User-Developed Protocol:

Isolation of total RNA from plants using the RNeasy® 96 Kit

These guidelines have been adapted by customers from the RNeasy 96 Protocol for Isolation of Total RNA from Cultured Cells, the RNeasy Plant Mini Protocol for Isolation of Total RNA, and the DNeasy® 96 Plant Protocol for Isolation of DNA from Fresh Leaf Tissue. These guidelines should be considered as recommendations for adapting the RNeasy 96 protocol for use with plant cells and tissues. **These procedures have not been tested or optimized by QIAGEN**.

Please be sure to read the *RNeasy 96 Handbook* or the *RNeasy Mini Handbook* and the *DNeasy 96 Plant Handbook* carefully before beginning the procedure.

Equipment and reagents to be supplied by the user

- Equipment for disrupting plant tissue. We recommend the TissueLyser* with TissueLyser Adapter Set 2 x 96 and reusable 3 mm stainless steel beads for optimal disruption.
- Centrifuge 4-15C or 4K15C with Plate Rotor 2 x 96 (cat. no. 81031)
- Multichannel pipet with extended tips
- Reagent reservoirs for the multichannel pipet
- Ethanol (96–100%)
- DNase I (optional; see step 13)

Important notes before starting

- For initial experiments, do not use more than 25 mg plant material per sample. With optimization, it may be possible to use larger amounts of starting material. The RNA content of plant tissues can vary due to tissue type, developmental stage, growth conditions used, and other factors.
- Add 10 µl β-mercaptoethanol per 1 ml of Buffer RLT before use. The solution is stable for one month.

  Note: β-mercaptoethanol is toxic; dispense in a fume hood and wear appropriate protective clothing.
- Take time to familiarize yourself with the TissueLyser and the Centrifuge 4-15C/Centrifuge 4K15C before starting this protocol.
- Do not use tungsten carbide beads with this procedure as they will react with Buffer RLT.

* All disruption steps can also be performed using the Mixer Mill MM 300 without modification.

- This protocol describes processing of fresh plant leaf tissue. Frozen plant leaf tissue can also be used: in this case, perform the disruption using liquid nitrogen, and do not add Buffer RLT until the samples have been disrupted (i.e., add Buffer RTL at step 5). Do not allow the TissueLyser Adapter Plates to come into contact with liquid nitrogen.
- This protocol describes processing of 192 samples (2 x 96). If you wish to process 96 samples or less, provide a balance for the TissueLyser by assembling a second plate sandwich using a rack of collection microtubes without samples or buffers, but containing the stainless steel beads, and fixing this second sandwich into the empty clamp.
- All centrifugation steps should be performed at room temperature.

Procedure

1. **Place up to 25 mg fresh leaf sample into each tube in two collection microtube racks.**

   Unless the optimal amount of starting material has been determined, do not use more than 25 mg (wet weight) per sample.

   We recommend using the racks of collection microtubes provided with the RNeasy 96 Kit for disrupting samples. The contents of collection microtubes can be seen more easily than those of standard 96-well blocks, making it easier in step 8 to avoid carryover of cell debris (which could clog the RNeasy 96 membrane).

2. **Add one stainless steel bead to each collection microtube.**

3. **Pipet 450 µl Buffer RTL into each collection microtube. Seal the microtubes with caps.**

   Note: Ensure that 10 µl β-mercaptoethanol was added per 1 ml of Buffer RLT before use.

   Note: It may be necessary to double the amount of Buffer RLT to 900 µl to ensure that enough lysate can be transferred at step 8 if the particulate plant material does not form a tight pellet after centrifugation in step 7.

4. **Disrupt the samples using the TissueLyser as follows:**

   a) **Sandwich each rack of collection microtubes between adapter plates and fix into the TissueLyser clamps as described in the TissueLyser instruction manual.**

      Note: Ensure that the microtubes are properly sealed with caps.

      IMPORTANT: Two plate sandwiches must be clamped to the TissueLyser to provide balance. To process 96 samples or less, assemble a second plate sandwich using a rack of collection microtubes containing stainless steel beads but no samples or buffers, and fix it into the empty clamp.

   b) **Shake for 1.5 min at 30 Hz.**

c) **Remove and disassemble the plate sandwiches. Ensure that the collection microtubes are tightly closed. Reassemble the plate sandwiches so that the collection microtubes nearest the TissueLyser in steps 4a and 4b are now outermost. Reinsert the plate sandwiches into the TissueLyser.**

Rotating the racks of collection microtubes in this way ensures that all samples are thoroughly disrupted. More foam will have formed in the tubes that were outermost during the initial disruption step.

IMPORTANT: Merely rotating the entire plate sandwich so that the QIAGEN logos are upside down when reinserted into the TissueLyser is not sufficient, since the same samples that were outermost during the initial disruption will remain outermost in the second disruption step.

d) **Shake for a further 1.5 min at 30 Hz.**

5. **Remove the plate sandwiches from the TissueLyser and remove the adapter plates from each rack of collection microtubes. Vortex the racks of collection microtubes vigorously.**

6. **Optional: Incubate the samples at 56°C for 1–3 min.**

This step may help disrupt the tissue. However, for samples with a high starch content, incubation at high temperature should be avoided to prevent swelling of the sample.

7. **Centrifuge the racks of collection microtubes for 10 min at 6000 rpm.**

Compact pellets should have formed after this centrifugation. If many particles remain in suspension, it may be necessary to extend this centrifugation step to 20 min.

