Protocol: In vitro Transcription of 16S rRNA

Step1: Digest the plasmid pUC118-16SrRNA encoding *E. coli* 16S rRNA with Bsa I

Water	xx μl	
10X NEB4 buffer	10 μl	
Bsa I (NEB)	10 μl	
Plasmid	xx μl	(80 μg)
	100 μl	

1. Incubate at 50 °C for 5 hours. Analyze an aliquote on 0.8 % agarose gel.

2. Add 10 μl of 0.5 M EDTA, pH8.0 and 10 μl of 3M Sodium acetate pH 5.2.

3. Add 120 μl of phenol-chloroform-isoamylalcohol mix, extract by vortexing rapidly for 30 sec. Spin in microfuge for 1 min at max speed.

4. Transfer upper aqueous phase to new tube. Add chloroform and repeat step 3.

6. To aqueous phase add 350 μl of 100% Ethanol. Precipitate DNA at –80 °C for atleast 30 min or –20 °C for 12 hours.

7. Spin tubes at 4 °C for 30 minutes.

8. Carefully remove and discard ethanol without disturbing the DNA pellet.

9. Add 500 μl of 70% ethanol, spin at max for 30 seconds. Carefully remove all the ethanol. Dry pellet for 5 minutes in the SpeedVac without heat.

10. Resuspend in 50 μl of TE.

Step2: Reaction mix for 500 µl transcription of 16S rRNA

(Refer: Ryder & Strobel (1999) METHODS, vol 18, 38-50; Ortoleva-Donnelly et al & Strobel (1998) RNA 4: 498-519)

		Final Conc.
Water	xxx µl	
1M Tris-HCl (pH7.5)	20 µl	(40 mM)
50 mM Spermidine	40 µl	(4 mM)
50 mM DTT	100 µl	(10 mM)
1M MgCl2	7.5 µl	(15 mM)
1% Triton X-100	25 µl	(0.05%)
25 mM AUGC (NTP mix)	**20 µl**	**(1 mM Each)***
T7 RNA Polymerase (JF prep)	10 µl	(0.08 µg/µl)
Digested Plasmid	25 µl	(40 µgs)
ONE NTPαS (Optional)*	**xx µl**	**(0.05 mM)**
	500 µl	

***Note:** For standard transcription reactions without NTPαS, use 4 mM NTP final conc.

1. Incubate for 3 hours at 37 °C.

2. Add 10 µl of RQI DNaseI (Promega), incubate for 37 °C for 30 minutes.

3. Add 50 µl of 0.5M EDTA, pH8.0 and 50 µl of 3M Sodium acetate pH 5.2.

4. Add 600 µl of chloroform, extract by vortexing rapidly for 30 sec. Spin in microfuge for 1 min at max speed.

5. Transfer upper aqueous phase to new tube. Add chloroform and repeat step 4.

6. To aqueous phase add 1.2 mls of 100% Ethanol. Precipitate RNA at –80 °C for atleast 30 min or –20 °C for 12 hours.

7. Spin tubes at 4 °C for 30 minutes.

8. Carefully remove and discard ethanol without disturbing the RNA pellet.

9. Add 500 µl of 70% ethanol, spin at max for 30 seconds. Carefully remove all the ethanol. Dry pellet for 5 minutes in the SpeedVac without heat.

10. Resuspend in 100 µl of water.

11. Purify on Sephadex G-50 column equilibrated with water using the ISCO UV-detector.

12. Collect the RNA fraction and recover RNA by ethanol precipitation.

13. Resuspend 16S rRNA in water or in 1X reconstitution buffer A.

14. Determine the conc. by measuring A260. For 30S reconstitution the 16S rRNA must be in 1X reconstitution buffer A.

15. Analyze the 16S rRNA on 4% denaturing polyacrylamide gel. Store at –80 C.

Notes:

1. For modification-intereference studies transcripts are synthesized incorporating only a single type of phosphorothioate analog within the RNA (say ATPαS). Reference: RNA-Protein Interaction Protocols, page 84.

2. Purchasing NTPαS:

NEN	2.0 micromole	$249	80 Trx*
Amersham	0.5 micromole	$70	20 Trx
Glen	0.05 micromole	$75	2 Trx

*Need 0.025 micromole of NTPαS per 500 μl transcription reaction

3. Perform Mn2+ Rescue Expt before trying Nucleotide Analog Interference Mapping.

4. Nucleotide Analog Interference Mapping using modified base analogs (available from Glen Research & Amersham): Transcription conditions are described in Ryder & Strobel (1999) METHODS, vol 18, 38-50; Ortoleva-Donnelly et al $ Strobel (1998) RNA 4: 498-519.

5. Incorporation of deoxy-analogs using mutant T7 RNA polymerase Y639F available from Epicenter or from Sousa. Sousa & Padilla (1995) EMBO J 14: 4609-4621.