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Supplementary Protocol

RNeasy® Midi Protocol for Isolation of Total Cellular RNA from Whole Blood

This protocol is designed for isolation of total cellular RNA from 1.5–10 ml of human whole blood. For smaller amounts of blood (up to 1.5 ml), the QIAamp® RNA Blood Mini Kit already comes complete with spin columns, RNase-free reagents, and detailed protocols. See the *RNeasy Midi/Maxi Handbook* for ordering information. Frozen whole blood cannot be used in the protocol.

Equipment and reagents to be supplied by user

- 14.3 M β-mercaptoethanol (β-ME)* (stock solutions are usually 14.3 M)
- Sterile, RNase-free pipette tips
- Laboratory centrifuge (capable of 3000–5000 x *g*)†
- Equipment for disruption and homogenization (see the *RNeasy Midi/Maxi Handbook*)
- Vessels for homogenization (e.g., 10–15 ml centrifuge tubes for the RNeasy Midi Kit; 50 ml centrifuge tubes for the RNeasy Maxi Kit)
- Ethanol (96–100%)
- Disposable gloves
- Buffer EL (cat. no. 79217)
- Ethanol (70% in water)

Important points before starting

- Blood and body fluids of all human subjects are considered potentially infectious. All necessary precautions recommended by the Food and Drug Administration (in the United States), the Bundesseuchengesetz (in Germany), or the appropriate regulatory authorities in the country of use should be considered when working with whole blood.

* β-ME must be added to Buffer RLT before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10 µl of 14.3 M β-ME per 1 ml of Buffer RLT. The solution is stable for 1 month after the addition of β-ME.

† All centrifugation steps are carried out in a conventional laboratory centrifuge, e.g., QIAGEN Centrifuge 4-15C, Centrifuge 4K15C, Beckman® CS-6KR, or equivalent, with a swinging bucket rotor for 15 ml (Midi) or 50 ml (Maxi) centrifuge tubes (the maximum speed of 3500–5000 rpm corresponds to 3000–5000 x *g* for most rotors). RNeasy Midi columns supplied with the kit fit into 15 ml centrifuge tubes. RNeasy Maxi columns supplied with the kit fit into 50 ml centrifuge tubes. These fit into the rotor of almost every standard laboratory centrifuge available. In the unlikely event that the tubes do not fit, the RNeasy columns can also be inserted into different 12–15 ml (Midi) or 50 ml (Maxi) RNase-free glass or polypropylene tubes. All centrifugation steps are carried out at 20–25°C.

- If using RNeasy Midi Kits for the first time, read "Important Points Before Using RNeasy Kits" in the *RNeasy Midi/Maxi Handbook*.
- If working with RNA for the first time, read Appendix A of the *RNeasy Midi/Maxi Handbook*.
- The maximum amount of whole blood that can be processed (10 ml) has been determined for blood from healthy adults (approximately 4000–7000 leukocytes per µl). Reduce the amount appropriately if using blood with elevated numbers of leukocytes. A maximum of 1×10^8 leukocytes can be processed on the RNeasy Midi column.
- **β-ME must be added to Buffer RLT before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10 µl β-ME per 1 ml Buffer RLT. Buffer RLT is stable for 1 month after addition of β-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.**
- Generally, DNase digestion is not required, because the RNeasy silica-membrane technology efficiently removes 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® RT-PCR analysis with a low-abundant target). In these cases, the small residual amounts of DNA remaining can be removed using the RNase-Free DNase Set (cat. no. 79254) for the optional on-column DNase digestion (see the *RNeasy Midi/Maxi Handbook*) or by a DNase digestion after RNA isolation. For on-column DNase digestion with the RNase-Free DNase Set, prepare the DNase I stock solution as described on in the *RNeasy Midi/Maxi Handbook* before beginning the procedure.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature (18-25°C).
- Buffer RLT and Buffer RW1 contain guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. Guanidine is an irritant. Take appropriate safety measures and wear gloves when handling.
- Erythrocyte lysis is performed on ice. Unless otherwise indicated, all subsequent steps of the RNeasy protocol should be performed at room temperature. During the procedure, work quickly.
- Centrifugation in steps 3 and 5 of this protocol is performed at 4°C in a standard laboratory centrifuge with a swinging bucket rotor capable of ≥3000 x *g* (see "Equipment and reagents to be supplied by user"). All remaining centrifugation steps are performed at 20–25°C. Ensure that the centrifuge does not cool below 20°C.
- Frozen whole blood cannot be used in the protocol.

Procedure

1. Mix 1 volume of whole blood with 5 volumes of Buffer EL in an appropriately sized tube. (Do not use more than 10 ml of whole blood.)

   For example, add 25 ml of Buffer EL to 5 ml of whole blood in a 50 ml centrifuge tube.

   Although it is preferable to use 5 volumes of Buffer EL per 1 volume of blood, the amount of Buffer EL may be reduced to 4 volumes if necessary in order to process 10 ml of whole blood in a single 50 ml centrifuge tube. However, in this case, the incubation time (step 2) may need to be increased.

2. Incubate for 10–15 min on ice. Mix by vortexing briefly twice during the incubation.

   The cloudy suspension becomes translucent during incubation, indicating lysis of red blood cells.

3. