# Synthesis of H3 with modified tail by one pot native chemical ligation

**Expressing H3(15-135, A15C)-6xHis-SUMO protein**

Harvest construct:

1. Pipet 5mL LB into culture tube, turn on flame for sterilization. Add 5uL of Kanamycin(-20 fridge, let thaw first) to LB.

2. Use a pipet tip to scrap some frozen DH5 cells with pET28b-H3(15-135, A15C)-6xHis-SUMO construct. (Idelisse’s inventory, #111) Eject tip into LB with Kan. Grow cell in 37 incubator overnight.

3. Miniprep to harvest plasmid. Use water to elute DNA. Concentration above 10ng/ul should be good enough for transformation.

Make 4L LB:

4. Take 4 plastic flasks, fill with 1L MilliQ water. Add 25g LB broth to every bottle. Cap bottles with aluminum foil, tag sterilize tape to foil cap and autoclave for 1h. Add antibiotics after cooled down or before adding cells to prevent heat inactivation.

Transformation to Rosetta cell:

5. Take Rosetta-pLysS competent cell from -80 fridge and thaw on ice.

6. Add 5uL of plasmid to cell near flame, flick the tube gently with finger to mix. Sit on ice for 10min.

7. Heat shock at 42 for 45s.

8. Add 250uL of SOC near flame.

9. Incubate at 37 with turning for 40min.

10. Take KanR CamR plate and streak the plate. Grow in 37 incubator overnight.

11. Pick a colony with pipet tip and inoculate in 60mL of LB with Kanamycin and Chloramphenicol. Grow in 37 overnight.

Inoculate and grow Rosetta cell:

12. For each 1L bottle of LB, add 1mL Kanamycin and 1mL Chloramphenicol. Take 1mL of LB as blank for absorbance checking.

13. Add 10mL of cell culture to each bottle. Incubate at 37, RPM=195.

14. Measure OD600 after 2h growth. Wait until the absorbance is about 0.5-0.6 (doubling time is about 20min, Induce after 20min if OD­600=0.3), add IPTG to each bottle such that each bottle has 300uM IPTG. Induce for 2h.

15. Harvest cell by centrifuging at 8000g at room temperature for 5min. Discard supernatant and flash freeze the cell pellet with liquid N2 and save cells in -80 fridge.

Cell lysis and H3 extraction:

15. Make following buffer

Wash buffer day 1 (500mL)

50mM Tris-HCl, pH 7.5

100mM NaCl

1mM EDTA

1mM benzamidine

TW buffer (500mL)

Wash buffer with 1% TritonX-100

Unfolding buffer (500mL)

7M GnHCl pH 7.9

50mM TrisHCl

2mM DTT (add before use)

Wash buffer day 2 A (200mL)

6M GnHCl pH 7.9

50mM Tris HCl

150mM NaCl

5mM imidazole

Wash buffer day 2 B (200mL)

50mM TrisHCl pH 8.2

150mM NaCl

6M urea

Elution buffer (200mL)

50mM TrisHCl pH 8.2

150mM NaCl

4.5M urea

500mM imidazole

Dilution buffer (1000mL)

50mM TrisHCl pH 7.5

150mM NaCl

1.5M urea

100mM L-arginine

10mM L-cysteine

2mM DTT (add before use)

* Remember to add DTT especially for the tag cleaving step.

16. Retrieve half of the cell, thaw in 37. Take a 50mL beaker and add wash buffer day 1 to about 30 mL volume, resuspend the cell by pipetting.

\* If the cell lysed it would become gooey due to DNA, try to make it homogeneous as possible.

17. Transfer to a small beaker for sonication. Keep the beaker on ice, try to let the sonicator tip submerge as much as possible but do not contact the beaker bottom. Sonicate with 75% amp, 0.5s on 0.5s off, 630s, rest 1min between each sonication.

\* readjust the tip height every time for best efficiency

18. centrifuge for 20min at 4, 25000rpm.

\* use Ti-45 rotor, balance the tubes before centrifuging.

19. Take ~1mL of sample from supernatant and discard.

20. Wash with 25mL TW buffer by resuspending and pipetting. Centrifuge for 10min, 25000 rpm, 4. Save sample of the supernatant and discard.

21. Wash and centrifuge again with TW buffer, Repeat washing twice with wash buffer day 1. Save sample each time.

\* do not forget to save sample for every wash for gel analysis.

22. (optional) Freeze half of the pellet and save.

\* depend on how much starting material you would like to start with. Half of the pellet is enough to make 10mg of purified H3 fragment. If not freezing half (2L culture), may consider scale up in extraction.

23. Add 400uL DMSO to the pellet, mince with spatula. Soak the pellet at room temperature for 30min.

24. Add 7mL of unfolding buffer, stir at room temperature overnight to extract H3.

\* I did not add DTT in this step and extraction went well.

