

Purification of Total RNA From Tissues Using the RNeasy® Plus 96 Kit And Vacuum/Spin Technology

This supplementary protocol is intended for users of the RNeasy Plus 96 Kit who would like to purify total RNA from animal and human tissues using vacuum/spin technology. The protocol is intended for easy-to-lyse tissues, such as liver, kidney, and lung. Up to 5 mg of fresh or frozen tissue can be processed. If the tissue is stabilized in Allprotect Tissue Reagent or RNAprotect® Tissue Reagent, 2–3 mg of tissue should be processed, as the tissue is partially dehydrated.

IMPORTANT: Please consult the “Safety Information” and “Important Notes” sections in the *RNeasy Plus 96 Handbook* before beginning this procedure. For safety information on the additional chemicals mentioned in this protocol, please consult the appropriate safety data sheets (SDSs), available from the product supplier. The kit is intended for research use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

Equipment and reagents

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

- RNeasy Plus 96 Kit (cat. no. 74192)
- Multichannel pipet with tips. For efficient liquid handling, we recommend using an electric multichannel pipet with a minimum capacity of 650 µl per pipet tip. Matrix Technologies Corporation (www.matrixtechcorp.com) provides cordless electronic multichannel pipets with a unique expandable tip-spacing system, allowing transfer of liquid between different types of multiwell plate. (This is not a complete list of suppliers and does not include many important vendors of biological supplies.)
- Reagent reservoirs for multichannel pipets
- Centrifuge 4-15C or 4K15C (for ordering information, please inquire)
- Plate Rotor 2 x 96 (cat. no. 81031)
- 96–100% ethanol and 70% ethanol in water (do not use denatured alcohol, which contains other substances such as methanol or methylethylketone)
- 14.3 M β-mercaptoethanol (β-ME) (commercially available solutions are usually 14.3 M) or, alternatively, 2M dithiothreitol (DTT) in water

- **Optional:** Tape Pads (cat. no. 19570) (for sealing unused wells)
- **Optional:** S-Blocks (cat. no. 19585) (if performing several 96-well preps per day, it may be convenient to have additional S-Blocks)
- **Optional:** Reagent DX (cat. no. 19088) (to reduce foaming of Buffer RLT Plus)
For stabilization of RNA in tissues: RNAprotect Tissue Reagent (cat. no. 76104 [50 ml] or cat. no. 76106 [250 ml]), Allprotect Tissue Reagent (cat. no. 76405 [100 ml]), or liquid nitrogen
- For disruption and homogenization of tissues (low-throughput): TissueRuptor® (for ordering information, please inquire) and TissueRuptor Disposable Probes (cat. no. 990890)
- For disruption and homogenization of tissues (medium- to high-throughput): TissueLyser or TissueLyser II (cat. no. 85300), TissueLyser Adapter Set 2 x 96 (cat. no. 69984), Stainless Steel Beads, 5 mm (cat. no. 69989), Collection Microtubes (cat. no. 19560), and Collection Microtube Caps (cat. no. 19566)
- QIAvac 96 vacuum manifold (cat. no. 19504)
- QIAGEN Vacuum Pump (for ordering information, please inquire) or other vacuum pump capable of generating a vacuum pressure of –800 to –900 mbar
- QIAGEN Vacuum Regulator (cat. no. 19530) or pressure gauge

Important points before starting

- If using the RNeasy Plus 96 Kit for the first time, read “Important Notes” in the *RNeasy Plus 96 Handbook*.
 - If preparing RNA for the first time, read Appendix A in the *RNeasy Plus 96 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*.
 - All vacuum steps are performed on the QIAvac 96 vacuum manifold. If using the manifold for the first time, read “QIAvac 96 vacuum manifold” in the *RNeasy Plus 96 Handbook*.
 - All centrifugation steps are performed in the Centrifuge 4-15C or Centrifuge 4K15C with the Plate Rotor 2 x 96. If using the centrifuge for the first time, read “Centrifuge 4-15C and Centrifuge 4K15C” in the *RNeasy Plus 96 Handbook*.
- Use of a multichannel pipet is recommended. Pour buffers and RNase-free water into reagent reservoirs for multichannel pipets. Use reservoirs from a freshly opened package or clean them as described for S-Blocks (see the *RNeasy Plus 96 Handbook*).
- For optimal results, stabilize harvested tissues immediately in RNAprotect Tissue Reagent (see the *RNAprotect Handbook*) or Allprotect Tissue Reagent (see the *Allprotect Tissue Reagent Handbook*). Tissues can be stored in the reagent at 37°C for up to 1 day, at 15–25°C for up to 7 days, or at 2–8°C for up to 4 weeks (RNAprotect) or 6 months (Allprotect). Alternatively, tissues can be archived at –30 to –15°C or –90 to –65°C.

