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APPENDIX I

Reducing ribonuclease contanination

General consideration : The surface of the skin and dust particles contain abundant amounts of RNases so it is important to wear latex or vinyl gloves when carring out the following precedures(changing the gloves frequenty) and to keep bottles and tubes closed as much as possible

Glass - and plastic- ware: Ribonucleases are stable at 120^{o} C so autoclaving alone is insufficient to completely inactivate any ribonucleases contaminating solutions and equipment. Sterile disposable plasticware can be cosidered ribonuclease-free but nondisposable glass- and plasticware can be treated with diethyl pyrocarbonate (DEPC) which is an efficent inactivator of ribonuclease. Glassware can be treated by immersion in a solution of 0.2% (v/v) DEPC followed by autoclaving to remove any residual traces of DEPC, which can carboxymethylate purine residue in the RNA.

Chemicals: New bottles of chemicals should be set aside specifically for use in RNA work and when weighing out chemicals it is preferable to avoid the use of spatulas, instead carefully tap the chemicals out of the bottles.

Solutions: Solutions and water should also be treated with DEPC by making the solutions 0.2% (v/v) with respect to DEPC and incubating for 12 h at 37^{o}C. Solutions must be autoclaved for 30 min to remove the DEPC. Buffers containing Tris cannot be treated in this way as DEPC is highly unstable in Tris solutions. Tris buffers, therefore, need to be prepared with DEPC-treated water. For other solutions, which cannot be autoclaved, filtering the solutions water through a sterile 0.22 μm filter may reduce ribonuclease contamination. (90)

APPENDIX II

Table 3 Primers for specific cytokine/chemokines and β-actin amplification

mRNA transcripts	Product Size (bp)	Primer Sequences
IL-18 5′	342	5′ - GCTTGAATCTAAATTATCAGTC
IL-18 3′		5′ - GAAGATTCAAATTGCATCTTAT
RANTES 5′	195	5′ - CCTCGCTGTCATCCTCATTG
RANTES 3′		5′ - ACTTGGCGGTTCTTTCGGGT
MIP-1α 5′	229	5′ - AACATATGGCATCACTTGCTGCTGACACGCCG
MIP-1α 3′		5′ - AAGGATCCTCAGGCACTCAGCTCTAGGTCGCT
β-actin 5′	548	5′ - GTGGGGCGCCCCAGGCACCA
β-actin 3′		5′ - CTCCTTAATGTCACGCACGATTTC

APPENDIX III

Flow chart for RNA Extraction

PBMC pellet (~ 4 x 10^6 cells)

1 ↓ +500 µl GTC lysis buffer

2 ↓ +50 µl 2 M NaOAc pH4

3 ↓ +500 µl saturated Phenol/Tris
+100 µl Chloroform/isoamyl alcohol

4 ↓ incubate in 4 °C 5 min

5 ↓ 14,000 rpm 4 °C 15 min

RNA in aqueous phase (or repeat at step 2) (61)

↓ +500 µl isopropanol (4 °C)

↓ Keep at –70 °C for 1 hr for RNA precipitation

↓ 14,000 rpm 4 °C 20 min

RNA pellet

↓ wash with 70%EtOH 2 times
at 14,000 rpm 15 min

RNA in DEPC-H_2O plus RNase inhibitor

↓

determining RNA concentration
at 260 nm in µg/µl

APPENDIX IV

CHEMICAL AGENTS AND INSTRUMENTS

A. Chemical substances

Agarose (GIBCO ; Grand Island, N.Y. USA)

Bromphenol blue (Sigma, MO, USA)

2-Mercaptoethanol (Sigma, MO, USA)

Diethylpyrocarbonate : DEPC (Sigma, MO, USA)

dNTPs (Promega, USA)

Ethanol (C_2H_5OH) (Sigma, MO, USA)

Ethidium bromide (Sigma, MO, USA)

Ficoll-Hypaque (Robbins scientific, Norway)

Guanidinium thiocyanate (GTC) (Sigma, MO, USA)

Isopropanol (Sigma, MO, USA)

MMLV-Reverse Transcriptase (Promega, USA)

Oligo dT (Promega, USA)

RNasin ribonuclease inhibitor (Promega, USA)

RPMI 1640 (GIBCO, Grand Island, NY)

Sarcosyl (Sigma, MO, USA)

Sodium acetate [CH_3COONa] (Sigma, MO, USA)