Ni-NTA column:

25. Set up a Ni-NTA column. Resin is stored in 4 fridge. Rinse the column before use. Take 10mL of mixture which should contain 5mL of resin. Wash the column with ~30mL DI water after loading resin to wash off ethanol. Wash the resin with ~20mL unfolding buffer.

26. Spin down the overnight mixture. (15min, 4, 25000rpm) Save the pellet and incubate the supernatant with resin.

\* transfer the resin and supernatant to a 15mL conical, turn for 1h.

27. Pour the mixture back to the column, collect flowthrough.

28. Wash with wash buffer day 2 A 215mL (3 column volume). Wash again with wash buffer day 2 B 15mL. Collect and save the washes.

29. Elute with 37.5mL elute buffer, use the same incubation method as step 26, incubate for 15min for each elution. Collect elutions in 15mL conicals.

30. Analyze all the samples by SDS gel. Samples include: Sonicate supernatant, TW1, TW2, D1W1, D1W2, pellet, D2 FT, D2WA1, D2WA2, D2WB, D2E1, D2E2, D2E3.

\*For gel sample, some may precipitate due to high urea concentration. Also for the pellet, pick a little bit and resuspend in ~15uL water. Try to load to wells for these sample with solid.

\*1:1 mix sample and loading buffer, heat at 95 for 5min. Take 20% SDS gel, load 4uL ladder for every gel, run at 200V for 30min. Stain for 20min. Destain until bands are visible.

31. Major band should be found at about 30kDa for all elutions. Pool the elutions and determine concentration by Nanodrop or Bradford assay.

\* Nanodrop: use protein A280, 30kDa, . Nanodrop often underestimate the protein concentration from experience.

\* Bradford: Make 20uL of 2, 1.5, 1, 0.7, 0.5, 0.3, 0.1 ug/uL BSA standard and 1:2, 1:4, 1:10, 1:50, 1:100 dilutions of sample. Add 1mL dye to each sample, vortex then add 150uL to the plate to measure absorbance.

**Cleaving of the SUMO tag**

1. If the concentration is high (>5mg/mL) Dilute to 1mg/mL protein with dilution buffer. If otherwise, TCA precipitate the protein first then dissolve with dilution buffer.

\*For TCA precipitation, add 15% (w/v) TCA. Sit on ice for 30min. Spin down the protein (3min, 3000rpm, 4) Save a sample of supernatant. Wash with 2mL cold H2O or acetone by gentle pipetting, centrifuge shortly and save wash for gel analysis.

2. Add SUMO protease Senp1 to a final concentration of 0.03mg/ml. Turn at room temperature overnight to cleave the tag.

\* Senp1 is in Fatima’s box at -80 fridge. Concentration is 15mg/mL.

3. Run gel to see if the protein is cleaved completely. H3(15-135) has MW=13.8kDa, the band is about the same level as 15kDa ladder. SUMO tag protein is about 17kDa in gel. Protease is about 20kDa.

**HPLC purification of H3 fragment**

1. Add 15%w/v TCA to the cleaved mix, sit on ice for 30 min to precipitate all the protein. Spin at 3000 rpm for 5 min, discard the supernatant. Wash the pellet with ~ 3 volume of cold water by mixing and resuspending. Spin at 3000 for 2 min. Collect the water wash.

\* Use cold water to minimize loss to water wash.

2. Dissolve the protein pellet with 40:60 acetonitrile:H2O. Spin down the insoluble part. Each HPLC run can load 10mL of sample. And the optimal amount of protein loaded is about 12.5mg. Dissolve to concentration of 1.25mg/mL if you want best yield. Or use more concentrated solution to reduce HPLC runs.

\* From experience, load 30-40mg protein, final H3 fragment protein after lyopholyzing is less than 5mg. If 5mg of H3 fragment can be harvest from each HPLC run, the yield is fairly good.

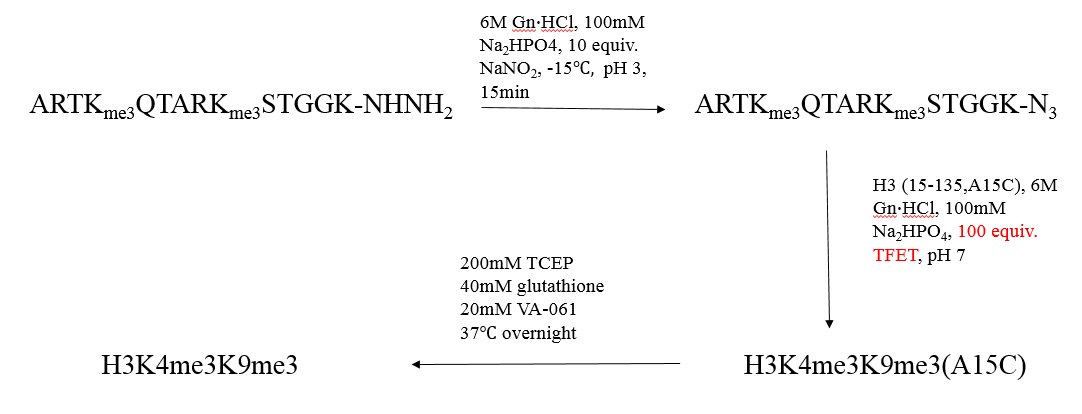
2. For HPLC, first open the top valve and prime the pumps. Then close the valve and start to wash the column with 100% acetonitrile, flowrate=4.7mL/min. After 5min, change gradient to 40% acetonitrile. Wash the loop by injecting H2O and acetonitrile through the loop. Turn on both lamps. Run the protocol “LRP\_40-60..”

