# 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 (4/28-5/2)**

1) Testing His-Tev-Sec18 for fusion. Made without ATP. (Dead!)

2) Production of His-Tev-Sec18 correctly! Induction. Harvest. Affinity purification. Gel

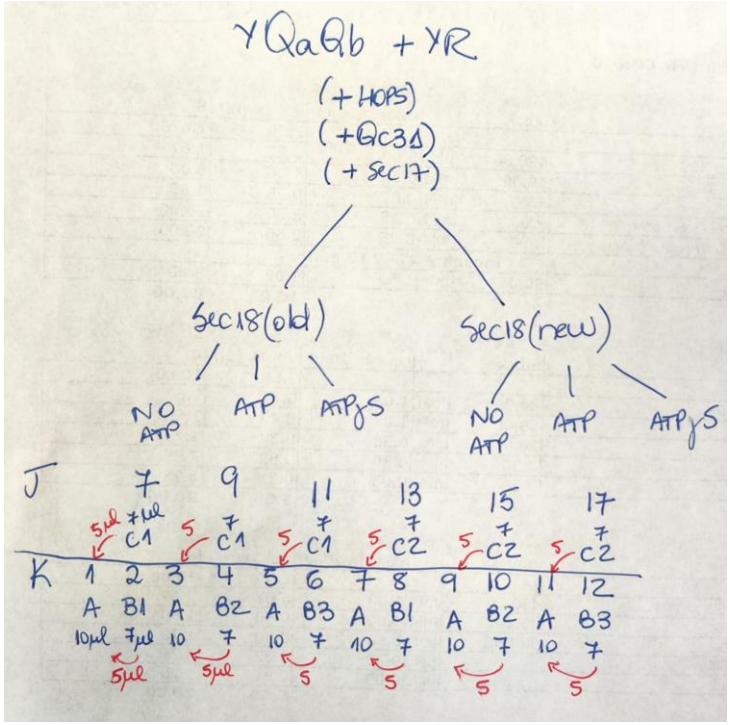
3) Cross-linking optimization: solve buffer exchange problem. First try: freshly add EDTA to buffer. Western. (Nope)

4) Made more ATP free His-Sec18.