- Fresh, frozen, or RNAprotect/Allprotect stabilized tissues can be used. Tissues can be stored at -90 to -65°C for several months. Flash-frozen tissues in liquid nitrogen, and immediately transfer to -90 to -65°C . Do not allow tissues to thaw during weighing or handling prior to disruption in Buffer RLT Plus. Homogenized tissue lysates from step 2 can also be stored at -90 to -65°C for several months. Incubate frozen lysates at 37°C in a water bath until completely thawed and salts are dissolved before continuing with step 3. Avoid prolonged incubation, which may compromise RNA integrity.
- Buffer RLT Plus and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See the *RNeasy Plus 96 Handbook* for safety information.
- Perform all steps of the procedure at room temperature (15 – 25°C). During the procedure, work quickly.
- Perform all centrifugation steps at 20 – 25°C . Ensure that the centrifuge does not cool below 20°C .

Disruption and homogenization in Buffer RLT Plus

The RNeasy Plus 96 Kit is supplied with Buffer RLT Plus, a lysis buffer that provides optimal sample lysis as well as appropriate conditions for DNA binding to gDNA Eliminator plates. When disrupting and homogenizing tissues in Buffer RLT Plus, excessive foaming may occur. This foaming is substantially reduced by adding Reagent DX to Buffer RLT Plus at a final concentration of 0.5% (v/v) before starting disruption and homogenization. Reagent DX has been carefully tested with RNeasy Plus Kits, and has no effect on RNA purity or on downstream applications such as real-time RT-PCR. Buffer RLT Plus containing Reagent DX can be stored at room temperature (15 – 25°C) for at least 9 months. Reagent DX is supplied separately (cat. no. 19088).

Things to do before starting

- β -mercaptoethanol (β -ME) must be added to Buffer RLT Plus before use. Add $10\ \mu\text{l}$ β -ME per 1 ml Buffer RLT Plus. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT Plus containing β -ME can be stored at room temperature (15 – 25°C) for up to 1 month. Alternatively, add $20\ \mu\text{l}$ of 2M dithiothreitol (DTT) per 1 ml Buffer RLT Plus. The stock solution of 2M DTT in water should be prepared fresh, or frozen in single-use aliquots. Buffer RLT Plus containing DTT can be stored at room temperature for up to 1 month.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add the appropriate volume of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Buffer RLT Plus may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.

Procedure

1. Excise the tissue sample from the animal or remove it from storage. Determine the amount of tissue. Do not use more than 5 mg. Proceed immediately to step 2.

Weighing tissue is the most accurate way to determine the amount. If necessary, cut the tissue on a clean surface and weigh the piece to be used.

For RNAprotect or Allprotect stabilized tissues: Remove the tissue from the RNAprotect Tissue Reagent using forceps and be sure to remove any crystals that may have formed. RNA in RNAprotect or Allprotect stabilized tissues is protected during cutting and weighing of tissues at ambient temperature (15–25°C). It is not necessary to cut the tissues on ice or dry ice or in a refrigerated room. Remaining tissues can be stored in RNAprotect or Allprotect Reagent. Previously stabilized tissues can be stored at –90 to –65°C without the reagent.

For unstabilized fresh or frozen tissues: RNA in harvested tissues is not protected until the tissues are treated with RNAprotect or Allprotect Reagent, flash-frozen, or disrupted and homogenized in step 2. Frozen tissues should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible. Remaining fresh tissues can be placed into RNAprotect Reagent to stabilize RNA or in Allprotect Tissue Reagent to stabilize DNA, RNA, and protein. However, previously frozen tissues thaw too slowly in the reagent, preventing the reagent from diffusing into the tissues quickly enough to prevent RNA degradation.

2. Disrupt the tissue and homogenize the lysate in Buffer RLT Plus (do not use more than 5 mg tissue) according to step 2a (TissueRuptor) or step 2b (TissueLyser). If preferred, tissues can be disrupted under cryogenic conditions: in this case, follow the guidelines in the Appendix: Cryogenic disruption of tissues (page 7).

Note: Ensure that β -ME (or DTT) is added to Buffer RLT Plus before use (see “Things to do before starting”).

After storage in RNAprotect or Allprotect Reagent, tissues may become slightly harder than fresh or thawed tissues. Disruption and homogenization using standard methods is usually not a problem.

