Appendix S1. Quality assessment data for RNA extractions using the protocols presented in this study.

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Appendix 1. Protocol for high-quality RNA extraction from ferns.

Equipment and materials

- Dewar to hold liquid nitrogen
- Nuclease-free micropipette filter tips
- Micropipettes
- Ice
- Microcentrifuge capable of cooling to 4°C
- Water bath capable of heating to 55°C
- Nuclease-free tubes (2.0 mL)

Reagents

- Lysis buffer: The lysis buffer should be prepared for each extraction immediately before use. It is composed of 3× CTAB buffer (0.1 M Tris-Cl pH 8.0, 1.4 M NaCl, 0.02 M EDTA, 30 mg/mL CTAB), 15 mM beta-mercaptoethanol (5 µL/mL), and 4% PVPP (4 mg/mL). Heat the buffer in the water bath at 55°C for about 10–15 min and vortex immediately prior to use.
- 24:1 Chloroform:isoamyl alcohol
- >99% Chloroform
- Nuclease-free water

Option A:
- Chilled isopropanol (−20°C)
- Qiagen RNeasy Plant Mini Kit (including on-column DNase) (Qiagen, Hilden, Germany) or TURBO DNase (Thermo Fisher Scientific, Waltham, Massachusetts, USA)

Option B:
- Spectrum Plant Total RNA Kit (Millipore Sigma, Darmstadt, Germany) or Zymo RNA Clean and Concentrator Kit (Zymo Research, Irvine, California, USA)
- 200-proof (100%) molecular-grade ethanol

Preparation

1. Precool centrifuge to 4°C and preheat water bath to 55°C.
2. Fill the Dewar with liquid nitrogen.
3. Gather required samples (kept at −80°C or in liquid nitrogen until ground), equipment, and reagents.
4. Clean workspace of clutter and possible contaminants. RNase Away or RNase Zap may be used to eliminate exogenous RNases in the workspace.

Protocol

1. Grind 25–100 mg of fresh tissue in a mortar with liquid nitrogen. Ensure that the tissue is ground to a fine powder. In a 1.7-mL Eppendorf tube, this corresponds to approximately 0.2 to 0.3 mL, although the weight of the sample should be determined directly from the sample. Transfer the ground tissue to a 2.0-mL tube and immediately add 1 mL of the pre-heated lysis buffer; do not let the sample thaw. Vortex to suspend all tissue in the buffer and place on ice.

   Note: If working with small amounts of tissue (e.g., gametophytes), the tissue can be mechanically disrupted by placing the tissue in a screw-cap tube with nuclease-free beads (e.g., 1.5-mm high-impact zirconium molecular-biology-grade beads), flash-freezing, pulverizing in a bead blaster for 30 s, and then resubmerging the tubes in liquid nitrogen. Repeat this step until the tissue is a fine powder (up to eight bead blastings). Ensure that the tissue remains frozen and does not thaw.
2. Centrifuge at 8000 × *g* at 4°C for 5 min to remove debris. Without disturbing the pelleted material, transfer the liquid to a new 2.0-mL tube.
3. Incubate at 55°C for 10 min. Invert/shake every 3 min.
4. Add 0.5 mL of 24:1 chloroform:isoamyl alcohol to each 2.0-mL tube and vortex to produce a homogenous solution. On a small cooler, invert/shake for 5 min.
5. Centrifuge at 13,000 × *g* at 4°C for 10 min. Transfer the aqueous phase to a new 2.0-mL tube.
6. Add 0.5 mL of >99% chloroform to each 2.0-mL tube and vortex to produce a homogenous solution. On a small cooler, invert/shake for 5 min.
7. Centrifuge at 13,000 × *g* at 4°C for 10 min. Proceed to either option A or option B.

Option A:

A1. Transfer the aqueous phase to a new 2.0-mL tube.

A2. Add an equal volume of chilled isopropanol to the tube and invert/mix a few times.

A3. Centrifuge the samples at 10,000 × *g* or higher at 4°C for 10 min.

A4. Remove the isopropanol by pouring, being careful not to lose the pellet, which may be difficult to see. Spin down samples for 1 min at 4°C and remove any remaining isopropanol with a micropipette.

A5. Dry the pellet at room temperature for 3 min.

A6. Dissolve the pellet with 15 µL or less of nuclease-free water. Flip the tubes to confirm that the pellets are well-dissolved. Note: Stopping at A6 will result in a solution containing both RNA and DNA. Proceed to A7 for DNase treatment and clean-up.

A7. Bring the volume of the extraction up to 100 µL with RNase-free water. Then follow the RNA Clean Up

protocol provided in the RNeasy Mini Handbook (Protocol "RNA Cleanup" in the RNeasy Mini Handbook 04/2023) including the DNase treatment, and then follow steps A8–A11 or proceed with the DNase treatment with TURBO DNase following the manufacturer's protocol.

A8. After the final wash, discard the collection tube and apply the column to a new collection tube. Dry the column by centrifuging for 1 min at 10,000 × *g*.

A9. Discard the collection tube and apply the column to a new RNase-free 2.0-mL tube.

A10. Add at least 25 μL of nuclease-free water directly onto the membrane of the column. Let the column sit for 1 min at room temperature.

A11. Centrifuge at 10,000 × *g* for 1 min.

Option B (Recommended):

B1. Transfer the aqueous phase to a new 2.0-mL tube. The following steps can be performed at room temperature unless otherwise noted. The aqueous phase is your sample that will be bound to the column.

B2a. If purifying using the Spectrum Plant Total RNA Kit, proceed to Step 4: Bind RNA to Column, Protocol A, which is recommended for difficult tissues. At this step, add 3–5× volume of binding buffer provided in the kit and allow the tube to sit at room temperature for 1 min prior to applying the solution to the column. If there is any precipitate that forms after adding the binding buffer, mix and transfer the solution with the precipitate to the column, then proceed with the washes and on-column DNase treatment. Complete steps 4 through 8 of the Spectrum Plant Total RNA Kit and then proceed to step B3 of this protocol.

B2b. If purifying with the Zymo RNA Clean and Concentrator Kit, proceed directly to Step 1 of the Total RNA Clean-up protocol (manual version 3.1.0) and add 2× volume of binding buffer provided in the kit. Complete steps 1 through 6 of the Zymo Total RNA Clean-up protocol and then procced to step B3 of this protocol.

B3. After the final wash, discard the collection tube and apply the column to a new collection tube. Dry the column by centrifuging for 1 min at 10,000 × *g*.

B4. Discard the collection tube and apply the column to a new RNase-free 2.0-mL tube.

B5. Add at least 15–25 μL of nuclease-free water (minimum 15 μL for Sigma, 25 μL for Zymo) directly onto the membrane of the column. Let the column sit for 1 min at room temperature.

B6. Centrifuge at 10,000 × *g* for 1 min and save the elute. This is your purified RNA extraction.

B7. Optionally, repeat steps B5 and B6 for a second elution. This may be recommended for tissues that are RNA-rich. Discard the column.

Quality control

Aliquot 4 μL of RNA elution into a new 2.0-mL RNase-free tube for quality control by TapeStation/Bioanalyzer, NanoDrop, and Qubit. The remaining sample should be kept at −80°C and freeze–thaw cycles should be avoided to preserve RNA integrity. Optimal RNA extractions should have OD 260/280 of 2.0, OD 260/230 > 2.0, and RINs above 7.0, without signs of degradation.