User-developed protocol

User-Developed Protocol:

Purification of total RNA from fatty tissues using the RNeasy® Lipid Tissue Mini Kit and MaXtract High Density

This protocol has been adapted by customers for the purification of total RNA from fatty tissues using the RNeasy Lipid Tissue Mini Kit and MaXtract High Density. This protocol can also be used with all other types of tissues. **This protocol has not been thoroughly tested and optimized by QIAGEN.**

IMPORTANT: Please be sure to read the *RNeasy Lipid Tissue Handbook* and the *MaXtract Low and High Density Handbook* before starting, paying careful attention to the Safety Information section.

Equipment and reagents to be supplied by user

- RNeasy Lipid Tissue Mini Kit (50) (cat. no. 74804)
- MaXtract High Density (200 x 2 ml) (cat. no. 129056)
- Also refer to "Equipment and Reagents to Be Supplied by User" in the *RNeasy Lipid Tissue Handbook*

Determining the correct amount of starting material

It is essential to use the correct amount of tissue in order to obtain optimal RNA yield and purity. With the RNeasy Lipid Tissue Mini Kit, a maximum of 100 mg brain or adipose tissue can generally be processed. For these tissues, the RNA binding capacity of the RNeasy Mini spin column and the lysing capacity of QIAzol Lysis Reagent will not be exceeded by these amounts. For other tissues, a maximum of 50 mg tissue can generally be used. For tissues with high RNA content such as liver, spleen, and thymus, we recommend using no more than 30 mg tissue to ensure optimal RNA yields and to avoid exceeding the binding capacity of the RNA spin column. Average RNA yields from various tissues are given in Table 2 (page 11) in the *RNeasy Lipid Tissue Handbook*.

If there is no information about the nature of your starting material, we recommend starting with no more than 30 mg tissue. Depending on RNA yield and purity, it may be possible to use up to 100 mg tissue in subsequent preparations.

Do not overload the RNeasy spin column, as this will significantly reduce RNA yield and quality.

Weighing tissue is the most accurate way to quantify the amount of starting material. As a guide, a 4 mm cube (64 mm^3) of most animal tissues weighs 70–85 mg.

Important points before starting

- If using RNeasy Lipid Tissue Kits for the first time, read "Important Notes" (page 10) in the *RNeasy Lipid Tissue Handbook*.
- If working with RNA for the first time, read Appendix A (page 28) in the *RNeasy Lipid Tissue Handbook*.

- If using the TissueRuptor, ensure that you are familiar with operating it by referring to the *TissueRuptor User Manual* and *TissueRuptor Handbook*.
- If using the TissueLyser, ensure that you are familiar with operating it by referring to the operating instructions and *TissueLyser Handbook*.
- To freeze tissue for long-term storage (several months), flash-freeze in liquid nitrogen, and immediately transfer to –70ºC. Do not allow tissues to thaw during weighing or handling prior to disruption in QIAzol Lysis Reagent. Homogenized tissue lysates from step 3 can also be stored at –70ºC for several months. Incubate frozen lysates at 37ºC in a water bath until completely thawed and salts are dissolved before continuing with step 4. Avoid prolonged incubation, which may compromise RNA integrity.
- Generally, DNase digestion is not required since integrated QIAzol and RNeasy technologies efficiently remove most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan® or LightCycler® RT-PCR analysis with a low-abundance target). In these cases, residual DNA can be removed by optional on-column DNase-digestion using the RNase-Free DNase Set (see Appendix C, page 32, in the *RNeasy Lipid Tissue Handbook*). Alternatively, for real-time, two-step RT-PCR applications, the QuantiTect® Reverse Transcription Kit provides cDNA synthesis with integrated removal of genomic DNA contamination.
- QIAzol Lysis Reagent and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See page 6 in the *RNeasy Lipid Tissue Handbook* for safety information.
- Except for phase separation (step 8), all protocol and centrifugation steps should be performed at room temperature (15–25ºC). During the procedure, work quickly.

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.
- If performing optional on-column DNase digestion, prepare DNase I stock solution as described in Appendix C (page 32) in the *RNeasy Lipid Tissue Handbook*.

Procedure

1. **If using the TissueLyser, add one stainless steel bead (5 mm mean diameter) per 2 ml microcentrifuge tube (not supplied). If working with tissues that are not stabilized in RNA*later*® RNA Stabilization Reagent, place the tubes on dry ice.**
2. **Excise the tissue sample from the animal or remove it from storage. Determine the amount of tissue. Do not use more than 100 mg. Proceed immediately to step 3.**

   Weighing tissue is the most accurate way to determine the amount.

   If the tissue sample was stored in RNA*later* RNA Stabilization Reagent, remove it from the reagent using forceps and be sure to remove any crystals that may have formed.

   RNA in harvested tissues is not protected until the tissues are treated with RNA*later* RNA Stabilization Reagent, flash-frozen, or disrupted and homogenized in step 3. Frozen tissues should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.

3. Disrupt the tissue and homogenize the lysate using either the TissueRupter (follow step 3a) or TissueLyser (follow step 3b).

See “Disrupting and homogenizing starting material”, page 12, in the *RNeasy Lipid Tissue Handbook* for more details on disruption and homogenization.

