Punjab Univ. J. Zool., Vol. 21 (1-2), pp. 19-27, 2006

AN IMPROVED METHOD FOR THE EXTRACTION OF TOTAL RNA FROM RIBONUCLEASE RICH TISSUES

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Abstract: The ability to isolate clean, intact RNA has important uses in cloning genes and is essential to analyze gene expression. To get useful clones, it is critical that full-length RNA is used as the starting material. To obtain good quality of eukaryotic mRNA it is necessary to minimize the activity of ribonuclease liberated during cell lysis by using their inhibitors. Guanidinium thiocyanate and 2-mercaptoethanol containing buffer ensures thorough denaturing of macromolecules and inactivation of RNases. Many different protocols and commercial kits are available for isolation of RNA from tissue but all have their own limitations. A single step isolation method for RNA was modified to get good quality total RNA from RNases rich tissue *e.g.*, pancreas. Tissue samples (chick and bovine pancreas) were collected under RNases free conditions. Total RNA was extracted using guanidinium thiocyanate and acid phenol. Two discrete bands of 28S and 18S rRNA were appeared on formaldehyde agarose gel that suggest isolation of good quality intact RNA.
Keywords: *Nucleic acid extracts, Tissue RNA extraction.*

INTRODUCTION

A number of methods are available for the extraction of total RNA and poly (A)+ mRNA. Many of these methods are tedious, time and labor intensive and do not provide the total spectrum of pure, undegraded RNA. Successful poly (A)+ mRNA selection from total RNA depends on high quality total RNA preparation (Hourfar *et al.*, 2005). The first step in all RNA isolation protocols involves lysing the cell in a

0079-8045/06/0019-0027 $ 03.00/0

chemical environment that results in denaturation of ribonucleases. The RNA is then fractionated from the other cellular macromolecules. The use of guanidinium to lyse cells was originally developed to allow purification of RNA from cells high in endogenous ribonucleases (Cox, 1968; Chirgwin *et al*., 1979). The method using hot phenol and guanidinium thiocyanate has also been described (Ferimisco *et al*., 1982). The ability to isolate clean, intact RNA has important uses in cloning genes and is essential to analyzing gene expression (Lake and Willows, 2003; Schlomm *et al.,* 2005; Cui *et al.,* 2005). Ribonuclease acid from any cell can be copied into double stranded DNA and cloned. To get useful clones, it is critical that full-length RNA is used as the starting material. Formaldehyde agarose gel electrophoresis of good quality total RNA preparation gave two discrete bands of 28S and 18S rRNA and mRNA appeared as a light smear between the 28S and 18S rRNA (Sambrook, *et al.,* 1989; Wick, 1986). Ribonucleases (RNases) that degrade RNA are very stable and active enzymes that generally require no cofactor to function. Minute amounts are sufficient to destroy RNA.

It is important to use RNase free conditions during all aspects of tissue collection, RNA isolation and further manipulation. To block nuclease activity, the isolation methods for mRNA using 6M urea 3m LiCl is insufficient (Ausubel, 1995). Many different protocols and commercial kits are available for isolation of RNA from tissue but all have their own limitations (Puissant and Houdebin, 1990; Wieslander, 1979). Best procedure to choose will be that which have less manipulations and less completion time. A single –step isolation method for RNA (Chomczynski and Sacchi, 1987) was modified to get good quality total RNA from RNases rich tissue *e.g.*, pancreas.

MATERIALS AND METHODS

Sample collection

Tissue was removed within 15 minutes of slaughter and quickly cut into cubes no longer than 3cm. The cubes were immediately transferred directly into liquid nitrogen. After the tissue had been completely frozen the

samples were stored at -70 °C. Care was taken that sample should not thaw during transfer.

Tissue processing

Frozen tissue sample (100mg) was homogenized in 1ml denaturing solution (4M guanidinium thiocyanate, 25mM sodium citrate pH 7.0, 0.1M 2-mercaptoethanol, 0.5% N-Lauro-sarcosine) in pestle and mortar or Glass-Teflon homogenizer.

