A robust plant RNA isolation method suitable for Affymetrix GeneChip analysis and quantitative real-time RT-PCR

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Microarray analysis and quantitative real-time RT-PCR are the major high-throughput techniques that are used to study transcript profiles. One of the major limitations in these technologies is the isolation of large quantities of highly pure RNA from plant tissues rich in complex polysaccharides, polyphenolics and waxes. Any contamination of the isolated RNA affects the downstream applications and requires extra cleaning procedures that result in a reduced RNA yield, especially the low molecular weight molecules. The protocol presented here is suitable for isolating high yield and clean total RNA from field-grown plants. Unlike current methods, such as LiCl and TRIZOL, with this new method, the isolated RNA can be used directly for Affymetrix GeneChip labeling or real-time RT-PCR without further purification. This fast and simple protocol provides ready-to-use RNA within 4–5 h after sampling. Additionally, the protocol described here maintains the isolation of small RNA molecules, making it an ideal choice for plant RNA preparation prior to high-throughput sequencing methods to study gene expression.

INTRODUCTION

Recent progress in plant genome sequencing and the parallel development of global gene expression analysis platforms provide the opportunity to understand developmental and physiological events at the genome level[1–5]. Microarrays/gene chips and quantitative real-time RT-PCR assay platforms are now widely used to quantify RNA levels[6–8]. Although these technological advances have made global gene analysis more feasible and reproducible, monitoring large numbers of transcripts in miniaturized assays makes the analysis a considerable challenge because of the fragile chemical nature of RNA molecules. Thus, a successful gene expression analysis largely relies on obtaining abundant, intact and pure RNA molecules suitable for reverse transcription, labeling and hybridization.

Several protocols[7,9–11] and commercial kits (i.e., Qiagen RNeasy Plant Kit, Invitrogen TRIZOL reagent and Ambion RNAqueous Kit) are used for the extraction of ribonucleic acids from different plant tissues. However, these generally do not allow maximum recovery of RNA, are time consuming or need specialized kits and/or equipment. Moreover, the differences in the biochemical compositions of plant tissues from different species have resulted in protocols that require special chemicals or extraction practices that are far from a 'generic' application approach. Several methods have been reported for the isolation of RNA from plant tissues (such as TRIZOL reagent extractions, and Chomczynski and Sacchi single-step RNA isolation[11]), but these methods have failed to produce either maximum quality or yield of RNA from field-grown soybean plants rich in polysaccharide. Contaminating polysaccharides and proteins may co-precipitate with the RNA during the extraction, affecting yield and quality and subsequently contributing to RNA degradation or enzyme fidelity in subsequent applications. Commonly used lithium salts, even when present in minute amounts, require long incubation periods and interfere with downstream enzymatic applications (e.g., reverse transcription). The presence of contaminating DNA is another problem often requiring enzymatic removal that otherwise causes false-positive results in amplification-dependent analyses such as RT-PCR. The use of a silica column to provide clean cytoplasmic RNA eliminates smaller molecular weight RNA species, including miRNA precursors, and other functional small RNA species making their global analysis impossible.

We describe here a robust plant RNA isolation protocol (DB protocol), primarily developed for soybean plants, suitable for GeneChip and real-time RT-PCR analyses (**Fig. 1**). The DB protocol consistently provides high yields of RNA from field-grown soybean (*Glycine max*) and *Nicotiana attenuata* tissues free of DNA, polysaccharide and other contaminants[14–16]. Both plant species are polysaccharide rich and have leaves with thick surface wax. The DB protocol is designed to be easily applied, process many samples ($\sim$16–24) at a time and provide high-quality RNA with high yield. Different from urea, LiCl- and NaCl-based RNA isolation protocols that are performed under basic pH conditions[11–13], in this protocol, RNA isolation depends on acidic pH. The presence of acidic phenol in the extraction buffer increases the strength of the homogenization buffer as an RNase denaturant. The integrity and size distribution of purified RNA were verified with analytical agarose gels and Bioanalyzer (Agilent) microfluidic electrophoresis chips. Compared with common preparations using a kit (i.e., RNeasy Plant RNA Kit), or commercial extraction buffer (i.e., TRIZOL reagent), the RNA purified with the DB protocol consistently yields purer RNA, typically with A_{260}/A_{280} ratios of 1.9–2.3 optical densities (**Fig. 2**). As some organic contaminants absorb light at 230 nm, 260/230 nm absorbance ratios were also measured. On average, 260/230 absorbance ratios >2.2 are attained for samples isolated by the DB protocol, whereas TRIZOL reagent and precipitation with 2-propanol yield samples with ratios ≤ 1.8, indicating improved removal of organic contaminants with the DB protocol. Ideal absorbance ratios of 260/230 are particularly important for successful *in vitro* transcription in Affymetrix experiments and are often

