*Plant Molecular Biology Reporter* **18:** 109–115, 2000
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*Protocols*

# A Simple Procedure for the Isolation of High Quality RNA from Ripening Banana Fruit

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**Abstract.** Isolation of high quality RNA from ripening banana fruit tissue is difficult due to high levels of polysaccharides and other substances that interfere when using conventional procedures for RNA isolation. These substances not only decrease the yield but the quality of RNA is almost unusable. We describe here a simple RNA procedure that effectively removes these contaminating substances without affecting the yield. Following this procedure, we routinely obtained 80-150 μg of total RNA per g fresh tissue. The RNA is of good quality and suitable for northern analysis, RT-PCR and cDNA library construction.

**Key words:** Banana, fruit tissue, *Musa acuminata*, polysaccharides, RNA extraction

**Abbreviations:** AMVRT, Avian Myeloblastosis Virus Reverse Transcriptase; CIP, Calf Intestine Alkaline Phosphatase; CTAB, cetyltrimethylammoniumbromide; DEAE, diethylaminoethyl; DEPC, diethyl pyrocarbonate; EtBr, ethidium bromide; EtOH, ethanol; RT-PCR, reverse transcription polymerase chain reaction; SDS, sodium dodecyl sulphate; SSC, saline sodium citrate.

## Introduction

Isolation of good quality RNA from tissues rich in polysaccharides and polyphenols is often difficult. The presence of these substances can affect the quality and/or quantity of the RNA isolated. Previous plant RNA isolation procedures often fail to work when applied to tissues rich in secondary products (Logemann et al., 1987; Levi et al., 1992; Lopez-Gomez and Gomez-Lim, 1992). We have been interested in studying the molecular biology of banana fruit tissue that is rich in polyphenols and polysaccharides. Our aim has been to study differential expression of genes during the course of ethylene-induced ripening. Hence, we needed a protocol that not only gives the same quality and quantity of RNA from each ripening stage but is also high yielding in order not to miss the rarely/weakly expressed genes. Banana fruit tissue, especially pulp, changes its characteristics as ripening progresses. We have modified the CTAB/NaCl method of Chang (1993) by removing PVP from the extraction buffer and including a simple polysaccharide precipitation step that does not affect the RNA yield but removes contaminating polysaccharides. With these and other modifications, we have developed a

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simple isolation procedure that yields high quality RNA from banana tissue, is cost effective, and does not require ultracentrifugation.

## Materials and Methods

### *Plant material*

Mature green and unripe Banana (*Musa acuminata* var. Harichal) fruits were obtained from a local farm and treated with 100 ppm ethylene for 24 h and then allowed to ripen for 7 d at 22°C. The banana fingers were harvested at various stages of ripening. The tissue was sliced, immediately frozen in liquid nitrogen, and stored at -70°C until further use.

### *Solutions and reagents*[1]

Extraction buffer: 100 mM Tris-Cl pH 8.2, 1.4 M NaCl, 20 mM EDTA (pH 8), 2% CTAB
10 M LiCl
3 M Na Acetate pH 5.2
2-mercaptoethanol
DEPC treated and autoclaved MilliQ grade water
Absolute EtOH
70% EtOH
Water saturated phenol
Chloroform : isoamyl alcohol (24:1)

### *RNA extraction protocol*

- Use a ratio of 10 mL extraction buffer / gram of tissue, add 1 µl of 2-mercaptoethanol per mL of buffer just before use
- To the frozen tissue add preheated (65°C) extraction buffer and homogenize in a warring blender at maximum speed giving two 30 s bursts.
- Transfer the homogenate to a clean 30 mL centrifuge tube and incubate at 65°C for 1 h, with gentle vortexing every 15 min.
- Cool the tube to room temperature and add an equal volume of chloroform[2]. Shake vigorously until the two phases form an emulsion, vent the tube during mixing.
- Centrifuge at 12000 *g* for 15 min at room temperature.
- Collect the aqueous phase and re-extract with an equal volume of chloroform[2]. Centrifuge as above.
- Collect the aqueous phase and add 10 M LiCl to a final concentration of 3 M and allow the RNA to precipitate at 4°C overnight.
- Recover the RNA by centrifugation at 17000 *g* at 4°C for 20 min.

[1] All the reagents were DEPC treated and autoclaved except Tris-Cl which was prepared in RNase free water. All transfers and manipulations have been done under RNase free conditions.
[2] Chloroform is always used as mixture of chloroform : isoamyl alcohol (24:1).

