**LEE LAB RESEARCH LABORATORY**

**GENERATION OF RECOMBINANT αS AND PFF PROTOCOL**

**Recombinant αS purification Protocol**

##

## Culture Growth and Induction

1. Day 1: Inoculate 5mL of LB/Amp [100ug/mL: 1uL of 100mg/mL Amp stock (freezer) for every 1mL of culture] with 1 colony from LB/Amp plate and put in shaker at 370C w/250 rpm overnight.
	* Modification:
		+ Inoculate 10mL of LB/Amp with 1 colony (allows more rapid growth)
	* If no viable plate:
		+ Take a stab from glycerol stock (-800C Freezer) and grow overnight
		+ Streak LB/Amp agar plate with overnight culture
2. Day 2: Inoculate 500mL of LB/Amp (100ug/mL: 1uL of 100 mg/mL stock for every 1mL of culture) with 5 mL of overnight culture and put in shaker at 370C until O.D = 0.5 – 0.6 (~1.5-3 hours)
	* Modification:
* Inoculate 1000mL of LB/Amp with 10mL of overnight culture and put in shaker.
* Nano-drop:
	+ Use 2μl drop
	+ Use LB or LB/Amp as a blank.
* Formula to determine the time it will take the culture to hit an OD of 0.5 (assuming 30 min doubling time):

For example: for a culture with an ODi = 0.052

t=97.3 minutes

1. **Induce with IPTG (400ug/mL: 6.4uL of 0.5M) for every 10mLs of culture) for 4-6 hours at 370C**
2. Spin down culture at 4600xg for 20 minutes at 40C in 500mL buckets (max volume 350ml) in JLA-10.5 rotor (blue rotor, standing centrifuge).

\*\*\*First Stopping Point: Pellet can be frozen at -200C. For > 500mL culture, split pellet into 500mL equivalent fractions\*\*\*

1. Day 3: Resuspend in 50mLs of lysis buffer (40mM NaOH, 20mM Tris pH 8.0, 1mM EDTA, 0.1% Triton X-100). Add 1 tablet of protease inhibitor cocktail (NOT MINI) and 50uL of 500mM PMSF (500mM stock in floor -20C; Final conc. 500nM; incubate at 370C without shaking for 35-40min.
2. Add 500uL of 1M MgCl2, 500uL of 1 M CaCl2, and 20uL of DNase from Roche (200U total) and incubate at 370C with 250 rpm shaking for one hour.
3. Add 1 mL 0.25 M EDTA, mix well, and remove cellular debris by centrifugation at 16,900xg for 15 minutes. (JA 25.50 rotor)
4. Add 0.116g of ammonium sulfate per mL of supernatant and stir at 40C for 1 hour. Centrifuge at 20,000xg for 30 minutes, use new tubes. (~5.8g; Toss pellet)
5. Add 0.244g of ammonium sulfate per mL of supernatant and stir at 40C for 1 hour (or overnight). Centrifuge at 20,000xg for 30 minutes, use new tubes. (~12.9g; \*\*Keep pellet)
	* Quality Control Checkpoint: Pellet should be at bottom of tube. Excessive proteolysis is indicated by floating pellet or significant smearing of pellet along the side of the tube.
6. Day 4: Resolubilize in 25mLs Buffer A (25mM Thris pH8, 20mM NaCl, 1mM EDTA) and add PMSF to 1 mM. Stir in refrigerator for ~30min.
7. Dialyze against 1L of Buffer A with 0.5mM PMSF overnight.
8. Day 5: Filter through 0.22um filter, Milex, Millipore.
9. LPLC: Run over Anion Exchange column HiTrap Q FF (program: SC IExAlphasS, Buffer A, Buffer B: 25 mM Tris pH 8, 1M NaCl, 1mM EDTA, filtered), aS will start to come off at ~20% Buffer B). Keep samples at 4C. **SEE DETAILED QFF PROTOCOL**
10. Concentrate αS positive fractions with Amicon Ultracel-3KD MWCO, Millipore, and exchange into Buffer C (25mM Tris pH 8, 1M NaCl, 1mM EDTA, filtered). -Perform during Day 6 equilibration
	* Samples spun at 4500xg for ~20 min till final sample volume <= 1mL (lower volume better e.g. 750uL).
11. Day 6: LPLC: Run over size exclusion column in Buffer C. aS will start to come off at ~80mL. **SEE DETAILED SEC PROTOCOL – Freeze tubes until Conc./BCA**
12. Concentrate aS positive fractions with Amicon Ultracel-3KD MWCO, Millipore, and exchange into sterile PBS.
	* BCA samples and aliquot at desired concentration/volume (recommended at 5ug/ul).
	* Freeze aliquots at -800C

**αS PFF Assembly Protocol**

## Aggregation

1. Remove monomer stock from -80 and thaw on ice.
2. Dilute stock to desired concentration (typically 5 ug/uL) to a final volume of 300 ul in autoclaved 2 mL centrifuge tubes.
3. Set thermocycler for continuous shaking (1000 RPM @ 370C)
4. Install 2ml plate
5. Run PBS and Protein samples. Take 2uL aliquot at 0 hr time point. Run ThioT Assay.
6. Take 2uL aliquots twice per day for up to 120 hours (72-96 hours may be sufficient)
7. Check ThioT Fluorescence
8. Aliquot and store at -80C.

## ThioT Assay

1. Prepare ThioT stock mixture (8ug ThioT in 10uL milliQ H2O)
2. Prepare working ThioT mixture, 20uL stock into 1 mL PBS.
3. Dispense 200uL working solution into each 2uL aliquot.
4. Dispense 200uL per sample into 96-well, black, corning glass-bottomed plate
5. Read Fluorescence Intensity (Ex 450 +/-10; Em 490 +/- 10)