required by microarray core facilities as a prerequisite for initiating labeling reactions. **Table 1** summarizes the 230, 260 and 280 nm readings of total RNA isolated with various methods. The readings were taken with a Nanodrop ND-1000 spectrophotometer. Although the protocol works efficiently with various tissues (leaves, stems and roots), plant tissues such as dry seeds that contain excessive amounts of secondary metabolites and storage proteins may require inclusion of polyvinylpyrrolidone (PVP-40) up to 4% (wt/vol) to improve RNA purity and yield. The DB protocol has not been tested for microscale high-throughput format RNA purification. For a feasible application of the method, homogenization and phase separation steps may require optimization.

RNA preparations from the DB protocol also contain more RNA species of small molecular weight (<300 ribonucleotides) compared with Qiagen cleaned total RNA (**Fig. 3**) and are free of any DNA contamination (**Fig. 4**). Without further purification, RNA isolated with the DB protocol is directly applicable for use in an Affymetrix probe labeling reaction and real-time RT-PCR (**Figs. 5** and **6**).

To date, our labs have successfully isolated total RNA using the DB protocol from more than 500 soybean samples and used >300 purified RNA preparations for Affymetrix GeneChip hybridizations and quantitative real-time RT-PCR assays[14–16]. The purified RNA readily dissolved in RNase-free water or 10 mM Tris buffer and was stable over the test period of 3 years when stored at −80 °C. The isolated RNA is suitable for downstream applications, including northern blot analysis, microarray hybridization (cDNA and Affymetrix GeneChip), reverse transcription and quantitative real-time PCR analysis without further processing[15]. We were able to purify RNA from mature aerial tissues of dicot plants, some of which contain significant amounts of polyphenolics, polysaccharides or secondary metabolites[14–16]. The protocol is also suitable for library construction, and rapid amplification of cDNA ends (D.D.B., unpublished results). The RNA isolation from storage tubers, fruits or seeds of exotic plant species may require additional purification steps or alternative methods specifically addressing recalcitrance due to complex tissue composition.

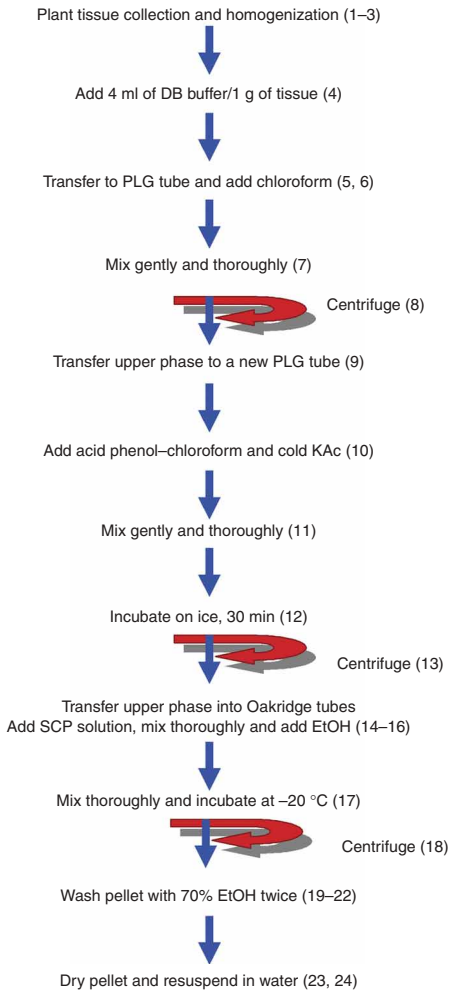


Figure 1 | Flow diagram to summarize the major steps of total RNA isolation with the DB protocol. Each step in parentheses is described in detail in the PROCEDURE section.

