

Tn5 Purification Protocol

Day 1- Prepare antibiotic stock, LB agar plates.

Antibiotic stock- β

Kanamycin: Final concentration of 30ug/ml

Make up 10ml of a 1000X stock of 30mg/ml, dissolved in H₂O.

-Add 300mg of Kanamycin to 10ml of H₂O

-Vortex until mixed thoroughly

-Filter using a 0.22micron filter and aliquot 1ml into Eppendorf tubes.

-Store aliquots at -20°C.

LB agar plates (1L)-

-Add 20 g/L of Bacto Agar and 25 g/L of LB Broth, Miller to 500ml

-Bring up to 1L

-Add stir bar before autoclaving.

-Autoclave on liquid cycle for 30 minutes (~1 hr total for complete cycle).

-Remove LB Agar from autoclave and allow to cool (<50 °C); bottle should be warm to the touch.

-Add 270 ul Kanamycin antibiotic from 1000X stock

-Mix well on stir plate

-Prepare a steril work area to pour plates, keep a Bunsen burner on while pouring to prevent contamination

-Grab a plate with your other hand and slide it towards the edge of the table, while keeping it closed.

-Once the plate is at the edge, open the lid as if there is an imaginary hinge at one end; so the plate opens like a clamshell.

-Pour the media into the bottom of the plate until it just covers the surface, use a serological pipette ~20-25 mL of media per plate. Do not over fill.

-Close the lid and allow to cool. The media will be solid.

-Leave the plates out for a day if possible so the condensation will evaporate from the plate.

You may place the plates in a 25C incubator overnight.

-Stack plates and slide the plastic sleeve over the top.

-Flip the stack over and seal the plastic sleeve with masking tape.

-Label the tape with the type of media, date produced, and name of individual that produced the stack.

-Store sealed stacks in the refrigerator at 4 °C until use.

Day 2- Transformation- set up on plate overnight (O/N) ~2 hours hands on

1. Add 1ul pETM11-Sumo3-Tn5 construct to 50ul CaCl₂-competent E.coli BL21(DE3) codon + RIL cells—add directly into a test-tube for easy addition of SOC.
2. Incubate on ice for 30 min, 30 sec in 42C, 5 min on ice.
3. Add 250ul of SOC medium, incubate for 1 hour at 37C.

4. Add 50ul and 100ul transformation mixture directly to two separate plates (this allows for either volume to enable the bacteria to grow separate colonies) incubate overnight (O/N) at 37C.
 - Instruction for plating liquid culture
 - I. Ignite the Bunsen burner
 - II. Remove desired culture from the test-tube and pipette directly on agar plate.
 - III. A beaker with 200ml of 70% ethanol and a spreader bar dipped in ethanol, grab and allow excess to drip off.
 - IV. Touch the spreader to the flame to ignite the alcohol and remove. Slowly turning the spreader will prevent burning drops from falling while the alcohol burns. Allowing it to cool slightly before touching culture.
 - a. Tip- touch the side of the agar slightly to determine if the bar is too hot.
 - V. Spread the culture around the plate and recover plate. Put spreader bar back in ethanol.
 - VI. Immediately turnoff the flame and clean up.

Make LB media (2L and 30ml Preculture)-

To make 2L of media, split into 500ml

-Add 12.5g of LB broth to 350ml

-Adjust volume to 500ml

-Autoclave and cool

-Once cool add antibiotic to have 30ug/ml (This can be done the next day)

To make 30ml of preculture media

-Add 0.75g of LB broth to 15ml

-Adjust volume to 30ml

-Autoclave and cool

-Once cool add antibiotic to have 30ug/ml (This can be done the next day)

Day 3- Preculture O/N ~30min hands on

1. Pick a single colony with pipette tip on a P1000 and inoculate in preculture LB.
2. Shake preculture overnight at 37C on 200rpm, no longer than 18 hours.