- Dissolve the pellet in DEPC treated water and extract once with phenol, phenol : chloroform (1:1), and chloroform, sequentially.
- Collect the aqueous phase and add to it 1/30 volume of 3 M Na acetate pH 5.2 and 0.1 volume of 100% EtOH, mix well and keep on ice for 30 min. Centrifuge in a microfuge at 4°C for 25 min. A white jelly-like pellet consisting mostly of polysaccharides is obtained and discarded. This step effectively removes any contaminating polysaccharides without any substantial loss in the amount of RNA.
- This step may be repeated if the problem of polysaccharide contamination persists.
- To the clear supernatant add 3 M Na acetate pH 5.2 to a final concentration of 0.3 M and 3 volumes of 100% ethanol.
- Allow the RNA to precipitate at -70°C for 3 h to overnight. Recover the RNA by centrifugation in a microfuge at 4°C for 20 min. Wash the pellet with equal volume of 70% EtOH. Vacuum dry the pellet and re-suspend in an appropriate volume of DEPC treated water (100 µl/g starting material).
- Quantitate the RNA spectrophotometrically at wavelengths of 240,260,280,300 and 320 nm.

In order to test the quality of RNA, we carried out northern analysis, RT-PCR analysis and prepared a cDNA library.

*Northern analysis*

40 µg of total RNA from various ripening stages of banana pulp were electrophoresed on 1% agarose-formaldehyde gels and blotted onto a nylon membrane (Hybond NX, Amersham) according to the manufacturer's instructions. The membrane was probed with $\alpha^{32}$P dATP labelled partial cDNA of β-fructosidase gene of banana. The probe was labelled by random priming using Klenow DNA polymerase as described in Sambrook et al. (1989). Hybridisation was carried out for 16 h at 42°C in 6x SSC, 1% SDS, 5x Denhardt's solution, and 50% formamide with $>1X10^6$ cpm of labelled probe per mL of hybridisation mixture. The blot was washed twice in 0.2x SSC (0.3 M NaCl and 30 mM Na citrate) and 0.1% SDS at 65°C. The blot was exposed to Kodak X-Omat film between two intensifying screens at -70°C. The autoradiogram was developed after 72 h of exposure.

*RT-PCR analysis*

RT-PCR was carried out using the Promega Access RT-PCR System in a single tube reaction protocol. The reverse transcriptase reaction was carried out at 48°C for 1 h, followed by heat inactivating the AMVRT at 94°C for 2 min. The PCR reaction was carried out for 40 cycles; conditions for each cycle being denaturation at 94°C for 30 s, annealing at 50°C for 1 min, and extension at 68°C for 2 min 30 s. The final extension was carried out at 68°C for 7 min. An amplicon of the expected size was obtained for the desired gene as confirmed later by sequencing.

*cDNA library construction*

The mRNA was purified by oligo dT cellulose column chromatography as described by Sambrook et al. (1989). The yield was around 1% of the total RNA applied. Two micrograms of mRNA was used for cDNA library construction. The cDNA was prepared using the Promega Universal cDNA Synthesis System. Radioactive incorporation was used to monitor the first strand and the second strand synthesis. The cDNA produced were ligated to pUC18 *Eco*R I/ CIP treated vector, and transformed in *Ecoli* XL1 Blue MRF' cells.

## Results and Discussion

Several methods reported for the isolation of RNA from plant failed to give either high quality or yield of RNA from banana tissue. During banana ripening, insoluble starch is converted to soluble polysaccharides and these polysaccharides display physicochemical properties similar to those of RNA. They may co-precipitate and contaminate the RNA during the extraction affecting the yield and quality (Logemann et al., 1987; Mc Neil et al., 1984). For the successful isolation of intact RNA from tissues rich in polysaccharides and polyphenols, it is important to prevent these contaminating substances from binding to nucleic acids. Additionally, the presence of pectic substances in the cell wall of ripening banana and the high content of polyphenols may complicate nucleic acid extraction. Recently a protocol was described by Liu et al. (1998), where ice cold potassium acetate was used to precipitate genomic DNA and the secondary metabolites in the banana fruit tissue. However, in our experience, this step greatly affects the RNA yield. In our protocol, the polysaccharide precipitation step effectively removes the polysaccharides without affecting the yield of RNA. In the presence of 0.1 volume of ethanol and 1/30 volume of 3 M Na acetate, the RNA remains in the solution, whereas the polysaccharides precipitate forming a jelly-like precipitate. The RNA in the supernatant can now be precipitated by adding Na acetate to a final concentration of 0.3 M and 3 volumes of 100% ethanol. This method consistently gave good RNA yield in the range of 80-150 μg/g fresh weight of tissue. The polyphenols are removed by the 20 mM EDTA and 2-mercaptoethanol. We have not used PVP in the extraction buffer since it is incompatible with phenol extraction and binds to nucleic acids.

