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PROTOCOL NOTE

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An optimized RNA extraction method for diverse leaves of Hawaiian *Metrosideros*, a hypervariable tree species complex

Maryam Hadi | **Elizabeth A. Stacy**

School of Life Sciences, University of Nevada Las Vegas, Las Vegas, Nevada 89154, USA

Correspondence

Maryam Hadi, School of Life Sciences, University of Nevada, Las Vegas, Mail Stop 4004, 4505 S. Maryland Pkwy., Las Vegas, Nevada 89154, USA.
Email: Maryam.hadi@unlv.edu

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Abstract

Premise: The isolation of RNA from trees is challenging due to the interference of polyphenols and polysaccharides with downstream processes. Furthermore, many RNA extraction protocols are time consuming and involve hazardous chemicals. To address these issues, we aimed to develop a safe protocol for high-quality RNA extraction from diverse *Metrosideros* taxa representing a broad range of leaf toughness, pubescence, and secondary metabolites.

Methods and Results: We tested popular RNA isolation kits and protocols that were effective on other recalcitrant trees, including a broad range of optimization and purification steps. We optimized a protocol involving two silica-membrane column-based kits that yielded high-quantity RNA with an RNA integrity number >7 and without DNA contamination. All RNA samples were used successfully in a follow-on RNA-Seq experiment.

Conclusions: We present an optimized high-throughput RNA extraction protocol that yielded high-quality and high-quantity RNA from three contrasting leaf phenotypes within a hyperdiverse woody species complex.

KEYWORDS

Metrosideros, protocol development, RNA extraction, trees, woody species

The isolation of high-quality RNA (i.e., RNA that is free of genomic DNA, phenols, polysaccharides, and secondary metabolites and that performs well in downstream applications [Ouyang et al., 2014]) from non-model plants remains a challenge. Non-kit-based protocols for RNA isolation from plants are highly time consuming (Porto et al., 2010; Yin et al., 2011), and most commercial kits for rapid RNA extraction are optimized for model plants such as *Arabidopsis* Heynh. and *Oryza sativa* L. (Li and Trick, 2005; Oñate-Sánchez and Vicente-Carbajosa, 2008). Furthermore, both kit-based and crude (i.e., non-kit-based) approaches for plant RNA extraction often involve hazardous chemicals such as guanidinium isothiocyanate, guanidinium chloride, sodium dodecyl sulfate (SDS), phenol, chloroform, and TRIzol. The development of a relatively rapid and safe protocol for extracting high-quality RNA from diverse woody species is needed.

Hawaiian *Metrosideros* Banks ex Gaertn. is a hypervariable, long-lived, and highly dispersible woody species complex that spans a striking range of environments from wet and subalpine forests to bogs, deserts, riparian zones, new lava flows, and windy cliffs. The more than 20 races, varieties, and species of *Metrosideros* in Hawai'i differ in morphology, physiology, and environment (Dawson and Stemmermann, 1990; Cordell et al., 1998; Sur et al., 2018; Stacy and Sakishima, 2019; Stacy et al., 2020) and are well suited for studies of local adaptation and stress tolerance in trees (e.g., Hoof et al., 2008; Morrison and Stacy, 2014; Ekar et al., 2019). However, transcriptomic studies of stress-tolerance mechanisms and the genes involved in stress response in this group will first require the isolation of high-quality RNA from a broad range of leaf types and chemistries. The polyphenols and polysaccharides in the leaves of *Metrosideros* (Martin and Asner, 2009) and other

woody taxa often interfere with the isolation of high-quality RNA.

The present study was motivated by the lack of a protocol that will extract sufficient quantities of high-integrity RNA from leaves of diverse taxa of Hawaiian *Metrosideros* for RNA-Seq experiments. Our goal was to test several commercially available kits and crude protocols to optimize a fast and reproducible protocol for three *Metrosideros* taxa that represent contrasting leaf phenotypes and ecologies and, presumably, leaf chemistries. We evaluated two methods of leaf sample preservation and then optimized a new protocol for RNA isolation from snap-frozen leaf tissue using two silica-membrane column-based kits.

