A Method for Extracting High-Quality RNA from Diverse Plants for Next-Generation Sequencing and Gene Expression Analyses

Authors: Yockteng, Roxana, Almeida, Ana M. R., Yee, Stephen, Andre, Thiago, Hill, Colin, et al.

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Protocol Note

A method for extracting high-quality RNA from diverse plants for next-generation sequencing and gene expression analyses[1]

Roxana Yockteng[2,3], Ana M. R. Almeida[2], Stephen Yee[2], Thiago Andre[4], Colin Hill[2], and Chelsea D. Specht[2,5]

[2]Department of Plant and Microbial Biology, Department of Integrative Biology, and the University and Jepson Herbaria, University of California, Berkeley, California 94720 USA; [3]Origine, Structure et Evolution de la Diversité (UMR 7205 CNRS), Muséum National d'Histoire Naturelle, CP39, 16 rue Buffon, 75231 Paris Cedex 05, France; and [4]Laboratório de Taxonomia Vegetal e Biologia Reprodutiva, Departamento de Botânica, Universidade Federal do Rio de Janeiro, 21941-902 Rio de Janeiro, Rio de Janeiro, Brazil

- *Premise of the study:* To study gene expression in plants, high-quality RNA must be extracted in quantities sufficient for subsequent cDNA library construction. Field-based collections are often limited in quantity and quality of tissue and are typically preserved in RNA*later*. Obtaining sufficient and high-quality yield from variously preserved samples is essential to studies of comparative biology. We present a protocol for the extraction of high-quality RNA from even the most recalcitrant plant tissues.
- *Methods and Results:* Tissues from mosses, cycads, and angiosperm floral organs and leaves were preserved in RNA*later* or frozen fresh at −80°C. Extractions were performed and quality was measured for yield and purity.
- *Conclusions:* This protocol results in the extraction of high-quality RNA from a variety of plant tissues representing vascular and nonvascular plants. RNA was used for cDNA synthesis to generate libraries for next-generation sequencing and for expression studies using quantitative PCR (qPCR) and semiquantitative reverse transcription PCR (RT-PCR).

Key words: cDNA library; gene expression; Illumina; RNA-Seq; RNA extraction; transcriptome.

High-throughput, next-generation sequencing of genomes and transcriptomes requires high-quality, clean, and concentrated genetic material. While getting high-quality genomic extractions from plants is not trivial, it is even more challenging to consistently obtain sufficient quantity of pure RNA for RNA-Seq (transcriptome) or chromatin immunoprecipitation followed by next-generation sequencing (ChIP-Seq) experiments. Due to its instability, RNA is more delicate to manipulate than genomic DNA. It is often single stranded and contains ribose sugars; the 2′-hydroxyl group attached to the ribose makes RNA more prone to hydrolysis. For studies of plant biology, high-quality RNA extraction is further complicated when plant samples contain large quantities of polysaccharides, high levels of RNases, various different kinds of phenolics including tannins, low concentrations of nucleic acids (high water content), and/or fibrous tissues such as lignin (wood) that are difficult to break up and remove (MacRae, 2007).

A large number of plant-specific protocols are available, recommending a diversity of strategies for tissue storage, maceration, preparation, and extraction to obtain sufficient RNA from plants depending on the species or lineage of plant, the tissue type being extracted, and the presence of secondary metabolites and inhibitors for any given plant lineage or plant part (for reviews see MacRae, 2007; Accerbi et al., 2010). For example, Ghawana et al. (2011) propose a phenol-based method to isolate RNA in species with high presence of secondary metabolites such as rheum (*Rheum austral* D. Don) and arnebia (*Arnebia euchroma* (Royle) I. M. Johnst.). A method proposed by Gehrig et al. (2000) uses high-molecular-weight polyethylene glycol (PEG) to extract RNA from species with high presence of polyphenols and polysaccharides (e.g., *Aloe* L., *Ananas* Mill., *Clusia* L., *Euphorbia* L.). Other protocols are designed to extract RNA from a specific type of tissue, such as the trizol-based methods proposed to extract RNA from siliques and seeds of *Arabidopsis* (DC.) Heynh. (Meng and Feldman, 2010) or the seeds of *Davidia involucrata* Baill. (Qi et al., 2009).