5) Re-re-submitted ATP paper

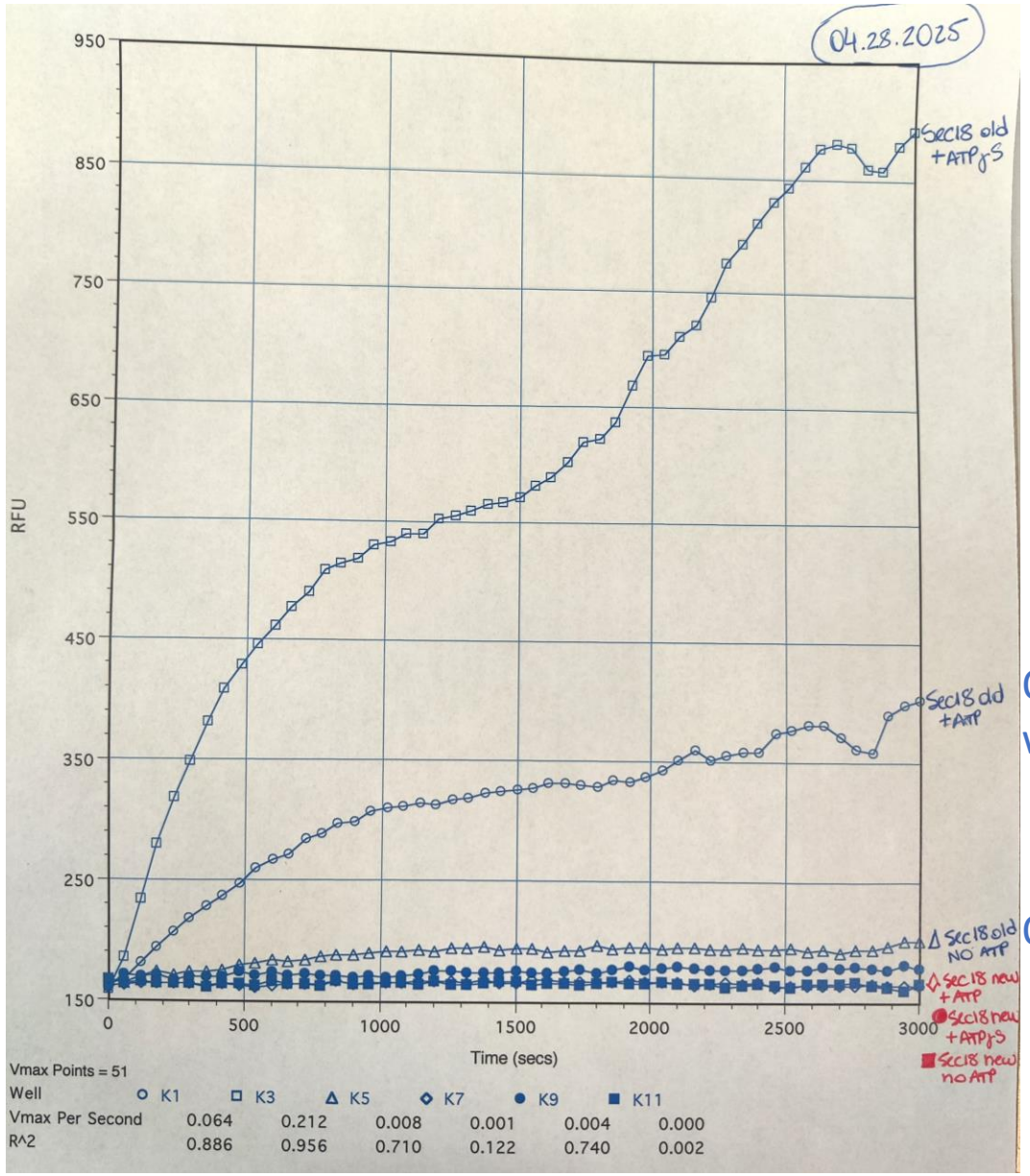
6) Sent GST-QaΔ3 for sequencing

# Testing activity of His-Tev-SEC18 made without ATP




| | FRET Ex:565 Em:670 CO:630 | | | | | |
|---|---|---|---|---|---|---|
| Mix A | **YR+YQab** | | | | | |
| | | Stock | | conc in 20 ul | Vol 1x (µl) | **8** |
| | RB150 | | | | 2.59 | 20.73 |
| | Streptavidin prep 389 box 91 | 480.00 | µM | 5.00 | 0.21 | 1.67 |
| | EDTA pH 8.0 in Rb150 | 10.00 | mM | 0.50 | 1.00 | 8.00 |
| | GTP box 92 | 1.00 | mM | 0.01 | 0.20 | 1.60 |
| | YQab | 2.00 | mM | 0.25 | 2.50 | 20.00 |
| | YR | 2.00 | mM | 0.25 | 2.50 | 20.00 |
| | | **Incubate 27˚C 10 min** | | | | |
| | MgCl2 in Rb150 | 30.00 | mM | 1.50 | 1.00 | 8.00 |
| | | | | | **10.00** | **80.00** |
| Mix B1 | **ATP mix** | | | | | |
| | | Stock | | conc in 20 ul | Vol 1x (µl) | **4** |
| | Rb150 | | | | 2.50 | 10.00 |
| | MgCl2 in Rb150 | 30.00 | mM | 1.00 | 0.67 | 2.67 |
| | ATP box 93 | 50.00 | mM | 1.00 | 0.40 | 1.60 |
| | Sec17 box 89 | 20.00 | µM | 0.30 | 0.30 | 1.20 |
| | Qc3D box 9 Dilute 1:10 | 7.30 | µM | 0.30 | 0.82 | 3.29 |
| | HOPS box 115 | 3.20 | µM | 0.05 | 0.31 | 1.25 |
| | | | | | **5.00** | **20.00** |
| Mix B2 | **ATPgS Mix** | | | | | |