MATERIALS

REAGENTS

▲ CRITICAL Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

- Acid phenol–chloroform (Ambion Inc., Austin, TX, cat. no. AM9722)
  ! CAUTION Phenol should be handled in a fume hood.
- Acidic phenol (nucleic acid grade) (Sigma Aldrich, St Louis, MO, cat. no. P4557) ! CAUTION Phenol should be handled in a fume hood. ▲ CRITICAL Do not equilibrate phenol with 10 mM Tris HCl pH 8, 1 mM EDTA.
- Agarose (molecular biology grade) (Fisher Scientific, Pittsburgh, PA, cat. no. PI-17850)
- Ammonium thiocyanate (Sigma Aldrich, St Louis, MO, cat. no. A7149)
- Bromophenol blue (sodium salt) (Sigma Aldrich, St Louis, MO, cat. no. B8026)
- Chloroform (Fisher Scientific, Pittsburgh, PA, cat. no. C606SK-1)
- Citric acid (anhydrous) (Sigma Aldrich, St Louis, MO, cat. no. 27488)
- Ethanol (EtOH)
- Ethidium bromide (Sigma Aldrich, St Louis, MO, cat. no. E1385.)
  ! CAUTION This chemical is harmful, avoid contact with eyes and skin.
- Glycerol (Fisher Scientific, Pittsburgh, PA, cat. no. G33-500)
- Guanidine thiocyanate (Ambion Inc., Austin, TX, cat. no. AM9422)
- Phase lock gel-heavy tubes (PLG; Brinkmann Instruments Inc., Westbury, NY)
- 3 M Potassium acetate pH 5.5 (Ambion Inc., Austin, TX, cat. no. AM9610)
- RNA Zap (Ambion Inc., Austin, TX, cat. no. AM9780)
- 3 M Sodium acetate pH 5.5 (Ambion Inc., Austin, TX, cat. no. AM9740)
- Sodium chloride (nucleic acid grade) (Sigma Aldrich, St Louis, MO, cat. no. S3014)
- Sodium citrate (tribasic dihydrate) (Sigma Aldrich, St Louis, MO, cat. no. S1804)
- Tris borate EDTA (TBE) buffer (Fisher Scientific, Pittsburgh, PA, cat. no. 610769-35-2)
- Sucrose (Sigma Aldrich, St Louis, MO, cat. no. S0389)
- TRIZOL (Invitrogen, Carlsbad, CA, cat. no. 15596-018) ! CAUTION TRIZOL should be handled in a fume hood.
- Water RNase-free, molecular-biology grade (Ambion Inc., Austin, TX, cat. no. AM9933)

EQUIPMENT

- Bioanalyzer (Agilent, Santa Clara, CA, Model 2100). Alternatively, agarose gel electrophoresis system can be used instead of Bioanalyzer microfluidics-based platform
- Eppendorf Centrifuge (Fisher Scientific, Pittsburgh, PA, Eppendorf Model 5810R)
- Eppendorf Microcentrifuge (Fisher Scientific, Pittsburgh, PA, Eppendorf Model 5430)
- Bidirectional rotary shaker (Fisher Scientific, Pittsburgh, PA, Lab-line 14-512-28)

- Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Other low-volume spectrophotometers can be used to measure absorbance at 230, 260 and 280 nm.
- Electrophoresis system (Bio-Rad, Hercules, CA, Sub Cell GT)
- GT Power Pac 300, power supply (Bio-Rad, Hercules, CA)
- Gel Doc XR system (Bio-Rad, Hercules, CA)

REAGENT SETUP

DB buffer for homogenization For 100 ml of homogenization buffer, weigh 3.19 g of ammonium thiocyanate (0.4 M final concentration), 9.45 g of guanidine thiocyanate (0.8 M final concentration) in a 250-ml clean RNase-free bottle. Dissolve the contents in 50 ml of water. Add 38 ml of phenol (38% vol/vol final concentration), 3.34 ml of 3 M sodium acetate pH 5.5 (0.1 M final concentration) and 5 ml glycerol (5% vol/vol final concentration) and adjust the volume to 100 ml with water. Mix the solution well and store at 4 °C. The solution is stable for up to 6 months if it is stored at 4 °C. ! CAUTION DB buffer should be handled in a fume hood as it contains phenol.

Citric acid (1 M final) For 100 ml solution, dissolve 19.21 g of citric acid in water. Keep the solution at room temperature (15–25 °C).

Sodium citrate stock solution (1 M final) Dissolve 58.82 g of sodium citrate in 50 ml of water and adjust the pH to 7 with 1 M citric acid. When the pH reaches 7, adjust the volume to 100 ml with water. Keep the solution at room temperature (15–25 °C).

Sodium citrate–sodium chloride precipitation (SCP) solution Dissolve 7 g of sodium chloride (1.2 M final) in 60 ml of water in a clean RNase-free bottle. Add 40 ml of 2 M sodium citrate stock solution (0.8 M final) to adjust the solution volume to 100 ml with water. Mix the solution well and store at room temperature (15–25 °C).

6× gel loading buffer Prepare 0.25% (wt/vol) bromophenol blue, 40% (wt/vol) sucrose mixture by using RNase-free water. Store at 4 °C.

EQUIPMENT SETUP

Porcelain mortar, pestle, glassware and Oakridge tube All the mortars, pestles, glassware and Oakridge tubes should be thoroughly washed and rinsed with water. Wrap the mortars and pestles separately with aluminum foil. Autoclave all the glassware, mortars and pestles for 20 min at 121 °C.

