User-Developed Protocol

Purification of total RNA from FFPE cores using the RNeasy® FFPE Kit

As an alternative to sectioning using a microtome, biopsy needles can be used to obtain core punches from FFPE specimens. These core punches can be used for RNA purification. However, as the diameter of these cores is typically 1–2 orders of magnitude greater than the thickness of microtome sections, the RNeasy FFPE protocol provided in the *RNeasy FFPE Handbook* should be modified. This User-Developed Protocol has been adapted by customers from the RNeasy FFPE protocol and is intended as a guideline for the purification of total RNA from FFPE cores using the RNeasy FFPE Kit. **This protocol has not been thoroughly tested and optimized by QIAGEN.**

IMPORTANT: Please consult the "Safety Information" and "Important Notes" sections in the *RNeasy FFPE Handbook* before beginning this procedure. For safety information on the additional chemicals mentioned in this protocol, please consult the appropriate material safety data sheets (MSDSs), available from the product supplier. The RNeasy FFPE Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

Equipment and reagents to be supplied by user

- RNeasy FFPE Kit (50) (cat. no. 73504)
- Ethanol (96-100%) (do not use denatured alcohol, which contains other substances such as methanol or methylethylketone)
- Sterile, RNase-free pipet tips
- Disposable gloves
- 1.5 ml or 2 ml microcentrifuge tubes
- >3 ml tubes
- Microcentrifuge(s) (with rotor for 2 ml tubes)
- Vortexer
- Mechanical disruption device (e.g., TissueLyser II or TissueLyser LT)
- Heating block or water bath capable of incubation at 80°C
- For deparaffinization of FFPE tissue sections: Deparaffinization Solution (cat. no. 19093) or alternative reagent (e.g., heptane, xylene, limonene, CitriSolv).

Starting material

Starting material for nucleic acid purification should be one FFPE core with a maximum diameter of 3 mm and a length of 3 mm after excess paraffin is trimmed off. If there is no information about the nature of your starting material, we recommend starting with no more than 10 mg of FFPE core per preparation. Do not use more than 25 mg of FFPE core per preparation. Do not overload the RNeasy MinElute® spin column, as this will significantly reduce RNA yield and quality. The maximum binding capacity of the RNeasy MinElute spin column is 45 µg RNA.

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Important points before starting

- If using RNeasy FFPE Kits for the first time, read “Important Notes” in the *RNeasy FFPE Handbook*.
- If working with RNA for the first time, read Appendix B in the *RNeasy FFPE Handbook*.
- Buffer RBC contains a guanidine salt and is therefore not compatible with disinfecting reagents containing bleach. See the *RNeasy FFPE Handbook* for safety information.

Things to do before starting

- 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.
- Equilibrate all buffers to room temperature (15–25°C). Mix reconstituted Buffer RPE by shaking.
- Set a thermal mixer or heated orbital incubator to 56°C for use in steps 3 and 8.
- Prepare DNase I stock solution by dissolving the lyophilized DNase I (1500 Kunitz units) 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. DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the vial. Insoluble material may remain after dissolving DNase. Due to the production process, insoluble material may be present in the lyophilized DNase. This does not affect DNase performance.

Procedure

1. **Place FFPE cores into a 2 ml microcentrifuge tube and close the lid.**

   Do not use more than the recommended maximum amount of starting material; see section “Starting material”.

2. **Disrupt and homogenize the FFPE core according to step 2a or 2b.**

   2a. Disruption and homogenization using the TissueLyser II:

   Place a stainless steel bead (5 mm in diameter) in the microcentrifuge tube containing the sample.

   Place the tubes in the TissueLyser Adapter Set 2 x 24.

   Operate the TissueLyser for 30 s at 20 Hz.

   Complete disruption and homogenization may not be possible. However, small amounts of debris are usually digested in the proteinase K step.

   2b. Disruption and homogenization using the TissueLyser LT:

   Place a stainless steel bead (5 mm mean diameter) in the microcentrifuge tube containing the sample.

   Place the microcentrifuge tubes into the insert of the TissueLyser LT Adapter and put insert into the base of the TissueLyser LT Adapter, which is attached to the TissueLyser LT. Place the lid of the TissueLyser LT Adapter over the insert, and screw the knob until the lid is securely fastened.

   Operate the TissueLyser LT for 30 s at 30 Hz.

   Complete disruption and homogenization may sometimes not be possible. However, small amounts of debris are usually digested in the proteinase K step.