| | | Stock | | conc in 20 ul | Vol 1x (µl) | **4** |
| | Rb150 | | | | 2.50 | 10.00 |
| | MgCl2 in Rb150 | 30.00 | mM | 1.00 | 0.67 | 2.67 |
| | ATPgS box 148 | 50.00 | mM | 1.00 | 0.40 | 1.60 |
| | Sec17 box 89 | 20.00 | µM | 0.30 | 0.30 | 1.20 |
| | Qc3D box 9 Dilute 1:10 | 7.30 | µM | 0.30 | 0.82 | 3.29 |
| | HOPS box 115 | 3.20 | µM | 0.05 | 0.31 | 1.25 |
| | | | | | **5.00** | **20.00** |
| Mix B3 | **NO ATP Mix** | | | | | |
| | | Stock | | conc in 20 ul | Vol 1x (µl) | **4** |
| | Rb150 | | | | 2.90 | 11.60 |
| | MgCl2 in Rb150 | 30.00 | mM | 1.00 | 0.67 | 2.67 |
| | Sec17 box 89 | 20.00 | µM | 0.30 | 0.30 | 1.20 |
| | Qc3D box 9 Dilute 1:10 | 7.30 | µM | 0.30 | 0.82 | 3.29 |
| | HOPS box 115 | 3.20 | µM | 0.05 | 0.31 | 1.25 |
| | | | | | **5.00** | **20.00** |
| Mix C1 | **Old Sec18** | | | | | |
| | | Stock | | conc in 20 ul | Vol 1x (µl) | **5** |
| | Rb150 | | | | 4.10 | 20.52 |
| | Sec18 box 393 | 6.70 | µM | 0.30 | 0.90 | 4.48 |
| | | | | | **5.00** | **25.00** |
| Mix C2 | **New HIS-Tev-Sec18** | | | | | |
| | | Stock | | conc in 20 ul | Vol 1x (µl) | **5** |
| | Rb150 | | | | 3.54 | 17.68 |
| | Sec18 box 406 Dilute 1:10 | 4.10 | µM | 0.30 | 1.46 | 7.32 |
| | | | | | **5.00** | **25.00** |

