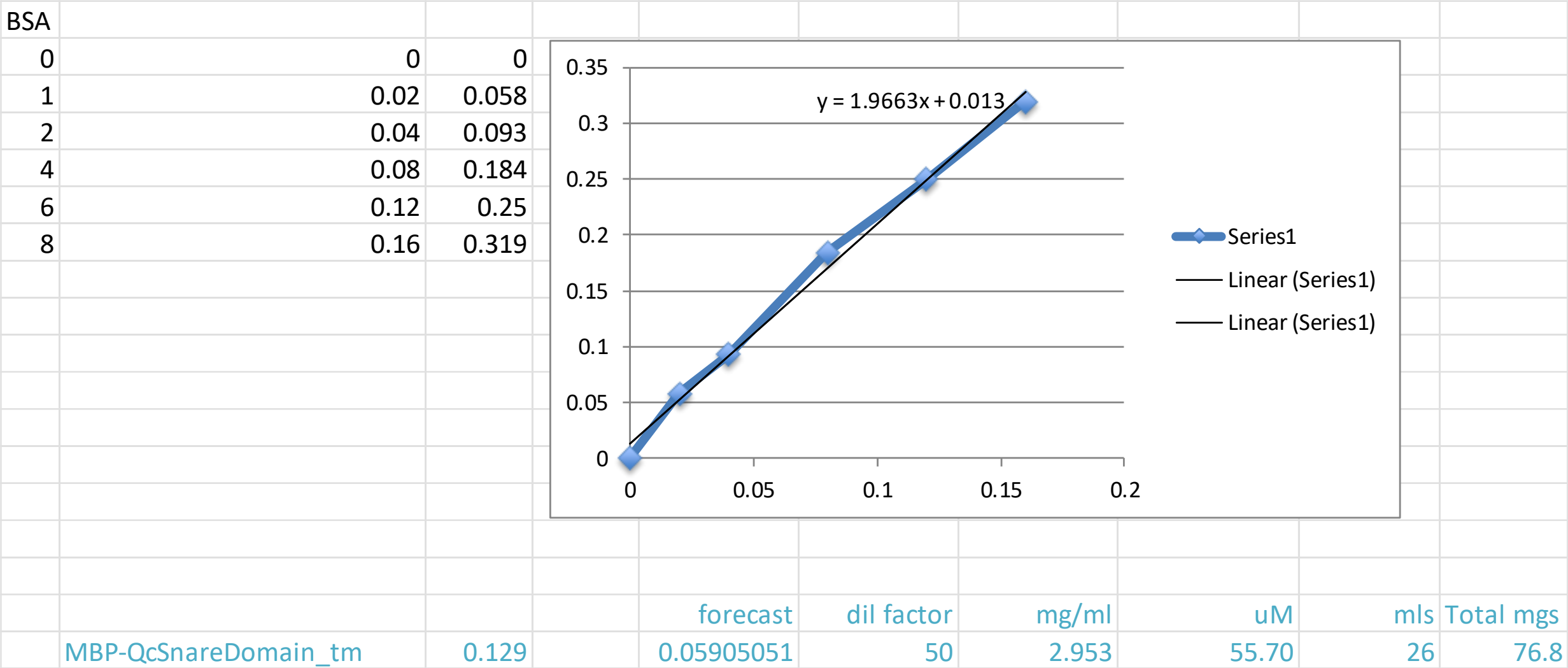
Samples that should have the protein of interest.
 Samples that should NOT have the protein of interest.

| MBP-Qc-SD-tm | | | | | | | | | | | |
|------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| F: 1-14 = 26 uLs | | | | | | | | | | | |
| 0.629 | 0.620 | 0.648 | 0.672 | 0.722 | 0.797 | 0.813 | 0.790 | 0.875 | 0.774 | 0.685 | 0.702 |
| 0.486 | 0.477 | 0.505 | 0.529 | 0.578 | 0.654 | 0.669 | 0.647 | 0.732 | 0.630 | 0.541 | 0.559 |
| 0.751 | 0.643 | 0.476 | 0.410 | 0.341 | 0.299 | 0.282 | 0.258 | 0.241 | 0.143 | | |
| 0.608 | 0.500 | 0.333 | 0.266 | 0.197 | 0.156 | 0.139 | 0.114 | 0.097 | 0.000 | | |

MBP-QcSnareDomain_tmQb purification



MBP-QcSnareDomain_tmQb purification



Next steps:

- Keep working on optimization of crosslink experiments with JxCys proteins. Solve buffer exchange problem.
- His-Tev-Sec18: test for fusion first/ test tag removal/ filter remove His/ western to check presence of His tag
- Samples for Chuchu: we will try with nanodiscs
- UT-Sec17_D1-30 production