METHODS AND RESULTS

Plant material and sampling

Young leaves were collected from four 9-year-old trees of each of three *Metrosideros* taxa (*M. polymorpha* Gaudich. var. *incana* H. St. John, *M. polymorpha* race L [Stacy et al., 2020], and *M. tremuloides* (A. Heller) Rock) from a greenhouse at the University of Hawai'i Hilo in Hilo, Hawai'i (12 trees total). Trees were derived from seeds produced through controlled crosses in natural populations on O'ahu (E. A. Stacy and J. M. Ekar, unpublished data). These three taxa differ in habitat, morphology, and leaf type. *Metrosideros polymorpha* var. *incana*, a tree with appressed pubescent leaves, colonizes new lava flows on active volcanoes (i.e., on Hawai'i Island) and otherwise is restricted to dry areas on all islands except Kaua'i. The O'ahu-endemic tree or shrub *M. tremuloides* dominates steep windy slopes and has glabrous, lanceolate leaves, and the glabrous shrub *M. polymorpha* race L, also endemic to O'ahu, is restricted to wet montane forest at high elevations (Dawson and Stemmermann, 1990; Stacy et al., 2020).

We initially collected young leaf material in RNA*later* reagent (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for shipping to the lab and storage at −20°C following product guidelines. We then followed the manufacturer's protocol for RNA*later* removal and attempted RNA