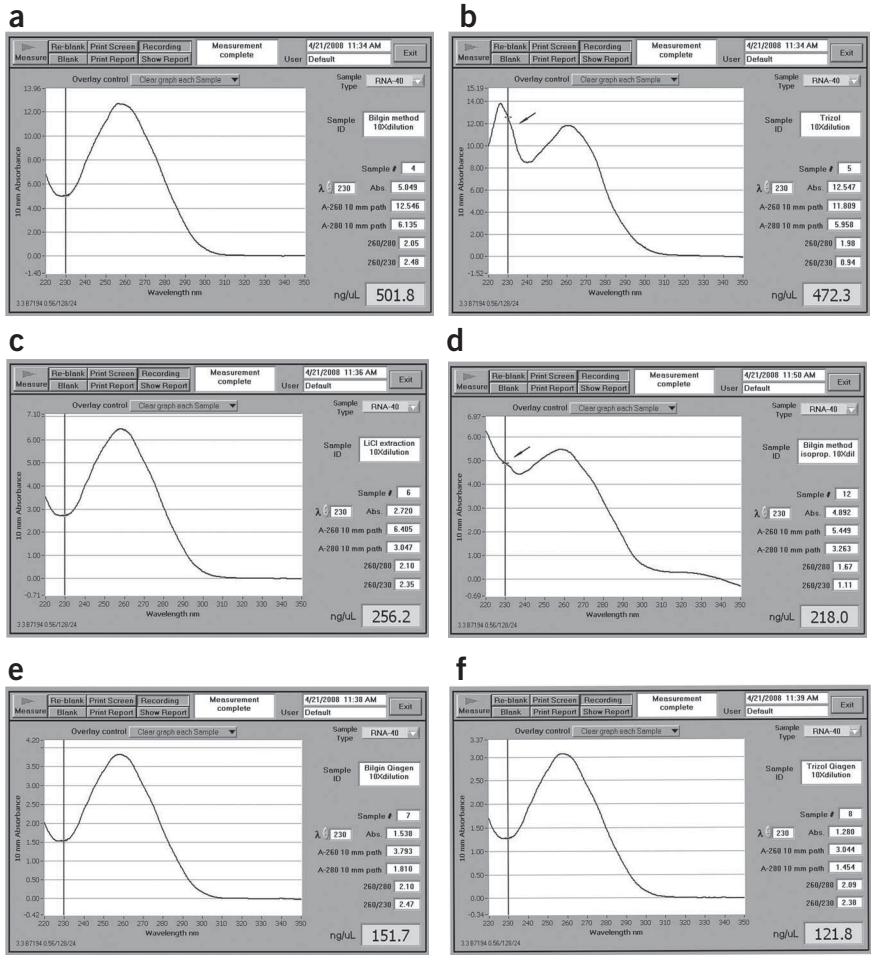


Figure 2 | Nanodrop spectrophotometric measurements at A_{230}, A_{260} and A_{280} of RNA purified using various methods. Soybean leaf RNA isolated with the DB protocol (**a**), TRIZOL reagent (**b**), $LiCl_2$ precipitation (**c**), with the DB protocol but precipitated with isopropanol instead of ethanol (**d**), the DB protocol and cleaned with Qiagen RNeasy RNA Cleanup Kit (**e**) or TRIZOL reagent and cleaned with Qiagen RNeasy RNA Cleanup Kit (**f**). Arrows show the impurities that have an absorbance at 230 nm.

PLG tubes Prior to use, spin 50-ml PLG tubes for 1 min at 1,500*g* in a swinging rotor to collect the gel at the bottom of the tubes.

PROCEDURE

Tissue collection and homogenization ● TIMING 1 h for eight samples

1| Transfer the frozen tissue from a −80 °C freezer to liquid nitrogen to prevent thawing prior to the next step.

▲ CRITICAL STEP Collection of the tissue is very important for the quality of the RNA isolation. Tissues should be frozen immediately in liquid nitrogen after collection and should be kept at −80 °C. Tissues should not be thawed prior to the addition of homogenization buffer.

TABLE 1 | Soybean leaf RNA quality measurements.