Make IPTG- working concentration is 1 M in H2O

-2.38g IPTG in 5ml H2O

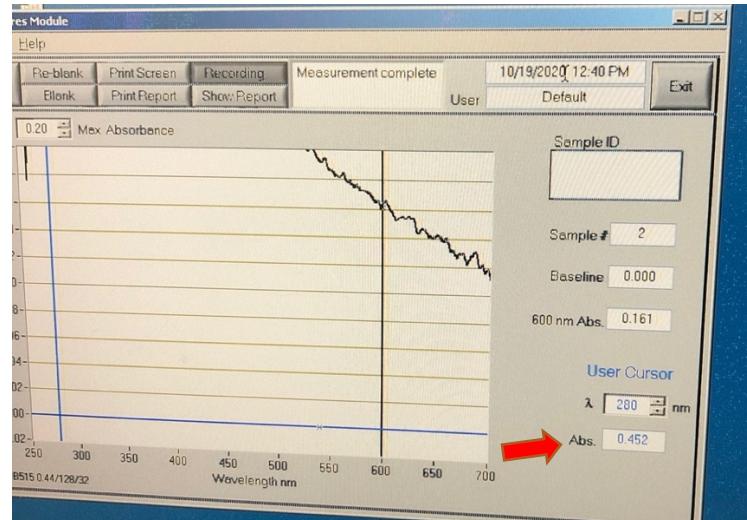
-Adjust volume to 10ml

-Distribute in 1 ml aliquots.

Day 4- Grow up and Express O/N at 18C ~Full day of active set up and checking.

1. Add preculture to LB media, add 7.5ml to each 500ml LB media.
2. Grow culture at 37C and 200rpm until OD600 ~ 0.5 (approximately 3.5 hours).

- a. 1st check after 2 hours. Then check every 30 minutes until ~OD 0.4, then check every 10 minutes.
 - i. Open nanodrop program
 - ii. Select cell culture.
 - iii. Blank with LB.
 - iv. The arrow is pointing to the window to determine the OD of the sample. This need to be ~0.5.



1. Combine the two 500 LB media to make two 1-liter of culture and reduce temperature to 18C (used Mike Schmale's large incubator) for 30 minutes.
2. After 30 minutes, induce the production of recombinant Tn5 by adding 0.2mM IPTG to culture (~200ul/1-liter culture). Allow to continue growing overnight at 18C.

During the two hours wait for the growing of the bacteria make buffers-

Running/wash buffer (500ml)

20mM Hepes-NAOH pH 7.2 – 2.38g

800mM NaCl – 80ml

20 mM Imidazole – 0.68g

1mM EDTA – 1ml

10% glycerol – 50ml

Filter through the 250ml filter bottles, twice (DO NOT AUTOCLAVE)

2mM DTT – 0.154g (DO NOT ADD UNTIL COLUMN PURIFICATION AND RIGHT BEFORE USE)

Lysis Buffer (250ml)

20 mM Hepes-NAOH pH 7.2 – 1.41g

800 mM NaCl – 40ml

20 mM Imidazole – 0.34g

1 mM EDTA – 500ul

10% glycerol – 25ml

0.2% Triton X100 – 500ul

Filter through the 250ml filter bottles (DO NOT AUTOCLAVE)

2 mM DTT – 0.07g (DO NOT ADD UNTIL RIGHT BEFORE USE)

Elution buffer (500ml)

20mM Hepes-NAOH pH 7.2 – 2.38g

800mM NaCl – 80ml

300 mM Imidazole – 10.2g

1mM EDTA – 1ml

10% glycerol – 50ml

Filter through the 250ml filter bottles, twice (DO NOT AUTOCLAVE)

2mM DTT – 0.154g (DO NOT ADD UNTIL RIGHT BEFORE USE)

Day 5- Harvest

1. Harvest cells by centrifugation 4600g for 30 min at 4C. (Use Mike Shmales centrifuge, schedule this time with him to help with setting the centrifuges compensation temperature and conversion of speed)
 - a. Balance 250ml bottles with a balance beam (Clean bottles by first with ethanol, then 3 times with nanopore water). Set lids on first to compensate for the difference in weight.
2. Combine pellets into 50ml conical tubes by using approx. 50 ml (approximately 10-12 ml per bottle) of running buffer to slowly resuspend pellet.
 - a. Tip- use a 10ml serological pipette to resuspend pellet.
 - b. Always keep on ice.
3. Centrifuge at 3000rcf for 30 minutes, store pellet at -80C.
 - a. Storing at -80C aids in the lysis.

Make 10% PEI-

-Add 1g of PEI to 8ml

-Adjust volume to 10ml

-Heat up gradually to dissolve

-Filter using a 0.22micron filter

-Store at -80C

Day 6- Lysis and 1st Nickel Affinity Chromatography

1. Add cold lysis buffer to pellet ~25ml for each pellet to have 50 ml in total and resuspend pellet. Add one pellet of the cOmplete protease inhibitors and shake to dissolve. Save 20ul of sample as a “Lysis” sample
2. Incubate on ice for 30minutes, rotating. Can put a small stir bar in the 50ml conical tube in a plastic cup with ice.
3. Sonicate (Used Danielle McDonalds) 4-6 times for 30 sec intervals on ice, shaking in-between sonication intervals. Save 20 ul lysis sample.

