3

Isolation of Messenger RNA from Plant Tissues

Alison Dunn

1. Introduction

The starting material for any representative plant cDNA library is a supply of good-quality messenger RNA from the plant tissue of choice. Extraction of RNA can be made from several grams of tissue or as little as 50 mg. However, large samples are generally more representative of the genes expressed in a population of plants in response to environmental cues or at a defined stage of development. Therefore, large-scale extraction of RNA is the method of choice for preparations to be used for cDNA libraries.

Several published protocols describe the rapid extraction of RNA from small quantities of plant tissue *(1,2)*, and a number of small scale RNA extraction kits are commercially available (Gibco-BRL Life Technologies, Middlesex, UK; Dynal, Oslo, Norway; Qiagen, Germany). Small-scale methods can be used to extract RNA for the preparation of cDNA libraries when quantities of suitable plant material are severely limited. However, for reasons already given, they are not ideal and are generally more useful for the analysis of expression of large numbers of individual plants. They are not further considered here.

The greatest obstacle to obtaining good-quality RNA is the ubiquitous and persistent nature of highly active RNases in plant tissues. All RNA extraction techniques are therefore based on initial inactivation or inhibition of RNases by chemical or physical means, such as suboptimal pH and temperature, followed by separation of nucleic acids from proteins, usually by extraction with phenol, thereby separating the RNA from RNases. The extraction buffer described here contains 4*M* guanidinium thiocyanate and β-mercaptoethanol, both of which irreversibly inactivate RNases *(3)*, and subsequent steps in the protocol employ high-pH buffers (pH 9.0) and a temperature of 50°C, which

From *Methods in Molecular Biology, Vol 69 cDNA Library Protocols*
Edited by I G Cowell and C A Austin Humana Press Inc , Totowa, NJ

inhibits RNase activity. Proteins are subsequently removed by phenol/chloroform extraction. Extraction of RNA is inevitably accompanied by extraction of cellular DNA, and enrichment for total cellular RNA is desirable, although not essential. Only approx 10% of total cellular RNA is messenger RNA; the rest is mainly ribosomal RNA. Since mRNA is the substrate for cDNA synthesis, enrichment for this fraction of total RNA is highly desirable and is achieved by affinity chromatography on oligo (dT)-cellulose. Polyadenylated mRNA (polyA$^+$ RNA) binds to oligo (dT)-cellulose at high salt concentrations and is eluted in a salt-free buffer. This is the basis of the method described here. It is worth noting that a kit for polyA$^+$ RNA purification is commercially available (Pharmacia, Uppsala, Sweden), which has the advantage of prepacked RNase-free columns and reagents reducing sources of RNase contamination.

Since RNases are ubiquitous and are not inactivated by autoclaving, there is a danger of contamination from laboratory glassware, reagents, and handling. Steps should be taken:

1 To decontaminate, as far as possible, all equipment to be used in the extraction,
2 To ensure inactivation of RNases in laboratory-prepared solutions, and,
3. To avoid recontamination

All work should be carried out wearing clean, disposable plastic gloves and, if possible, equipment and chemicals kept only for RNA extraction and analysis should be used, e.g., pestles, mortars, spatulas, Corex® tubes (DuPont, Wilmington, DE), reagent bottles. Sterile, disposable plasticware, which has not been handled, is RNase-free. RNA preparation requires a degree of paranoia!

2. Materials

2.1. RNA Extraction

2.1.1. Equipment for RNA Extraction

1. Corex tubes (12 × 40 mL): Siliconized by soaking in dichlorodimethylsilane (5% [v:v]) in chloroform for 15 min, rinsing four times with deionized water and once with 100% ethanol. The siliconizing solution can be stored for further use.
2. Glassware and nondisposable plasticware: glass rod, measuring cylinder, 10-mL pipets, bottles (4 × 100 mL), filter funnel, sterile universal. Pretreat by soaking overnight in a 2% (v/v) solution of Absolve (DuPont; *see* Note 1) and thoroughly rinse before sterilization, preferably by baking at 180°C for several hours or by autoclaving for 15 min at 15 psi.
3 Pestle (not wooden handles) and mortar: pretreat as for glassware (*see* Notes 1 and 2).
4. Sterile Miracloth (Calbiochem, La Jolla, CA).

2.1.2. Chemicals for RNA Extraction

1. Guanidinium thiocyanate (GT) (Fluka, Buchs, Switzerland).
2. β-Mercaptoethanol.
3. Sodium citrate.
4. Sodium lauryl sarcosine
5. Oligo (dT)-cellulose (Pharmacia)
6. Diethylpyrocarbonate (DEPC).
7. Liquid nitrogen.
8. Phenol (equilibrated in 0.1*M* Tris-HCl, pH 8.0).

