

Purification of H₆-TEV-Rop (and variants)

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DAY 1

1. Grow a 25 mL overnight culture (2YT/Kan). 1X Kan is 35 µg/mL from a sterile-filtered 1,000X stock (35 mg/mL) in water. 2YT is 16 g Bacto-Tryptone, 10 g yeast extract and 5 g NaCl per liter, autoclaved.

DAY 2

2. Inoculate 1 L of 2YT/Kan with the 25 mL overnight. Grow to an OD₆₀₀ of approximately 0.75 (0.6-1.0 is fine). Induce with a final concentration of 0.1 mM IPTG. Grow for 3-4 more hours at 37 °C or overnight at 30 °C.
3. Spin down in GS3 tubes (6k rpm 10 min) and decant media. Store overnight at -80 °C.

DAY 3

4. Resuspend pellet in 25 mL of lysis buffer (50 mM Tris•HCl pH 8, 300 mM NaCl, 10 mM imidazole, 2 mM β-mercaptoethanol).
5. Add the following in this order, mixing gently after each addition (assuming a 30 mL final volume):

2 M MgCl ₂	75 µL (5 mM final)
100 mM CaCl ₂	150 µL (0.5 mM final)
DNase I	5 µL
RNase A	5 µL

6. Dissolve 30 mg of HEW lysozyme in 1 mL lysis buffer. Add to the lysis solution and mix gently. Add 300 µL of 10% Triton X-100 (0.1% final). Incubate on ice 30 min.
7. Sonicate three times at 50% power for 30 sec on ice. Allow 2 minutes between each pulse to cool on ice. Sonicate further if the viscosity of the solution is not acceptably low.
8. Transfer to SS34 tubes. Spin 20k rpm (30k g) for 1 h, 4 °C.
9. Decant the supernatant (S30) to a fresh 50 mL conical tube. (Save 10 µL of S30 and a small amount of P30 for gel analysis.) Add 1.5 mL of Qiagen 50% slurry of Ni-NTA agarose. Allow to mix in the cold room for 1 h.
10. Add to a large, pre-fritted column. Save 10 µL of the flow-through for gel.
11. Wash the media twice with 6 mL of wash buffer (50 mM Tris•HCl pH 8, 300 mM NaCl, 20 mM imidazole, 2 mM βME). Save 10 µL of each wash for gel.
12. Elute three times with 1 mL of elution buffer (50 mM Tris•HCl pH 8, 300 mM NaCl, 250 mM imidazole, 2 mM βME).

13. Run a gel of the following (18% SDS-PAGE, 200V). Denature the sample at 95 °C for 10 minutes and then spin for 10 minutes before loading.

RPN755 marker	2.5 μ L + 2.5 μ L SDS Loading Buffer (SLB)
S30	1.5 μ L + 1.5 μ L SLB
P30	Add an equal volume of SLB and load about 3 μ L
Flow-through	1.5 μ L + 1.5 μ L SLB
Wash 1	10 μ L + 10 μ L SLB
Wash 2	10 μ L + 10 μ L SLB
Elution	2.5 μ L + 2.5 μ L SLB

14. Add 1 mL of lysis buffer to the 3 mL pooled elutions. Add DTT to 5 mM. Add half an aliquot (50 μ L) of AcTEV protease (Invitrogen) and incubate overnight (> 8h) at room temperature or 3 hours at 30 °C without agitation. Then, add the second half of the aliquot and another 5 mM DTT and incubate at 30 °C another 1-3 h.
15. Check the progress of the scission reaction on a gel. If necessary, add another aliquot of 5 mM DTT and another half-aliquot of protease and incubate again.