3. Locate the fractions with H3 peak (usually about 60). Take 30uL sample from each fraction and speedvap until all liquid is gone. Add H2O and loading buffer to test these fractions with gel. Only collect the fractions without uncleaved band.

4.Pool the fractions, blow off the acetonitrile before lyopholyzing. Use the AIR blower in the hood to blow air to the surface of solution. As the volume decrease to about 1/3 of original volume, nanodrop and lyopholyze.

\* Important: Remember to weigh the tube before lyopholyzing to determine the weight of H3 fragment.

**Ligation**



1. Make buffers:

6M Gn HCl

100mM NaH2PO4.

100mL, Split to two 50mL tubes. For one tube adjust pH to 3.0-3.1, for the other one adjust pH to 6.8-7.0.

1. Save a little bit of H3 and dissolve in water as a sample for protein gel and masspec. Determine the stoichiometry. MW(H3 fragment)=13800Da, MW(K4me3,K9me3 peptide)=1587.7Da. Try to make a 2mM concentration of H3. Peptide should be 3 equivalence. Determine the total volume of reaction. Dissolve the peptide in half of the volume with pH=3 buffer in 1.5mL Eppendorf tube. Dissolve H3 in half of the volume with pH=7 buffer in the smallest glass vial.

\*If the amount of peptide is too low to be weighed, Make a 1mg/uL peptide stock solution with water. The stock solution can be saved in 4 for next time.

2. Make 1M stock solution of NaNO2 in water.

3. In a Dewar, mix ice and NaCl in roughly 3:1 proportion. Use a thermometer to monitor temperature until it drops to -15. Some ice may melt during mixing, Pipet out water. Keep the pipet aside to exclude water frequently to keep the temperature low.

4. Cool the peptide and NaNO2 for 10 min. Add 10 equiv. of NaNO2 to the peptide. Pipet to mix. Let react for 15min.

\* Do not let the reaction proceed over 20 min.

\* I left the pipet tip inside the tube. Every 5 min, Use the tip to pipet and mix the peptide solution to mix.

5. Transfer all the activated peptide to the glass vial with H3. Add 100 equiv. of TFET and adjust pH to 7. Place a small magnetic stir bar. Stir at room temperature overnight.

\* TFET is liquid and very hard to pipet, use syringe instead. MW(TFET)=116.11 Density of TFET= 1.305g/mL.

\* Be careful when adjusting pH. Pipet 0.5uL of reaction mix to a pH paper to determine pH. Use 1N NaOH when close to neutral pH. Do not overshoot pH about 10, it will cause hydrolysis of peptide.

\* Can also react at 37 using oil bath. Increasing the temperature can probably increase the yield.

6. Save 2uL sample of reaction mix for masspec. Take 1uL of reaction mix and dilute to 10uL with water. Make a sequential 1:5 dilution and load both samples and H3 before ligation to gel. The new band above H3 band is ligated product. Estimate yield by comparing the band intensity. Expected yield is >60%.

\* can make one or two more dilutions for better comparison of bands.

**Desulfurization**

1. Make 1mL stock solution of 2M TCEP in water, 400mM glutathione in water, 200mM VA-061 in methanol. Degas briefly by bubbling Argon through the solution.

2. Transfer all the reaction mix to an Eppendorf tubel, degas the reaction mix by blowing Argon.

\*If the volume is low, gently blow Argon gas to the surface of the solution. If the volumn is high try gently bubbling Argon gas through the solution. If the protein concentration is high, the solution can become viscous and degassing through the reaction mix may cause massive bubbling and loss of reaction mix.

3. Add reagent to reaction mix to final concentration of 200mM TCEP, 40mM glutathione, 20mM VA-061.

4. Put the Eppendorf tube in a plastic culture tube, incubate at 37 overnight to desulfurize the ligated product.

5. Mix 1uL of pre-desulfurization product and 50uL of water. Do the same for the H3 sample and post-desulfurization product. Add TCEP to 200mM concentration and incubate at 37 for 5-10min. Centrifuge at high speed for 1min to prevent solid to block masspec machine. Transfer the sample to wells in masspec plate and run masspec.

\*For the masspec, use the file name Eriko. Make sure the sample is taken at the right plate position. (1 is on the left, 2 is on the right.) Running sample should take ~10min. For the spectrum, use mouse right click to select only peak portion. Set range to 15000-15600 for the ligated products. A decrease of 32Da should be observed for the main peak.