# Testing activity of His-Tev-SEC18 made without ATP




Old friend His-Sec18 with ATPgS

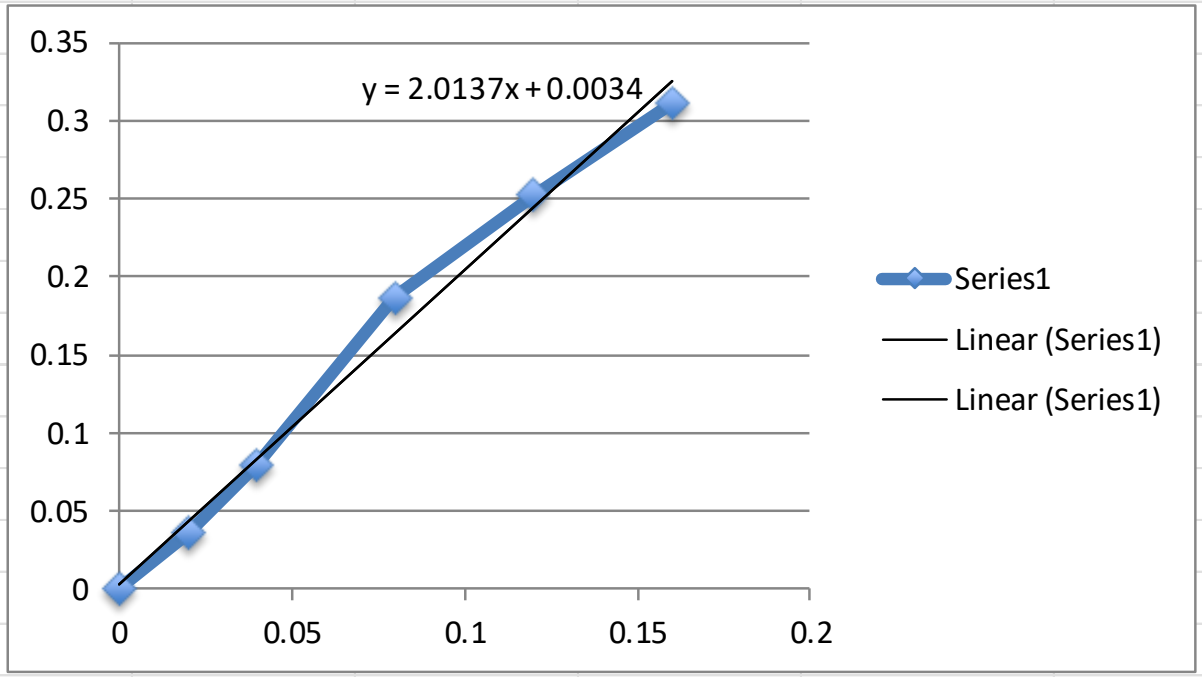
Old friend His-Sec18 with ATP

Old friend His-Sec18 NO ATP

With ATP, ATPgS or NO ATP…is DEAD!

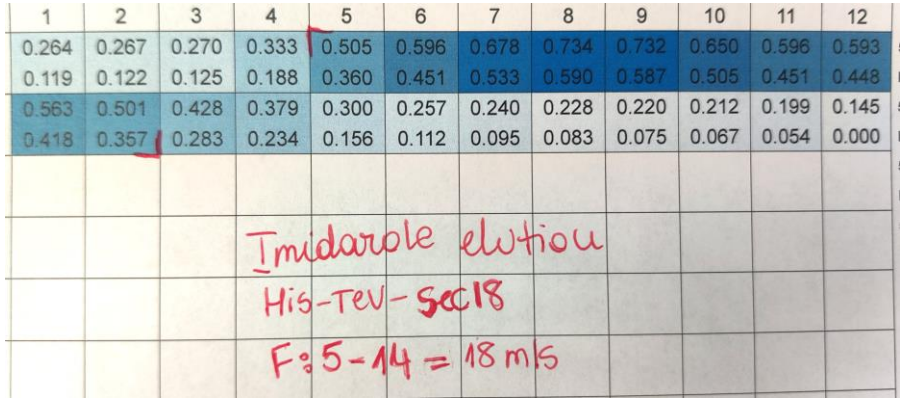
# ATP free SEC18

| BSA | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0 | | | | | | | |
| 1 | 0.02 | 0.036 | | | | | | | |
| 2 | 0.04 | 0.08 | | | | | | | |
| 4 | 0.08 | 0.187 | | | | | | | |
| 6 | 0.12 | 0.252 | | | | | | | |
| 8 | 0.16 | 0.311 | | | | | | | |




| | | | | forecast | dil factor | mg/ml | uM | mls | Total mgs |
|---|---|---|---|---|---|---|---|---|---|
| | Sec18 ATP free | 0.12 | | 0.05804846 | 5 | 0.290 | 3.4 | 1.4 | 0.4 |

# HIS-Tev-SEC18 purification



| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.264 | 0.267 | 0.270 | 0.333 | 0.505 | 0.596 | 0.678 | 0.734 | 0.732 | 0.650 | 0.596 | 0.593 |
| 0.119 | 0.122 | 0.125 | 0.188 | 0.360 | 0.451 | 0.533 | 0.590 | 0.587 | 0.505 | 0.451 | 0.448 |
| 0.563 | 0.501 | 0.428 | 0.379 | 0.300 | 0.257 | 0.240 | 0.228 | 0.220 | 0.212 | 0.199 | 0.145 |
| 0.418 | 0.357 | 0.283 | 0.234 | 0.156 | 0.112 | 0.095 | 0.083 | 0.075 | 0.067 | 0.054 | 0.000 |