Sample ID	ng µl^{-1}	230 nm	260 nm	280 nm	260/280	260/230
(a) DB protocol	501.8	5.049	12.546	6.135	2.05	2.48
(b) TRIZOL reagent	472.3	12.547	11.809	5.958	1.98	0.94
(c) $LiCl_2$ extraction	256.2	2.720	6.405	3.047	2.10	2.35
(d) DB protocol isopropanol precipitation	218.0	4.897	5.449	3.263	1.67	1.11
(e) DB protocol Qiagen cleaned	151.7	1.538	3.793	1.810	2.10	2.47
(f) TRIZOL reagent Qiagen cleaned	121.7	1.280	3.044	1.454	2.09	2.38

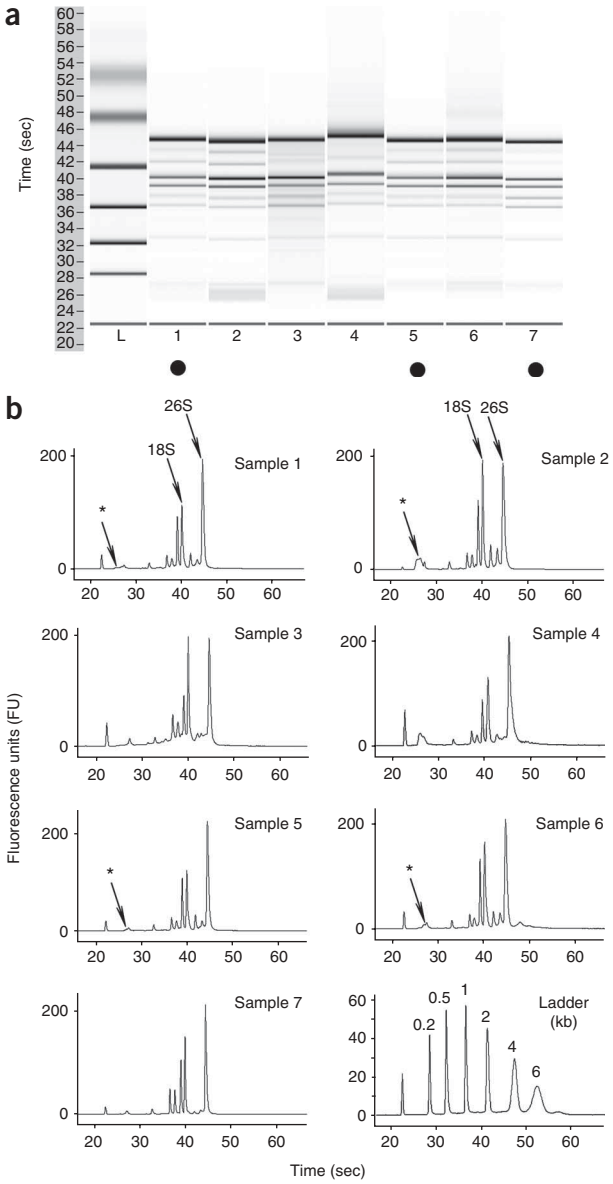


Figure 3 | The quality of the RNA isolated using various methods measured with an Agilent 2100 Bioanalyzer microfluidic electrophoresis chip. (**a**) The first lane contains the RNA ladder (L), sample lanes 1–6 contain soybean RNA isolated with (1) the DB protocol, (2) TRIZOL reagent, (3) $LiCl_2$ precipitation, (4) the DB protocol but precipitated with isopropanol instead of ethanol, (5) the DB protocol and cleaned with Qiagen RNeasy RNA Cleanup Kit and (6) TRIZOL reagent and cleaned with Qiagen RNeasy RNA Cleanup Kit; sample lane (7), *N. attenuata* leaf RNA isolated with the DB protocol. The dots at the bottom of the lanes indicate good quality RNA samples. (**b**) The electropherogram of the same samples helps to determine the intensity of each band on the gel. The arrows with asterisk indicate where the small molecular weight RNA should be. The arrows without asterisks indicate 26S and 18S ribosomal bands.

2| Pre-cool the mortars by pouring liquid nitrogen into them.

3| Add the tissue into the mortar containing liquid nitrogen and grind with a pestle until it is a fine powder.
▲ **CRITICAL STEP** Do not let the tissue thaw during grinding. If necessary, add extra liquid nitrogen to the mortar during grinding, but this addition has to be done very slowly to avoid splashes.

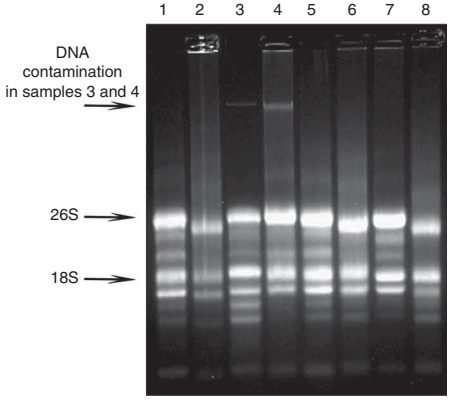
4| Once the tissue is ground sufficiently, add 4 ml of DB homogenization buffer for each 1 g of ground tissue. The tissue/buffer ratio can be approximate; however, the mixture should not be viscous. Excess buffer will only increase the volume of the extraction but will not interfere with the yield of the RNA.
▲ **CRITICAL STEP** If less than the required volume of buffer is added, the efficiency of the extraction decreases.

