Poison Primer Extension--- Purity of MS2-tagged subunit Preps

1. rRNA extraction:

25pmols ribosome (crude or purified)
+ 2 ul 0.5M EDTA pH 8.0 (5mM)
+ 5 ul 20% SDS (0.5%)
+ 20ul 3M NaoAc pH 5.2 (0.3M)
+ H2O up to
200ul

3 phenol extractions (H2O saturated,pH4.5) (Balance at RT for 30 mins)
3 chloroform extractions(~180ul)

- Add same volume of organic solution
- Shake at RT for 5 min on eppendorf shaker .
- Spin down for 1 min at max speed
- Recover the aqueous phase(top), or remove organic solution from the bottom
- +2.5 volumes of 100% ethanol(~500ul)
- 15 min precipitation at 4C
- 15min spinning down at 16000g at 4C
- Wash pellets with 200ul 70% ethanol
- Spin 1min at max speed
- Dry at RT for 2 mins
- Resuspend in 25ul of H2O
- Store at –20C

2. MS2-primer labeling

16S Primer sequence: 5'-CCCGTCCGCCACTCGTCAGC -3' (complementary to 113-94) (MW=)
with ddCTP stop at +13(MS2) and +8(WT)
23S primer sequence: 5'-TCA ACG TTC CTT CAG GAC CCT -3'(complementary to 2820-2800) (MW=6317)
with ddCTP stop at +5(MS2) and +9(WT)

11ul H2O
4ul 10uM 16S-MS2-primer
2ul PNK 10Xbuffer
2ul γ32P-ATP
1ul T4 PNK
20uL

- Incubate at 37°C for 15min.
- Add 10ul 3M NaAc (pH5.2) + 2ul 10mg/ml Glycogen + 70ul water (~100ul)
- 2x chloroform extraction.
- EtOH precipitate DNA by adding 2.5x 100% EtOH (250ul)
- Precipitate at -20°C o/n, or 4C for 30min.
- Spin at top speed for 30min.
- Discard radioactive EtOH to radioactive waste container.
- Resuspend pellet in 40ul water

3. PE reaction

Buffers

4.5x hybridization buffer

	stock	volume	1X
225mM K-Hepes, pH7.0	1M	225ul	50mM
450mM KCl	3M	150ul	100mM
water		625ul	
		1000ul	

10x extension buffer

	stock	volume	1X conc.
1.3M Tris-HCl,pH8.5	2M	650ul	130mM
100mM $MgCl_2$	1M	100ul	10mM
100mM DTT	1M	100ul	10mM
Water		150ul	
		1000ul	

Stop buffer

	Stock	volume	
85mM NaOAc	0.3M	14ml	4.2ml
2.5X ethanol	95%	35ml	10.8ml
		50ml	15.0ml

Urea loading buffer(8M urea,0.1x TBE,0.03% bromphenol blue&Xylene cyanol)

0.25mM d/ddNTP: mix same volume of 1mM dATP, dGTP, dTTP and ddCTP

AMV Reverse transcriptase (Cat# 70041Z,USB,15u/ul)

Procedure

- **Hybridization :** (4.5ul/reaction)
  2.0 ul 4.5x hybrid buff &^{32}P-primer (1:1)
  2.5 ul RNA template or H2O for control
  4.5ul

Spin samples in microcentrifuge at top speed for 5-10 sec to mix samples
Denaturalize at 90C for 1 min
Transfer to tray containing hot water and let cool to 47C (should take about 10min)
Spin down tubes briefly (5sec) to retrieve condensate on lid and sides

- **Extension**
  To make extension mix: (5.5uL/reaction)

	6rex	8rex	10rex	12rex	14rex	16rex
10x extension buffer	6	8	10	12	14	16
0.25mM d/ddNTP	24	32	40	48	56	64
AMV-RT(15u/ul)	0.5	0.5	1	1	1	1
water	2.5	3.5	4	5	6	7
Total volume	33	44	55	66	77	88

Add 5.5ul to hybridization reaction. Total volume will be 10ul
Mix properly by pipetting up & down 5-10 times.
Incubate at 42C incubator for 30min

- **Stop**

Add 120ul precipitation buffer
Vortex to mix and incubation at RT for 10'
Spin at RT for 10min.
Dry pellets at RT for 2.5min
Resuspend in 10ul of urea loading buffer.
Heat for 2' at 90C

- **Gel analysis**

Prepare 10% acrylamide/urea gel (0.4mm, BRL sequencing gel, 34 combs).
Pre-run for 30mins at 50 watts.
Load 5ul of each sample.
Run the gel for 2h 15mins at 50 watts until BRB reach the bottom of gel.
Wrap it in plastic film/filter paper and dry gel at 80C for 1.5h.