Imidazole elution
His-Tev-Sec18
F: 5-14 = 18 mls

**BILL'S SUPER Sec18 PURIFICATION PROTOCOL**

- Inoculate 300mls LB+200ug/ml Amp, 20ug/ml Tet in a 2L flask with pQE9-His6-Sec18/XL1 Blue (aka CBB214)
- Grow 37˚C O/N with shaking at 200rpm
- Transfer 3x 90mls into 3x 3L of LB/Amp/Tet in 6L flasks.
- Grow to OD600 ~ 1.0
- Add IPTG to 0.5mM and grow additional 4 hrs, shaking at 37˚C
- Spin 5min, 5K, 23˚C in JA10.5 rotor
- Resuspend in total vol of 300mls Lysis Buffer +0.5mM PMSF
- Spin 5min, 5K, 23˚C in JA10.5 rotor
- Resuspend in total vol of 150ml Lysis Buffer +0.5mM PMSF
- Freeze dropwise in liquid Nitrogen
- Thaw in glass beaker by swirling in RT water. Keep an eye on it so it stays cold.
- French Press 2 passes at 4˚C
- Spin in 60Ti, 2˚C, 50K, 1hr
- Freeze cytosol dropwise in liquid Nitrogen
- Thaw as before, mix with 1/50$^{th}$ volume of 1M Imidazole-Cl pH 7.0
- Add 12mls Ni-NTA beads equilibrated in Buffer A at 4˚C.
- Nutate 1hr at 4˚C
- Pour into a 1.5cm column, drain, then wash with 100ml Buffer A at 4˚C.
- Step-elute with 50ml Buffer B at 4˚C, collecting 25x2ml fractions.
- Do a quick Bradford (10ul fraction + 190ul Bradford reagent) to find peak, and pool.
- Snap-freeze in 1ml aliquots for future gel filtration step.

**Lysis Buffer:** 100mM HEPES-KOH pH 7.0, 0.5M KCl, 5mM ATP, 5mM MgCl2, 0.014% B-Mercaptoethanol, 1x PIC

**Buffer A (wash):** 20mM HEPES-KOH pH 7.0, 480mM KCl, 0.5mM ATP, 0.014% B-Me, 10% glycerol, 20mM ImidazoleCl pH 7.0, 1mM MgCl2

**Buffer B (elution):** 20mM HEPES-KOH pH 7.0, 0.5mM ATP, 0.014% B-Me, 10% glycerol, **500mM** ImidazoleCl pH 7.0, 1mM MgCl2