2.1.3. Solutions for RNA Extraction (see Notes 3 and 4)

Except where noted, prepare 100 mL of each of the following:

1. 200 m*M* Sodium citrate
2. 10% (w/v) Sodium lauryl sarcosine.
3. 1*M* Acetic acid.
4. 50 m*M* Tris-HCl, pH 9.0, 0.2*M* NaCl, 5 m*M* EDTA, 1% (w/v) sodium dodecyl sulfate (SDS).
5. 3*M* Sodium acetate, pH 5.5
6. 0.2*M* Sodium acetate, pH 5.5.
7. Chloroform.pentan-2-ol (24:1 [v/v]).
8. TE Buffer 10 m*M* Tris-HCl, pH 7.4, 1 m*M* EDTA.
9. 1 L DEPC water
10. 4*M* GT medium: Dissolve 50 g guanidinium thiocyanate in 50 mL sterile deionized water and heat to 60 or 70°C to dissolve if necessary Add 10.6 mL 200 m*M* sodium citrate solution and 10 6 mL 10% sodium lauryl sarcosine, make up to 106 mL with sterile water Filter if necessary (only if sediment is visible) and divide between two sterile dark bottles (wide-necked 100-mL are best so that ground plant tissue can be added directly to the bottle). Store at 4°C until required (stable indefinitely). Add β-mercaptoethanol to 0.1*M* just before use (0.43/50 mL GT medium).

2.2. Purification of PolyA⁺ RNA

2.2.1. Equipment for PolyA⁺ Purification

1. Two plastic mini-columns (5 mL).
2. 15-mL Corex centrifuge tubes.

2.2.2. Solutions for PolyA⁺ Purification

Prepare 20 mL of each of the following from stock solutions.

1. 10 m*M* Tris-HCl, pH 7.5, 1*M* KCl.
2. 10 m*M* Tris-HCl, pH 7.5, 0.5*M* KCl (loading buffer).
3. 10 m*M* Tris-HCl, pH 7.5, 0.1*M* KCl (wash buffer).
4. 10 m*M* Tris-HCl, pH 7.5, preheated to 60°C (elution buffer).
5. 3*M* Sodium acetate, pH 5.5.

3. Methods

3.1. RNA Extraction

This method is a modification of that described in ref. *4*.

1. Add 4–5 g plant tissue to liquid nitrogen in precooled mortar and pestle, when N_2 has almost evaporated, grind vigorously—keep cool and add more N_2 if necessary The ground tissue should be a very fine powder (*see* Note 5)
2. Scrape frozen powder with a sterile spatula into 55-mL GT medium in a sterile bottle and shake for 2 min. Filter through two layers of sterile Miracloth into a clean bottle or directly into Corex tubes
3 Dispense into Corex tubes, and centrifuge for 10 min at 12,000*g* at 10°C.
4. Collect supernatant, add 0.025 vol 1*M* acetic acid and 0 75 vol absolute ethanol at room temperature, invert to mix (cover with Nescofilm while mixing) and centrifuge for 15 min at 12,000*g* at 10°C
5 Remove supernatant, dry pellet, and redissolve in 4 mL 50 m*M* Tris-HCl, pH 9 0, 0.2*M* NaCl, 5 m*M* EDTA, 1% SDS (*see* Note 6)
6 Transfer to a sterile universal tube, warm to 50°C (pellet from previous step may not fully dissolve until now, but make sure it is dissolved before proceeding) Add an equal volume of phenol and shake vigorously.
7 Transfer to a fresh Corex tube, centrifuge for 10 min at 4500*g* Transfer the aqueous phase to a fresh Corex tube, extract with an equal volume of chloroform:pentan-2-ol, recentrifuge as previously, and remove the aqueous phase to a fresh Corex tube.
8. Add 0.1 vol 3*M* sodium acetate, pH 5.5 and 2.5 vol absolute ethanol, mix, and leave at 20°C overnight to precipitate nucleic acids.
9. Centrifuge for 30 min at 12,000*g*, decant supernatant, and dry the pellet. Add 2 mL 0.2*M* sodium acetate, pH 5.5, and allow to stand for 1 h with gentle agitation occasionally (*see* Note 7).
10. Remove solution to a fresh Corex tube (leaving behind any undissolved nucleic acid), add 2.5 vol absolute ethanol, and precipitate overnight at –20°C.
11 Centrifuge for 30 min at 12,000*g*, decant supernatant and dry the RNA pellet. Redissolve in either TE buffer or loading buffer for polyA$^+$ purification (*see* Notes 8 and 9).