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

- 2a. Disruption and homogenization using the TissueRuptor:

- Place the tissue in a suitably sized vessel. Add 350 μ l Buffer RLT Plus.

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 homogeneous (usually 30 s). Proceed to step 3.

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. If this happens, let the homogenate stand at room temperature for 2–3 min until the foam subsides before continuing with the procedure.

2b. Disruption and homogenization using the TissueLyser:

- Place the tissues in collection microtubes (cat. no. 19560) containing 1 stainless steel bead (5 mm mean diameter).

If handling fresh or frozen tissue samples, keep the tubes on dry ice.

- Place the tubes at room temperature. Immediately add 350 μ l Buffer RLT Plus per tube.
- Attach collection microtube caps (cat. no. 19566), and place the tubes in the TissueLyser Adapter Set 2 x 96.
- Operate the TissueLyser for 2 min at 25 Hz.

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

- Disassemble the adapter set, rotate the rack of tubes so that the tubes nearest to the TissueLyser are now outermost, and reassemble the adapter set. Operate the TissueLyser for another 2 min at 25 Hz.

Rearranging the tubes allows even homogenization.

- Proceed to step 3.

Do not reuse the stainless steel beads.

3. Place a gDNA Eliminator 96 plate on top of a new S-Block. Mark the plate for later identification.

4. Centrifuge the lysates from step 2 at 6000 rpm (~5600 x g) for 4 min at 20–25°C. Carefully remove the supernatants by pipetting, and transfer them to the wells of the gDNA Eliminator 96 plate.

Note: Take care not to wet the rims of the wells, as this could lead to cross-contamination.

5. Seal the gDNA Eliminator 96 plate with an AirPore tape sheet. Place the S-Block and gDNA Eliminator 96 plate into the metal holder, and place the whole assembly into the rotor bucket. Centrifuge at 6000 rpm (~5600 x g) for 4 min at 20–25°C. Discard the gDNA Eliminator 96 plate, and save the flow-through.

Centrifugation with sealed plates prevents cross-contamination.

Note: Make sure that no liquid remains on the membranes after centrifugation. If necessary, repeat the centrifugation until all liquid has passed through the membranes.

6. Assemble the QIAvac 96 vacuum manifold: first place the waste tray inside the QIAvac base, then place the QIAvac 96 top plate squarely over the QIAvac base. Place an RNeasy 96 plate in the QIAvac 96 top plate, making sure that the plate is seated tightly. Attach the vacuum manifold to a vacuum source. Keep the vacuum switched off.

Note: Always place the RNeasy 96 plate into the vacuum manifold with the beveled edges pointing to your right-hand side.

7. Add 1 volume (350 μ l) of 70% ethanol to each well of the S-Block containing the flow-through from step 5. Mix well by pipetting up and down 3 times.

8. Transfer the samples (700 μ l) to the wells of the RNeasy 96 plate, and switch on the vacuum. Apply the vacuum until the samples have completely passed through the membranes (15–60 s). Switch off the vacuum, and ventilate the manifold.

Make sure the QIAvac 96 vacuum manifold is assembled correctly before loading the samples. The flow-through is collected in the waste tray.

Note: Take care not to wet the rims of the wells, as this could lead to cross-contamination.

Note: Tape unused wells with adhesive tape or Tape Pads (cat. no. 19570). Do not use the AirPore tape sheets supplied with the RNeasy Plus 96 Kit.

Note: The vacuum must be switched off and the manifold ventilated between pipetting steps to maintain uniform conditions for each sample.

9. Add 800 μ l Buffer RW1 to each well of the RNeasy 96 plate, and switch on the vacuum. Apply the vacuum until the buffer has completely passed through the membranes (10–30 s). Switch off the vacuum, and ventilate the manifold.

The flow-through is collected in the same waste tray from step 8.

10. Lift the QIAvac 96 top plate carrying the RNeasy 96 plate from the QIAvac base, and empty the waste tray. * Reassemble the QIAvac 96 vacuum manifold.

11. Add 800 μ l Buffer RPE to each well of the RNeasy 96 plate, and switch on the vacuum. Apply the vacuum until the buffer has completely passed through the membranes (10–30 s). Switch off the vacuum, and ventilate the manifold.

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

12. Place the RNeasy 96 plate on top of an S-Block (either new or reused). Mark the plate for later identification.

If reusing an S-Block, make sure it is cleaned as described in the *RNeasy Plus 96 Handbook*.