5| Transfer the tissue-buffer mixture to a 50-ml PLG tube by simply pouring it from the mortar. Make sure that all of the tissue is transferred.

RNA extraction ● TIMING 1.5–2 h

6| Add 1/5 volume of chloroform (0.2 ml of chloroform/1 ml of DB hybridization buffer).
! CAUTION Chloroform- and phenol-containing steps should be handled in the fume hood wearing protective clothing.

7| Mix the solution thoroughly by inverting the tubes or by placing them for 5 min on a rotary shaker (slow speed <70 r.p.m.).
▲ **CRITICAL STEP** Avoid vigorous shaking or vortexing because the phase lock gel may break into small pieces.
? TROUBLESHOOTING



8| Centrifuge for 10 min at 2,000*g*, 4 °C. Use a swinging rotor.

9| Transfer the upper aqueous phase into new 50-ml PLG tubes.
? TROUBLESHOOTING

Figure 4 | Agarose (1.2%) (wt/vol) gel electrophoresis of the RNA (5 μg) isolated with various methods and from various plant species. Soybean RNA isolated with (1) the DB protocol, (2) TRIZOL reagent, (3) $LiCl_2$ precipitation, (4) the DB protocol but precipitated with isopropanol instead of ethanol, (5) the DB protocol and cleaned with Qiagen RNeasy RNA Cleanup Kit, (6) TRIZOL reagent and cleaned with Qiagen RNeasy RNA Cleanup Kit; (7) *N. attenuata* leaf and (8) *N. attenuata* root RNA isolated with the DB protocol was run on an agarose gel. From top to bottom, arrows show DNA contamination in samples 3 and 4, 26S ribosomal band and 18S ribosomal band.

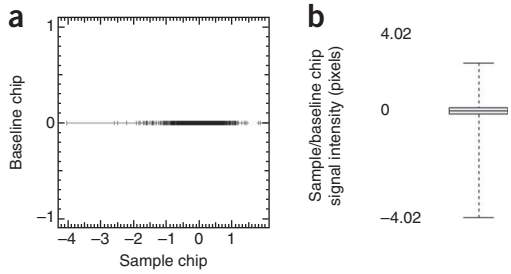


Figure 5 | Results of Affymetrix hybridization statistics. The RNA isolated with DB protocol was used directly for probe labeling and hybridization. A separate aliquot of the RNA was cleaned with Qiagen RNeasy Cleanup Kit and used for probe labeling and hybridization. The results of the hybridizations were compared to see whether there are any differences between the two chips due to the purity of RNA. The results from the Qiagen cleaned RNA chip were used as a baseline in the analysis. (**a**) Scatter plot analysis of hybridizations and (**b**) ratio of the signal distribution of the two chips.

10| Add an equal volume of acid phenol–chloroform (1 ml of acid phenol–chloroform/1 ml of aqueous phase). Add 3 M cold (0–4 °C) potassium acetate, pH 5.5 (1 ml of potassium acetate/3 ml of aqueous phase).

11| Mix the solution by inverting the tubes.

12| Incubate on ice for 30 min to 1 h.

13| Centrifuge for 10 min at 2,000*g*, 4 °C.

RNA precipitation ● TIMING 1.5 h

14| Transfer the upper aqueous phase into Oakridge tubes. Add 0.25 ml of SCP solution per 1 ml of the aqueous phase.

15| Mix thoroughly.
▲ **CRITICAL STEP** Failure to mix the SCP with the aqueous phase before adding the ethanol will decrease the precipitation efficiency.

16| Add 0.25 ml of EtOH per 1 ml of aqueous phase transferred.
▲ **CRITICAL STEP** Most of the RNA isolation protocols use 1 volume of isopropanol; however, in the DB protocol, precipitation with isopropanol decreases the efficiency of precipitation and causes DNA contamination (**Fig. 2**).

17| Mix thoroughly. Incubate for at least 1 h at −20 °C.
■ **PAUSE POINT** The samples can be stored overnight at −20 °C.

Washing RNA ● TIMING 30 min

18| Centrifuge for 10 min at 11,000*g*, 4 °C. Use a fixed-angle rotor.

19| Discard the supernatant.
▲ **CRITICAL STEP** Be careful not to lose the RNA pellet.

20| Add 2 ml of 70% EtOH, and vortex.

21| Centrifuge for 10 min at 11,000*g*, 4 °C.

22| Repeat the 70% EtOH wash step one more time.