3.2. Preparation of PolyA$^+$ RNA

1 Equilibrate 0.3 g oligo(dT)-cellulose with loading buffer (*see* Section 2.2.2) and load onto a minicolumn. Allow to drain.
2. Heat 0.5 mg total RNA (*see* Note 10) dissolved in 1 mL loading buffer at 65°C for 5 min, cool on ice, and then load onto the column. Collect the eluant, and reload it onto the column. Allow to drain and discard eluant.
3. Wash column with 5 mL wash buffer (*see* Section 2.2.2.) and discard eluant.
4 Have ready elution buffer (*see* Section 2.2.2.) at 60°C Elute polyA$^+$ RNA with 5 mL elution buffer at 60°C

5 Collect eluant in Corex tube, add 0.1 vol 3*M* sodium acetate, pH 5.5, 2 5 vol absolute ethanol, and precipitate for 2 h or overnight at –20°C
6. Centrifuge for 30 min at 12,000*g* at 10°C, to recover polyA$^+$ RNA Dissolve in TE buffer or DEPC-treated sterile water.
7. To prepare RNA that is 90% polyA$^+$, steps 1–6 should be repeated with a fresh oligo(dT) cellulose column The final pellet should be washed in 2 mL 70% ethanol and recentrifuged for 5 min at 12,000*g* at 10°C, dried, and redissolved in 50 µL DEPC-treated water

4. Notes

1. Absolve is an alkaline detergent that is effective in removing RNases, but it can cause degradation of RNA if glassware is not thoroughly rinsed.
2. Pestles and mortars should be precooled at –70°C following sterilization.
3. Common laboratory chemicals are not listed.
4. DEPC is a powerful RNase inhibitor that can be used to treat water and solutions for RNA preparation. DEPC should be added to water and solutions (except those containing Tris buffers) in the ratio 1 mL:1 L (0.1%). Solutions should be thoroughly shaken, then left overnight in a fume hood, and autoclaved at 15 psi for 15 min to remove residual DEPC DEPC carboxymethylates purine residues in RNA, which affects efficiency of in vitro translation, but not hybridization DEPC is volatile and a suspected carcinogen It should be handled with care in a fume hood. Where DEPC treatment is inadvisable, chemicals kept separate from general laboratory stocks should be used.
5. This is probably the most important step in the procedure. Well-ground plant tissue gives high yields of RNA.
6. This step can be slow, and it may be necessary to disrupt the pellet gently by pipeting
7. This preferentially redissolves RNA leaving most of the DNA in the pellet. It may be necessary to recentrifuge briefly (5 min at 12,000*g*) if the residual pellet is not sticking to the tube
8 Use TE buffer or manufacturer's recommendation if using a kit for polyA$^+$ purification
9. All ethanol precipitations are incubated overnight at –20°C, but 2 h (minimum) at –20°C can be substituted if you are in a hurry.
10. RNA concentrations can be determined using a spectrophotometer (1 A_{260} U = 40 µg/mL RNA) or by visualization of a diluted aliquot on an ethidium bromide-impregnated agarose gel against tRNA standards. (Use 1 µL of each dilution.)
11. The described RNA extraction has been used to make good-quality RNA to prepare cDNA libraries from barley (*Hordeum vulgare*) *(5)*, white clover (*Trifolium repens*) (unpublished), and cassava (*Manihot esculenta*) *(6)*, and typically yields 1–2 mg total RNA/extraction (0.5 mg/g of starting material). More recently, polyA$^+$ RNA has been prepared using an oligo(dT) cellulose kit (Pharmacia) as an alternative to the method described here

References

1 Jakobsen, K. S , Breiwold, E., and Hornes, E. (1990) Purification of mRNA directly from crude plant tissues in 15 minutes using magnetic oligo dT microspheres. *Nucleic Acids Res* **18,** 3669.

2 Verwoerd, T. C , Dekker, B. M M, and Hoekema, A (1989) A small scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Res* **17,** 2362.

3. Chirgwin, J M., Przbyla, A E , McDonald, R J , and Rutler, W J (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease *Biochemistry* **18,** 5294–5299

4 Broglie, R., Coruzzi, G., Keith, B , and Chua, N -H. (1986) Molecular biology of C4 photosynthesis in Zea mays: differential localisation of proteins and mRNAs in two leaf cell types. *Plant Mol Biol* **3,** 431–444.

5 Dunn, M. A , Hughes, M. A., Pearce, R. S , and Jack, P. L. (1990) Molecular characterisation of a barley gene induced by cold treatment. *J Exp. Bot* **41,** 1405–1413.

6. Hughes, M A , Brown, K., Pancoro, A., Murray, B S., Oxtoby, E., and Hughes, J (1992) A molecular and biochemical analysis of the structure of cyanogenic β-glucosidase (linamarase) from cassava (*Manihot esculenta* Crantz) *Arch Biochem. Biophys* **295,** 273–279.