- a. Clean the sonicator with 70% ethanol first and then 2 times with dH₂O. The peddle control the sonicator, press when inserted into sample.
4. Centrifuge at 30,000g for 30 min at 4C, combine and save supernatant.
5. Add 2ml 10% PEI dropwise.
 - a. This should be done dropwise over 30 minutes. Making sure the PEI is fully incorporated before adding more drops. This is an important step.
6. Centrifuge at 20,000g for 10 minutes at 4C.
7. Filter supernatant with 0.44uM Millipore filter, save 20 ul as “post-PEI sample”.

In cold room, 1st Nickel affinity chromatography, cOmplete His-Tag purification column, 5ml.

- a. Add 5ml of resin to the column.
8. Equilibrate column with water first, then with running buffer 5 column volume (CV), this means 25ml.
9. Load all supernatant slowly to column, incubate for 30 minutes.
10. Collect flow through in 50 ml conical tube, save 20ul as a “FT” sample.
11. Wash the column with 10 CV (50ml) of wash buffer.
12. Elute column with elution buffer collecting in 5ml fractions a total of 10 CV (50ml).
13. Run SDS gel with lysis, post-PEI, and FT and the 10 fractions.
 - a. See page 8 on how to make gels.
 - b. 20ul of sample with 10ul of 3X buffer Laemli Sample Buffer.
 - c. Place gel in the cassette with combs facing away from you and lower into the box.
 - d. Pour buffer into the center of the gel cassette and take out comb carefully.
 - e. Use protein loading tips to get into the wells and do this slowly.
 - f. Once you see the blue bands run all the way off the gel, remove the gel cassette.
 - g. Use the gel opener to carefully open the two glass plates holding the gel together.
 - h. Take the gel and put it into an old pipette tip box and pour Coomassie blue die to just cover the gel.
 - i. Put in microwave for 15 seconds and shake for 15 minutes.
 - j. Pour off the stain (This can be reused).
 - k. Pour Destaining solution to cover the gel and add a kimtech wipe to the side if the box, touching the liquid.
 - l. Microwave for 15 seconds, let sit for approximately 5 minutes. Add more destain if necessary.
14. Pool fractions that have good bands on the gel. Save 20 ul of this sample.



L- Ladder	8- E5
1- Pellet	9- E6
2- Lysate	10- E7
3- FT	11- E8
4- E1	12- E9
5- E2	13- E10
6- E3	14- E11
7- E4	

pooled 8-12

Make dialysis buffer 1L

-11.3g Hepes pH 7.2

-320ml NaCl
 -2.72g Imidazole
 -10ml EDTA
 -200 ml glycerol
 -0.6g DTT

15. Add 1 aliquot of His6-senp2 to remove the tag and dialyze against 1000ml (1L) dialysis buffer O/N at 4C. 1:100 ratio for SENP2: Protein (for ~30ml of sample, 5ul of His6-senp2)
- Obtain dialysis membrane, clamps, and floats from Dr. Crawford's drawer.
 - Get a plastic cup full on dH₂O.
 - Cut membrane according to the volume conversion for the length of the membrane.
 - Place membrane in water to get soft.
 - Place two clamps on close to one of the openings.
 - Pinch the other opening with fingertips and start trying to open slowly.
 - Have a P1000 ready with sample to start loading as soon as a little pocket opens.
 - Load the rest of the sample.
 - Use two clamps on the end, leave enough room to be able to cut after the dialysis.
 - Place floats on ends where the clamp is and place in the dialysis buffer O/N in cold room.

Day 7- 2nd Nickel Affinity Chromatography

- Obtain new column
- Equilibrate column with the 5ml (nickel resin) with water first, then wash column with running buffer 5CV.
- Load the pooled samples after dialysis.
- Collect FT in 50 ml fraction (THIS IS WHERE THE PROTEIN IS)
- Wash column with wash buffer 5CV.
- Elute with elution buffer 50ml and collect in 10 ml fractions.
- Run a SDS gel to determine if the size has changed since the removal of the tag.



L- Ladder	8- E7
1- Pellet	9- E8
2- E1	10- E9
3- E2	11- E10
4- E3	
5- E4	
6- E5	
7- E6	

pooled 2-6

- While the gel is running make the Final Buffer for the second dialysis

Final Buffer (1L)

-25mM Tris pH 7.5 – 25ml
 -800mM 5M NaCl – 160ml

-0.1mM EDTA – 200ul
 -50% glycerol – 500ml
 -1mM DTT – 0.154g

8. Use the Pierce Coomassie Plus (Bradford) Assay Kit to check the concentration of protein.
 - a. Make the standards to know in what range of protein concentration our sample is.

