

# Protocol: Total RNA Isolation from Microdissected Formalin-Fixed Tissues

This protocol is designed for the isolation of RNA from microdissected, formalin-fixed animal tissue samples. For frozen samples, see “Protocol: Total RNA Isolation from Microdissected Cryosections” (page 18).

Laser-microdissected tissue specimens present a particular challenge for molecular analysis, as nucleic acids must be purified from very small amounts of starting material. In addition, fixation and staining steps may compromise the integrity of RNA, and it may be necessary either to modify fixation protocols or to use cryosections from flash-frozen specimens to minimize this problem.

In order to remove proteins, which can interfere with the procedure, the standard RNeasy Micro protocol for total RNA isolation from animal tissues has been adapted to include a proteinase K digest. Samples are lysed in a guanidine-isothiocyanate-containing lysis buffer (Buffer RLT). After dilution of the lysate, the sample is treated with proteinase K. Debris is pelleted by centrifugation. Ethanol is then added to the cleared lysate and RNA is bound to the RNeasy MinElute silica-gel membrane. Traces of DNA that may copurify are removed by a DNase treatment on the RNeasy MinElute Spin Column. DNase and any contaminants are washed away, and total RNA is eluted in RNase-free water.

Depending on the fixation protocol, the age of the samples, the staining procedure, and the storage conditions used, RNA can be highly fragmented into pieces smaller than 300 nucleotides, thus limiting the size of RNA fragments isolated. Furthermore, as the RNeasy procedure removes RNA smaller than 200 nucleotides, this can lead to an overall loss in yield if the RNA is highly degraded.

A wide range of equipment and consumables for sectioning, staining, and microdissection of specimens is available from Leica ([www.leica-microsystems.com](http://www.leica-microsystems.com)).

## Additional reagents to be supplied by user

- QIAGEN Proteinase K, >600 mAU/ml (cat. no. 19131 or 19133). Proteinase K must be used in the procedure. If using proteinase K from another supplier, use a 20 mg/ml solution in water.

## Important points before starting

- If using RNeasy Micro Kits for the first time, read “Important Notes” (page 11).
- If working with RNA for the first time, read Appendix A (page 58).

- To minimize RNA degradation, avoid prolonged storage of unstabilized samples at room temperature. RNA in tissues is not protected before fixation, stabilization in RNA<sup>later</sup> RNA Stabilization Reagent,\* or flash-freezing in liquid nitrogen.†
- Homogenized tissue lysates (in Buffer RLT, step 4) can be stored at  $-70^{\circ}\text{C}$  for several months. To process frozen lysates, thaw samples at room temperature or at  $37^{\circ}\text{C}$  in a water bath until they are completely thawed and salts in the lysis buffer are dissolved. Avoid extended treatment at  $37^{\circ}\text{C}$ , which can cause chemical degradation of the RNA. Continue with step 5.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 5 for safety information. Take appropriate safety measures and wear gloves when handling.
- Unless otherwise indicated, all protocol steps, including centrifugation, should be performed at room temperature. During the procedure, work quickly.

### Things to do before starting

- Before using the kit for the first time, prepare 80% ethanol by mixing 24 ml ethanol (96–100%) and 6 ml RNase-free water (supplied with the kit).
- $\beta$ -Mercaptoethanol ( $\beta$ -ME) must be added to Buffer RLT before use. Add 10  $\mu\text{l}$   $\beta$ -ME per 1 ml Buffer RLT. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT is stable at room temperature for 1 month after addition of  $\beta$ -ME.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- Prepare DNase I stock solution before using the RNase-free DNase for the first time. Dissolve the solid DNase I (1500 Kunitz units) in 550  $\mu\text{l}$  of the RNase-free water provided. Take care that no DNase I is lost when opening the vial. Mix gently by inverting the tube. **Do not vortex.**

For long-term storage of reconstituted DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at  $-20^{\circ}\text{C}$  for up to 9 months. Thawed aliquots can be stored at  $2$ – $8^{\circ}\text{C}$  for up to 6 weeks. Do not refreeze the aliquots after thawing.

\* A modified protocol for preparation of RNA<sup>later</sup> preserved tissues for histological studies is available from QIAGEN Technical Services; please inquire.

† When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- When processing <5000 cells, carrier RNA should be added to the lysate before the proteinase K digest. The RNeasy Micro Kit contains poly-A RNA to be added as carrier RNA (see “Carrier RNA”, page 17). Before using for the first time, dissolve the carrier RNA (310 µg) in 1 ml RNase-free water. Store this stock solution at –20°C, and use it to make fresh dilutions for each set of RNA preps.

The concentration of this stock solution is 310 µg/ml (= 310 ng/µl). To make a working solution (4 ng/µl) for 10 preps, add 5 µl of the dissolved RNA to 34 µl of Buffer RLT and mix by pipetting. Take 6 µl of this diluted solution, and add it to 54 µl Buffer RLT. The final concentration is 4 ng/µl. Add 5 µl of this solution to the lysate in step 4.

### Procedure

1. **Heat a water bath or heating block to 55°C for proteinase K digestion in step 6.**
2. **Collect the sample directly into an appropriate volume of Buffer RLT (the volume depends on the collection vessel used for the microdissection, but should not be greater than 140 µl).**
3. **If necessary, transfer the sample and Buffer RLT into a larger reaction vessel (such as a 1.5 ml or 2.0 ml microcentrifuge tube).**
4. **Adjust the sample volume to 150 µl with Buffer RLT.**

**Note:** When processing <5000 cells, add 20 ng of carrier RNA (5 µl of a 4 ng/µl solution) to the lysate before proceeding to step 5. Prepare a dilution of the carrier RNA provided, as described in “Things to do before starting”.