13. Add 800 μ l Buffer RPE to each well of the RNeasy 96 plate, and seal the plate with an AirPore tape sheet. Place the S-Block and RNeasy 96 plate into the metal holder, and place

* The waste liquid contains Buffer RLT Plus and Buffer RW1 and is therefore not compatible with bleach. See the RNeasy Plus 96 Handbook for safety information.

the whole assembly into the rotor bucket. Centrifuge at 6000 rpm (~5600 x *g*) for 10 min at 20–25°C to dry the membranes.

Centrifugation with sealed plates prevents cross-contamination.

It is important to dry the RNeasy membranes, since residual ethanol may interfere with downstream reactions. The 10 min centrifugation ensures that residual traces of salt are removed such that no ethanol is carried over during RNA elution.

14. Remove the AirPore tape sheet. Place the RNeasy 96 plate on top of a rack of Elution Microtubes CL. Add 45–70 µl RNase-free water to each well, and seal the plate with a new AirPore tape sheet. Incubate for 1 min at room temperature (15–25°C). Then centrifuge at 6000 rpm (~5600 x *g*) for 4 min at 20–25°C to elute the RNA.

Note: Be sure to pipet the RNase-free water directly onto the RNeasy membranes. Elution will be incomplete if some of the water sticks to the walls or the O-rings of the RNeasy 96 plate.

15. Remove the AirPore tape sheet. Repeat step 14 with a second volume of 45–70 µl RNase-free water.

Note: Repeating step 14 is required for complete recovery of RNA. The eluate volume will be approximately 15 µl less than the volume of RNase-free water added to the membrane (the 15 µl corresponds to the membrane dead volume).

Use the caps provided with the kit to seal the microtubes for storage. Store RNA at –30 to –15°C or –90 to –65°C.

Appendix: Cryogenic disruption of tissues

The above procedure provides instructions for disrupting tissues in Buffer RLT Plus. If preferred, tissues can be disrupted under cryogenic conditions without Buffer RLT Plus. After disruption, the samples are mixed with Buffer RLT Plus, and then homogenized. This appendix provides guidelines on cryogenic tissue disruption and subsequent homogenization. There are 2 alternative methods of disruption and 2 alternative methods of homogenization.

Disruption using the TissueLyser

- Precool the TissueLyser Adapter Set 2 x 96 at –90 to –65°C for at least 2 h.
- Place the collection microtubes (cat. no. 19560) on dry ice, and add 1 stainless steel bead (5 mm diameter) to each tube.
- Place the frozen tissues in the collection microtubes, attach the collection microtube caps (cat. no. 19566), and place the tubes in the TissueLyser Adapter Set 2 x 96.
- Operate the TissueLyser for 1 min at 25 Hz. Disassemble the adapter set, rotate the rack of tubes so that the tubes nearest to the TissueLyser are now outermost, and reassemble the adapter set. Operate the TissueLyser for another 1 min at 25 Hz.
- Immediately add 350 µl Buffer RLT Plus to each tube.

- Proceed with one of the 2 homogenization methods described in this appendix.
Important: Do not freeze the TissueLyser Adapter Set 2 x 96 and the collection microtubes in liquid nitrogen, as this may result in breakage of the tubes.

Disruption using a mortar and pestle

- Place the tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle.
- Decant the tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen-cooled, 2 ml microcentrifuge tube. Allow the liquid nitrogen to evaporate, but do not allow the tissue powder to thaw.
- Immediately add 350 µl Buffer RLT Plus to the tissue powder.
- Proceed with one of the 2 homogenization methods described in this appendix.

Homogenization using the TissueLyser

- If necessary, transfer the lysates to collection microtubes (cat. no. 19560), each containing 1 stainless steel bead (5 mm diameter).
- Attach collection microtube caps (cat. no. 19566) to the collection microtubes, and place the tubes in the TissueLyser Adapter Set 2 x 96.
- Operate the TissueLyser for 1 min at 20 Hz. Disassemble the adapter set, rotate the rack of tubes so that the tubes nearest to the TissueLyser are now outermost, and reassemble the adapter set. Operate the TissueLyser for another 1 min at 20 Hz.
- Proceed to step 3 of the above procedure.

Homogenization using a needle and syringe

- Pass the lysate at least 5 times through a blunt 20-gauge needle fitted to an RNase-free syringe.
- Proceed to step 3 of the above procedure.

Document Revision History

Date	Changes
11/2019	Change in product names; clarification on temperatures for tissue storage; update of safety information from "MSDS" to "SDS",

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