User-Developed Protocol:

Purification of total RNA from plant tissue using the RNeasy® Lipid Tissue Mini Kit

This protocol has been adapted by customers from the RNeasy Lipid Tissue Mini protocol and is intended as a guideline for the purification of total RNA from plant tissue using the RNeasy Lipid Tissue Mini Kit. Plant materials contain large amounts of substances such as starch that can interfere with RNA purification using RNeasy technology. **This protocol has not been thoroughly tested and optimized by QIAGEN.**

IMPORTANT: Please read the "Safety Information" and "Important Notes" sections in the *RNeasy Lipid Tissue Handbook* before beginning this procedure. For safety information on the additional chemicals mentioned in this protocol, please consult the appropriate material safety data sheets (MSDSs), available from the product supplier. RNeasy Lipid Tissue Kits are intended for research use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

Equipment and reagents to be supplied by user

- RNeasy Lipid Tissue Mini Kit (cat. no. 74804)
- Chloroform
- Ethanol (70%) (do not use denatured alcohol, which contains other substances such as methanol or methylethylketone)
- Sterile, RNase-free pipet tips
- Disposable gloves
- Equipment for tissue disruption and homogenization: we recommend either the TissueRuptor with TissueRuptor Disposable Probes (cat. no. 990890), or the TissueLyser with TissueLyser Adapter Set 2 x 24 (cat. no. 69982) and Stainless Steel Beads, 5 mm (cat. no. 69989); for ordering information for the TissueRuptor or TissueLyser, please refer to the QIAGEN Product Guide or contact your local QIAGEN office
- 1.5 ml or 2 ml microcentrifuge tubes
- Microcentrifuge(s) (with rotor for 2 ml tubes) for centrifugation at 4ºC and at room temperature (15–25ºC)
- Optional: Liquid nitrogen (required for disrupting frozen tissues using the TissueRuptor)
- Optional: RNase-Free DNase Set (cat. no. 79254)

Important points before starting

- If using RNeasy Lipid Tissue Kits for the first time, read "Important Notes" (page 10) in the *RNeasy Lipid Tissue Handbook*.
- If working with RNA for the first time, read Appendix A (page 28) in the *RNeasy Lipid Tissue Handbook*.

- If using the TissueRuptor, ensure that you are familiar with operating it by referring to the *TissueRuptor User Manual* and *TissueRuptor Handbook*. Especially hard tissues, such as roots or seeds, could cause the disposable probes to break and may not be well-suited for use with the TissueRuptor.
- If using the TissueLyser, ensure that you are familiar with operating it by referring to the operating instructions and *TissueLyser Handbook*. It is important that both adapter plates are balanced before starting operation of the TissueLyser.
- We recommend using no more than 25 mg plant tissue per prep. In some cases, it may be possible to use up to 100 mg plant tissue per prep. RNA content varies due to factors such as tissue type, developmental stage, and growth conditions.
- Do not use tungsten carbide beads, as they react with QIAzol Lysis Reagent.
- Fresh or frozen plant tissues can be used in the procedure. If processing frozen tissues with the TissueLyser, precool the TissueLyser Adapter Set by storing it at –80ºC for at least 2 h, and keep it on dry ice until it is assembled. Samples should also be kept on dry ice until they are assembled into the TissueLyser Adapter Set. After sample disruption, immediately add QIAzol Lysis Reagent. Do not freeze the adapter set or samples in liquid nitrogen.
- Generally, DNase digestion is not required since integrated QIAzol and RNeasy technologies efficiently remove most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., real-time RT-PCR analysis of a low-abundance target). In these cases, residual DNA can be removed by optional on-column DNase-digestion using the RNase-Free DNase Set (see Appendix C, page 32, in the *RNeasy Lipid Tissue Handbook*). Alternatively, for real-time two-step RT-PCR applications, the QuantiTect® Reverse Transcription Kit provides cDNA synthesis with integrated removal of genomic DNA contamination.
- QIAzol Lysis Reagent and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 6 in the *RNeasy Lipid Tissue Handbook* for safety information.
- Except for phase separation (step 5), all protocol and centrifugation steps should be performed at room temperature (15–25ºC). During the procedure, work quickly.

Things to do before starting

- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- If performing optional on-column DNase digestion, prepare DNase I stock solution as described in Appendix C (page 32) in the *RNeasy Lipid Tissue Handbook*.

Procedure

1. Disrupt plant tissues and homogenize the lysates using either the TissueRuptor (follow step 1a) or TissueLyser (follow step 1b).

See “Disrupting and homogenizing starting material”, page 12, in the *RNeasy Lipid Tissue Handbook* for more details on disruption and homogenization.

Note: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNeasy spin column. Homogenization with the TissueRuptor or TissueLyser generally results in higher RNA yields than with other methods.

1a. Disruption and homogenization using the TissueRuptor:

- **Place 25 mg fresh tissue (wet weight) in a suitably sized vessel, and add 1 ml QIAzol Lysis Reagent. Alternatively, place 25 mg fresh or frozen tissue (wet weight) in a suitably sized vessel, and add liquid nitrogen.**

  For efficient disruption, the size of the sample must not be greater than half the diameter of the disposable probe. If necessary, cut the sample into smaller pieces.