5. **Add 295 µl RNase-free water to the lysate. Then add 5 µl QIAGEN Proteinase K solution and mix thoroughly by pipetting.**
6. **Incubate at 55°C for 10 min.**
7. **Centrifuge for 3 min at 10,000 x g at room temperature.**

A small pellet of tissue debris will form, sometimes accompanied by a thin layer or film on top of the supernatant.

8. **Pipet the supernatant (approximately 450 µl) into a new tube (not provided).**

Avoid transferring any of the pellet. If unavoidable, however, a small amount of pelleted debris may be carried over without affecting the RNeasy Micro procedure. Hold the pipet tip under the thin layer or film on top of the supernatant, if present. This layer will usually adhere to the outside of the pipet tip and should not be transferred.

9. **Add 0.5 volumes (usually 225  $\mu$ l) of ethanol (96–100%) to the cleared lysate. Mix well by pipetting. Do not centrifuge.**

A precipitate may form after the addition of ethanol, but this will not affect the RNeasy procedure.

10. **Pipet the sample, including any precipitate that may have formed, into an RNeasy MinElute Spin Column in a 2 ml collection tube. Centrifuge for 15 s at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm). Discard the flow-through.\***

Reuse the collection tube in step 11.

11. **Pipet 350  $\mu$ l Buffer RW1 into the RNeasy MinElute Spin Column, and centrifuge for 15 s at  $\geq 8000 \times g$  to wash. Discard flow-through.\***

Reuse the collection tube in step 13.

**Optional:** If on-column DNase treatment using the RNase-free DNase and Buffer RDD is not desired, increase the amount of Buffer RW1 in this step to 700  $\mu$ l, centrifuge for 15 s at  $\geq 8000 \times g$  to wash, and discard flow-through\* and collection tube. Continue the protocol with step 15.

12. **Add 10  $\mu$ l DNase I stock solution to 70  $\mu$ l Buffer RDD. Mix by gently inverting the tube.**

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

13. **Pipet the DNase I incubation mix (80  $\mu$ l) directly onto the RNeasy MinElute silica-gel membrane, and place on the benchtop at room temperature for 15 min.**

**Note:** Make sure to pipet the DNase I incubation mix directly onto the RNeasy MinElute silica-gel membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the RNeasy MinElute Spin Column.

14. **Pipet 350  $\mu$ l Buffer RW1 into the RNeasy MinElute Spin Column, and centrifuge for 15 s at  $\geq 8000 \times g$ . Discard flow-through and collection tube.\***

15. **Transfer the RNeasy MinElute Spin Column into a new 2 ml collection tube (supplied). Pipet 500  $\mu$ l of Buffer RPE onto the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 15 s at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to wash the column. Discard the flow-through.**

Reuse the collection tube in step 16.

**Note:** Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Things to do before starting").

\* Flow-through contains Buffer RL1 or Buffer RW1 and is therefore not compatible with bleach. See page 5 for safety information.

16. **Add 500  $\mu$ l of 80% ethanol to the RNeasy MinElute Spin Column. Close the tube gently, and centrifuge for 2 min at  $\geq 8000 \times g$  ( $\geq 10,000$  rpm) to dry the RNeasy MinElute silica-gel membrane.**

Prepare the 80% ethanol with ethanol (96–100%) and the RNase-free water supplied with the kit.

**Note:** Following the centrifugation, remove the RNeasy MinElute Spin Column from the collection tube carefully so the column does not come into contact with the flow-through as this will result in carryover of ethanol.

17. **Transfer the RNeasy MinElute Spin Column into a new 2 ml collection tube (supplied). Open the cap of the spin column, and centrifuge in a microcentrifuge at maximum speed for 5 min. Discard the flow-through and collection tube.**

To avoid damage to the caps, place the columns into the centrifuge with at least one empty position between each column. Place the caps so that they point in the opposite direction to the rotation of the rotor (i.e., if the rotor rotates in a clockwise direction, orient the caps in a counter-clockwise direction).

It is important to dry the silica-gel membrane since residual ethanol may interfere with downstream reactions. Centrifuging with the caps open ensures that no ethanol is carried over during elution.

18. **To elute, transfer the spin column to a new 1.5 ml collection tube (supplied). Pipet 14  $\mu$ l RNase-free water directly onto the center of the silica-gel membrane. Close the tube gently, and centrifuge for 1 min at maximum speed to elute.**

Smaller volumes of RNase-free water can be used to obtain a higher total RNA concentration, but this will influence the overall yield. The yield will be approximately 20% less when using 10  $\mu$ l RNase-free water for elution. Elution with less than 10  $\mu$ l may not be enough to sufficiently hydrate the silica-gel membrane and is therefore not recommended.

The dead volume of the RNeasy MinElute Spin Column is 2  $\mu$ l; elution with 14  $\mu$ l of RNase-free water results in an eluate with a volume of 12  $\mu$ l.

**Note:** When performing RT-PCR with RNA isolated using this procedure we recommend using the QIAGEN OneStep RT-PCR Kit. This kit contains a specially formulated blend of Omniscript Reverse Transcriptase, designed for RNA amounts greater than 50 ng, and Sensiscript Reverse Transcriptase, for very small amounts of RNA (<50 ng). See page 70 for ordering information. For quantitative real-time RT-PCR we recommend QIAGEN QuantiTect RT-PCR Kits.