# RNA extraction ( ≤ 1 $\mu$g )

RNeasy® Micro Kit ( Manual )

## Preparation

Usage amount per sample:

| Chemical | Concentration | Amount | Location |
|---|---|---|---|
| $\beta$-mercaptoethanol ( $\beta$-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 $\mu$L $\beta$-mercaptoethanol ( $\beta$-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 $\beta$-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 $\beta$-mercaptoethanol ($\beta$-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 $\mu$L **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 );

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