Dilution Scheme for Standard Test Tube and Microplate Protocols (Working Range = 125–1500µg/mL)			
Vial	Volume of Diluent	Volume and Source of BSA	Final BSA Concentration
A	0	300µL of Stock	2000µg/mL
B	125µL	375µL of Stock	1500µg/mL
C	325µL	325µL of Stock	1000µg/mL
D	175µL	175µL of vial B dilution	750µg/mL
E	325µL	325µL of vial C dilution	500µg/mL
F	325µL	325µL of vial E dilution	250µg/mL
G	325µL	325µL of vial F dilution	125µg/mL
H	400µL	100µL of vial G dilution	25µg/mL
I	400µL	0	0µg/mL = Blank

- b. Perform this to know what the sample is.

Microplate Procedures

A. Standard Microplate Protocol (Working Range = 100-1500µg/mL)

1. Pipette 10µL of each standard or unknown sample into the appropriate microplate wells (e.g., Thermo Scientific™ Pierce™ 96-Well Plates, Product No. 15041).
2. Add 300µL of the Coomassie Plus Reagent to each well and mix with plate shaker for 30 seconds.
3. Remove plate from shaker. For the most consistent results, incubate plate for 10 minutes at room temperature (RT).
4. Measure the absorbance at or near 595nm with a plate reader.
5. Subtract the average 595nm measurement for the Blank replicates from the 595nm measurements of all other individual standard and unknown sample replicates.
6. Prepare a standard curve by plotting the average Blank-corrected 595nm measurement for each BSA standard vs. its concentration in µg/mL. Use the standard curve to determine the protein concentration of each unknown sample.

Note: When compared to the Standard Test Tube Protocol, 595nm measurements obtained with the Microplate Protocols are lower because the light path is shorter. Consequently, this may increase the minimum detection level of the assay. If higher 595nm measurements are required, use 15µL of standard or sample and 300µL of Coomassie Plus Reagent per well.

Note: If using curve-fitting algorithms associated with a microplate reader, a four-parameter (quadratic) or best-fit curve will provide more accurate results than a purely linear fit. If plotting results by hand, a point-to-point curve is preferable to a linear fit to the standard points.

9. Due to the imidazole concentration being too high creating a interference, another dialysis was perform using 1L of the Final Buffer
10. Check protein concentration again to determine if concentration needs to be performed.
11. Concentrate to ~1.4ml using 30K Amicon (sample may become yellow, due to protein accumulation).
 - The Pierce Protein Concentrator found where the dialysis materials are
 - Add 5ml to tube and centrifuge at 3,000g for 15 min
 - Discard flow through and pipette concentrated sample up and down.
 - Perform until all sample is done.

Protocol for making gels:**30% Acrylamide**

*** Acrylamide is a neurotoxin. Be very careful, wear gloves/PPE and prepare under the hood.

29g acrylamide

1g of N,N'-methylbisacrylamide

Dissolve in 60ml of water, adjust the volume to 100ml with water.

Filtration through 0.45-micron pore size filter.

Store at 4°C.

You should try to make 10% gels for the protein prep. Let's make these on Friday to check the reagents work. If they work fine, they can be saved in paper towels and buffer for a few days. We can go over how to cast the gels together.

10% Tris-Glycine Resolving Gel**(5.0ml per gel)**

30% Acrylamide/Bis	1.7 ml
1.5 M Tris pH 8.8	1.25 ml
10% SDS	50 uL
ddH2O	1.9725 ml
10% APS	25 uL
TEMED	2.5 ul

4% Tris-Glycine Stacking Gel**(3.75 ml)**

30% Acrylamide/Bis	0.5 ml
0.5 M Tris pH 6.8	0.945 ml
10% SDS	37.5 ul
ddH2O	2.245 ml
10% APS	18.75 ul
TEMED	3.75 ul

Here's the recipe for the running buffer:

10X Tris Glycine Running Buffer: for 1L stock

30g Tris

144g Glycine

10g SDS

H2O to 1L

Dilute to 1X for running gels.