Other methods focus on rapid methods for RNA extraction, although these are typically proposed only for model organisms (e.g., *Arabidopsis* leaves) and are limited to PCR-based

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[5]Author for correspondence: cdspecht@berkeley.edu

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downstream use of the extracted RNA (e.g., Berendzen et al., 2005). Between October 2011 and September 2013, a total of 53 references for plant RNA extraction methods are found in Web of Science. Of these methods, 90% have only been tested in one plant lineage (e.g., Kumar and Singh, 2012; Gudenschwager et al., 2012) and the majority are relatively straightforward modifications of the cetyltrimethylammonium bromide (CTAB) method with polyvinylpyrrolidone (PVP; e.g., Japelaghi et al., 2011; Yin et al., 2011).

Given the variability of extraction techniques and the quality of their products, the ability to perform comparative analyses of genomes or transcriptomes across plant lineages and tissue types remains a challenge for researchers interested in questions of plant evolution. Here, we present a general protocol that can be used for a wide range of plant tissues and across a broad range of taxa. Our intention in presenting this protocol is not to discount other protocols that work well in particular cases, but rather to present a single protocol that works well across a broad variety of plant tissue types and plant species. This modified protocol uses the proprietary Plant RNA Reagent from Life Technologies (Carlsbad, California, USA) that is composed in part of 2-mercaptoethanol (10–30% weight) and sodium azide (0.1–1.0% weight).

We demonstrate the efficacy of this extraction pipeline in different plant species including mosses, gymnosperms, and angiosperms, and demonstrate its ability to generate successful sequencing product from different plant tissue types, including individual floral and reproductive organs as well as combined reproductive and vegetative tissues.

METHODS AND RESULTS

***Sampling*—**Plant material was collected from greenhouses and botanical gardens (Table 1) and either stored in RNA*later* (Ambion, Carlsbad, California, USA) or frozen immediately in liquid nitrogen. Preserved tissue was placed in long-term storage at −80°C. For storage in RNA*later*, approximately 3× the volume of RNA*later*:tissue is used. Tissue stored in RNA*later* and frozen (−80°C) was defrosted just enough to remove the tissue from the RNA*later* prior to extraction.

***Basic protocol*—**The following protocol was modified from the manufacturer's provided instructions for effective use of the Plant RNA Reagent from Life Technologies. As indicated, all solutions are prepared with sterile RNase-free water, and all supplies and handling materials are cleaned with RNase *AWAY* (Ambion) prior to dissection and storage. This protocol is optimized to isolate RNA from approximately 0.1 g of plant tissue. If the amount of plant tissue is increased, reagent volumes must be scaled appropriately.

Grinding the tissue—One of the critical points to obtain high yield in the extraction of genetic material is the grinding. It is essential to grind the tissue as finely as possible, maintaining samples as cold as possible during grinding to avoid degradation. Selection of FastPrep or mortars/pestle depends on the hardness of the tissue being processed.

A FastPrep FP120 Homogenizer (Thermo Savant, Carlsbad, California, USA) was used for grinding floral organs and soft leaf tissue. Approximately 0.1 g of frozen floral organs, whole flowers, and leaves or herbaceous stems were added to FastPrep 2-mL tubes (MP Biomedicals, Santa Ana, California, USA) 1/5 filled with bulk Lysing Matrix D (MP Biomedicals). FastPrep tubes containing the frozen tissue plus Lysing Matrix beads were shaken in the homogenizer (FastPrep) for 40 s at speed 6 (6 m/s) without buffer at room temperature.

For grinding hard tissue or ligneous tissue, such as cone scales from gymnosperms, the tissue was ground under liquid nitrogen in a mortar and pestle that was sterilized and baked (minimum 12 h at 150°C). The tissue was ground as finely as possible, and the powdered material was placed in a 1.5-mL tube. The manually ground sample can be added to the FastPrep tube with Lysing Matrix beads (see above) for additional pulverization.