# HIS-Tev-SEC18 purification

| BSA | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 0 | 0 | 0 | | | | | | | |
| 1 | 0.02 | 0.049 | | | | | | | |
| 2 | 0.04 | 0.099 | | | | | | | |
| 4 | 0.08 | 0.168 | | | | | | | |
| 6 | 0.12 | 0.237 | | | | | | | |
| 8 | 0.16 | 0.302 | | | | | | | |
| | | | | | | | | | |
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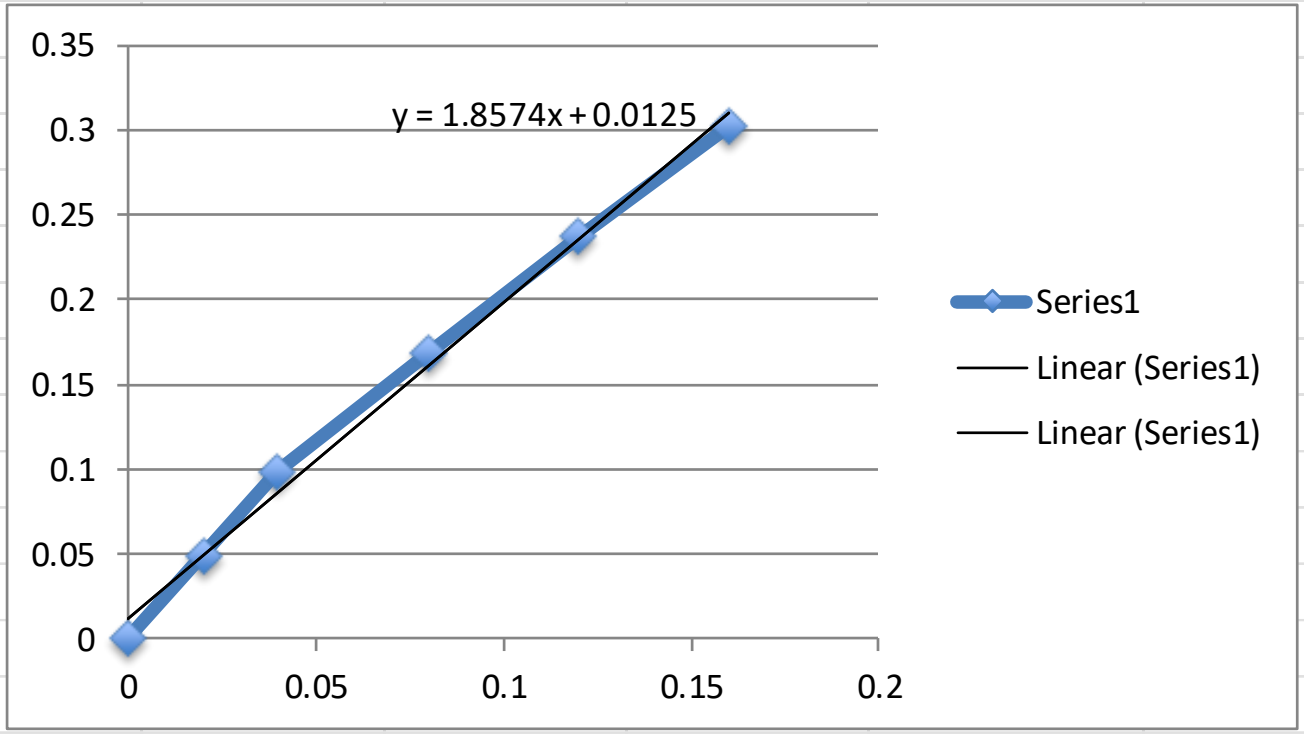
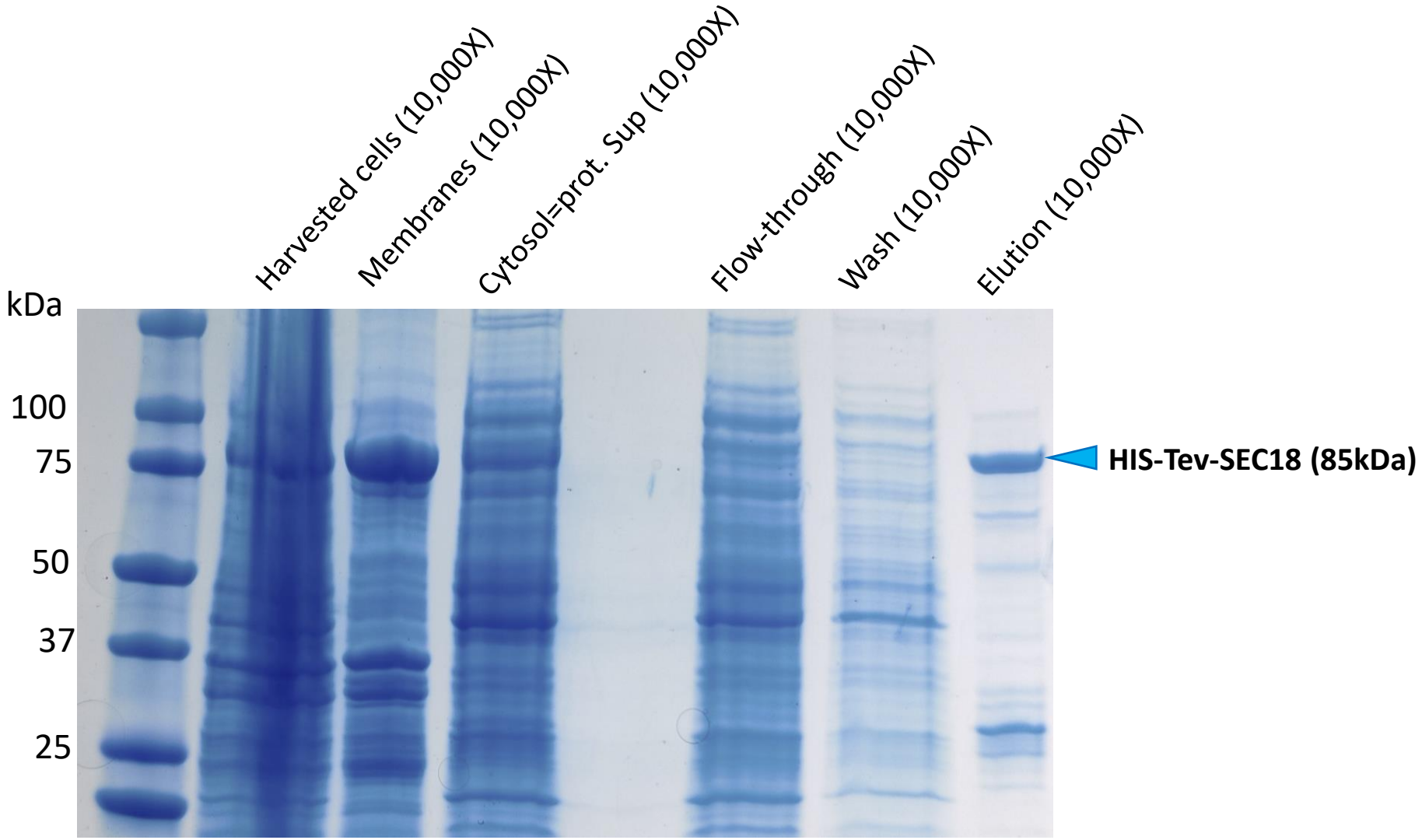
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | forecast | dil factor | mg/ml | uM | mls | Total mgs |
| | His-Tev-Sec18 | 0.077 | | 0.03495691 | 50 | 1.748 | 20.6 | 18 | 31.5 |



