RNA extraction (≤ 1 μg)

RNeasy® Micro Kit (Manual)

Preparation

Usage amount per sample:

Chemical	Concentration	Amount	Location
β-mercaptoethanol (β-ME)	–	3.5 µL	Toxic (b)
Buffer RLT mixture [α]	–	350 µL	RNA bench
Buffer RW1 [β]	–	700 µL	RNA bench
Buffer RPE mixture [γ]	–	500 µL	RNA bench
Buffer RDD	–	70 µL	4°C (b)
DNAse I incubation mix [δ]	–	80 µL	fresh-prepared
Ethanol (EtOH) (for RNA use)	≥ 96%	—	V
Ethanol (EtOH)	80%	500 µL	fresh-prepared
Ethanol (EtOH)	70%	350 µL	fresh-prepared
RNase-free water	–	14 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. Dissect ≤ 5 mg tissue (around 30 root tips) and dab the surface dry gently (better to use KimWipes paper).

- 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. Close the eppendorf lid tightly, and then immediately put the eppendorf into liquid nitrogen (it will float up by itself, no worry).
4. The drill of the TissueLyser instrument should be pre-cold, by inserting it into the liquid nitrogen.
5. Take out the eppendorf and powderize the tissue with the TissueLyser instrument (this step is extremely important to yield high quantity and quality of RNA, and this step also should be finished as quick as possible).
6. Add **350 µL Buffer RLT mixture** into the eppendorf and immediately vortex to homogenize the buffer and the powderized tissue (10-15 secs)
7. Now we get the **raw lysate**. Place the eppendorf on ice to inhibit enzymatic activity of the lysate.

Step 2: Collect the lysate supernatant

1. Centrifuge the raw lysate (3 mins, 15000 rpm).
2. Carefully take the supernatant to a new eppendorf, and then place on ice.
3. Add **350 µL 70% EtOH** into the sample, mix well with pipetting. ***DO NOT centrifuge***. Proceed to the next step immediately.

Step 3: Wash (1)

1. Transfer the sample (including any precipitate that may have formed) to an **RNeasy MinElute® spin column** in a **2 mL collection tube**. Close the lid and centrifuge (**4°C, 1 min, ≥ 15000 rpm**). **Discard the flow-through and reuse the collection tube** for the next step.
2. Add **350 µL Buffer RW1** to the RNeasy MinElute® spin column. Close the lid and centrifuge (**4°C, 1 min, ≥ 15000 rpm**). **Discard the flow-through and reuse the collection tube** for the next step.

Step 4: Digest DNA

1. Add the **DNase I incubation mix (80 µL)** to the RNeasy MinElute® spin column membrane. Place on benchtop at room temperature for **15 mins ~ 1 hour**.

> - Be sure to add the DNase I incubation mix directly to the RNeasy MinElute 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

> - The **DNase I incubation mix (80 µL)** should be fresh-prepared. Add 10 µL DNase I stock solution to 70 µL Buffer RDD, mix by inverting the tube, and put on ice. Please refer to the first page for details.

Step 5: Wash (2)

1. Add **350 µL Buffer RW1** to the RNeasy MinElute® spin column. Close the lid and centrifuge (**4°C, 1 min, ≥ 15000 rpm**). **Discard the flow-through and collection tube**.

2. Place the RNeasy MinElute® spin column in a **new 2 mL collection tube** (supplied). Add **500 µL Buffer RPE** to the spin column. Close the lid and centrifuge (**4°C, 1 min, ≥ 15000 rpm**). **Discard the flow-through and reuse the collection tube**.

3. Add **500 µL 80% EtOH** to the spin column. Close the lid and centrifuge 2 times (**2 × 2 mins, ≥ 15000 rpm**). **Discard the flow-through and collection tube**.

> - After centrifugation, carefully remove the RNeasy MinElute 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 MinElute® spin column in a **new 2 mL collection tube** (supplied). **Open the lid** and centrifuge at full speed (**4°C, 15 mins, ≥ 15000 rpm**) to dry the membrane. **Discard the flow-through and collection tube**.

> - To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise)

Step 6: Elute RNA

1. Place the RNeasy MinElute® spin column in a **new 1.5 mL RNase-free low-binding collection tube** (supplied).

2. Add **14 µL RNase-free water** directly to the center of the spin column membrane. Close the lid gently.

3. Centrifuge at full speed (**4°C, 1 ~ 15 mins, ≥ 15000 rpm**) to elute the RNA.

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

Step 7: RNA quality & quantity measurement

	NanoDrop™	Qubit™
Concentration (> 100 ng / *u*L)	✓	✓
Concentration (< 10 ng / *u*L)		✓
Require high precision		✓

Materials Provided by the kit

> Buffer RLT (store at 15 - 25°C)
> Buffer RPE (working solution) (store at 15 - 25°C)
> Buffer RDD (store at 15 - 25°C)
> Buffer RW1 (store at 15 - 25°C)
> DNase Set (RNase-Free) (store at 2 - 8°C)
> RNeasy MinElute® spin columns (store at 2 - 8°C)
> 2 mL collection tube
> 1.5 mL collection tube