RNA extraction—Once the tissue is sufficiently homogenized:

1. Add 0.6 mL of cold (4°C) Plant RNA Reagent (Life Technologies) to pulverized tissue. Mix by brief vortexing or flicking the bottom of the tube until the sample is thoroughly resuspended. If tissue was ground with a FastPrep, homogenize with the cold buffer for an additional 40 s. If the tissue is not completely ground, repeat 1–2× until the tissue is pulverized. If the tissue was ground with a mortar and pestle and does not need additional grinding, continue with the next step.
2. Incubate 5 min at room temperature. Placing the samples on a rotator or nutator will help to maximize surface area of the tissue with the extraction buffer.
3. Clarify the solution by centrifuging for 2 min at 12,000 × *g* in a microcentrifuge at room temperature. Transfer the supernatant to a tube with Phase Lock Gel (5 Prime, Gaithersburg, Maryland, USA). Although the Phase Lock Gel tubes are not required, they greatly facilitate separation of the organic and aqueous phases and help ensure cleanliness of the sample.
4. Add 0.1 mL of 5 M NaCl to the sample, tap tube to mix, and add 0.3 mL of chloroform–isoamyl alcohol (24 : 1). Mix thoroughly by inversion and centrifuge the sample at 4°C for 10 min at 12,000 × *g* to separate the phases. Transfer the aqueous (top) phase to an RNase-free tube.
5. Add to the aqueous phase an equal volume of a mix of LiCl (4 M) (3/4 v) and isopropyl alcohol (1/4 v). Mix and let stand at −20°C for 30 min to overnight. If the tissue was stored in RNA*later*, mix and let stand at −20°C for a maximum of 3 h (not overnight). If the precipitation is longer, salts from the RNA*later* solution could also precipitate. (Note: We have also let the sample stand at −80°C for 3 h, and this works as well.)
6. Centrifuge the sample at 4°C for 20 min at 12,000 × *g*.
7. Decant or remove supernatant with a pipette, taking care not to lose the pellet. Add 1 mL of 75% ethanol to the pellet. The pellet may be difficult to see. To help to see the pellet, you can add 1 µL of GlycoBlue (Ambion) in step 5.
8. Centrifuge at 4°C for 5 min at 12,000 × *g*. Decant the liquid carefully, taking care not to lose the pellet. Briefly centrifuge to collect the residual liquid, and remove it with a pipette.
9. Let dry on ice for 15 min at room temperature and elute pellet in 10–30 µL of RNase-free water. Pipette the water up and down over the pellet to dissolve the RNA. If the pellet is difficult to dissolve, add more water or warm to 37°C to facilitate the dissolution. It is important to resuspend the pellet completely to obtain an accurate measure of the concentration of your RNA. If the sample is not clean, it can be purified by the cleanup step suggested later. Although some protocols have suggested that performing an additional step of chloroform would clean the RNA samples (e.g., Accerbi et al., 2010), we found that an additional chloroform step decreases the RNA yield substantially.

A NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) can be used to verify the concentration and purity of the RNA obtained. To assess the presence and purity of extracted RNA, use the ratio of absorbance at 230 nm, 260 nm, and 280 nm. If the RNA is pure, we expect a 260/280 ratio around 2, although this ratio does not guarantee pure RNA (see below). If the ratio is appreciably lower, it is an indication of the presence of protein, phenol, or other contaminants that absorb strongly at or near 280 nm. The ratio 260/230 is expected to be around 2–2.2. If this value is appreciably lower, it is an indication that contaminants such as carbohydrates, EDTA, guanidine isothiocyanate, and phenol that absorb at 230 nm are present in the sample. Ratios lower than expected could indicate that additional cleaning is necessary and the optional cleanup should be followed. While a more accurate assessment of the quality will be determined with a bioanalyzer prior to sequencing, this initial NanoDrop read will provide an indication of the presence of RNA, enabling the researcher to continue.

***Optional cleanup*—**If the sample is not clean, the following modified cleanup procedure will help to purify the total RNA. This protocol is adapted from that published for DNA cleanup by Rohland and Reich (2012), using magnetic beads to capture nucleotide material and permit additional washing steps that aid in the removal of undesirable metabolites. All stock solutions and reagents must be prepared with RNase-free water.

Preparing Sera-Mag beads solution—Begin by preparing 50 mL of Sera-Mag beads. Add 9 g of PEG-8000, 10 mL of 5 M NaCl, 500 µL of 1 M Tris-HCl (pH 8.0), and 100 µL of 0.5 M EDTA (pH 8.0) to a 50-mL Falcon tube. Add 1 mL (9 g) of the carboxyl-modified Sera-Mag Magnetic SpeedBeads (Thermo

TABLE 1. RNA yield from tested land plants using the method described in this study. Tissue type and taxon names are indicated.