# HIS-Tev-SEC18 purification



# GST-Pull down of 4-Snare complex

## 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 µl)

| Qa$_{1b2a}$ + R$_{1b2a}$ (R0C228) | | | | |
|---|---|---|---|---|
| **Protein amount** | **Box** | **Prep** | **Concentration** | **Volume to add in 100µl** |
| 4 µM GST-His-Vam7 (uncleavable) | 65 | 392 | 138 µM | 3.2 µl |
| 4 µM cleaved MBP_Qa-3C262 | 342 | 526 | 15 µM | 26.7 µl |
| 4 µM cleaved MBP_R0C228 | 380 | 580 | 14 µM | 28.6 µl |
| 4 µM untagged Vti1 | 195 | 421 | 44 µM | 9.2 µl |
| 50mM DTT (1M stock) | | | | 5.0 µl |
| 1X Buffer P + 1% β-OG | | | | 27.3 µl |

| Qa$_{1b2a}$ + R$_{1b2a}$ (R0C227) | | | | |
|---|---|---|---|---|
| **Protein amount** | **Box** | **Prep** | **Concentration** | **Volume to add in 100µl** |
| 4 µM GST-His-Vam7 (uncleavable) | 65 | 392 | 138 µM | 3.2 µl |
| 4 µM cleaved MBP_Qa-3C262 | 342 | 526 | 15 µM | 26.7 µl |
| 4 µM cleaved MBP_R0C227 | 375 | 561 | 10.2 µM | 39.2 µl |
| 4 µM untagged Vti1 | 195 | 421 | 44 µM | 9.2 µl |
| 50mM DTT (1M stock) | | | | 5.0 µl |
| 1X Buffer P + 1% β-OG | | | | 16.7 µl |

| Qa$_{1b2a}$ + R$_{1b2a}$ (R0C229) | | | | |
|---|---|---|---|---|
| **Protein amount** | **Box** | **Prep** | **Concentration** | **Volume to add in 100µl** |
| 4 µM GST-His-Vam7 (uncleavable) | 65 | 392 | 138 µM | 3.2 µl |
| 4 µM cleaved MBP_Qa-3C262 | 342 | 526 | 15 µM | 26.7 µl |
| 4 µM cleaved MBP_R0C229 | 381 | 570 | 7.8 µM | 51.3 µl |
| 4 µM untagged Vti1 | 195 | 421 | 44 µM | 9.2 µl |
| 50mM DTT (1M stock) | | | | 5.0 µl |
| 1X Buffer P + 1% β-OG | | | | 4.6 µl |

