Growth and induction of tagged 50S (Noller Lab)

Cell line MOPOP, RecA-

Once the tagged construct has been assembled into a plasmid under pL promoter control the culture should not be grown above 30^{o}C unless you want to induce expression. Even for wt tagged constructs.

Prepare 4 L of culture per sample.

Pre-warm 4 x 1 l to 42^{o}C, at the same time put up 30^{o}C overnight.
Grow an over night culture at 30^{o}C, 5 ml of your MOPOP cells transformed with your tagged construct. [Although the MOPOP are inherently tet resistant, only use AMP selection because the growth becomes very slow.] Also the growth at 30^{o}C is considerably slower (than 37 ^{o}C) and you'll want incubations of 20-24 h, ideally.

Dilute your saturated O/N from an A_{550} of ~5 to about 0.025 in fresh LB (250 ml) with amp and continue to grow at 30^{o}C, until the OD reaches ~0.5, about 2.5 h. Use this 'log phase' culture, 50 ml into 1 l, to seed into your pre-warmed 42^{o}C culture.

Shake at 42^{o}C until the OD reaches 0.5 A_{550}. *wild-type* usually take 1.5 h and lethal mutants longer, ~2h.

Once the culture reaches 0.5 A_{550}, pellet. In 1 L flasks in a Sorvall (RC-5B+),

10 min, 6 K.

Re-suspend the pellets in 50 ml 10A and pellet again in 50 ml falcons.

10 min, 6K.

Store at –80^{o}C. Unless your running your sucrose cushions same day then simply crack and spin.

Affinity purification of MS2 aptamer tagged 50S

Buffers

Loading Buffer A1 (1 l)

		Final
Tris-HCl pH 7 (1 M)	20 ml	20 mM
NH_4Cl (4 M)	25 ml	100 mM
$MgCl_2$ (1 M)	1 ml	1 mM
2-Mercaptoethnol (14.4 M)	0.416 ml	6 mM

10 mM Mg^{2+} (A 10) 500 ml

Tris-HCl pH 7 (1 M)	10 ml	20 mM
NH_4Cl (4 M)	12.5 ml	100 mM
$MgCl_2$ (1 M)	5 ml	10 mM
2-Mercaptoethnol (14.4 M)	0.208 ml	6 mM

Elution Buffer 250 ml

Tris-HCl pH 8 (1 M)	12.5 ml	50 mM
NH_4Cl (4 M)	6.25 ml	100 mM
$MgCl_2$ (1 M)	0.25 ml	1 mM
Reduced Glutathione powder	0.76 g	10 mM
2-Mercaptoethnol (14.4 M)	0.104 ml	6 mM

Check the pH especially if you use a lower pH of Tris.

Salt Wash Buffer (B) 500 ml

Tris-HCl pH 7 (1 M)	10 ml	20 mM
NH_4Cl (4 M)	62.5 ml	500 mM
$MgCl_2$ (1 M)	5 ml	10 mM
2-Mercaptoethnol (14.4 M)	0.208 ml	6 mM

0 mM Mg^{2+} (A0) 50 ml

Tris-HCl pH 7 (1 M)	1 ml	20 mM
NH_4Cl (4 M)	1.25 ml	100 mM
2-Mercaptoethnol (14.4 M)	0.021 ml	6 mM

Filtered through 0.2 μm, and chill to 4°C
Aliquote 10A, 1A, 0A, and B into 50 ml falcons.
Assemble french press and chill to 4°C

Sucrose Cushion

Pour 2 x Ti60 sucrose cushions (11 ml each) [Ti45s are also fine, 35 ml each]
Load supernatant from up to 4 L of lysate on each Ti60 cushion

Cushions are typically: 20 mM Tris pH 7, 10 mM MgCl, 100 mM NH_4Cl, 0.5 mM EDTA, 37.7% sucrose, 6 mM 2-mercaptoethanol.

5X Buffer B 10ml

		5X Final
Tris-HCl pH 7 (1 M)	1 ml	100 mM
NH_4Cl (4 M)	6.25 ml	2.5 M
$MgCl_2$ (1 M)	0.5 ml	50 mM
H_20	2.2 ml	

Cushion mix 30 ml

5X Buffer B	6 ml	
70% sucrose	16.16 ml	1.1 M (37.7%)
2-Mercaptoethnol (14.4 M)	0.0125 ml	6 mM

Chill cushions to 4^oC

Crack cells and cushion

Take all samples to be purified through the cushion, salt wash stage in parallel

(Defrost cell pellet and) re-suspend in 12 ml of 10 A buffer.

Pass through the french press, twice.

Spin 30,000 g x 10 min (14,000 rpm) in a JA20 rotor.

