RNA extraction (≥ 5 μg)

RNeasy® Plant Mini Kit (Manual) or RNeasy® Plus Mini Kit (Manual)

Preparation

Usage amount per sample:

Chemical	Concentration	Amount	Location
β-mercaptoethanol (β-ME)	–	4.5 μL	Toxic (b)
Buffer RLT mixture [α]	–	450 μL	RNA bench
Buffer RW1 [β]	–	700 μL	RNA bench
Buffer RPE working solution [γ]	–	1000 μL	RNA bench
DNAse I incubation mix [δ]	–	80 μL	fresh-prepared
Ethanol (EtOH) (for RNA use)	≥ 96%	225 μL	V
RNase-free water	–	30 μL	RNA bench

[α] **Buffer RLT mixture**: Add 10 μL β-mercaptoethanol (β-ME) to 1 mL Buffer RLT. The mixture can be stored at room temperature for up to 1 month. Buffer RLT contains a high concentration of guanidine isothiocycanate, which supports the binding of RNA to the silica membrane. The β-mercaptoethanol should be added to Buffer RLT to inactivate RNases.

[β] **Buffer RW1** contains a guanidine salt, as well as ethanol, and is used as a stringent washing buffer that efficiently removes biomolecules such as carbohydrates, proteins, fatty acids etc., that are non-specifically bound to the silica membrane. At the same time, RNA molecules larger than 200 bases remain bound to the column.

[γ] **Buffer RPE working solution**: Add 4 volumes of ≥ 96% ethanol to Buffer RPE. In this case, add 2000 µL ethanol to 500 µL Buffer RPE. It is a mild washing buffer for removing traces of salts, which are still on the column due to buffers used earlier in the protocol.

[δ] **DNase I incubation mix**: Add **10 µL DNase I stock solution** to **70 µL Buffer RDD**, mix by inverting the tube, and put on ice.

> - **DNase I stock solution**: Located at 4°C (b). Dissolve the lyophilized DNase I in 550 µL 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 vial. Do not vortex. Store the stock solution as single use aliquots at −20°C (9 months) or 4°C (1 month). Do not refreeze after thawing.
> - **Buffer RDD**: Located at 4°C (b). It provides efficient on-column digestion of DNA and also ensures that the RNA remains bound to the column.

Procedure

- Ensure that all instruments are properly sterilized before starting the experiment to prevent RNA contamination and eliminate residual RNase activity.
- Perform all procedures prior to the elution step in a fume hood due to the hazardous nature of β-mercaptoethanol (β-ME).

Step 1: Cell lysis

1. Put in 5 beads in each 2 mL microcentrifuge tube. Prepare **450 µL Buffer RLT mixture** (contains 1% β-ME) into the eppendorf and put in ice to pre-cold the buffer.

2. Flash freeze samples with liquid nitrogen, dissect ≤ 100 mg samples and immediately submerge them into the pre-colded Buffer RLT mixture.

> - The rice root tip weight is approximately: [17.7, 20.6, 19.7, 21.2, 22.3] mg cm^{-1}/ 10 root-tips ≈ **0.2 mg / mm**. So the maximum is 100 root tips (cut 5 mm).
> - Put the tissue into an eppendorf, slightly centrifuge to spin down the tissue to the bottom of the eppendorf. If the tissue is not used immediately, store the sample at −80°C.

3. Disrupt the tissue using Tissue Grinder (**30 hits / sec**, **10 mins**).

> - Place a thick layer of tissue paper between the tissue grinder adapter cover and the eppendorf tube caps to boost grinding efficiency and prevent the tube caps from popping open during high-speed reciprocal shaking.

4. (optional) Water bath (**56°C**, **1 min**). Help to disrupt the tissue and release RNA.

5. Now we get the **raw lysate**.

Step 2: Collect the lysate supernatant

1. Transfer the **raw lysate** (≈ 450 µL) to a **QIAshredder spin column** (purple lid) placed in a **2 mL collection tube**. Centrifuge (**full speed**, **2 mins**).

> - The QIAshredder spin column is used for removing cell debris and simultaneously homogenizes the lysate.