Dissolving RNA ● TIMING 20 min

23| Decant the supernatant, place the tubes horizontally, and air dry the residual alcohol from the pellets for at least 15 min.
▲ **CRITICAL STEP** Before air drying the pellet, mark the location of the pellet on the tube from outside with a marker. As the pellet dries, it becomes slightly transparent, so marking the location helps to locate the pellet once it is dissolved in RNase-free water. Avoid drying the pellet completely, as overdried pellets are difficult to dissolve and remove from plastic.
? TROUBLESHOOTING

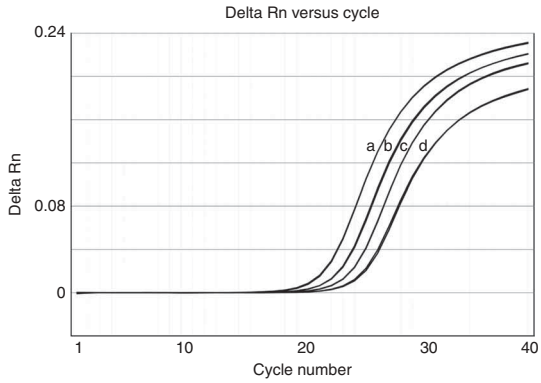


Figure 6 | Amplification plot showing the expression level of small subunit of ribulose bisphosphate carboxylase gene (*Rubisco SSII*) measured by Sybergreen qPCR. The cDNA was prepared by using the total RNA isolated with the procedure described in this paper. Primers were designed using Primer 3 software[18]. The reactions were set up with serial dilutions of the cDNA, (a) 1:5, (b) 1:10, (c) 1:25 and (d) 1:50.

24| Dissolve the pellet in 50–200 µl of RNase-free water.

■ **PAUSE POINT** The samples can be stored at −20 °C for short-term or −80 °C for long-term storage. Avoid thawing the RNA stock several times.

? TROUBLESHOOTING

Measuring RNA concentration ● TIMING 10 min for eight samples

25| Determine the RNA concentration spectrophotometrically[17] by measuring the absorbance at 230, 260 and 280 nm. The purity of the RNA can be estimated by calculating the A_{260}/A_{280} ratio. Pure RNA has a A_{260}/A_{280} ratio between 1.8 and 2.2. Calculate the A_{260}/A_{230} ratio to determine whether organic impurities, such as polysaccharides and polyphenolics, are present.

▲ **CRITICAL STEP** To measure RNA concentration accurately with a Nanodrop spectrophotometer, the samples must be diluted to 2–3000 ng μl^{-1}. For a typical RNA preparation using the DB protocol, a 10-fold dilution of the sample with water or 10 mM Tris EDTA usually gives concentrations within this range. Use 1.5–2 µl of sample for spectrophotometric measurements with the ND-1000 for best results.

? TROUBLESHOOTING

Checking RNA quality by electrophoresis

26| Checking the RNA quality can be performed using option (A), Bioanalyzer microfluidic electrophoresis chips or option, (B) an agarose RNA gel.

(A) Bioanalyzer microfluidic electrophoresis chips ● TIMING 30 min

(i) For total RNA, RNA 6000 Nano chip can be used. Prepare the Bioanalyzer microfluidic electrophoresis chips according to the manufacturer's protocol (http://www.chem.agilent.com/Library/usermanuals/Public/G2938-90034_KitRNA6000Nano_ebook.pdf).

(ii) After determining the RNA concentration spectrophotometrically, use the remaining 10-fold diluted RNA from Step 25 to analyze with an Agilent 2100 Bioanalyzer microfluidic electrophoresis chip.

(iii) Load 1 µl of the sample onto the gel, the RNA concentration should be between 25 and 500 ng μl^{-1}. **Figure 3** shows the Bioanalyzer microfluidic electrophoresis chip and individual graphic presentations of RNA isolated with various RNA isolation protocols. The mature leaves of soybean plants grown in the field were used as tissue material.

(B) Agarose RNA gel ● TIMING 1 h

(i) Prepare a 1.2% (wt/vol) agarose gel, weigh 1.2 g of molecular biology grade agarose, and mix with 100 ml of RNase-free 0.5× TBE buffer in RNase-free glassware.

(ii) Boil the contents until the agarose dissolves in the buffer completely. Add ethidium bromide (final concentration 0.5 µg ml^{-1}); pour and run the gel as described in Sambrook and Russell[18]. **Figure 4** shows the results of electrophoresis of RNA isolated with different isolation techniques. The gel image was taken by Gel Doc XR system.

▲ **CRITICAL STEP** The 6× gel loading buffer and the running buffer (0.5× TBE) must be RNase free. The gel box, the gel comb and the gel tray can be RNase decontaminated with RNA Zap followed by an RNase-free water rinse prior to use.

