ISOLATION OF TOTAL RNA FROM *E. COLI* FOR MICROARRAYS

*(Phenol/EtOH stop developed by Jon Bernstein (***october@leland.stanford.edu)***, S.N. Cohen's lab, Stanford)*

Note – perform all steps swiftly to minimise RNA degradation. Use RNase free or DEPC treated solutions, RNase free microfuge tubes and RNase free filter pipette tips.

A/ Harvesting total RNA

- Grow o/n cultures of MG1655 based strains from which you intend to harvest RNA.
- Dilute the o/n cultures 1:100 into 100 ml of appropriate media and grow to appropriate OD.
- **Harvest cells** by pipetting 10 ml of culture into a 15 ml conical tube containing 1.25 mls of ice-cold EtOH/Phenol stop solution (5 % water-saturated phenol (pH<7.0) in ethanol). This step stops the degradation of mRNA.
- Spin down the cells at 8,000 rpm for 2 min at 4 °C. Aspirate off media and freeze pellet in liquid nitrogen. Store at –80 °C until required.
- Lyse the cells by resuspending the pellet in a final volume of a fresh solution of 800 µl 0.5 mg/ml lysozyme, TE pH 8.0. Add 80 µl 10% SDS, mix and place in water bath at 64 °C for 1-2 min. The sample should clear.
- After incubation, add 88 µl 1 M NaOAc, pH5.2 and mix.
- **Hot Phenol Extraction:** Add the samples to an equal volume (1 ml) of water saturated phenol (pH<7.0) in RNase-free 2 ml microfuge tubes. Invert 10 times and incubate in 64 °C waterbath for 6 min. Invert 6-10 times every 40 s or so.
- Place tubes on ice to chill. Spin at max speed (14,000 rpm) for 10 min at 4 °C.
- **Chloroform Extraction**: Transfer the aqueous layer to a fresh 2 ml microfuge tube containing an equal volume of chloroform. Invert 6-10 times, and then spin at max speed (14,000 rpm) for 5 min. at 4 °C.
- **Ethanol precipitation:** For each sample, transfer the aqueous layer equally into two 1.5 ml microfuge tubes. Add 1/10 volume of 3M NaOAc pH 5.2, 1 mM EDTA (DEPC-treated), and 2-2.5 volumes of cold 100% EtOH. Incubate at –80 °C for 20 min (or o/n if required). Centrifuge at 14,000 rpm for 25 min. at 4 °C. Carefully remove ethanol. You should see a small white pellet
- Wash pellet with 1ml 80% cold ethanol (made with DEPC-treated water). Centrifuge at 14,000 rpm for 5 min at 4 °C. Carefully remove ethanol and air dry pellet (15-20 min in the fume hood).

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- Resuspend each pellet in 100 µl RNase-free H_2O (DEPC treated) and pool each pair of tubes.

B/ DNase I treatment of RNA samples

Note – it is essential to remove all contaminating DNA before the reverse transcriptase reaction

1) Treat each 200 µl sample with DNaseI as follows:

Volume (µl)	Reagent	
0.5	**RNase inhibitor**	(40 U/µl) Boehringer Mannheim; catalog #799 017
50	**5× DNase I Buffer**	50 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 5 mM DTT
1	**RNase-free DNase**	(10 U/µl) Boehringer Mannheim; catalog #776 785

Incubate the reactions at 37 °C for 30 min.

2) **1 phenol extraction** (use water-saturated phenol, pH<7.0). Invert 6-10 times and spin 2-3 min at RT.

3) **1 phenol (water-saturated)/chloroform (50:50) extraction**. Invert 6-10 times and spin 2-3 min at RT.

4) **2 chloroform extractions**. Invert 6-10 times and spin 2-3 min at RT.

5) **Ethanol precipitation**. Add 1/10 volume of 3M NaOAc pH 5.2, (DEPC-treated) and 2-2.5 volumes of cold 100% EtOH. Incubate at –80 °C for 20 min. or o/n if required. Centrifuge at 14,000g for 25 min. at 4°C. Carefully remove ethanol.

6) Wash pellet with 1 ml 80% EtOH (made with DEPC-treated water). Centrifuge at 14,000 rpm for 5 min at 4°C. Carefully remove ethanol and dry pellet at RT.

7) Resuspend pellet in 50 µl RNase-free H_2O (DEPC treated)

8) Calculate the concentration of RNA.

a) **Quantitation**: Take 2 µl RNA sample and add to 800 µl DEPC-treated water. Use clean quartz cuvette to avoid RNase contamination. Read absorbance at 260nm. Calculate concentration using the following formula:

$$\text{Concentration } (\mu g/\mu l) = A_{260} \times 400 \times 40{,}000 ug/\mu l.$$

b) **Purity**: Take 2 µl RNA sample and add to 800 µl 10 mM Tris-HCl pH 7.5. Read absorbance at 260 and 280nm to determine A_{260}/A_{280} ratio. Good values are between 1.8-2.1 for clean RNA.

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Notes:

- *Since the A_{260}/A_{280} ratio is affected by pH, it is more accurate to determine this value in a Tris buffer. However, the quantitation* ***must*** *be determined in H_20 (ref: QIAGEN RNAeasy handbook).*
- *Typical RNA yields vary from 70 – 300 μg RNA, depending upon strain, growth conditions and OD upon harvesting.*
- *If required, the integrity of the RNA can be analysed on a denaturing formaldehyde 1% agarose gel (ref: QIAGEN RNAeasy handbook). Analyse 2μl each RNA sample, and upon visualisation of the gel, the 23S and 16S ribosomal RNA should be easily observed. The 23S rRNA should be twice as intense as the 16S rRNA species. Large smearing towards smaller RNAs indicate significant sample degradation during preparation.*

Notes on primer annealing and cDNA synthesis

– *In order to synthesize amino-allyl-UTP labelled cDNA, we recommend using 16 μg RNA sample with 10 μg random hexamer (ref:* Reverse Transcription and aa-UTP Labeling of RNA; www.microarrays.org/protocols.html*).*