Sodium citrate (Sigma, MO, USA)

Phenol/Tris (Amresco, Solon, OHIO)

Chloroform (J.T.Baker, USA)

Isoamyl alcohol (Sigma, MO, USA)

Tag DNA polymerase (Promega, USA)

Disodium ethylene diamine tetraacetate dihydrate [$Na_2EDTA.2H_2O$] (E.Merch, Darmstadt, W., Germany)

B. Instruments

Agarose submarine gel apparatus (Biorad, CA, USA)

Automatic pipette (Gilson, Lyon, France)

Analytical balance

Electrophoresis power supply (Biorad, CA, USA)

Glover, non-sterile

Incubator (Forma Scientific, Ohio, USA)

Microcentrifuge (Eppendorf, USA)

Mixer-Vertex-Genic (Scientific industries, N.Y., USA)

Pipette tip (Costar Corp., Cambridge, USA)

pH meter, Model 10 (Corning, N.Y., USA)

PCR machine GeneAmp PCR System 9600 (Perkin elmer)

Refrigerate centrifuge, Model Centra 7-R (IEC, Boston, MA., USA)

Speed Vac Dryer (Savant Holbrock, NY)

Spectrophotometer (Spectronic Genesys 5, Milton Roy USA)

UV trans-illuminator (ULTRA-LUM, Carson, California)

APPENDIX V

REAGENTS AND PREPARATIONS

1. Reagents for sample preparation

1.1 Guanidinium thiocyanate (GTC) lysis buffer

Stock solution :	final concentration
250 g GTC plus 293 ml H_2O	4 M GTC
17.6 ml 0.75 M sodium citrate (pH 7)	25mMsodium citrate
26.4 ml 10% sarcosyl	

Dissolve at 65o C, then store at room temperature for up to 3 months

Working solution :

Add 0.18 ml 2-mercaptoethanol (2ME) to 25 ml of stock GTC solution to make working solution (final concentration of 2ME = 0.1 M)

1.2. 2 M Sodium acetate, pH 4.0

$CH_3COONa.3H_2O$	6.75	g
H_2O	20	ml

Adjust pH to 4.0 using around 4 ml of acetic acid

1.3. Water-saturated phenol or Phenol/Tris

Commercially prepared "Buffer-saturated Phenol"

1.4. Chloroform/isoamyl alcohol (49:1)

Chloroform	4.9	ml
Isoamyl alcohol	0.1	ml

1.5. Diethylpyrocarbonate (DEPC) -treated H_2O

Commercially prepared DEPC	1	ml
H_2O	500	ml

Incubate overnight at 37o C in water bath, then autoclave at 121o C for 15 minutes.

1.6. DEPC-H_2O plus RNase inhibitor

RNase inhibitor	0.5	µl
DEPC- H_2O	20.0	µl

Make sufficient volume for samples to be resuspended plus a little extra, prepare immediately before use.

2. Reagents for agarose gel electrophoresis

2.1. 0.5 M EDTA (pH 8.0)

Add 93.05 g $Na_2EDTA.2H_2O$ to 300 ml distilled water. Adjust the pH to 8.0 with 10 M NaOH (about 25 ml). (Note : EDTA will not go into solution completely until pH is greater than about 7.0) Adjust the final volume to 500 ml, and autoclave. Store at room temperature.

2.2. 50X Tris-acetate buffer (TAE)

Tris-base	242.0	g
Glacial acetic acid	57.1	ml
0.5 M EDTA pH 8.0	100.0	ml

Adjust the volume to 1 litter with deionized distilled water and sterilize by autoclaving at 121° C for 15 min

2.3. 10 mg/ml Ethidium bromide

Ethidium bromide	1	g
DDW	100	ml

Stir on a magnetic stirrer for several hours to ensure that dye has dissolved. Wrap the container in aluminium foil or transfer to a dark bottle and stores at 4°C

2.4. 1.5% Agarose gel

Agarose ultrapure	0.3	g
1X TAE	100.0	ml
10 mg/ml ethidium bromide	2.0	µl

BIOGRAPHY

Palabodeewat, Somnuek Mr was born on February 5, 1963 in Chonburi, Thailand. He graduated with a Bachelor degree of Science (Biology) from the Faculty of Science at Ramkhamhaeng University in 1986. He has worked as a scientist in Center for Vaccine Development, Mahidol University since 1987 to present.