An RNA isolation procedure can be judged by the quantity, quality and the integrity of the RNA obtained. The $A_{260}/A_{230}$ absorbance ratio indicates polysaccharide or polyphenolic contamination and the $A_{260}/A_{280}$ ratio indicates the protein contamination (Logemann et al., 1997; Manning 1990). Ribosomal RNA represents >90% of the total RNA, and any degradation in the RNA preparation can be easily visualized on an EtBr agarose gel. We repeatedly obtained $A_{260}/A_{240}$ ratios greater than 1, and the $A_{260}/A_{280}$ ratios were always between 1.8 and 2.0, indicating that there was no polysaccharide, polyphenol or protein contamination in our preparation. Apart from the integrity of the ribosomal bands (Figure 1), the intactness of the RNA can also be monitored by northern analysis, RT-PCR and cDNA library construction. Expression of the β-fructosidase gene was monitored during the ripening period (Figure 2). The expression of this gene was represented as a single band without any smearing. Also the expression shows a characteristic

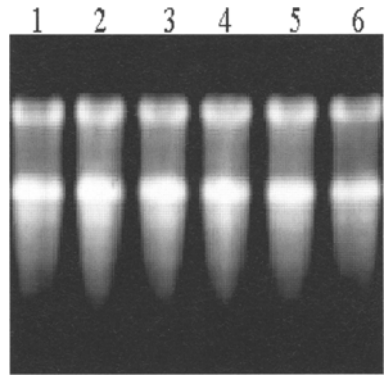


*Figure 1.* Visualization of total RNA from tissues of various ripening stages. Total RNA was separated on 1% non-denaturing agarose gel containing EtBr and photographed under ultraviolet light. Lane 1 represents RNA isolated from control untreated banana fruit, lanes 2-6 represent RNA isolated from banana fruits after 1,2,3,4 and 5 d after ethylene treatment respectively.

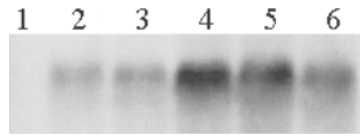


*Figure 2.* Autoradiogram of northern analysis of the β-fructosidase gene. 40 µg of total RNA from tissues of different ripening stages was loaded in each lane. Lanes 1-6 represent different ripening stages as in Figure 1.

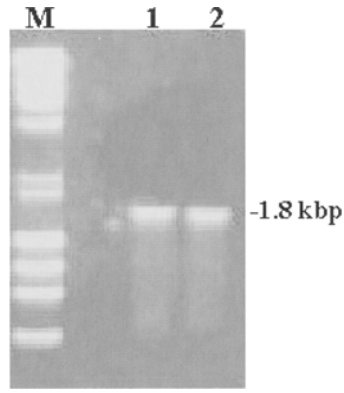


*Figure 3.* Agarose gel electrophoretic analysis of RT-PCR amplified cDNA of a ripening related gene. Lane M represents DNA marker, lanes 1 and 2 are RT-PCR amplicons from total RNA and mRNA respectively.

pattern comparable to enzyme activity. This indicates that the RNA was intact, that the procedure is not affected by developmental stage, and that even a low expression of the gene can be monitored. Using gene specific primers and an RT-PCR technique we have cloned four different genes from ripening banana. Figure 3 shows the RT-PCR amplicon of one of the ripening related genes. A

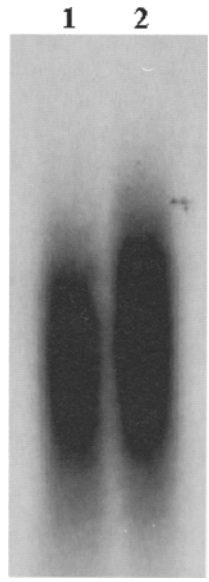


*Figure 4.* Autoradiogram of labelled cDNA resulting from reverse transcription of Total RNA with oligo dT primers. $^{32}P$ labelled reaction products were electrophoresed on a 1% alkaline agarose gel, transferred to DEAE paper and autoradiographed overnight. cDNAs range from 4 kb to 200 bp. Lane 1 represents cDNA/RNA hybrids of first strand synthesis and lane 2 represents cDNA of second strand synthesis.

similar amplicon was obtained from total RNA as well as mRNA when it was used as template in different experiments. For the cDNA library construction, 2 μg of mRNA was used and the first and second strand synthesis was monitored by radioactive incorporation assays. The RNA preparation was not biased for any specific size as the cDNAs varied from 500 bp to 4 kb (Figure 4). More than 58% of the polysomal RNA was transcribed into the first strand of which 98% was copied to the second strand. Though the complexity of the library has not yet been fully evaluated, on preliminary screening of recombinant clones, we found inserts ranging from 400 bp to 2 kb (data not shown).

Thus, this procedure is highly useful for the isolation of good quality and quantity of total RNA from various ripening stages of banana pulp tissue. This procedure has also been applied to banana peel tissue and the results were found comparable. We believe that this procedure can successfully applied to other tissues that are rich in polysaccharides.

## Acknowledgements

We thank the Council of Scientific and Industrial Research, India for the fellowship to MHA and PD. Financial assistance by Department of Biotechnology, India is also acknowledged.

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