3. **Add 320 µl Deparaffinization Solution, vortex vigorously for 10 s, and centrifuge briefly to bring the sample to the bottom of the tube.**

Deparaffinization Solution is not supplied with the RNeasy FFPE Kit and should be ordered separately (cat. no. 19093). If using an alternative deparaffinization method, see Appendix A of the *RNeasy FFPE Handbook* for further details.

4. **Incubate at 56°C for 3 min, then allow to cool at room temperature.**

   If too little Deparaffinization Solution is used or if too much paraffin is carried over with the sample, the Deparaffinization Solution may become waxy or solid after cooling. If this occurs, add additional Deparaffinization Solution and repeat the 56°C incubation.

5. **Remove the stainless steel bead.**

   For bead removal, a magnet can be slid along the outside of the reaction tube so that the bead is indirectly moved to the rim of the tube. Avoid removing any tissue fragments.

6. **Add 240 µl Buffer PKD.**
7. **Centrifuge for 1 min at 11,000 x *g* (10,000 rpm).**
8. **Add 10 µl proteinase K to the lower, colorless phase. Mix gently by pipetting up and down.**
9. **Incubate at 56°C for 15 min, then at 80°C for 15 min. If a lot of tissue debris is still visible after 15 min at 56°C, it is possible to prolong the incubation time at 56°C to up to 3 hours.**

   Small amounts of insoluble material may remain after the 56°C incubation. Proteinase K digestion to the point of complete solubilization is not required to achieve maximum RNA yields

   If using only one heating block, leave the sample at room temperature after the 56°C incubation until the heating block has reached 80°C.

   IMPORTANT: Ensure that the heating block has reached 80°C before starting the 15 min incubation. The 15 min incubation at 80°C is critical for reversal of crosslinks and optimal RNA performance in downstream applications such as real-time RT-PCR.

   The incubation at 80°C in Buffer PKD partially reverses formaldehyde modification of nucleic acids. Longer incubation times or higher incubation temperatures may result in more fragmented RNA, but may also result in slightly lower C_T values in downstream applications such as real-time RT-PCR.

10. **Transfer the lower phase into a new tube.**
11. **Incubate on ice for 3 min. Then, centrifuge for 15 min at 20,000 x *g* (13,500 rpm).**
12. **Transfer the supernatant to a new tube (>3 ml) taking care not to disturb the pellet. The pellet contains insoluble tissue debris, including crosslinked DNA.**
13. **Add DNase Booster Buffer equivalent to a tenth of the total sample volume (approximately 25 µl) and 10 µl DNase I stock solution. Mix by inverting the tube. Centrifuge briefly to collect residual liquid from the sides of the tube.**

    DNase I is supplied lyophilized and should be reconstituted as described in “Things to do before starting”.

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

14. **Incubate at room temperature for 15 min.**
15. **Add 500 µl Buffer RBC to adjust binding conditions, and mix the lysate thoroughly.**
16. **Add 1200 µl ethanol (100%) to the sample, and mix well by pipetting. Do not centrifuge. Proceed immediately to step 16. Precipitates may be visible after addition of ethanol. This does not affect the procedure.**

17. **Transfer 700 µl of the sample, including any precipitate that may have formed, to an RNeasy MinElute spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.* Reuse the collection tube in step 17.**
18. **Repeat step 16 until the entire sample has passed through the RNeasy MinElute spin column.**

    Reuse the collection tube in step 18.
19. **Add 500 µl Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.**

    Buffer RPE is supplied as a concentrate. Ensure that ethanol is added before use as described in the *RNeasy FFPE Handbook*.

    Reuse the collection tube in step 19.
20. **Add 500 µl Buffer RPE to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the collection tube with the flow-through.**

    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.
21. **Place the RNeasy MinElute spin column in a new 2 ml collection tube. Open the lid of the spin column, and centrifuge at full speed for 5 min. Discard the collection tube with the flow-through.**

    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).It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.
22. **Place the RNeasy MinElute spin column in a new 1.5 ml collection tube. Add 14–30 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.**

    Elution with smaller volumes of RNase-free water leads to higher total RNA concentrations, but lower RNA yields.

    The dead volume of the RNeasy MinElute spin column is 2 µl: elution with 14 µl RNase-free water results in a 12 µl eluate.

* Flow-through contains Buffer RBC and is therefore not compatible with bleach. See the safety information in the *RNeasy FFPE Handbook* for details.