Note: Incomplete homogenization leads to significantly reduced RNA yields and can cause clogging of the RNeasy spin column. Homogenization with the TissueRupter or TissueLyser generally results in higher RNA yields than with other methods.

3a. Disruption and homogenization using the TissueRuptor:

- **Place the tissue in a suitably sized vessel containing 1 ml QIAzol Lysis Reagent.**

  Note: Use a suitably sized vessel with sufficient extra headspace to accommodate foaming, which may occur during homogenization.

  Generally, round-bottomed tubes allow more efficient disruption and homogenization than conical-bottomed tubes.

- **Place the tip of the disposable probe into the vessel and operate the TissueRuptor at full speed until the lysate is uniformly homogeneous (usually 20–40 s). Proceed to step 4.**

  Note: To avoid damage to the TissueRuptor and disposable probe during operation, make sure the tip of the probe remains submerged in the buffer.

  Foaming may occur during homogenization, especially of brain tissue. If this occurs, let the homogenate stand at room temperature for 2–3 min until the foam subsides before continuing with the procedure.

3b. Disruption and homogenization using the TissueLyser:

- **Place the tissues in the tubes prepared in step 1.**
- **If the tubes were stored on dry ice, place them at room temperature. Then immediately add 1 ml QIAzol Lysis Reagent per tube.**
- **Place the tubes in the TissueLyser Adapter Set 2 x 24.**
- **Operate the TissueLyser for 2 min at 20 Hz.**

  The time depends on the tissue being processed and can be extended until the tissue is completely homogenized.

- **Rearrange the collection tubes so that the outermost tubes are innermost and the innermost tubes are outermost. Operate the TissueLyser for another 2 min at 20 Hz.**

  Rearranging the tubes allows even homogenization.

- **Carefully pipet the lysates into new microcentrifuge tubes (not supplied). Proceed to step 4.**

  Do not reuse the stainless steel beads.

4. Place the tube containing the homogenate on the benchtop at room temperature (15–25ºC) for 5 min.

This step promotes dissociation of nucleoprotein complexes.

5. **Centrifuge a 2 ml tube of MaXtract High Density at 12,000–16,000 x *g* for 20–30 s. Transfer the homogenate to the MaXtract tube.**

   Note: If the homogenate was derived from RNA*later* stabilized tissue, add 50–100 µl RNase-free water to the MaXtract tube as well.

6. **Add 200 µl chloroform to the MaXtract tube. Securely cap the tube, and shake it vigorously for 15 s. Do not vortex.**

   Thorough mixing is important for subsequent phase separation.

7. **Place the MaXtract tube on the benchtop at room temperature for 2–3 min.**

8. **Centrifuge at 12,000 x *g* for 15 min at 4ºC. After centrifugation, heat the centrifuge to room temperature (15–25ºC) if the same centrifuge will be used in the later steps of this procedure.**

   After centrifugation, the sample separates into 3 phases: an upper, colorless, aqueous phase containing RNA; a white interphase (MaXtract High Density gel); and a lower, red, organic phase. For tissues with an especially high fat content, an additional, clear phase may be visible below the red, organic phase. The volume of the aqueous phase should be approximately 600 µl.

9. **Transfer the upper, aqueous phase to a new tube (not supplied). Add 1 volume (usually 600 µl) of 70% ethanol, and mix thoroughly by vortexing. Do not centrifuge. Proceed immediately to step 10.**

   Note: The volume of lysate may be less than 600 µl due to loss during homogenization and centrifugation.

   Precipitates may be visible after addition of ethanol. Resuspend precipitates completely by vigorous shaking, and proceed immediately to step 10.

10. **Transfer up to 700 µl of the sample to an RNeasy Mini spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) at room temperature (15–25ºC). Discard the flow-through.***

    Reuse the collection tube in step 11.

11. **Repeat step 10 using the remainder of the sample. Discard the flow-through.***

    Reuse the collection tube in step 12.

    Optional: If performing optional on-column DNase digestion (see "Important points before starting"), follow steps C1–C4 (page 32) in the *RNeasy Lipid Tissue Handbook* after performing this step.

12. **Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the membrane. Discard the flow-through.***

    Reuse the collection tube in step 13.

    After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.*

    Note: Skip this step if performing optional on-column DNase digestion.

* Flow-through contains QIAzol Lysis Reagent or Buffer RW1 and is therefore not compatible with bleach. See page 6 in the *RNeasy Lipid Tissue Handbook* for safety information.

13. **Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the membrane. Discard the flow-through.**

    Reuse the collection tube in step 14.

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

14. **Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to wash the membrane.**

    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.

    Note: 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.

15. **Recommended: Place the RNeasy spin column in a new 2 ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.**

    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 after step 14.

16. **Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently. To elute the RNA, centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm).**

17. **Repeat step 16 using another volume of RNase-free water, or using the eluate from step 16 (if high RNA concentration is required). Reuse the collection tube from step 16.**

    If using the eluate from step 16, 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.

RNeasy Lipid Tissue Kits, RNA*later* RNA Stabilization Reagent, and QuantiTect Kits are intended for research use. No claim or representation is intended for their use to provide information for the diagnosis, prevention, or treatment of a disease.

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QIAzol Lysis Reagent is a subject of US Patent No. 5,346,994 and foreign equivalents.

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