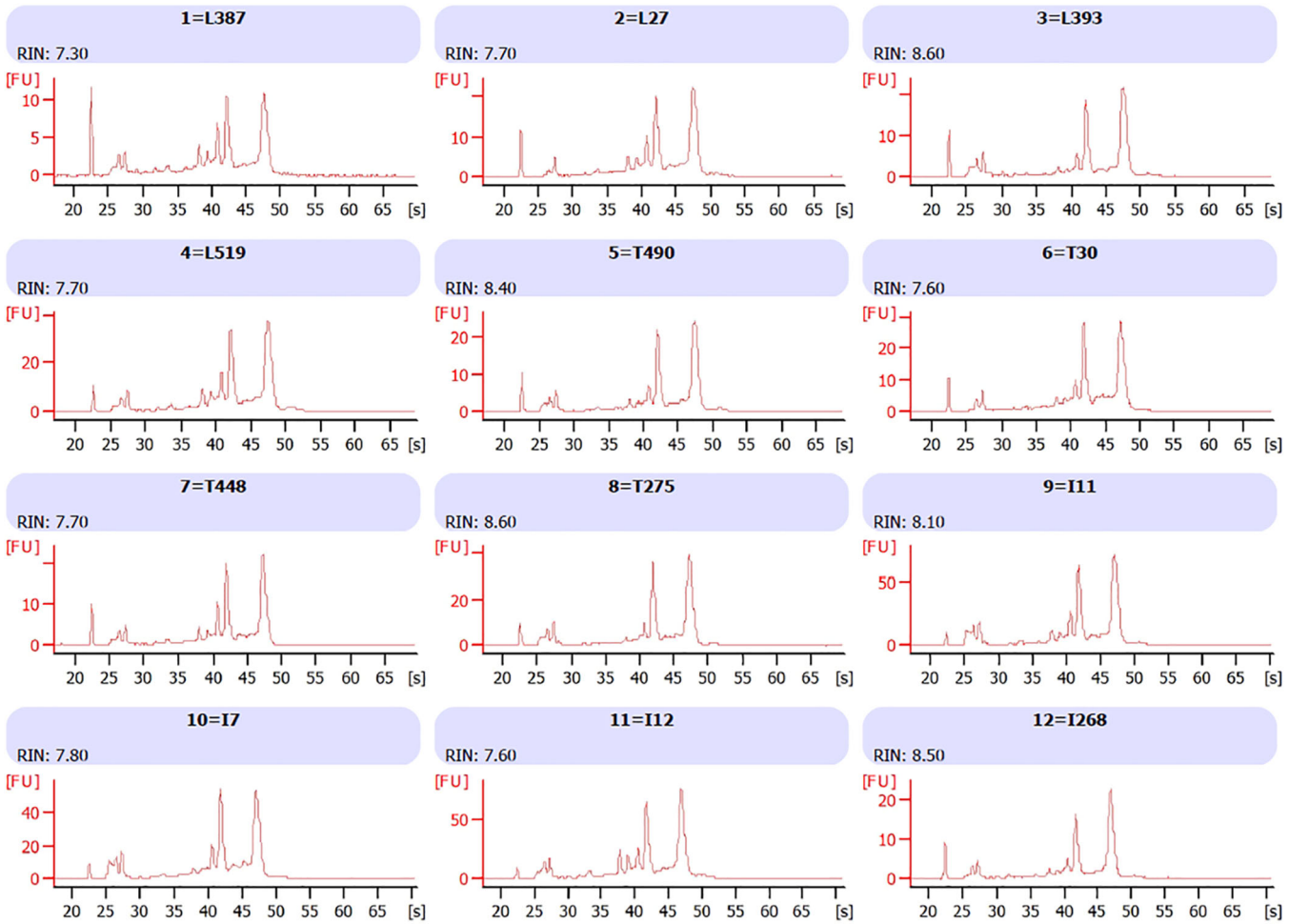

FIGURE 1 RNA integrity number (RIN) values of 12 *Metrosideros* samples measured by an Agilent 2100 Bioanalyzer indicate intact RNA with distinct peaks for 18S and 28S ribosomal RNA. Samples 1–4 are *M. polymorpha* race L (L), samples 5–8 are *M. tremuloides* (T), and samples 9–12 are *M. polymorpha* var. *incana* (I).

isolation from these samples (two samples per trial representing different taxa) using the Direct-zol RNA MiniPrep Kit (Zymo, Irvine, California, USA), TRIzol reagent (Invitrogen, Waltham, Massachusetts, USA), and RNeasy Mini Kit (Qiagen, Hilden, Germany) separately, along with fresh rice leaves as a positive control. Because the above protocols failed to yield RNA from any *Metrosideros* samples preserved in RNA*later*, to avoid any possible interference of RNA*later* reagent with the RNA extraction materials, subsequent extraction protocols were performed on either fresh leaf samples or leaf samples that were snap-frozen in liquid nitrogen for shipment and storage at −80°C. Thus, we did not test all possible combinations of leaf-sampling and RNA isolation protocols. In total, we tested 10 combinations of RNA isolation protocols and purification kits on the three taxa (Appendix S1). A positive control was included in any protocol for which no data were available on the expected RNA yield for a reference plant. The yield and purity of extracted RNA resulting from each protocol were assessed using the methods below (see "Determination of RNA quantity and integrity" and Appendix S1). Nine of these protocols failed to yield high-quality RNA from any of the three taxa. Only the combined NucleoSpin RNA Plant and Fungi Mini-kit (Macherey-Nagel, Düren, Germany) and RNA Clean & Concentrator Kit (Zymo) was successful in extracting RNA with high quantity and integrity from all three taxa and was selected for optimization.

Optimized RNA isolation protocol for Hawaiian *Metrosideros*

The single protocol that yielded high-quality RNA from all three taxa was a modification of the protocols for the NucleoSpin RNA Plant and Fungi Mini-kit, followed by the RNA Clean & Concentrator Kit. Compared to all other protocols, this protocol yielded higher concentrations and A_{260}/A_{280} and A_{260}/A_{230} absorbance values for extracted total RNA from all three taxa examined (Appendix S1). This optimized protocol was used successfully to extract RNA from all 12 individuals (four per taxon) of Hawaiian *Metrosideros* and is summarized in the following paragraphs; the step-by-step protocol and equipment list are provided as Appendix 1.

Mortars, pestles, and all glassware used for total RNA isolation were incubated overnight at 220°C; forceps and tweezers were treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC) for 2 h, autoclaved to inactivate RNases, air-dried, and kept at −20°C until RNA extraction (see Appendix 1).

From each sample, approximately 60 mg of snap-frozen, young leaf tissue was ground to a fine powder in a pre-cooled mortar and pestle with liquid nitrogen. The total RNA isolation was done according to the instructions provided for the NucleoSpin RNA Plant and Fungi Mini-kit with the following modifications. The first centrifugation step was carried out at 20,000 × *g* instead of 14,000 × *g*, and the time was increased to 3 min to maximize the separation of the supernatant (including the RNA) from the plant debris. Subsequent centrifugation steps were done at the maximum recommended speed (16,000 × *g*). At the washing stage, we added one additional wash each with Buffer PFW1 and Buffer PFW2 for a total of five washes (buffers are from the NucleoSpin RNA Plant and Fungi Mini-kit). Finally, at the final elution step, we used a single volume of 50 µL of DNase/RNase-Free Water (Sigma-Aldrich, St. Louis, Missouri, USA) passed through the column twice to increase the RNA concentration (Appendix 1).