Assemble the gel casting apparatus

1. Assemble the components that you will need for casting the gel: a tall glass plate with attached 1 mm spacers, a small glass plate, a green casting frame and a casting stand.
2. Place the green casting frame on the bench with the green "feet" resting firmly against the bench and the clamps open (perpendicular to the frame) and facing you.
3. Place the two gel plates in the frame. Insert the taller spacer plate with the "UP" arrows up and the spacers facing toward you into the casting frame (the BioRad logo should be facing you). Insert the short glass plate in the front of the casting frame. ***There should be a space between the plates.***
4. Secure the plates in the casting frame by pushing the two gates of the frame out to the sides. IMPORTANT: the bottom edges of the two plates should be flush with the lab bench before you clamp the frame closed to ensure a watertight seal. ***To do this, rest the frame vertically on the bench BEFORE closing the gates.***
5. Clamp the casting frame with glass plates into the casting stand, with the gates of the casting frame facing you. ***Repeat steps 1-5 to prepare a second gel in the casting frame.***
6. Check to see if the assembled plates in the casting stand are sealed properly by pipetting a small amount of isopropanol into the gap between the plates. If the

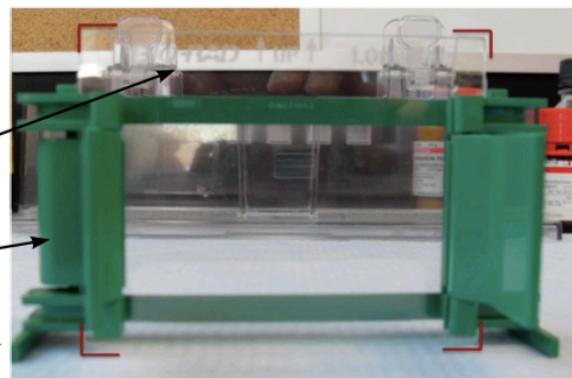
glass plates holds and doesn't leak, you are ready to make the gels.

Assembling plates in the casting frame.

Large spacer plate with spacer goes in rear, oriented so the arrows point up and the logo is readable. Small plate is placed in front. (Corners of the plates are marked with bracket marks.)

Green gates of casting frame are open.

Casting frame "feet" and bottom edges of plates are flush against the benchtop.



7. Label two 15 mL conical tubes "Resolving gel" and "Stacking gel".
8. Prepare resolving gel. Mix the acrylamide solution, **pH 8.8** Tris buffer and water, as shown in the chart above. Mix the ingredients gently, trying not to introduce air. Oxygen inhibits polymerization of acrylamide gels.
9. To the resolving gel mixture, 10% ammonium persulfate (APS) solution. Gently mix the solution, trying not to introduce air. Oxygen inhibits acrylamide polymerization.
10. Add TEMED catalyst. Once again, gently mix in the catalyst trying not to introduce air bubbles. CAUTION: TEMED has an unpleasant odor. Cover the tube immediately after you aliquot this reagent.
11. Working quickly, use a plastic transfer pipette to fill the space between the two plates until the resolving gel solution reaches a height just above the green clamps on the gel casting frame. Draw up any remaining acrylamide into the transfer pipet. (You will know that the acrylamide has polymerized when you can no longer push the acrylamide out of the pipet.)
12. Using a transfer pipet, add isopropanol so that it gently flows across the surface of the polyacrylamide mixture. The layer ensures that the polyacrylamide gel will have a level surface once it polymerizes.
13. Allow the gel to polymerize, which takes ~15-20 minutes. You will note that the interface between the polyacrylamide and isopropanol overlay disappears temporarily while the gel polymerizes. A sharp new interface then forms between the two layers, indicating that polymerization is complete. (You can also check the remaining polyacrylamide in the transfer pipette to see if it has polymerized.)
14. **When polymerization is complete, remove the isopropanol from the top of the resolving gel by tilting the gel to the side and using a paper towel or Kimwipe to wick out the isopropanol.**
15. Prepare the stacking gels. Mix the acrylamide solution, **pH 6.8** Tris buffer and water, as shown in the chart above.
16. Add 30 μ L 10% APS and 7.5 μ L TEMED to the stacking gel acrylamide mixture. Mix the contents by gently inverting the tube twice.

17. Use a transfer pipette to pipette the stacking gel on top of the resolving gel between the two glass plates. Add enough stacking solution until it just reaches the top of the small plate.
18. Carefully, but quickly, lower the comb into position, being careful not to introduce air bubbles. (The Bio Rad logo on the comb should be facing you.) Adding the comb will force some solution out of the gel, but this is fine. If air bubbles become trapped below the comb, remove the comb and reposition it.
19. Once stacking gel has solidified. Carefully remove the gels from the casting stand and then from their green frames.
20. Keeping the combs in the gel, wrap the gels in a paper towel that is wet with buffer. Then wrap the gels in plastic wrap to be used later. ***The gels will be ruined if they are not kept wet and properly wrapped!***