● **TIMING**

Steps 1–5: Tissue collection and homogenization, 1 h for 8 samples
Steps 6–13: RNA extraction, 1.5–2 h
Steps 14–17: RNA precipitation, 1.5 h
Steps 18–22: Washing RNA, 30 min
Steps 23 and 24: Dissolving RNA, 20 min
Step 25: Measuring RNA concentration, 10 min for eight samples
Step 26: Checking RNA quality by electrophoresis
Option (A): Bioanalyzer microfluidic electrophoresis chips 30 min
Option (B): Agarose RNA gel 1 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

	Problem	Possible reason	Possible solution
Step 7	Phase lock gel carry over	Vigorous shaking or vortexing during mixing	Mix gently when the samples are in PLG tubes. If small pieces of PLG start to float, transfer the aqueous phase to a clean regular tube and centrifuge for 5 min at 2,000*g*. The PLG pieces will remain as a pellet; collect the aqueous phase
Step 9	Small volume of aqueous phase during phenol extractions	Not enough DB buffer was added to the homogenized tissue	Collect both aqueous and organic phases (by poking a hole in the PLG). Add $\frac{1}{2}$ volume of DB buffer mix and centrifuge in PLG tubes
Steps 23 and 24	Insoluble RNA pellet	Organic phase or PLG might have carried over (Steps 9 and 14)	Add 1 volume of chloroform and extract the aqueous phase and precipitate with 2 volumes of EtOH
		RNA might have dried too much	Dry RNA until EtOH evaporated, then dissolve
Step 25	Low RNA yield	SCP and EtOH were not thoroughly mixed in the correct order (Step 15)	SCP should be thoroughly mixed with the aqueous phase before EtOH was added
		The homogenization was not efficient (Step 3)	Homogenize the tissue until it is in powder form. Use autoclaved quartz sand if necessary

ANTICIPITATED RESULTS

The DB RNA isolation protocol described here produces high-purity, high-yield, DNA-free RNA from various polysaccharide-rich plant tissues (**Table 1**). The comparison of the total RNA isolated with DB protocol with various other protocols shows that the total RNA is intact and free of DNA and insoluble contaminants (**Figs. 2,3** and **4**). The electropherogram of the RNA samples run on the Bioanalyzer (**Fig. 3b**) helped to determine the intensity of each band on the gel. The arrows with asterisks in samples 1 and 5 point to 0.2–0.5 kb nucleic acid peaks between 25 and 30 s. The peak in sample 1 is wider than sample 5, indicating the loss of the small molecular weight RNA due to the Qiagen clean up step. The arrows with the asterisks in samples 2 and 6 point to similar small molecular weight RNA loss. The low 26S to18S ribosomal band ratio in sample 2 indicates a lower RNA quality compared with sample 1. The RNA run on an agarose gel (**Fig. 4**) shows that the RNA in samples 1, 5–8 are clean and without any visible DNA contamination. The RNA in sample 2 has impurities that are visible in the gel well and form a smeary background during the run. Samples 3 and 4 have DNA contamination demonstrated by extra bands of high molecular weight.

The protocol is cost efficient and reasonably quick to perform. The total RNA isolated with this procedure can be used for Affymetrix GeneChip analysis and quantitative real-time RT-PCR, high-throughput cDNA sequencing and small RNA analysis without additional RNA clean up. We compared total leaf RNA isolated with the DB protocol to total RNA that received an additional purification through a Qiagen RNeasy column (**Fig. 5**). Even though the hybridization results did not differ from each other, the total RNA prepared with the DB protocol without the subsequent Qiagen purification had a better labeling efficiency and fractionation than did the same preparation receiving Qiagen RNeasy column purification (**Supplementary Figure 1**) as visualized by increased dye incorporation measured by fluorescence. The scatter plot analysis and the '0' signal intensity ratio distribution of Affymetrix GeneChips, hybridized with targets from Qiagen column cleaned or DB protocol isolated RNA show that there was no difference between the number and the signal intensity of the hybridized probe sets (**Fig. 5a**,**b**). The quantitative real-time RT-PCR of ribulose bisphosphate carboxylase (*Rubisco SSII*) gene with serial dilutions of single strand cDNA prepared from the total RNA isolated with this method worked efficiently without further purification (**Fig. 6**). Generally, from 100 mg of leaf tissue, 80–100 μg of total RNA can be isolated. The use of PLG tubes increases the efficiency of phase separation and sample recovery and greatly reduces contamination with the organic phase. However, if care is taken, the protocol can be used without PLG tubes with comparable results.

Note: Supplementary information is available via the HTML version of this article.

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