RNA purification

The purification protocol was only slightly modified from the RNA Clean & Concentrator Kit protocol (Zymo). We set the centrifuge speed to 16,000 × *g* for all steps to improve the capture of RNA from the column. In addition, to increase the concentration of the eluted RNA, we passed the 50 µL of DNase/RNase-Free Water through the column twice (Appendix 1).

Determination of RNA quantity and integrity

The RNA concentration was determined by measuring the absorbance at 230, 260, and 280 nm, using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). The spectrophotometric images showed that the RNA extracted from all 12 samples using the optimized protocol was of high yield, ranging from 111.7 to 417.5 ng. The purity of the RNA was estimated by calculating the A_{260}/A_{280} and A_{260}/A_{230} ratios to

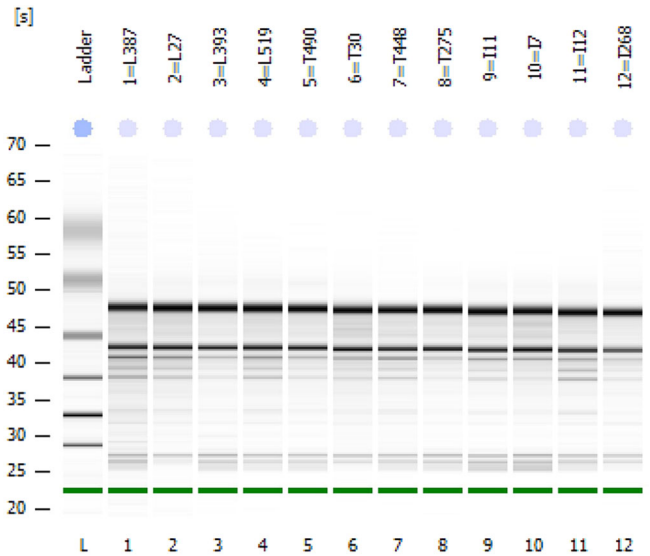

FIGURE 2 The electrophoresis gel showed clear bands for 18S and 28S ribosomal RNA and no genomic DNA contamination in RNA extracted from leaves of 12 individuals of Hawaiian *Metrosideros* (four replicates of three taxa). Lanes 1–4 are *M. polymorpha* race L (L), lanes 5–8 are *M. tremuloides* (T), and lanes 9–12 are *M. polymorpha* var. *incana* (I). Image obtained from an Agilent 2100 Bioanalyzer. The *y*-axis represents time in seconds.

evaluate the levels of protein and polysaccharide/phenolic compound contamination, respectively (Appendix S2).

The integrity of total RNA was verified with 5 µL of each extract using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA) through the calculation of RNA integrity number (RIN) values (Figure 1). The gel revealed clear, distinct 18S and 28S ribosomal RNA bands for all 12 samples (Figure 2), and these samples were successfully used in a follow-on RNA-Seq experiment. Twelve paired-end libraries were constructed using an Illumina P2 Reagents kit (Illumina, San Diego, California, USA) for 300 cycles and then sequenced on an Illumina NextSeq 2000 Sequencer in high-output mode at the Genomics Core facility at the University of Nevada Reno. The quality control of the run was 92.26%.

CONCLUSIONS

To extract RNA from leaves of three taxa of the woody genus *Metrosideros*, we tested four commercially available RNA isolation kits following kit guidelines, as well as the cetyltrimethlammonium bromide (CTAB) method (Chang et al., 1993), the SDS-Tris-saturated phenol method (Ghawana et al., 2011), phenol-based methods (Chomczynski and Sacchi, 1987; Hou et al., 2011), and the LiCl-isopropanol purification method (Rezadoost et al., 2016). RNA*later*-preserved leaves failed to yield quality RNA using any of five isolation protocols and thus were replaced by fresh or snap-frozen leaves for later trials. The NucleoSpin RNA Plant and Fungi Mini-kit produced RNA with the highest absorbance ratios relative to other RNA extraction kits or protocols and thus was selected for optimization of RNA clean-up, which was achieved using the RNA Clean & Concentrator Kit. We report here a time-saving and reproducible protocol that yielded high-quality RNA from snap-frozen leaf tissue from three *Metrosideros* taxa with contrasting leaf phenotypes, as indicated by acceptable $A_{260/280}$ and $A_{260/230}$ absorbance ratios, RIN values >7, and successful downstream RNA sequencing of all 12 samples. With all other protocols tested, RNA purity and integrity were compromised likely due to the presence of polysaccharides, polyphenols, and secondary metabolites. The optimized protocol reported here may be useful for RNA isolation from leaves of diverse woody taxa.