RNA extraction and precipitation

The homogenate was transferred to sterile 5ml polypropylene tube and 0.1 ml of 2M-sodium acetate, pH, and 4.0 was mixed thoroughly by inversion. Then 1ml of water saturated phenol and 0.2 ml of 49:1 chloroform/isoamylalcohal was mixed thoroughly and chilled for 5 minutes at 0-4°C. Aqueous phase was separated by centrifugation at 9000 rpm (10,000Xg) at 4°C and transferred to fresh tube. For RNase rich tissue such as pancreas two more phenol:choloroform extractions were repeated other tissue did not need. Total RNA was precipitated by adding an equal volume of 100% isopropanol to aqueous phase and was chilled at -20°C for 30 minutes. After centrifugation at 14000 rpm for 10 minutes at 4°C RNA precipitates formed white pellet on the side and bottom of the tube. RNA pellet was dissolved in 0.3ml denaturation solution, and transferred to 1.5ml microfuge tube. After this equal volume of phenol:choloroform (1:1) was mixed and centrifuge at 10,000Xg at 4°C. Aqueous phase was transferred to fresh tube and 0.3ml of 100% isopropanol was added and again chilled at -20°C for 30 minutes. Final RNA pellet was collected at the bottom of the tube by centrifugation at 10,000X*g* at 4°C for 10 minutes. Supernatant was aspirated and the total RNA pellet was washed twice with diethyl pyrocarbonate (DEPC) treated 75% ethanol by vortexing and subsequent centrifugation for 5 minutes at 9000 rmp at 4 °C.

Solubilization

Ethanol wash was removed and RNA pellet was briefly air dried for 3-5 minutes. Over dry will reduce the solubility of RNA pellet. RNA was

dissolved in 50 µl Diethyl DEPC treated water or formamide by incubating for 5 minutes at 55-60 °C.

Spectrophotometeric analysis

The extracted RNA was diluted (100 times) with TE buffer (10mM Tris-Cl, 1mM EDTA) pH 8.0 and absorbance was measured at 260 and 280 nm using a quartz microcuvette.. Purity of RNA preparation was determined by calculating 260/280 ratio. The absorbance should be between 0.1 and 1.0. An absorbance of 1.0 at 260nm corresponds to approximately 40 µg/ml of RNA.

Formaldehyde agarose gel (1.2%)

Agarose was melted in DEPC-treated water, cooled to 60 °C, 5X 3-N-morpholino propane sulfonic acid (MOPS) was added to final 1X and formaldehyde to final 2.2M concentration. Gel was poured in preset gel tray in chemical hood and allowed to set for at least 30 minutes at room temperature. The RNA sample for loading was prepared by mixing 4.5 µl (30-40µg) of RNA with 2 µl of 5X formaldehyde-gel running buffer (0.1M MOPS pH 7.0, 40mM sodium acetate, 5mM EDTA pH 8.0), 3.5 µl of formaldehyde and 10 µl formamide.

RNA was denatured by providing heat shock to the mixture at 65 °C for 15 minutes then chilled on ice. After a brief spin 2µl of sterile DEPC-treated gel loading buffer (50% glycerol, 1mM EDTA, 0.4% bromophenol blue) was added and kept on ice till loading. Before loading the samples gel was pre-run for 5 minutes in 1X MOPS (formaldehyde not included) at 5V/cm. Samples were immediately loaded in the wells and gel was run at 4V/cm till bromophenol blue was migrated to two third of the gel. RNA was visualized under UV transilluminator after staining with ethidium bromide. Hands and dust are major source of RNases contamination gloves should be worn at all time during the procedure to avoid the introduction of RNases.