Note: If 96-well blocks from another manufacturer are used, ensure that they can withstand the *g*-forces generated during centrifugation.

8. **Carefully transfer each supernatant to new racks of collection microtubes.**

Collection microtubes are connected in strips of 8. To avoid transferring particulate matter, it is helpful to remove the strips from the rack so that the contents of the microtubes are visible, and to use the multichannel pipet on its lowest speed setting.

If less than 450 µl (or 900 µl) supernatant is recovered, adjust the amount of ethanol in step 9 accordingly.

9. **Add 0.5 volumes (typically 225 µl) of ethanol (96–100%) to each sample. Mix by pipetting up and down 3 times.**

A precipitate may form upon addition of ethanol. This precipitate will not interfere with the RNeasy procedure or any subsequent application.

10. **Place two RNeasy 96 Plates on top of Square-Well Blocks (provided). Mark the RNeasy 96 Plates for later sample identification.**

11. Carefully transfer each sample to the RNeasy 96 Plates.

Take care not to wet the rims of the wells to avoid aerosols during centrifugation. Do not transfer more than 700 µl of sample at a time. If the volume of the sample exceeds 700 µl, load the first 700 µl, spin the samples as described in step 12, load a further 700 µl, and repeat the process until all of each sample, including any precipitate, has been transferred to the RNeasy 96 Plates.

Note: Lowering pipet tips to the bottoms of the wells may cause sample overflow and cross-contamination. Therefore, remove one set of caps at a time, and begin drawing up the samples as soon as the pipet tips contact the liquid. Repeat until all the samples have been transferred to the RNeasy 96 Plates.

12. Seal each RNeasy 96 Plate with an AirPore Tape Sheet (provided). Centrifuge for 4 min at 6000 rpm. Discard the flow-through from the square-well block.

Use of AirPore Tape prevents cross-contamination between samples during centrifugation.

After centrifugation, check that all of the lysate has passed through the membrane in each well of the RNeasy 96 Plates. If lysate remains in any of the wells, centrifuge for a further 2 min.

13. Optional: on-membrane DNase I treatment

Note: DNase I digestion is generally not required since RNeasy silica-gel–membrane, spin-technology efficiently removes most of the DNA. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA.

a) Prepare a DNase I stock solution by dissolving solid DNase I in 550 µl of the provided RNase-free water. To avoid loss of DNase I, do not open the vial. Inject RNase-free water into the vial using an RNase-free needle and syringe. Mix gently by inverting the tube. DO NOT VORTEX.

For long-term storage of the DNase I solution, store aliquots at –20°C. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze after thawing.

b) For each well to be treated with DNase I, mix 10 µl DNase I stock solution with 70 µl Buffer RDD. Mix by gently inverting.

c) Remove the tape from the RNeasy 96 Plates. Pipet the 80 µl of the DNase I incubation mix directly onto the membrane in each well of the RNeasy 96 Plates. Seal the plates with a new AirPore Tape Sheet, and incubate for 15 min at room temperature (15–25°C).

Note: Make sure to pipet the DNase I directly onto the RNeasy 96 membrane. DNA digestion will be incomplete if some of the mixture sticks to the walls or O-rings of the RNeasy 96 Plates.

14. Remove the tape. Add 800 µl Buffer RW1 to each sample, and seal the plates with a new AirPore Tape Sheet. Centrifuge for 4 min at 6000 rpm. Discard the flow-through.

Note: If an on-membrane DNase I digestion was performed in the previous step, incubate the plates for 5 min before centrifugation.

15. **Remove the tape. Add 800 µl Buffer RPE to each sample, and seal the plates with a new AirPore Tape Sheet. Centrifuge for 4 min at 6000 rpm. Discard the flow-through.**
16. **Remove the tape. Add another 800 µl Buffer RPE to each sample, and seal the plates with a new AirPore Tape Sheet. Centrifuge for 4 min at 6000 rpm. Discard the flow-through.**
17. **Centrifuge the plates for a further 10 min at 6000 rpm.**

    IMPORTANT: It is important to dry the RNeasy 96 membranes thoroughly as residual ethanol may interfere with downstream applications and reduce overall RNA yields. This 10 min centrifugation ensures that residual trances of salt are removed and that no ethanol is carried over during elution.
18. **Remove the tape. To elute the RNA, place each RNeasy 96 Plate in the correct orientation on a new rack of collection microtubes and add 45–70 µl RNase-free water (provided) to each sample. Seal each plate with a new AirPore Tape Sheet and incubate for 1 min at room temperature. Centrifuge for 4 min at 6000 rpm.**
19. **Repeat step 18 if the expected RNA yield is >20 µg.**
20. **Use the provided caps to seal the collection microtubes. Store RNA at –20°C or –70°C.**

Ordering Information

Product	Contents	Cat. No.
TissueLyser — for high-throughput disruption of a wide range of biological samples		
TissueLyser (220–240 V, 50/60 Hz)	Universal laboratory mixer-mill disruptor, 220–240 V, 50/60 Hz	85220
TissueLyser (120 V, 50/60 Hz)	Universal laboratory mixer-mill disruptor, 120 V, 50/60 Hz	85210
TissueLyser (100 V, 50/60 Hz)	Universal laboratory mixer-mill disruptor, 100 V, 50/60 Hz	85200
Accessory		
TissueLyser Adapter Set 2 x 96	2 sets of Adapter Plates for use with Collection Microtubes (racked) on the TissueLyser	69984

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