AUTHOR CONTRIBUTIONS

E.A.S. collected all leaf material; M.H. performed RNA isolation experiments; M.H. and E.A.S. wrote and edited the manuscript. All authors approved the final version of the manuscript.

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OPEN RESEARCH BADGES

This article has earned Open Data and Open Materials badges. All Data and Materials are provided in the article and Supporting Information.

DATA AVAILABILITY STATEMENT

All data that support the findings of this study may be found online in the Supporting Information section at the end of the article.

ORCID

Maryam Hadi http://orcid.org/0000-0001-5351-8397
Elizabeth A. Stacy http://orcid.org/0000-0001-7221-8744

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. NanoDrop 1000 spectrophotometric analysis of yield and purity of RNA isolated from young leaf tissue of Hawaiian *Metrosideros* using different protocols.

Appendix S2. NanoDrop 1000 spectrophotometric analysis of yield and purity of RNA isolated from Hawaiian *Metrosideros* using the optimized protocol for RNA isolation.

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Appendix 1. Optimized RNA extraction and purification protocol for Hawaiian *Metrosideros*.

Materials and reagents

1. Nuclease-free tubes (1.5 mL)
2. Nuclease-free micropipette tips
3. Diethyl pyrocarbonate (DEPC) water for treating glassware and mortars and pestles
4. Liquid nitrogen
5. NucleoSpin RNA Plant and Fungi Mini-kit (Macherey-Nagel, Düren, Germany)
6. 96–100% ethanol (for preparation of Buffer PFW2 in the NucleoSpin RNA Plant and Fungi Mini-kit and the RNA wash buffer in the RNA Clean & Concentrator Kit)
7. DNase/RNase-Free Water (Sigma-Aldrich, St. Louis, Missouri, USA)
8. RNA Clean & Concentrator Kit (Zymo, Irvine, California, USA)

Equipment

1. Pipettes
2. Oven
3. Mortar and pestle
4. Vortexer
5. Tube heater
6. −80°C freezer
7. NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA)
8. Centrifuge 5424R (Eppendorf, Hamburg, Germany)
9. Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California, USA)

RNA extraction protocol

NOTE: The following instructions are from the NucleoSpin RNA Plant and Fungi Mini-kit manual, except for the modifications that are mentioned in the Methods and Results section and indicated here in bold.

Before starting:

- Mortars, pestles, and all glassware used for the total RNA isolation were incubated overnight at 220°C; forceps and tweezers were treated with 0.1% (v/v) DEPC for 2 h, autoclaved to inactivate RNases, air-dried, and kept at −20°C until RNA extraction.
- Precool mortar and pestle with liquid nitrogen or at −70°C in a freezer.
- Add 96–100% ethanol to the Buffer PFW2 according to the NucleoSpin RNA Plant and Fungi Mini-kit instructions (for 6 mL of Buffer PFW2, we added 24 mL of ethanol).
- Add 500 µL of Buffer PFL into a 1.5-mL or 2-mL microcentrifuge tube. Add 50 µL of Buffer PFR to the tube.

Step 1: Grind roughly 60 mg of snap-frozen young leaf tissue to a fine powder.

Step 2: Transfer the sample to the Buffer PFL/PFR mixture and mix immediately. The plant material will only thaw within the lysis buffer.

Step 3: Incubate the lysis tube for 5 min at 56°C.

Step 4: Centrifuge for 3 min at 20,000 × *g* in order to sediment the cell debris. Continue with the clear supernatant.

Step 5: Insert a NucleoSpin RNA Plant and Fungi Filter Column (green ring) into a 2-mL microcentrifuge tube with the lid to facilitate mixing by vortexing in step 6.

Step 6: Load the clear lysate from step 5 onto the column. Centrifuge for 1 min at 16,000 × *g*.