RESULTS AND DISCUSSION

RNA retains much of its secondary structure during electrophoresis unless it is first denatured. The addition of formaldehyde to the agarose gel maintains the RNA in its linear (denatured) form (Lehrach *et al.,* 1977; Goldberg, 1980; Seed, 1982). Figure 1 showed that after resolving total RNA sample on formaldehyde agarose gel and ethidium bromide staining, 28S and 18S RNA appeared as discrete bands at approximately 5.3 and 2Kb respectively. To avoid the time consumed in staining, ethidium bromide can be added to the sample before loading in the agarose gel. Ethidium bromide concentration up to 50μg/ml do not significantly alter in mobility and denaturation temperature 60-85 °C do not affect the ethidium bromide. This was also tried and found equally effective during this research work. Low yield may be the result of poor resuspension of RNA pellet because it is not readily soluble and sufficient time and vortexing is required. Partially solubilized RNA has an A260/A280 ratio <1.6 (Ausubel, 1995). Degradation of RNA by ribonucleases can best be avoided by working quickly and keeping everything cold. RNA is very unstable in tissue once removed from the body so it is very critical to freeze the tissue quickly. For this purpose liquid nitrogen was used. Guanidinium thiocyanate and 2-mercaptoethanol containing buffer ensures thorough denaturing of macromolecules and inactivation of RNases. The final preparation of total RNA was free of protein contamination as indicated by the peak of RNA sample that was at 257nm and O.D. 260/280 ratio range was found 1.8- 2.3 (Fig. 2). For optimal spectroscopic measurement RNA aliquots should be diluted with water or buffer with a pH >7.5. Distilled water with a pH < 7.5 falsely decrease the 260/280 ratio and impedes the detection of protein contamination in RNA sample (Wilfinger *et al*., 1997). For good results sterile techniques should always be used when working with RNA. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent Rnase contamination from the surface of skin or from dusty laboratory equipments. Gloves should be frequently changed and tubes should be kept closed. The use of sterile, disposable polypropylene tubes is preferable or non-disposable plastic ware should be rinsed thorouly with 0.1 NaOH, 1mM EDTA followed by RNase free water (Ausubel, 1995; Sambrook *et al.,* 1989). Old protocols for RNA isolation were time consuming often needed 6 hours to two days, this modified protocol increases the chance of providing intact RNA and

reduces hand on time for the experimenter, can be used to isolate RNA from large number of samples as well it allows the recovery of total RNA from small quantity of tissue (1-10mg) making it suitable for gene expression studies whenever a limited quantity of tissue or cells are available.

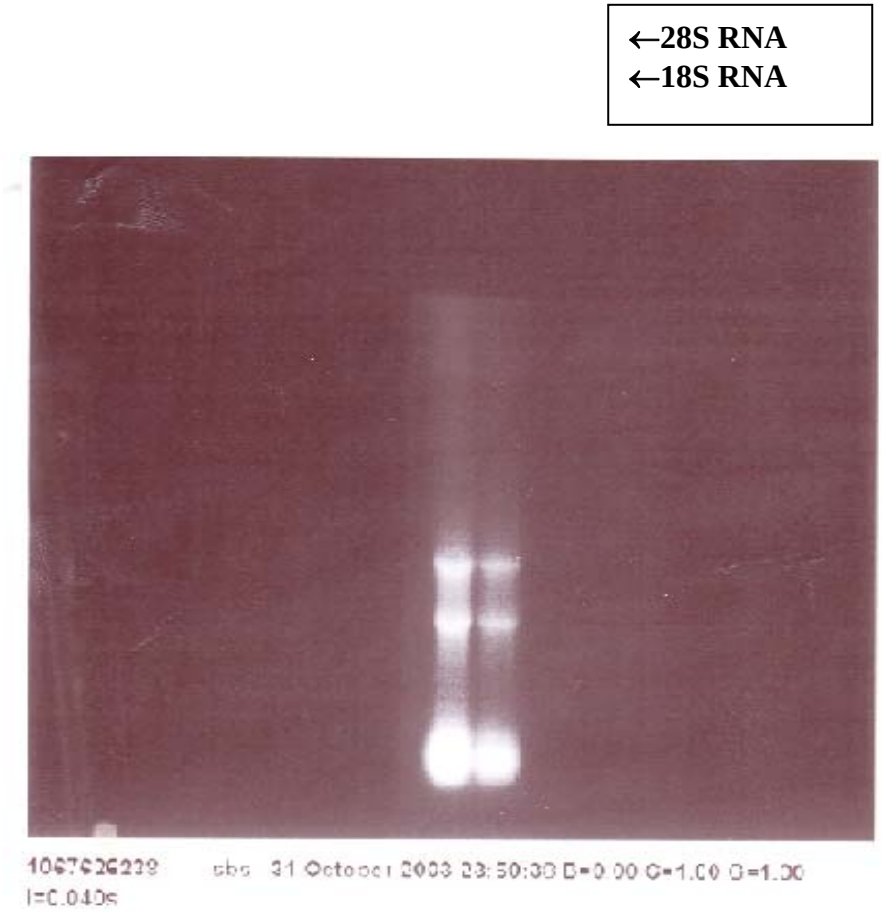

Figure 1: Resolution of total RNA on formaldehyde agarose gel.

