Purification of U1A Tagged 50S subunits (Jason Feinberg) version 2

This protocol is a morph of techniques from protocols provided by the Strobel lab and the Noller lab. It has been optimized for time and yield. Variations include usage of French press, deletion of pelleting spins and deletion of zonal centrifugation. It is important to note that purity of His-tagged 50S depends greatly on removing the 30S as can be seen in poison primer experiments. (J.S.F 2006)

- Inoculate 250 ml LB containing chloramphenicol and ampicillin with scrape from His_6-U1A cell stock.
- Grow/shake o/n @ 30°C
- Add 5 ml of o/n culture to (one or more) 1 l LB containing chloramphenicol and ampicillin **pre-warmed to 42°C.**
- Grow/shake at 42°C to a final OD_{600} of 0.5
- Ice for 20 min.

- Pellet cells at 6-8k rpm in Beckman JA-10.
- Wash (resuspend and spin as above) and resuspend cells in 15 ml of Cell Lysis Buffer.
- Lyse cells in French press (2 passes ~18000 psi)
- Add 10 µl RQ1-DNAse
- Pellet cell debris at 15k rpm in Beckman JA-17
- Transfer supernatant to fresh tubes and spin again @ 15k rpm

- Using "red capped" Ti-70 centrifuge tubes put 9 ml sucrose cushion under 14 ml of supernatant from previous spin (adjust volume w/wash buffer of supernatant for even # tubes).
- Spin C

- Wash pellets 1 ml of Washing Buffer and resuspend in 10 ml Washing Buffer.
- Pool resuspended pellets and place into "red capped" centrifuge tubes with a 1 ml sucrose cushion. Spin D.

- Wash pellets with 1 ml of **LOW Mg^{+2}** buffer and resuspend pellets in 10 ml of LOW **Mg^{+2}** buffer. (you now have a crude ribosome slurry with dissociated 30S and 50S)

Take OD_{260} of ribosomes and calculate nmoles.

((OD260xdil. Factor)/39.15)x volume in mls = nmoles of ribosomes

For every 40 nmoles of ribosomes, use 1 ml Ni-NTA beads

- Prepare Ni-NTA beads by rinsing with 2x in MiliQ H_2O in a 50 ml falcon. Then rinse in 1x PD buffer.
- Add ribosome slurry to Ni-NTA beads using the following formula:

For every ml of beads there should be 7.5x volume of solution. So: 5 ml of beads have a final volume of beads + ribosomes + buffer = 35mls
So:

10 mls ribosomes in LOW Mg^{2+} buffer
5 mls beads in 1X PD buffer
20 mls 1X PD buffer______.
35 mls final volume

- Mix ribosomes with beads gently rocking for 20 min @ 4°C
- Pour over a pre-chilled column
- Collect flow through and re-pour over column
- Wash with at least 20 x column volumes of 1x PD buffer
- Drain until just above beads and add 20ml of 1xEB
- Collect eluate and put into ultra "red capped" tubes. Balance with Ribosome Storage Buffer.
- Spin 3.5 hours at 60K
- Resuspend in ~0.1-1 ml Buffer A depending on pellet size
- Take O.D. and calculate concentration of 50S
- Aliquot into eppendorf tubes, flash freeze with liquid nitrogen and store at -80°C

Cell Lysis Buffer	Stock	500 ml
20 mM Tris-HCl (pH 7.6)	1M	10 ml
100 mM NH_4Cl	5M	10 ml
10.5 mM Mg acetate	1M	5.25 ml
0.5 mM EDTA	0.5M	0.5 ml
3 mM β-mercaptoethanol	14.4	10.5 µl

Sucrose Cushion	Stock	200 ml
20 mM Tris-HCl (pH 7.6)	1M	4 ml
100 mM NH_4Cl	5M	20 ml
10.5 mM Mg acetate	1M	2.1 ml
0.5 mM EDTA	0.5M	0.2 ml
Sucrose		75.24 g
3 mM β-mercaptoethanol	14.4	4.2 µl

Washing Buffer	Stock	500 ml
20 mM Tris-HCl (pH 7.6)	1M	10 ml
500 mM NH_4Cl	5M	50 ml
10.5 mM Mg acetate	1M	5.25 ml
0.5 mM EDTA	0.5M	0.5 ml
7 mM β-mercaptoethanol	14.4	10.5 µl

Low Mg^{+2} Washing Buffer	Stock	500 ml
20 mM Tris-HCl (pH 7.6)	1M	10 ml
500 mM NH_4Cl	5M	50 ml
1.5 mM Mg acetate	1M	0.74 ml

0.5 mM EDTA	0.5M	0.5 ml
7 mM β-mercaptoethanol	14.4	10.5 µl

Pull Down Buffer	Stock	200 ml
50 mM Tris-HCl (pH 7.6)	1M	10 ml
5 mM Imidazole	1M	1ml
70 mM NH_4Cl	5M	2.8 ml
1 mM $MgCl_2$	1M	200 µl
10 µg/ml total tRNA	10 mg/ml	200 µl
10 µg/ml Heparin	100 mg/ml	20 µl

Elution Buffer	Stock	20 ml
50 mM Tris-HCl (pH 7.6)	1 M	1 ml
70 mM NH_4Cl	5 M	0.7 ml
30 mM KCl	3 M	0.2 ml
10 mM $MgCl_2$	1 M	2 ml
200 mM Imidazole	1 M	4 ml

Ribosome Storage Buffer	Stock	100 ml
50 mM Tris-HCl (pH 7.6)	1 M	5 ml
70 mM NH_4Cl	5 M	3.5 ml
30 mM KCl	3 M	1.5 ml
7 mM $MgCl_2$	1 M	0.7 ml
DTT	1 M	100 µl
0.5 mM EDTA	0.5 M	100 µl

Spin	mls Sucrose	Spin Option
A	x	JA-20 rotor 10 min at 3000 rpm Then 30 min at 9000 rpm
B (4 tubes)	x	Ti-70 30 min at 16K
C (8 tubes)	9 ml	8.6h @ 50K 13.5h @ 40K 15h @ 38K 16.5h @ 36K 17.5h @ 35K
D (4-8 tubes)	1 ml	4.4h @ 65K 4.8h @ 62K 5.2h @ 60K 5.5h @ 58K 6.2 @ 55K 6.7h @ 53K 7.4h @ 50K 8h @ 48K 9h @ 45K 15h @ 35K