

Per Widlund's His-hsAurora Kinase A purification protocol v. 18.02.2009
(adapted from Tim's GST fusion protocol)

Transformation – Day 1

1. Transform vector into Rosetta (DE3) cells (contains pRARE plasmid). Select on LB plates containing 100ug/mL Kanamycin and 25ug/mL Chloramphenicol.

Expression – Day 2/3

2. Inoculate a 20mL LB-Kan/Chlor starter culture and incubate shaking overnight – 37C 180rpm. Book separate incubator (for 18C) for the next morning.
3. Set incubator to 18C
4. Prepare 1 liter of LB medium containing Kan/Chlor. Transfer 500mL of medium to a 2.8L Fernbach flask. Inoculate with 15mL of the overnight culture.
5. Shake for about 2.5 hours at 37C (OD600~1.0) at 180rpm. Store the remaining 500mL at 4C.
6. Add the cold 500mL LB to the culture
7. Shake for 20min at 18C
8. Take 1mL sample for SDS-PAGE – spin and freeze pellet.
9. Add 200uL of 1M IPTG to the culture (0.2mM final)
10. Shake overnight at 18C (18 hours). Book JLA8.1000 rotor for the next morning.

Harvest – Day 4

11. Take 1mL sample for SDS-PAGE – spin and freeze pellet.
12. Centrifuge culture at 5,000rpm for 10min at 4C in JLA 8.1000 rotor.
13. Resuspend bacteria in 30mL of cold lysis buffer. Freeze suspension in LN2 and store at -80C, or continue with purification.

Lysis and clarification – Day 4

14. Thaw cell suspension if necessary. Add protease inhibitors:
 - a. 1 complete tablet (roche)
 - b. 40uL 10mg/mL pepstatin (10ug/mL final)
 - c. 40uL 10mg/mL PMSF (10ug/mL final)
15. Add 0.5mg/ml lysozyme. Incubate on ice for 30min
16. Sonicate 30 sec at 50% amplitude on ice.
17. Load 6-8 tubes in MLA-80 rotor. Spin in Ultracentrifuge for 15min at 40,000rpm at 4C.
18. Pool supernatant and add imidazole (from 1M stock) to 10mM final.

Purification – Day 4

19. Perform first purification with a 5mL Nickel column on a peristaltic pump:
 - a. Wash with 5-10 CV of water at 5ml/min
 - i. Strip column if necessary
 1. -10 CV of 50mM EDTA, pH 8.0
 2. -8 CV water
 3. -1 CV of 100mM NiCl₂
 4. -8 CV water
 - b. Equilibrate in 5CV 3% buffer B/97% buffer A at 5ml/min
 - c. Load lysate at 1-2ml/min
 - d. Wash with 5CV of 3% buffer B/97% buffer A at 5ml/min
 - e. Wash with 5CV of 3% buffer B/97% buffer A high salt at 5ml/min
 - f. Wash with 5CV of 10% buffer B/90% buffer A at 5ml/min
 - g. Elute with 15ml of 100% buffer B at 5ml/min – collect 1mL fractions
 - h. Wash with 10CV water
 - i. Run 5CV 20% Ethanol for storage.
20. Check for peak protein fractions with using a quick Bradford assay (1-3ul in 500ul 5xdiluted Bradford reagent)
21. Check for quality of purification by running positive fractions along with samples of crude lysate, cleared lysate, flow-through and all wash steps on SDS-PAGE.
22. Pool peak fractions – avoid exceeding 2mL
23. Run size exclusion chromatography on a Superdex 200 16/60
 - a. Wash column with 2CV (240mL) water at up to 1mL/min
 - b. Equilibrate column with 2CV of Aurora A size exclusion buffer.
 - c. Load 2mL of protein and run at 1mL/min for 180 mL – protein should elute at ~72mL.
 - d. Wash with 2CV water
 - e. Run 2CV of 20% Ethanol for storage
24. Determine concentration:
 - a. Take 5uL of each fraction and add 5uL 8M Urea. Take A280 with nano-drop using Urea/size exclusion buffer mix as a blank. Be careful to remove all traces of urea when finished. Use extinction coefficient 38120 M⁻¹cm⁻¹ or 0.7713 (mg/mL)⁻¹cm⁻¹. Multiply by 2 (dilution factor).
25. Pool peak fractions (3-5mL). Add 80% glycerol to 10% final (140ul for every 1mL) and add 100mM DTT to 1mM final (11ul for every 1mL). Adjust concentration determination accordingly.
26. Snap freeze aliquots in LN2 and store at -80C.

Media

LB or TB medium

100mg/ml ampicillin in water

15mg/ml chloramphenicol in ethanol

Protease Inhibitors

Complete protease inhibitor tablets (Roche)

10mg/ml pepstatin in DMSO

10mg/ml PMSF in ethanol

Lysis buffer

50mM Tris-HCl pH7.2

500mM NaCl

0.1% TritonX-100

5% glycerol

Nickel column buffers/solutions

Buffer A

25mM Tris-HCl pH 8.0 (3.03g for 1L)

300mM NaCl (17.53g for 1L)

20% glycerol

Buffer B:

As above, but with 300mM imidazole (20.4g for 1L)

High Salt Wash

1.5mL Buffer B

48.5mL Buffer A

3.0g NaCl

Size Exclusion Buffers/solutions

Nanopure water

20% ethanol

Aurora A size exclusion buffer

50mM Tris pH 7.5

300mM KCl

100mM DTT

80% glycerol