- Buffer RLT is a lysis buffer for lysing cells and tissues
- Buffer RPE is a 55 ml concentrated buffer for washing membrane-bound RNA
- Buffer RDD provides efficient on-column digestion of DNA and also ensures that the RNA remains bound to the column
- Buffer RW1 is for washing membrane-bound RNA
- **RNeasy MinElute® spin columns** (pink color lid) stored at 4°C, b cabinet

RNA quantification with Qubit™

Qubit™ RNA HS Assay Kit

Procedure

Materials

> For best results, ensure that all materials and reagents are at room temperature.

1. Set up the required number of Qubit™ Assay Tubes for standards and samples. This assay requires 2 standards (Component C and D).

- ***Note***: *Use only thin-wall, clear, 0.5 mL PCR tubes (Cat. No. Q32856) for the Qubit™ 4 Fluorometer.*

2. Label the tube lids.

- ***Note***: *Do not label the side of the tubes, that will affect light penetration. Label the lid of each standard tube correctly, as the calibration of the Qubit™ Fluorometer requires the standards to be inserted into the instrument in the right order.*

Step 1: Make working solution

The working solution is a mixture of Component A (dye) and Component B (HS buffer), with a ratio of 1 : 199. For example, to make 10 tubes (8 samples + 2 standards), add 10 µL dye into 1990 µL HS buffer.

1. Prepare the **working solution** by diluting Component A 1:200 in Component B for each tube (sample + standard solutions). For example, 10 µL Component A + 1990 µL Component B for 10 tubes (8 samples + 2 standards).

- ***Note***: ***DO NOT*** *mix the working solution in a glass container. Use clean plastic tube*

Step 2: Prepare standard solutions

Step 3: Prepare sample solutions

2. Add the working solution to each tube such that the final volume is 200 µL.

	Standard assay tubes	User sample assay tubes
Volume of working solution	190 µL	180 – 199 µL
Volume of standard	10 µL	—
Volume of user sample	—	1 – 20 µL
Total volume in each assay tube	200 µL	200 µL

5. Add **10 µL** of Component C (**standard # 1**) and D (**standard # 2**) separately to the appropriate tubes.
6. Add **1 µL** of each user **sample** to the appropriate tubes.
7. Vigorously vortex for 3 ~ 5 seconds.
8. Incubate at room temperature for 2 minutes. Then proceed to read standards and samples with the Qubit™ 4 Fluorometer.

Reading standards and samples

1. On the `Home` screen, touch `RNA`, then select `RNA High Sensitivity` as the assay type. Touch `Read standards` to proceed.
2. Insert the tube containing **Standard # 1** into the sample chamber, close the lid, then touch `Read standard`. When the reading is comnplete, remove Standard # 1.
3. Insert the tube containing **Standard # 2** into the sample chamber, close the lid, then touch `Read standard`. When the reading is comnplete, remove Standard # 2.
4. Touch `Run samples`.
5. On the assay screen, select the `Sample volume` and `Unit`.

- Touch the + or - buttons on the wheel, or anywhere on the wheel itself, to select the sample volume added to the assay tube (1 µL in this protocol).
- From the `Unit` dropdown menu, select the units for the output sample concentration.

6. Insert a sample tube into the sample chamber, close the lid, then touch `Read tube`. When the reading is complete (~ 3 seconds), remove the sample tube.

- The top value (in large font) is the concentration of the original sample and the bottom value is the dilution concentration.

7. Repeat step 6 until all samples have been read.

Materials Provided by the kit

Component	Material name	Q32852 *	Concentration
A †	Qubit™ RNA HS Reagent	250 µL	200X in DMSO
B §	Qubit™ RNA HS Buffer	50 mL	-
C ※	Qubit™ RNA HS Standard # 1	1 mL	0 ng/µL in TE buffer
D ※	Qubit™ RNA HS Standard # 2	4 × 250 µL	10 ng/µL in TE buffer

* **Q32852**: Catalogue number. When stored as directed, kits are stable for 6 months.

† **Component A**: Stored at 2 ~ 8°C (short-term storage) or ≤ -20°C (long-term storage), desiccated, protect from light. Located at -80°C, upper door, 2nd layer, 2nd column, 3rd drawer, 10 μL **pale blue** aliquots in PCR tubes.

§ **Component B**: Stored at ≤ 30 °C. Located at VI.

※ **Component C & D**: Stored at 2 ~ 8°C (short-term storage) or ≤ -20°C (long-term storage). Located at -80°C, upper door, 2nd layer, 2nd column, 3rd drawer.

Materials NOT Provided by the kit

1. Plastic container (RNase-free, disposable) for mixing the Qubit™ working solution;
2. Qubit™ Assay Tubes (500 tubes, Cat. No. Q32856);