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QIAGEN RNeasy Plant RNA Extraction Protocol (Modified) V.2

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1 Works for me dx.doi.org/10.17504/protocols.io.bqnkmvcw

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ABSTRACT

This is a protocol for extraction of RNA from plant leaf tissue using a QIAGEN RNeasy Plant Mini Kit. The procedure largely follows the manufacturer's instructions but there are a few minor tweaks we introduced which in our hands were necessary for optimal results. This protocol also includes the on-column DNase I digestion step.

The original protocol is attached below

HB-0572-002 1101268_PCard_RNY_Plant_Mini_0316_WW_WEB.pdf

Kit:
https://www.qiagen.com/us/products/diagnostics-and-clinical-research/sample-processing/rneasy-plant-mini-kit/#orderinginformation

RNase free DNase kit:
https://www.qiagen.com/us/products/discovery-and-translational-research/lab-essentials/enzymes/rnase-free-dnase-set/#orderinginformation

DOI

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GUIDELINES

It is critical that you do not overload the spin columns to ensure high purity of final RNA. Therefore before sampling determine how much an individual leaf disk weighs then harvest and freeze only 100 mg or less of fresh leaf tissue per sample vial.

MATERIALS TEXT

- TissueLyser II (QIAGEN; 85300)
- 2 mL centrifuge tubes
- 4mm SPEXTM stainless steel grinding beads (SPEX; 2150)
- Humboldt brass cork borer set (07-865-10B; Fisher Scientific)
- 13.4 mm diameter, flash-frozen leaf disks
- RNeasy Plant Mini Kit (QIAGEN; 74904)
- RNase-Free DNase Set (QIAGEN; 79254)

SAFETY WARNINGS

Perform all steps within a fume hood and collect tips and tubes in thehazardous material collection bins. β-mercaptoethanol (β-ME) included in the extraction buffer is toxic, harmful to the environment and corrosive (it also stinks!)

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BEFORE STARTING

- Calculate the volume of extraction buffer RLT required for the number of samples to be processed. Each sample requires at least **450 µl** working on the assumption that **100 mg** of fresh tissue is disrupted. It is advisable to prepare **500 µl** of RLT per sample, to ensure sufficient buffer is made. (So for 10 samples, this would be **5 mL**)
- To prepare RLT buffer, in a fresh tube add **10 µl** β-ME per **1 mL** Buffer RLT.
- Add 4 volumes of ethanol (96–100%) to Buffer RPE for a working solution
- Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the lyophilized DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. 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 vial. Do not vortex (DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube.)
- For long-term storage of DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.
- Calculate the volume of DNase I working solution required for the number of samples to be processed. Each sample requires **80 µl** . Make a master mix containing **10 µl** of DNAse I per sample and **70 µl** buffer RDD. It is advisable to make up 10-20% extra than you need to account for pipetting inaccuracies. (So for 10 samples this would be **120 µl** of DNAse I and **840 µl** of buffer RDD). Prepare in a new nuclease-free

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tube

Tissue Lysis

1 Pre-cool 4mm SPEX stainless steel grinding beads at **-80 °C**

2 Pre-cool the TissueLyser inserts in LN_2 for **00:30:00** 30m

3 Using forceps cooled in LN_2, add one SPEX bead per sample tube.

As mentioned in the description a sample tube should contain 100 mg or less of flash-frozen leaf tissue that has been sored in LN_2 or at **-80 °C**

4 Insert tubes into pre-cooled TissueLyser II cassettes, ensuring a balanced number of samples between cassettes.

5 Grind tissue for **00:01:30** at 20 Hz 1m 30s

Warning: Do not exceed this frequency, higher frequencies increase the number of cases where the steel beads will break the lid of centrifuge tubes resulting in sample loss. Using reinforce centrifuge tubes is recommended.

If using the SPEX 2150 beads it is necessary to use 2 mL centrifuge tubes to ensure proper grinding, in 1.5 mL tubes the bead will not reach the tapered bottom leaving samples unground

6 Remove cassettes from TissueLyser II and submerge them in LN_2 to prevent thawing

7 Grind tissue for **00:01:30** at 20 Hz 1m 30s

This repeat is to ensure all tissue is correctly grounded. There will be odd instances where leaf tissue has not properly ground for reasons such as samples sticking to the edge of tubes, check for this on removal, it may be necessary to repeat again.

