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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 9 (8): 1601-1604, 2006
ISSN 1028-8880
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An Improvement in Total RNA Isolation Protocol for Ecologic Studies: Case Study Tobacco Cells

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Abstract: Many experimental techniques related to molecular biology such as the Northern blot analysis, RNA protection assays, *in situ* hybridization and RT-PCR need maximum-yielded, highly purified RNA samples. Researchers who study with RNA extraction are well known for being extremely sensitive about their chemical solutions, equipment, glassware and even laboratories. Taking certain precautions, which are given in protocol, is more convenient than obtaining a low 260/280 spectrophotometric ratio or a blank line in RT-PCR gel for ecologic studies. In the improved protocol, which is presented here, each step was tested many times and high-quality total RNA was determined. Using this protocol, 260/280 spectrophotometric ratio was found between 1.88-1.97 and total RNA was found between 174.6-184.3 μg RNA g cell FW^{-1} in tobacco cells. However, in order to obtain RNA with high purity levels, an additional step may be necessary to eliminate contaminating DNA depending on the sample characteristics.

Key words: RNA, extraction method, purification, RNAse elimination

INTRODUCTION

The most important protocols for RNA isolations are based on the use of guanidine salts as deproteinization agents (Cox, 1968; Chirgwin *et al*., 1979). Chomczynski and Sacchi developed a single-step procedure which has become very popular since its publication in 1987. All of these protocols can be modified and developed for different plant samples. But most of the data in literature on total RNA isolation (Montagu, 1987; Cisar and TeBeest, 1992; Brisco *et al*., 1977; Karp *et al*., 1998; Stull and Pisano, 2001; Huc *et al*., 2002) are limited to tobacco (*Nicotiana tobaccum*) cell suspension culture isolation (Smart *et al*., 1990; Çokuysal, 2000). Using this protocol, it is easy to determine total RNA at different growth stages of tobacco cells and due to purity and yield of total RNA it is possible to harvest poly (A)+ RNA from total RNA (Cokuysal, 2000). Although the easiest way to eliminate ribonuclease activity is by using commercial RNAse kits, the conventional method is given in this study.

According to studies, for successful results it is important to the take following points into consideration:

For protection from researcher-related contamination, which is the main potential source, latex or vinyl gloves should be worn while handling preparation materials and at all stages of the experiment.

Plastic tubes, glassware materials, pipettes and other materials should be used for only RNA analysis and never touched with bare hands.

All plastic material should be treated with 0.1% DEPC solution, which is a strong RNAse inhibitor, at least for 12 h. These materials should be autoclaved before being used to remove traces of DEPC. All glassware should be baked overnight at 180°C.

For preparing Tris buffers, glassware should be baked and autoclaved. DEPC treated water should be used. After preparation the buffer solutions should also be autoclaved. It is strongly recommended to use chemicals only for RNA analysis and all stages of the experiment should be done in cold conditions, on ice and cells should be disrupted with liquid Nitrogen, in order to minimize RNAse activity.

MATERIALS AND METHODS

Harvesting tobacco cells (Skokut and Filner, 1980; Guy *et al*., 1988; Guy and Heimer, 1993).

Cells of the XD line of tobacco, derivated from pith cells of *Nicotiana tobaccum* L. Cv. *Xanthi* were grown on nitrate-less M-ID medium. pH was adjusted to 6.2 with NaOH.

Filter-sterilized urea was used as the sole nitrogen source for supporting cell proliferation.

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The culture was grown in dark conditions on a gyrorotatory shaker (110 rpm) at 28°C.

Two milliliter of samples were pipetted, vacuum filtered through Whatman No.1 filter paper and the density of XD cells, total weight and necessary volume for the 1 g XD cells was calculated. Each 1 g sample was kept at -80°C

Solutions and buffers

Extraction buffer
50 mM TRIS-HCl (pH 8.3)
150 mM NaCl
10 mM EDTA
1% Lauryl sarcosine

PIC (24:1:24)
45.0 mL Phenol
37.5 mL Chloroform
1.5 mL Isoamyl alcohol
Use DEPC treated water
Stored at 4°C in a dark bottle

Stock solutions
1 M TRIS-HCl (pH 8.3)
0.5 M EDTA (pH 8.0)
3 M NaAOc (pH 5.2)

Experimental protocol

- Grind frozen sample in mortar with pestle, pour liquid Nitrogen and add some sand. Mix into a fine powder
- Transfer powder to corex tube with frozen spatula
- Add 500 μL extraction buffer (on ice)
- Add 500 μL PIC and Vortex
- Spin for 6 min at 9100 rpm in cold, RS5S centrifuge, temperature +4°C, fixed angle SS34, rotor code 05
- Carefully transfer the upper phase to a new corex tube
- Add 500 μL PIC
- Spin for 6 min at 9100 rpm in cold (RS5S centrifuge)
- Transfer and measure supernatant (SN) volume to a new corex tube
- Add 1/10 SN volume of 3 M NaOAc (pH 5.2)
- Add 1/1 total SN (including NaOAc) of isopropanol (-20°C) and Vortex
- Divide each sample into two Eppendorf tubes
- Put eppendorfs in an ice box
- Precipitate RNA for overnight at -20°C
- Spin Eppendorfs for 10 min in cold conditions
- Discard SN with narrow type Pasteur pipette
- Resuspend the pellet with 1125 μL of TE (10:10); for each Eppendorf 562.5 μL TE (10:10)
- Add 375 μL 8 M LiCl (for each Eppendorf 187.5 μL LiCl) and Vortex
- Leave samples for 4 h in ice box in a refrigerator
- Resuspend the pellet with 1125 μL of TE (10:10)
- Add 375 μL 8 M LiCl and Vortex
- Leave samples in an ice box in a refrigerator overnight
- Spin Eppendorfs for 15 min in cold conditions
- Transfer SN to a new tube
- Precipitate by adding 150 μL of cold (-20°C) 80% EtOH
- Spin for 1 min in cold conditions
- Dry pellet in incubator at 37°C (app. 30 min)
- Resuspend the pellet in 40 μL of TE (10:1)
- Take 4 μL of isolated RNA and add to 996 μL DEPC treated water
- Immediately store source of RNA (which was resuspended at step 31) at -80°C
- Measure in a spectrophotometer at a wavelength of 260 nm; 1 O.D unit corresponds to a concentration of 40 μg mL^{-1} RNA.