DAY 4

16. Equilibrate two PD10 columns with 25 mL of lysis buffer (each). Apply 2.5 mL (half) of the cleaved eluent to each PD10 column and let it run through to waste. Apply 3.5 mL of lysis buffer to each column and collect the eluents.
17. Set aside 10 μ L of the rTEV cleavage reaction for gel. Add 1.5 mL of 50% Ni-NTA agarose slurry. Allow to mix in the cold room for 1 h.
17. Load into a small, pre-fritted column. **Collect the flow-through.** (Note: this is where your protein is!)
18. Wash the media twice with 4 mL of lysis buffer. **Collect the washes.**
19. Elute twice with 1 mL of elution buffer. Save the eluent until you have completed the gel analysis.

20. Acetone precipitate the samples for gel analysis.

<u>Sample</u>	<u>Amount</u>	<u>Water</u>	<u>Acetone</u>
Eluent b/f cleavage	2 μ L	18 μ L	80 μ L
rTEV reaction	2 μ L	18 μ L	80 μ L
Flow-through	2 μ L	18 μ L	80 μ L
Flow-through	4 μ L	16 μ L	80 μ L
Flow-through	10 μ L	10 μ L	80 μ L
Wash 1	20 μ L	--	80 μ L
Wash 2	20 μ L	--	80 μ L
Eluent	10 μ L	10 μ L	80 μ L

Incubate the samples at -20 °C for at least 1 h. Spin 30 minutes and discard the supernatant. Allow to air dry or speed vac to dryness. Dissolve each sample in 2.5 μ L of water. Add 2.5 μ L of SLB, denature at 95 °C for 10 min, spin 10 minutes and then load the gel. Remember to denature and load 2.5 μ L of RPN755 marker with 2.5 μ L of SLB.

21. Obtain a mass spec of the protein (MALDI or ES). Obtain a UV-VIS spectrum from 200 to 500 nm (noting that 1 mg/mL will give an absorbance around 1 at 280 nm).

For crystallography, gel filtration chromatography may be necessary to remove the remaining contaminants. Also, if nucleic acid contamination is a problem, we might wish to add a second RNase A step after the sonication (5 μ L, 15 min, 37°C).

22. For storage, concentrate the protein to about 1 mL (about 5 mg/mL) using a Centricon 3. Dialyze the protein twice against 10% glycerol storage buffer and once against 50% glycerol storage buffer (use a 3500 mw Slide-A-Lyzer). Protein can then be stored at -20 °C or snap-frozen on N₂(l) and stored at -80 °C.

10X storage buffer, no glycerol or DTT

200 mM Tris•HCl pH 8

500 mM NaCl

10% glycerol storage buffer (1 L)

100 mL glycerol

100 mL 10X storage buffer

800 mL water

2 mL 1 M DTT* (2 mM final)

50% glycerol storage buffer (500 mL)

250 mL glycerol

50 mL 10X storage buffer

200 mL water

1 mL 1 M DTT* (2 mM final)

*Store 1 M DTT frozen at -20 °C.

DAY 4, LARGE PREP

16. Dialyze the TEV reaction into lysis buffer at 4 °C. The 10 kD Slide-a-lyzers work well for this. A minimum of two 30-fold dilutions should be accomplished.
17. Pre-cycle 1.5 mL of 50% Ni-NTA slurry with lysis buffer. Add it to the dialyzed TEV reaction. Allow to mix in the cold for 1 h.
18. Load into a pre-fritted column. **Collect the flow-through. This is where your protein is!**
19. Wash the media twice with 1.75 mL lysis buffer. Add DTT to 5 mM.
20. Concentrate to about 2.5 mL with a YM-10 concentrator.
21. FPLC. Gel filtration chromatography is carried out on an S75 16/60 column in 50 mM NaPhos (6.3), 300 mM NaCl, 5 mM DTT. The column should be washed with 45 mL water, 90 mL buffer, and then run for about 150 mL. After a void volume of about 35 mL, the protein elutes between 40-60 mL (i.e., 75-95 mL from injection).
22. Run a gel to confirm the location and purity of the protein. Pool fractions and concentrate to 2.5 mL with a YM-10 concentrator.
23. For crystallography, use a PD10 to exchange into 10 mM PIPES (6.5), 50 mM NaCl, 0.5 mM DTT.