Lineage	Plant tissue	Organ	Species	Accession no.	Yield (ng/μL)	260/280	260/230	After cleanup[a]		
								Yield (ng/μL)	260/280	260/230
Monocot	Flower	Petals	*Canna indica* L.	2011-777 (UCGH)	748	2.18	2.14	N/A	N/A	N/A
Monocot	Flower	Filament	*Canna indica* L.	2011-777 (UCGH)	3619	2.11	2.04	N/A	N/A	N/A
Monocot	Flower	Stamen	*Canna indica* L.	2011-777 (UCGH)	4843	1.37	1.34	N/A	N/A	N/A
Monocot	Flower	Staminodes	*Canna indica* L.	2011-777 (UCGH)	1114	2.16	1.86	N/A	N/A	N/A
Monocot	Flower	Total flower	*Zingiber officinale* Roscoe	90-1128 (UCGH)	2834	2.1	1.66	N/A	N/A	N/A
Monocot	Flower	Sepal	*Zingiber officinale* Roscoe	90-1128 (UCGH)	439	2.14	1.83	N/A	N/A	N/A
Monocot	Flower	Petal	*Zingiber officinale* Roscoe	90-1128 (UCGH)	654	2.17	2.09	N/A	N/A	N/A
Monocot	Flower	Labellum	*Zingiber officinale* Roscoe	90-1128 (UCGH)	2713	2.02	1.51	N/A	N/A	N/A
Monocot	Flower	Stamen	*Zingiber officinale* Roscoe	90-1128 (UCGH)	1816	1.99	1.22	N/A	N/A	N/A
Monocot	Flower	Gynoecium	*Zingiber officinale* Roscoe	90-1128 (UCGH)	4965	1.49	1.47	N/A	N/A	N/A
Monocot	Vegetative	Leaf meristem	*Costus spicatus* (Jacq.) Sw.	2002-127 (UCGH)	3908	2.07	1.94	N/A	N/A	N/A
Monocot	Vegetative	Leaf meristem	*Musa basjoo* Siebold	89.0873 (UCBG)	1461	2.13	1.56	N/A	N/A	N/A
Monocot	Vegetative	Leaf meristem	*Canna indica* L.	2011-777 (UCGH)	117	2	2.38	N/A	N/A	N/A
Monocot	Vegetative	Leaf meristem	*Monocostus uniflorus* (Poepp. ex Petersen) Maas	94-725 (UCGH)	1131	2.19	2.09	N/A	N/A	N/A
Monocot	Vegetative	Leaf meristem	*Orchidantha fimbriata* Holttum	1994-656 (UCGH)	2554	2.18	2.09	N/A	N/A	N/A
Monocot	Vegetative	Leaves	*Hordeum vulgare* L.	Jackson Laboratory (PMB-UC)	2434	2.17	2.2	N/A	N/A	N/A
Eudicot	Vegetative	Leaves	*Bursera* sp. Jacq. ex L.	71.0536 (UCBG)	601	1.84	0.78	352	2.01	1.84
Eudicot	Vegetative	Leaves	*Nicotiana benthamiana* Domin	Jackson Laboratory (PMB-UC)	1946	2.13	2.21	N/A	N/A	N/A
Eudicot	Vegetative	Lamina leaf	*Dionaea muscipula* J. Ellis	2009.0254 (UCBG)	1321	0.81	8.16	N/A	N/A	N/A
Eudicot	Vegetative	Leaf base	*Dionaea muscipula* J. Ellis	2009.0254 (UCBG)	1078	0.86	4.22	N/A	N/A	N/A
Gymnosperm	Reproductive	Cones	*Dioon sonorense* (De Luca, Sabato & Vázq. Torres) Chemnick, T. J. Greg. & Salas-Mor.	2004.1346 (UCBG)	997	1.99	0.87	2249	2.09	1.98
Gymnosperm	Reproductive	Cones	*Macrozamia lucida* L. A. S. Johnson	AQ0813814 (BRI)	289	1.67	0.7	454	2.08	1.98
Gymnosperm	Vegetative	Frond	*Dioon mejiae* Standl. & L. O. Williams	109266 (HBG)	218	1.98	1.03	183	2.05	2.46
Moss	Vegetative	Gametophyte	*Physcomitrella patens* (Hedw.) Bruch & Schimp.	Gransden ecotype (IVC)	254	2.41	2.19	N/A	N/A	N/A
Moss	Vegetative	1-wk-old protonema	*Physcomitrella patens* (Hedw.) Bruch & Schimp.	Gransden ecotype (IVC)	1145	2.46	2.21	N/A	N/A	N/A
Moss	Vegetative	3-wk-old protonema	*Physcomitrella patens* (Hedw.) Bruch & Schimp.	Gransden ecotype (IVC)	2661	2.31	2.24	N/A	N/A	N/A

