

Procedures

Overnight Culture.

1. Prepare 20 mL of LB medium in a flask.
2. Add 20 μ L of each antibiotic (ampicillin and chloramphenicol) to LB medium.
3. Poke a hole on top of the flask (if an opening is not already present).
4. Add 2 μ L of *E. coli* (BL21 (DE3) coco, pET5_SecA_WT) containing the plasmid (T5) with either the *WT-secA*- or *Y134S-secA*- gene to flask.
5. Place flask in the shaker overnight at 37°C.

Culture preparation.

1. Starting from autoclaved LB medium, add antibiotics at a final concentration of 1X (400 μ L antibiotic / 400 mL LB medium). Keep in mind that the bacteria need to breathe- poke a hole in the lid if not already present.
2. Add 8 mL to 10 mL (2 - 2.5% vol.) of overnight culture to large flask with LB medium and antibiotics.
3. Transfer the culture to a shaker at 37°C.
4. Monitor the absorbance of the culture at 600 nm using a spectrophotometer.
 - a. In a cuvette, transfer 1 mL of LB medium (without bacteria) and measure the absorbance at 600 nm (zero value).
 - b. Every 20 minutes, record the Optical Density (OD) of the culture.

Protein expression.

5. Once the OD reaches a value of 0.6 to 0.8, add 0.5 mM of IPTG (400 μ L from 0.5 M stock solution / 400 mL of LB medium).
6. Transfer the culture to a shaker at 30°C

7. Measure OD of solution every 30 minutes, collect a fraction from the solution (250 μ L for 2.5 OD), and centrifuge briefly at full speed (15 seconds).
8. Discard the supernatant, solubilize the pellet in 25 μ L of Loading buffer, and boil for about 10 minutes.
9. After 2 hours, centrifuge the entire culture for 15 minutes (4000 rpm at 4°C).
10. Remove the supernatant to isolate the pellet.
11. Add 60 mL of supernatant to the pellet and solubilize briefly.
12. Centrifuge the solution a second time for 10 minutes.
13. Remove the supernatant to isolate the pellet and freeze at -20°C until needed.
14. Add bleach to supernatant overnight to kill all remaining bacteria and discard accordingly.

Solubilization and bacterial lysis.

1. Add less than 48 mL of Buffer A to flask with pellet.
2. Shake, vortex, and mix flask with a magnet stirrer to solubilize.
3. Pour solubilized mixture into a French Pressure Cell.
4. Turn the knob on the side of the French Press, collecting lysed material with a new flask from the output tube of the French Press.
5. Transfer solution to the larger French Press Machine and collected lysed material with a flask (if the stream of lysed material is fast and the solution is not clear, repeat this step again).
6. Transfer 5 to 6 mL of the lysed material into a smaller flask.
7. Place flask in the sonication machine (output- 4, duty cycle- 50, time- 30 s, pulsed), raising the flask until the tip of the probe touches the solution in the flask.

8. Repeat steps 6 to 7 until the contents of the entire flask have undergone sonication.
9. Transfer the solution into 24 2-mL test tubes.
10. Centrifuge for 10 minutes (13,300 rpm and 4°C).
11. Consolidate the supernatant (should be clear, and pellet should be yellow) from the small test tubes into a larger flask.

Purification by Immobilized-Metal Affinity Chromatography.

1. Equilibrate the column with 3 column volumes (CV) of water followed by 3 CV of Buffer A (0.8 ml/min).
2. Solubilize one of the test tubes containing a pellet with 2 mL of Buffer A and label I (insoluble).
3. Transfer 2 mL of supernatant to a test tube and label S (soluble).
4. Load the remaining supernatant into column, changing speed to 0.4 mL/min to give SecA time to bind to the cobalt ions.
5. Collect output in a flask labeled FT (flow-through).
6. Wash the column with 3x1 CV of buffer A; collect flow-through from washes (flasks W1, W2, and W3).
7. Elute the protein with 2 CV of buffer B and collect 2-mL fractions.
8. Add 85 µL of Bradford's reagent to 12 new test tubes.
9. Transfer 15 µL of protein from first 12 test tubes (step 7) to corresponding tubes with Bradford's reagent (step 8).
10. Gently shake tubes with reagent and select 8 test tubes with the highest concentration of protein (brightest blue color).
11. Mark the corresponding 8 test tubes from step 7 (without Bradford's reagent).

12. Add 5 μ L of loading buffer to 8 new 1-mL test tubes.
13. Transfer 20 μ L from each of the 8 test tubes in step 11 to the test tubes with the loading buffer in step 12.
14. Transfer 20 μ L of solution from test tubes I, S, FT, W1, W2, and W3 and transfer into 1-mL test tubes; add 5 μ L of loading buffer to each tube.
15. Boil all 14 test tubes from steps 13 and 14, along with ladder for 5 to 10 minutes.
16. Run a SDS-PAGE to monitor the protein elution with all 15 test tubes from step 15.

Desalting.

1. Pool the fractions containing the protein into a centrifugal filter unit (CFU) with a cutoff membrane of 50 kDa.
2. Centrifuge CFU at full speed at 5°C for cycles of 10 minutes until the volume is 2.5 mL or less. Between each centrifugation cycle, ensure that the protein does not precipitate (milky or flocky solution).
3. Equilibrate a PD-10 desalting column with 25 mL of Buffer C.
4. Load 2.5 mL of the concentrated protein (from the CFU) onto the PD-10 column (do not need to collect the flow-through).
5. Once the protein sample is fully incorporated into the resin, load 3.5 mL of Buffer C and collect the flow-through (3.5 mL) in a new tube.
6. Store CFU in the fridge with water to keep the membrane moist.
7. Wash the PD-10 desalting column with 25 mL of water, store in the fridge.

Determining Protein Concentration.

1. Prepare standards as shown below:

	0	0.5	1	2	3	4
BSA (μL)	0	5	10	2	3	4
Water (μL)	10	5	0	8	7	6
BSA Stock 1 (1 mg/mL)				BSA Stock 10 (10 mg/mL)		

2. Prepare a tube with 5 μL of the purified protein with 5 μL of water.
3. Add 1 mL of Bradford's reagent to all tubes; shake, sit for 5 minutes.
4. Measure absorbance at 595 nm using spectrophotometer for all tubes.

Fluorescence.

1. Prepare the sample (200 μL): 0.5 mg/mL of WT or Y134S-SecA in buffer C and transfer to quartz cuvette.
2. Set emission wavelength to 280 nm.
3. Set fluorescence intensity to measure from 310 nm to 400 nm.
4. Set temperature range to monitor thermal unfolding from 20°C to 60°C.
5. Record tryptophan fluorescence for 3 samples each for WT-SecA and Y134S-SecA.