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8 Remove tubes with forceps cooled in LN_2. Samples can be stored at **-80 °C** for several months before processing further.

Loading samples

9 Add **450 µl** Buffer RLT to a maximum of 100 mg tissue powder. Vortex immediately until the powder is re-suspended.

This step can take up to a couple of minutes. If lumps form it can lead to partial thawing before the powder is completely dissolved in the buffer and degradation of RNA due to the activity of endogenous RNAses. It is therefore advisable to start with samples that are at **-70 °C** , (tubes on dry ice) rather than moving samples straight from LN2, which has a greater tendency for lumps to form.

It is critical to pre-weigh your samples so as not to overload the spin columns, using too much tissue will result in sub-standard purity of RNA after extraction

10 Spin samples **12000 x g, Room temperature , 00:01:00** to pellet any residual debris.

This is not included in the original protocol, ideally following proper grinding and re-suspension there should be little to no debris or clumps. However, in our experience, there can be aggregates of insoluble material, spinning will help keep these to the bottom of the centrifuge tube

11 Transfer the lysate to a QIAshredder spin column (lilac) placed in a **2 mL** collection tube

Be careful not to disturb any pellet, if you are having trouble with the tip getting blocked it can help to cut off the end

12 Spin **12000 x g, Room temperature , 00:02:00**

13 Transfer the supernatant of the flow-through to a new microcentrifuge tube (not supplied) without disturbing the cell-debris pellet.

Use nuclease-free centrifuge tubes

14 Add 0.5 volume of ethanol (96–100%) to the cleared lysate, and mix immediately by pipetting. Do not centrifuge.

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Typically the volume of EtOH to use will be **225 µl**

15 Transfer the sample (usually **650 µl**), with any precipitate, to an RNeasy Mini spin column (pink) in a **2 mL** collection tube (supplied).

16 Close the lid, and centrifuge for **12000 x g, Room temperature , 00:00:15**). Discard the flow-through.

On column DNAse I treatment 15m

17 Add **350 µl** Buffer RW1 to the RNeasy spin column. Close the lid, and centrifuge for **12000 x g, 00:00:15** . Discard the flow-through.

18 Add the DNase I incubation mix (**80 µl**) directly to the RNeasy spin column membrane, and incubate on the benchtop (20–30°C) for **00:15:00** . 15m

Be sure to add the DNase I incubation mix directly to the RNeasy spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column

19 Add **350 µl** Buffer RW1 to the RNeasy spin column. Close the lid gently, and spin **12000 x g, Room temperature , 00:00:15** . Discard the flow-through

Washing samples

20 Add **500 µl** Buffer RPE to the RNeasy spin column. Close the lid, and spin for **12000 x g, Room temperature , 00:00:15** . Discard the flow-through

21 ⚠

Repeat step 12 at least 4 times

*This differs from the original protocol and you will need to order an additional bottle of RPE buffer to supplement what is supplied in the kit *

This is critical to ensure a yield of highly pure RNA, reflected in A260/A230 ratios >1.8. Otherwise, samples will likely be contaminated with residual guanidium salts and leaf pigments.

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22 Transfer the column to a new 2 mL collection tube. Add **500 µl** Buffer RPE to the RNeasy spin column. Close the lid, and spin for **12000 x g, 00:00:15** . Discard flow-through after inspecting the color.

* This differs from the original protocol *

Samples are transferred to a new collection tube in this instance to allow you to see if the flow-through is colorless. If it is not, repeat the RPE washes until no coloration can be seen in the sample. Realistically this shouldn't be more than one additional spin, if your flow-through is still very green you probably have too much sample in the first place and should consider starting again.

23 Transfer the spin column to a fresh tube, spin for **12000 x g, 00:02:00**

This is to ensure removal of residual EtOH on the column, it is important to ensure high purity RNA samples for downstream analysis

24 Place the RNeasy spin column in a new nuclease-free **1.5 mL** collection tube (supplied). Add **50 µl** RNase-free water directly to the spin column membrane.

25 Close the lid, and spin for **12000 x g, Room temperature , 00:01:00** to elute the RNA.

26 Place samples on ice or store at **-80 °C**

For best results, it is advisable to proceed directly to downstream applications.

e.g. check RNA amount and purity using a Nanodrop. A260/A280 ratio should be >2 and A260/A230 ratio should be >1.8.

Check RNA integrity using Qubit Fluorometer and perform cDNA synthesis

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