There are a number of ways to store RNA but the easiest way is to store the RNA in RNAse free water at -80°C. This will keep it in good condition for years, although after several freeze-thaw cycles it will eventually start to break down.

Quality verification of total RNA: The quantity and quality of all RNA samples should be checked before cDNA synthesis. Firstly, the concentration is measured at 260 nm and the presence/absence of proteins and other contaminants is checked at 280 nm. The ratio between [260 nm] and [280 nm] should be 1.85-2.00 (Maniatis *et al.*, 1982). If the RNA had a particularly low 260/280 spectrophotometric ratio, this is very likely to be the cause. Generally, for phase separation protocols, only 2/3 of the aqueous phase should be taken. Secondly, the quality of the RNA is checked by agarose gel electrophoresis.

Gel-Electrophoresis of Isolated RNA (Maniatis *et al.*, 1982)

• Preparation of 1% Agarose gel (40 mL)
4.0 g Agarose
30.8 mL DEPC treated water
Melt in microwave oven
Add 2.0 mL of MOPS buffer (×20)
Add 7.0 mL Formaldehyde

• Preparation of Running Buffer
Take 20 mL of MOPS (×20)

Adjust the volume of the solution to 400 mL with DEPC treated water

3.Preparation of Sample
1.0 μL EtBr (1 mg mL^{-1})
1.5 μL MOPS ×10
7.5 μL Formamide
2.6 μL Formaldehyde
5.0 μL Isolated RNA

- Load sample on gel
- Run at 59 V

RESULTS AND DISCUSSION

Data represented here are five replication which is each replicate consisting of minimum 10 sample results. The RNA preparation had an average yield ranging from 174.6-184.3 μg of RNA g cell FW^{-1} (Table 1). Taking into account that the absorbance ratio (A260/280) ranged from 1.88-1.97 which indicates that RNA is quite pure (Maniatis *et al*., 1982), the results imply that improved protocol for tobacco cells was successfully worked in cell suspension culture. Also higher purity and yield of RNA was determined (Table 1). The integrity and quality of total RNA were confirmed by electrophoresis.

The following protocol can be applied to wide variety of plant tissues in variably yielding large amounts of undegraded, pure total RNA suitable for further analysis (Hunter *et al*., 2001; Hosein, 2001). However it can be easily improved and adapted for other plant materials, as well (Gehring *et al*., 2000; Jiang *et al*., 2000; Scotti *et al*., 2001).

The objective of this study was to examine the effectiveness of a improved protocol of RNA isolation on tobacco cells as a case study for ecologic research. Overall of present results are in agreement with previous studies that this protocol is effectively works, cheaper than commercial isolation kits and able for high quality RNA isolation, while adding some modification with the optimum results may use other plant species. These modifications on the extraction phase may provide useful tools for wide range plant material.

Table 1: Yield and quality of RNA isolation from tobacco cells

Replication	No. of samples	A260/280 ratio	μg RNA g cell FW^{-1}
I	10	1.950	178.80
II	15	1.970	184.30
III	13	1.920	175.90
IV	10	1.940	179.30
V	13	1.880	174.60
Average		1.930	178.60
Variance		0.012	14.08
Standard deviation		0.034	3.75

CONCLUSIONS

The earlier protocol named as overnight centrifugation of the GTC lysate through a cesium chloride cushion, capitalies more on the greater density of RNA than contaminating DNA and protein. Chomczynski and Sacchi (1987) used acid phenol/chloroform extraction instead of the previous protocol to accomplish this separation. In acidic phenol solutions, RNA will remain in the aqueous phase, while DNA and protein will partition to the interphase and organic phase, respectively. The quality of the RNA sample can be estimated by comparing the 28S and 18S bands. After running the RNA, the gel should be wrapped with it in cling film. There is no risk of RNAse contamination while or after the RNA is transferred to a solid support. The main the problem is drying out of the gel rather than RNAse contamination.

This modified protocol for RNA isolation is reliable, convenient for multiple samples and because it does not need any commercial RNA kits it is therefore cheaper; easier and especially the quality of RNA isolate is very high in *Nicotiana tobaccum*. The incremental success of that protocol indicates a potentially useful tool for other ecologic studies.

ACKNOWLEDGMENTS

This study was supported by grants from Mr. Cihat Kora and the authors would like to gratefully acknowledge the generosity of him.

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