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 µg of protein/ µl of resin
   Slurry is 75%, so 55µl of slurry = 40µl 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.1qq1

# Crosslink optimization: freshly add EDTA to buffer

05.01.2025

With 4-share samples 3Rs

* Buffer exchange with freshly add EDTA to Rb150

- wash resin 4x
- spin 2min 1,000xg
- Apply 40µl of each (will collect ~45µl)

* Cross-link with BMOE

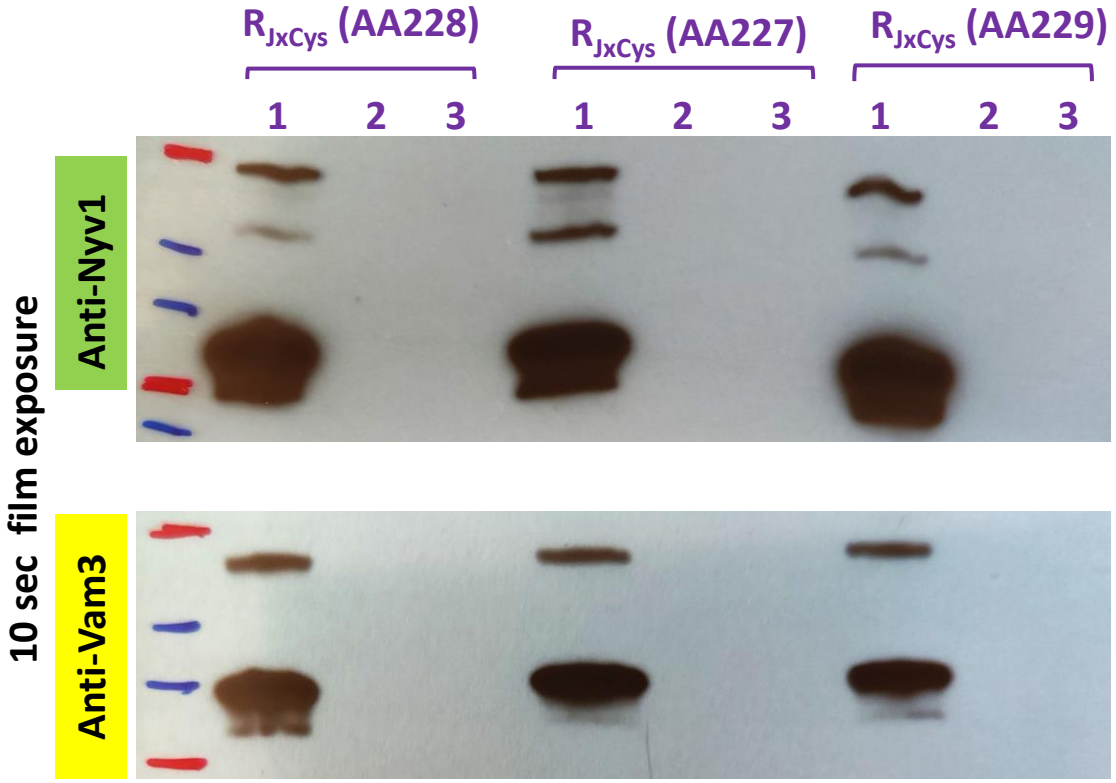
- Prepare 20mM in DMSO : 2.3mg / 500µl 2.0/435µl
- Diute 10x = 2mM
- Add 4µl to 20µl sample
- Incubate 1h, RT
- Add 1µl 1M DTT to stop Rxn

- Run samples for α-Nyv1 and α-Vam3

Gel

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|
| NW | BBE | ABE | CL | BBE | ABE | CL | BBE | ABE | CL |
| | R(228) | | | R(227) | | | R(229) | | |

1) Before buffer exchange
2) After buffer exchange
3) Crosslinked with BMOE



## Next steps:

- Statistics for Bill
- Check sequencing results of GST-QaDelta3
- 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