Note: BRI = Queensland Herbarium, Brisbane, Australia; HBG = Huntington Botanical Gardens, San Marino, California, USA; IVC = in vitro culture; PMB-UC = Plant and Microbial Biology Department, University of California, Berkeley, California, USA; UCBG = University of California, Berkeley, California, USA; UCGH = University of California, Berkeley, greenhouses, Berkeley, California, USA.

[a] N/A indicates that the cleanup method was not tested on these particular samples.

Scientific, Waltham, Massachusetts, USA; cat. no. 09-981-123) to a 1.5-mL tube. Pellet the beads for 5 min with a magnetic stand designed for 1.5–2-mL tubes. Remove the storage buffer, leaving the tubes in the magnetic stand. Wash beads 1× with 1 mL TE or water. Remove the tube from the magnetic stand and immediately resuspend the beads in an additional 1 mL TE or water.

Add the bead suspension to the prepared 50-mL Falcon tube and wrap the Falcon tube with aluminum foil. Store at 4°C for further use. The final concentration of the bead solution is 0.1% carboxyl-modified Sera-Mag Magnetic SpeedBeads, 18% PEG-8000 (w/v), 1 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), and 0.05% Tween 20.

RNA cleanup—Bring a measured amount of prepared Sera-Mag beads solution to room temperature at least 30 min prior to use. For maximum binding, measure out 3× the total RNA volume of the beads solution and place into a 1.5-mL tube along with the RNA. Place tubes on a rotator at room temperature for 10 min.

Spin the beads solution down before the beads dry permanently onto the wall of the tube. The beads must be spun down very quickly and at low speed to avoid bead precipitation; a mini benchtop centrifuge is recommended, with a spin so quick such that the lid does not need to be closed. Incubate without rotating at room temperature for an additional 10 min to maximize RNA binding. Place the 1.5-mL tubes in the magnetic stand for 5 min, then remove the buffer with a pipette and wash the beads 2× with 500 µL of fresh 80% ethanol. After the second wash, remove all ethanol and make sure that no ethanol is left in the samples. Remove the tubes from the magnetic stand and spin beads down from the sides of the wall before they dry. Air-dry the bead pellet for 10 min. Elute with RNase-free water using approximately the same volume as your original sample of RNA.

Vortex beads and water just to mix, and spin down quickly at low speed as before. Place tubes on a rotator at room temperature for 2–5 min for maximum RNA elution, and spin down quickly. Place tubes in the magnetic stand. Let stand for 5 min and recover eluted RNA in a separate tube.

A NanoDrop measure is recommended to check quality and yield of RNA.

DISCUSSION

***Diversity of samples and tissue types*—**High-yield and pure RNA was obtained for different plant lineages (Table 1), including mosses, gymnosperms, and various groups across angiosperms. Successful cDNA library construction was completed from various plant tissue types, including individual floral and reproductive organs as well as combined reproductive and vegetative tissues (Table 1).

In general, high yield was obtained even when the starting material measured less than 0.1 g. RNA obtained was used in several downstream experiments such as cDNA synthesis for RT-PCR and qPCR (Yockteng et al., 2013; Almeida et al., 2013; Almeida et al., unpublished), and for generating transcriptome libraries and high-quality RNA-Seq data using TruSeq RNA sample preparation kits (Illumina, San Diego, California, USA).

RNA extracted from specific floral organs from *Costus spicatus* (Jacq.) Sw., *Musa basjoo* Siebold, and *Canna indica* L. (Table 1) was labeled with TruSeq RNA sample preparation kits to generate and sequence RNA-Seq libraries, which yielded high-quality sequence data that contributed to analyses of the gene expression differences underlying evolution of flower morphology across the Zingiberales (Yockteng et al., 2013; Almeida et al., unpublished).