2. (optional) Transfer the supernatant to a **gDNA Eliminator Mini Spin Column** (colorless, with purple O-ring) placed in a 2 mL collection tube (supplied). Centrifuge (≥ **10,000 rpm** or 8000 × g, **30 secs**). Discard the column and save the flow-through.

> - The gDNA Eliminator Mini Spin Column is the component of RNeasy® Plus Mini Kit. It is not sold separately.

3. Transfer the supernatant of the flow-through to a new 1.5 mL microcentrifuge tube (not supplied) without disturbing the remaining cell-debris pellet.

4. Add **225 µL 96~100% ethanol** (≈ 0.5 volume) to the cleared lysate, and **mix well** immediately by pipetting. ***DO NOT centrifuge***. Proceed to the next step immediately.

> - The volume of lysate may be less than 450 µL due to loss during homogenization.
> - Precipitates may be visible after additon of ethanol. This does not affect the procedure.

Step 3: Bind RNA to column

1. Transfer the sample lysate (usually 650 µL), including any precipitate that may have formed, to an **RNeasy spin column** (pink lid) placed in a 2 mL collection tube (supplied). Close the lid gently.
2. Centrifuge (≥ **10,000 rpm** or 8000 × g, **15 secs**). **Discard the flow-through**. Reuse the collection tube.

> - If the sample volume exceeds 700 µL, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.

Step 4: DNA digestion (optional)

1. Add **350 µL Buffer RW1** to the **RNeasy spin column**. Close the lid gently.
2. (optional) Incubate on benchtop (25°C, 3–5 mins) before centrifuging to enhance removal of excess gDNA prior to applying the enzyme.
3. Centrifuge (≥ **10,000 rpm** or 8000 × g, **15 secs**) to wash the **RNeasy spin column membrane**. **Discard the flow-through**. Reuse the collection tube.
4. Add **10 µL DNase I** to **70 µL Buffer RDD**. Mix well by gently inverting the tube. Centrifuge briefly to collect residual liquid from the sides of the tube.

> - DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex.

5. Add the **80 µL DNase I incubation mix** directly to the **RNeasy spin column membrane**, and place on the benchtop (**25 ± 5°C**, **15 mins**).

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

Step 5: Wash

1. Add **350 µL Buffer RW1** to the **RNeasy spin column**. Close the lid gently. Centrifuge (≥ **10,000 rpm** or 8000 × g, **15 secs**) to wash the spin column membrane. **Discard the flow-through**. Reuse the collection tube.
2. Add **500 µL Buffer RPE** to the **RNeasy spin column**. Close the lid gently. Centrifuge (≥ **10,000 rpm** or 8000 × g, **15 secs**) to wash the spin column membrane. **Discard the flow-through**. Reuse the collection tube.
3. Add **500 µL Buffer RPE** to the **RNeasy spin column**. Close the lid gently. Centrifuge (≥ **10,000 rpm** or 8000 × g, **2 mins**) to wash the spin column membrane. **Discard the collection tube with the flow-through**.

> - 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.
> - 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.

4. Place the **RNeasy spin column** in a **new 2 mL collection tube** (supplied). Close the lid gently. Centrifuge (**full speed**, **1 ~ 5 mins**).

> - 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.

Step 6: Elute RNA

1. Place the **RNeasy spin column** in a **new 1.5 mL collection tube** (supplied).

2. Add 30–50 µL RNase-free water directly to the spin column membrane. Close the lid gently.

> - To increase RNA yield, incubate the spin column on the bench for 10 mins (optional).
> - If the expected RNA yield is >30 µg, repeat the elution step using another 30–50 µL RNase-free water. That means, you will get 60–100 µL eluate.

3. Centrifuge (≥ **10,000 rpm** or 8000 × g, **1 min**) to elute the RNA.

> - If high RNA concentration is required, repeat the elution step using the eluate. 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.

4. Store the RNA eluate at **−20°C** (few weeks) or **−80°C** (> 1 year).

Done !!!

If you encounter any issues with the protocol, please report them at:
https://github.com/P10911004-NPUST/jklai/issues.