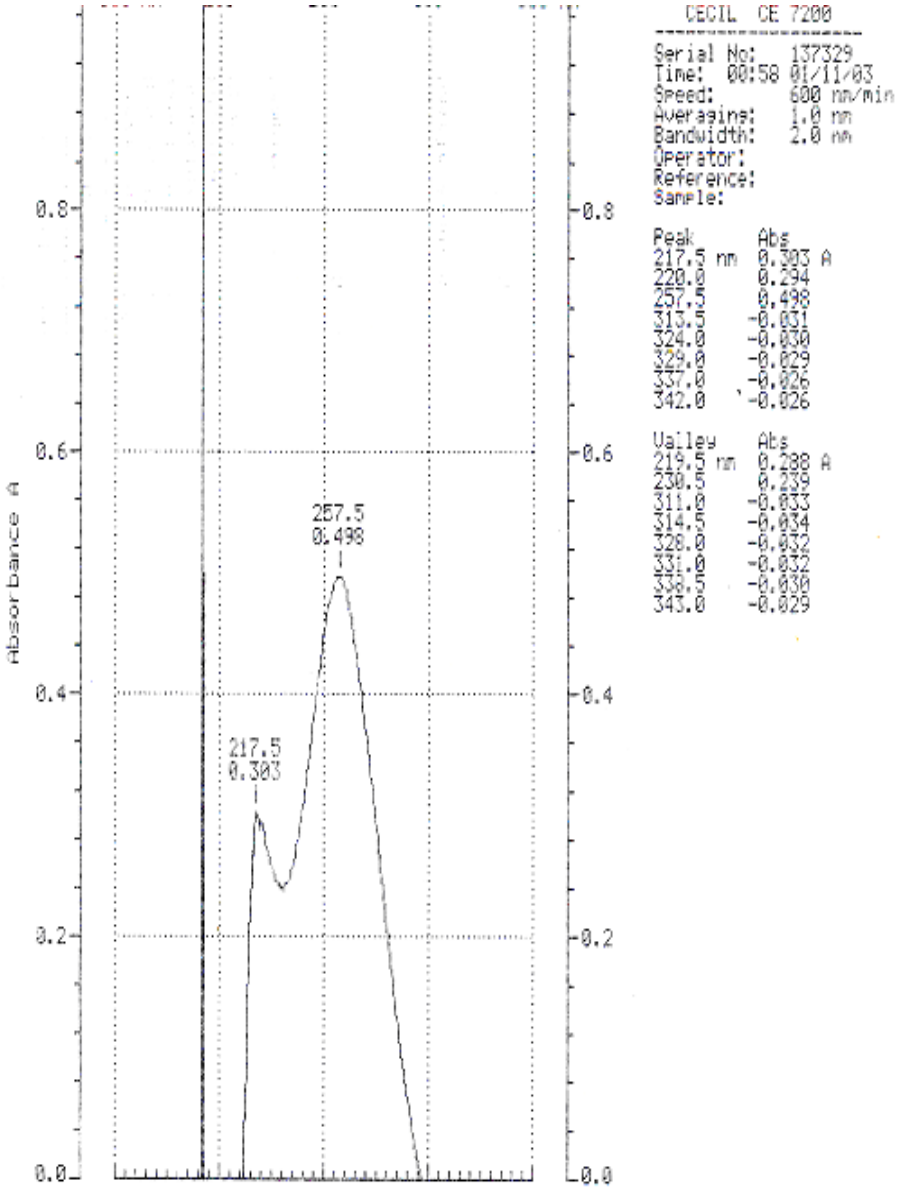

Figure 2: Spectrophotometeric analysis of total RNA from RNase-rich tissues

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Received: December 07, 2005; Revised: March 14, 2006)