RNA extracted from leaf material and cone scales of cycad species *Dioon mejiae* Standl. & L. O. Williams and *Macrozamia lucida* L. A. S. Johnson (Table 1) was cleaned with the optional cleanup method presented here. RNA-Seq libraries were successfully sequenced and transcriptome assemblies are being used to design gene targets for a transcriptome-based exon capture approach to identify markers for population genetic and phylogenetic analyses (e.g., Bi et al., 2012).

***Quality and quantity*—**Experiments using a PCR-based approach to sequence fragments of cDNA generated from RNA extractions typically only require checking the concentration of RNA via NanoDrop (or similar equipment) and running an aliquot of the RNA on an agarose gel to verify the appearance of ribosomal RNA bands indicating a lack of degradation.

In RNA-Seq or ChIP-Seq experiments, both the quality and the quantity of the RNA are very important. We have found that it is necessary to start cDNA synthesis and subsequent sequencing library preparation with at least 10 µg of pure RNA that is determined to have good integrity based on the entire electrophoretic trace. An Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) calculates the "RNA integrity number" (RIN) using all of the information present in the electrophoretic trace. In general, RNA with a value above 7 is required to produce good results in a next-generation sequencing analysis.

Bioanalyzer results are shown for four samples extracted with our detailed method (Fig. 1). The two peaks of 18S and 28S ribosomal RNA appear as indicated, and the trace in the background corresponds to other types of RNA with less concentration; i.e., the mRNA. All RIN values are above 8, indicating no degradation of the RNA. These traces represent typical results obtained from our method, indicating high concentration and good quality.

The purity of extracted RNA can depend on the amount of secondary metabolites produced by the plants being studied. Our extraction method consistently produced good yield for a diversity of plants; however, the recovered RNA was sometimes not pure enough for subsequent cDNA synthesis and sequencing library preparation. This was particularly true in plants such as *Dionaea muscipula* J. Ellis (Venus flytrap) where the presence of complex proteins affected the 260/280 ratio. In the case of *Bursera* sp. (Burseraceae) and in cone scales of cycads (*Dioon sonorense* (De Luca, Sabato & Vázq. Torres) Chemnick, T. J. Greg. & Salas-Mor. and *Macrozamia lucida*), the ratio 260/230 was affected by secondary metabolites and highly lignified tissue, respectively, and the values fell below 1, indicating the presence of carbohydrates.

In these plants we tried other methods of RNA extraction such as the RNAqueous (Ambion) protocol, based on silica columns that are reported to isolate pure RNA, and the TRIzol Reagent (Ambion), which is reported to be effective with a broad range of samples. Neither of these methods were able to produce a good yield (e.g., RNA recovered using RNAqueous was only 8.6 ng/µL from fresh, frozen, or RNA*later*-preserved leaves of *Bursera* spp.). Another recommendation to increase RNA purity commonly found across various protocols is to conduct two chloroform washes. After many attempts, we conclude that adding a chloroform step in our protocol significantly reduces the RNA yield without resulting in any improvement in RNA quality.

For recalcitrant samples with lignin and/or secondary metabolites, we propose several modifications to a method that has been previous published for cleaning DNA (Rohland and Reich, 2012). This low-cost method uses carboxyl-modified beads that have a high binding capacity for nucleic acids, permitting additional washes without significant loss and enabling the isolation of RNA from other components obtaining high yield and pure extraction product.