  Note: Use a suitably sized vessel with sufficient extra headspace to accommodate foaming, which may occur during disruption in QIAzol Lysis Reagent.

- **Place the tip of the disposable probe into the vessel and operate the TissueRuptor at full speed until no debris is visible (usually 30 s). If the sample was disrupted in QIAzol Lysis Reagent, proceed straight to step 2. If the sample was disrupted in liquid nitrogen, allow the liquid nitrogen to evaporate, add 1 ml QIAzol Lysis Reagent, and proceed to step 2.**

  For efficient disruption, move the disposable probe up and down during operation of the TissueRuptor.

  Note: To avoid damage to the TissueRuptor and disposable probe during operation, make sure the tip of the probe remains submerged in QIAzol Lysis Reagent or liquid nitrogen.

  If foaming occurs during disruption in QIAzol Lysis Reagent, leave the sample at room temperature for 2–3 min until the foam subsides. Alternatively, briefly centrifuge the sample.

1b. Disruption and homogenization using the TissueLyser:

- **If handling frozen tissues, precool the TissueLyser Adapter Set 2 x 24 by storing it at –80ºC for at least 2 h (do not freeze the adapter set in liquid nitrogen). If handling fresh tissues, do not precool the adapter set.**
- **Place 25 mg tissue (wet weight) in 2 ml microcentrifuge tubes containing one stainless steel bead (5 mm mean diameter). If handling frozen tissues, keep the tubes on dry ice (do not freeze the tubes in liquid nitrogen).**
- **If handling fresh tissues, add 1 ml QIAzol Lysis Reagent to each tube. If handling frozen tissues, do not add QIAzol Lysis Reagent. Be sure to seal each tube securely.**
- **Place the tubes in the TissueLyser Adapter Set 2 x 24.**
- **Operate the TissueLyser for 1 min at 20–30 Hz. Disassemble the adapter set, rotate the rack of tubes so that the tubes nearest to the TissueLyser are now outermost, and reassemble the adapter set. Operate the TissueLyser for another 1 min at 20–30 Hz.**

  The duration of disruption and homogenization depends on the tissue being processed and can be extended until no tissue debris is visible. If processing frozen tissues, keep the samples and the adapter set on dry ice for several minutes in between the individual disruption steps to avoid thawing.

Rearranging the tubes ensures uniform disruption and homogenization.

- **If fresh tissues were disrupted, proceed straight to step 2. If frozen tissues were disrupted, add 1 ml QIAzol Lysis Reagent, and proceed to step 2.**

  Do not reuse the stainless steel beads.

2. **Place the tube containing the homogenate on the benchtop at room temperature (15–25ºC) for 5 min.**

   This step promotes dissociation of nucleoprotein complexes.

3. **Add 200 µl chloroform to the tube. Securely cap the tube, and vortex vigorously.**

   Thorough mixing is important for subsequent phase separation.

4. **Place the tube on the benchtop at room temperature for 2–3 min.**

5. **Centrifuge at 12,000 x *g* for 15 min at 4ºC. After centrifugation, heat the centrifuge to room temperature (15–25ºC) if the same centrifuge will be used in the later steps of this procedure.**

   After centrifugation, the sample separates into 3 phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase. The volume of the aqueous phase should be approximately 500 µl.

6. **Transfer the upper, aqueous phase to a new tube (not supplied). Add 1 volume (usually 500 µl) of 70% ethanol, and mix thoroughly by vortexing. Do not centrifuge. Proceed immediately to step 7.**

   Note: The volume of lysate may be less than 500 µl due to loss during homogenization and centrifugation.

   Precipitates may be visible after addition of ethanol, but do not affect RNA purification.

7. **Transfer up to 700 µl of the sample to an RNeasy Mini spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) at room temperature (15–25ºC). Discard the flow-through.***

   Reuse the collection tube in step 8.

8. **Repeat step 7 using the remainder of the sample. Discard the flow-through.***

   Reuse the collection tube in step 9.

   Optional: If performing optional on-column DNase digestion (see “Important points before starting”), follow steps C1–C4 (page 32) in the *RNeasy Lipid Tissue Handbook* after performing this step, and then proceed to step 10.

9. **Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the membrane. Discard the flow-through.***

   Reuse the collection tube in step 10.

   After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.*

   Note: Skip this step if performing optional on-column DNase digestion.

* Flow-through contains QIAzol Lysis Reagent or Buffer RW1 and is therefore not compatible with bleach. See page 6 in the *RNeasy Lipid Tissue Handbook* for safety information.

10. **Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the membrane. Discard the flow-through.**

    Reuse the collection tube in step 11.

    Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see “Things to do before starting”).

11. **Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to wash the membrane.**

    The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

    Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

12. **Recommended: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.**

    Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 11.

13. **Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently. To elute the RNA, centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm).**

14. **If the expected RNA yield is >30 µg, repeat step 13 using another volume of RNase-free water, or using the eluate from step 13 (if high RNA concentration is required). Reuse the collection tube from step 13.**

    If using the eluate from step 13, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.