NOTE: If the sample does not pass the column completely, centrifuge at 20,000 × *g* for an additional 3 min.

Step 7: To adjust RNA binding conditions, add 500 µL of Buffer PFB to the flowthrough and mix by pipetting.

NOTE: Please refer to Table 2 of the NucleoSpin RNA Plant and Fungi Mini-kit instructions for recommendations on Buffer PFB increase for certain sample types.

Step 8: Incubate for 5 min at room temperature.

Step 9: To bind RNA for each preparation, take one NucleoSpin RNA Plant and Fungi Column (light blue ring) preassembled with a collection tube. Load 650 µL of the sample onto the NucleoSpin RNA Plant and Fungi column. Centrifuge for 30 s at 16,000 × *g*. Discard the flowthrough and reuse the collection tube. Load the residual sample volume (ca. 200 µL) onto the column. Centrifuge for 30 s at 16,000 × *g*. Discard the collection tube with the flowthrough and insert the column into a fresh 2-mL collection tube.

Step 10: Wash and dry the silica membrane: For the first wash, add 500 µL of Buffer PFW1 onto the column. Centrifuge for 1 min at 16,000 × *g*. Discard the flowthrough and reuse the collection tube.

Step 11: For the second wash, add 500 µL of Buffer PFW1 again onto the column. Centrifuge for 1 min at 16,000 × *g*. Discard the collection tube with flowthrough and insert the column into a fresh collection tube.

Step 12: For the third wash, add 500 µL of Buffer PFW2 onto the column. Centrifuge for 1 min at 16,000 × *g*. Discard the flowthrough and reuse the collection tube.

Step 13: For the fourth wash, add 500 µL of Buffer PFW2 again onto the column. Centrifuge for 1 min at 16,000 × *g*. Discard the flowthrough and reuse the collection tube.

Step 14: For the fifth wash, add 500 µL Buffer PFW2 onto the column one more time. Centrifuge for 1 min at 16,000 × g. Discard the collection tube and flowthrough.

Step 15: Elute RNA: Insert the column into a fresh collection tube (1.5 mL). Add 50 µL of RNase-free H_2O onto the column. Incubate for approximately 1 min at room temperature. Centrifuge for 1 min at 16,000 × *g*.

Step 16: Pass the RNA solution obtained in step 15 through the column matrix again and centrifuge for 1 min at 16,000 × *g* to increase RNA concentration.

NOTE: If higher RNA concentrations are desired, use 40 µL of RNase-free H_2O for elution. Overall yield, however, will decrease when using higher volumes.

RNA purification protocol

NOTE: The following instructions are from the RNA Clean & Concentrator Kit manual with modifications in centrifuge speed and final elution steps indicated in bold.

Before starting:

- Add 96 mL of 100% ethanol (104 mL of 95% ethanol) to the 24 mL of RNA Wash Buffer concentrate.

Step 1: Add two volumes of RNA Binding Buffer to each sample and mix. (We mixed 100 µL of buffer and 50 µL of sample.)

Step 2: Add an equal volume of ethanol (95–100%) and mix. (We added 150 µL of ethanol.)

Step 3: Transfer the sample to the Zymo-Spin IC Column in a collection tube and centrifuge for 30 s at **16,000 × *g***. Discard the flowthrough.

Optional: At this point, in-column DNase I treatment can be performed. (We did perform this step.)

Step 4: Add 400 µL of RNA Prep Buffer to the column and centrifuge for 30 s at **16,000 × *g***. Discard the flowthrough.

Step 5: Add 700 µL of RNA Wash Buffer to the column and centrifuge. Discard the flowthrough.

Step 6: Add 400 µL of RNA Wash Buffer to the column and centrifuge for 1 min at **16,000 × *g*** to ensure complete removal of the Wash Buffer. Carefully transfer the column into an RNase-free tube.

Step 7: Add 50 µL of DNase/RNase-Free Water directly to the column matrix and centrifuge for 30 s at **16,000 × *g***.

Step 8: Pass the RNA solution obtained in step 7 through the column matrix again and centrifuge for 30 s at 16,000 × *g* to increase RNA concentration. Alternatively, for highly concentrated RNA use ≥6 µL elution.

The eluted RNA can be used immediately or stored frozen.