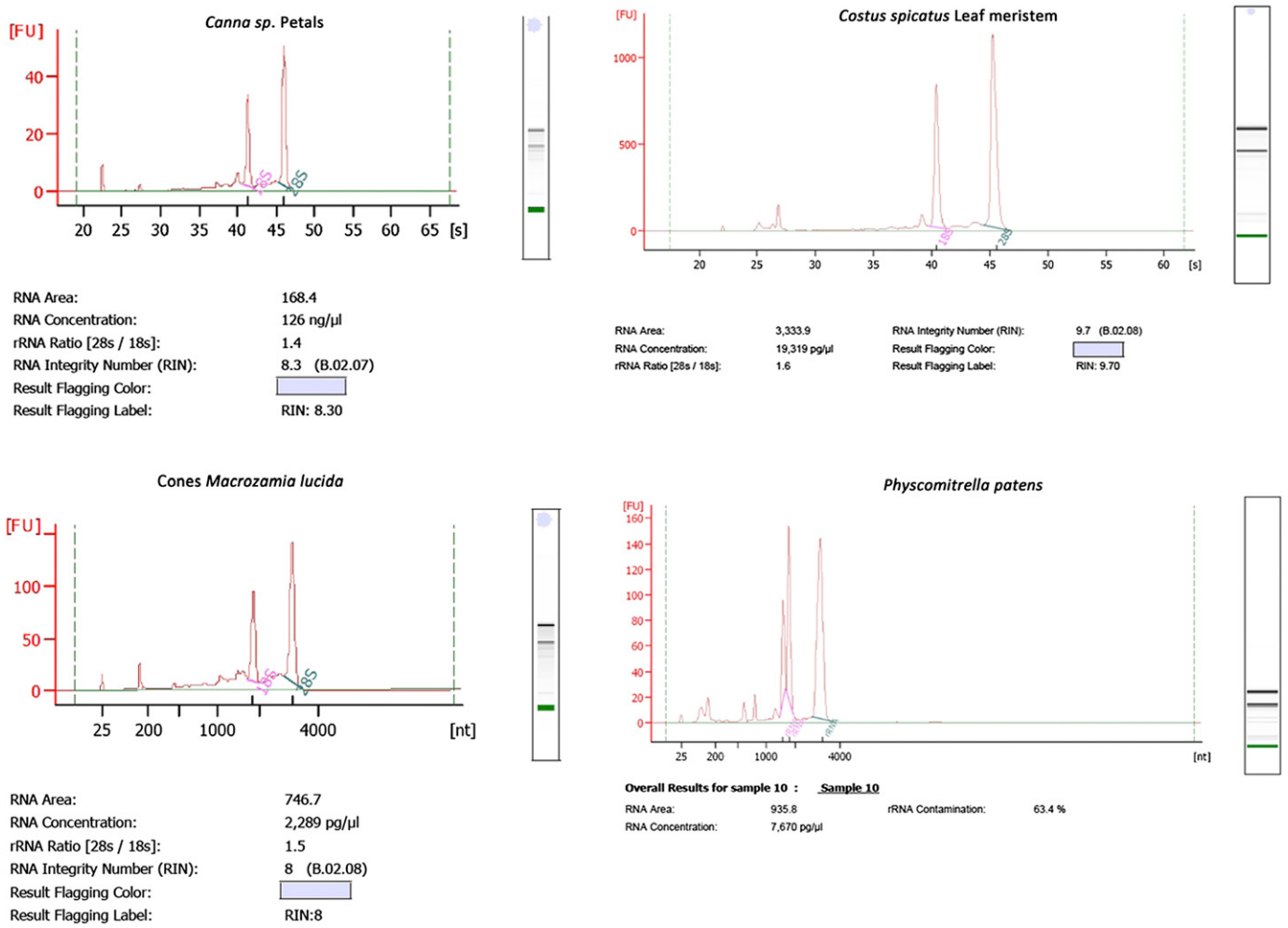

Fig. 1. Bioanalyzer results of total RNA extracted using the method described in this study. Extractions were made from four different species and tissues as indicated and run on an Agilent 2100 Bioanalyzer using the Total RNA Pico assay (Functional Genomics Laboratory, California Institute for Quantitative Biosciences [QB3], University of California, Berkeley).

CONCLUSIONS

This protocol produces high yield and quality of pure RNA from a variety of plant lineages and plant tissue types, as indicated by bioanalyzer results as well as successful downstream use of extracted RNA. The RNA obtained using this method has been used for diverse downstream experiments including RNA-Seq, RT-PCR, and qPCR. We have used extracted RNA to generate high-quality RNA-Seq libraries for both mRNA and microRNA (unpublished data). In addition to the basic protocol outlined here, we have incorporated an optional cleanup protocol using Sera-Mag magnetic beads. This procedure further purifies extracted RNA, removing metabolites and other contaminants. RNA extracted from leaves of the cycad *Dioon mejiae* and purified using this protocol was recently used successfully for RNA-Seq, and RNA from cone scales of the cycad *Macrozamia lucida* was successfully used for high-quality qPCR.

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