Transfer the supernatant into a fresh tube and repeat the spin.

Load supernatant on to sucrose cushions and spin, based on:

100,000 g x 21 h

Ti60 spin times			**Ti45 spin times**	
rpm (x 1000)	time (h)		rpm (x 1000)	time (h)
42.4	16.5		39.8	17
41.7	17		38.7	18
41.1	17.5		37.6	19
40	18.5		36.7	20

Salt wash

Salt wash ribosomes twice.

Pour away the sucrose and rinse the pellet and Ti60 tube with salt wash buffer. Invert and allow the 'goo' to roll off, 15 min. If it doesn't come off, then try and dissolve it in salt wash buffer and pour away. I don't think it really matters.

Re-suspend the pellet in 2 ml salt wash buffer, (use baked, small magnetic fleas) Once fully dissolved fill the tube with salt wash buffer and pellet.

100,000 g x 3.5 h

The shortest is: **57,400 rpm x 1.5 h** (Ti60)

Rinse the pellet, tube and repeat.

If you are going to do the purification on the same day as salt washing re-suspend the second salt washed pellet in 2.5 ml of loading buffer (1A). **Take a 'crude' sample**.

To store pellets, re-suspend the second salt washed pellet in 0.5 ml of 10A, store at –80^{o}C; don't store ribosomes in 1 mM Mg^{2+}.

Working from frozen salt washed (crude) 70S

Defrost 'crude' sample and dilute (5 fold) the Mg to 2 mM (with 0A). Take an A_{260}. **Take a 'crude' sample.**

Determine the total number of nmoles in your sample. Typically a purification requires at least 8 (to 12 max) nmoles of tagged 50S. *wild-type* tagged expression is approximately 40% of total, and dominant lethal mutants around 20%.

Add an 8 fold excess of GSTMS2(V75E;A81G) to the tagged sample in a 50 ml falcon and place on a rocking table at 4^{o}C for 1 h.

GST-MS2 (V75E;A81G) mol weight = 39.7 kDa
25 nmoles/mg

Before use, wash the loading needle and the filter unit with buffer 1A.

Filter the resuspended mixture through a disposable syringe filter 0.2 μM (UNIFLO-25, CA membrane- Schleicher & Schuell)

FPLC stage purification

GSTrap FF 5 ml column (Amersham Cat# 17-5130-01)

Set lamp to 254 nm, set lamp sensitivity to 2, set back pressure limit 0.3 MPa
Wash the pumps with ddH_20, wash pump A with 1A.

Assemble super loop, very lightly lubricate the o-rings with glycerol. Fill with 1A and attach (before connecting GST column). If it leaks: use teflon tape on connections, flange new connections. If it leaks, or alarms when the plug reaches the top, unscrew the cap and pull the top stopper out a little, flow over 1 ml and push it down again.

Run 10-15 ml of loading buffer (flow rate 0.5-1 ml/min) through the super loop.

Attach the GST column

Run 5ml_equ (pump A = A1 and pump B = ddH_20)

programme fraction collector to collect 5 min fractions, load collecter with 8 tubes, wash pump B with elution buffer.

Fill the syringe with 1A, remove all bubbles and most of the buffer. Place the needle in the falcon with filtered complex and inject a tiny ammount of buffer and then draw up the fluid. Remove any bubbles.

Set the valve to load, and load the superloop with ribosome/MS2 complex.

Run elution method (e_7_slow)

The method will collect 8 franctions the eluted 50S typically lie in 4 fractions (2-5). Pool complex fractions, take A_{260} of 10 µl in 1 ml, and concentrate in centricon plus-20 (amicon cat# UFC2BHK08). Typically it will take 25 min to concentrate the sampel (4000g), buffer exchange by adding 20 ml A10 and concentrate again, this spin will typically take 45 min and best split into two spins (25/20 min) pouring away the void volume after each stop. Do two buffer exchages.

Regenerate the GST column

pump wash A/B with ddH_20, pump wash A with 6 M Guandium hydrochloride. Run reg_5w.

pump wash A/B with ddH_20, pump wash A/B with 20% EtOH

wash the column (valve = load) with 25 ml 20% EtOH, store at 4^oC.

A_{260} conversions

70S

1 A_{260} = 26.69 pmol = 66.67 μg / ml
1 pmol = 2.5 μg
1 μg = 0.4 pmol
Mr = 2.5 MD

50S

1 A_{260} = 39.21 pmol = 66.67 μg / ml
1 pmol = 1.7 μg
1 μg = 0.59 pmol
Mr = 1.7 MD

30S

1 A_{260} = 83.38 pmol = 66.67 μg / ml
1 pmol = 0.8 μg
1 μg = 1.25 pmol
Mr = 0.8 MD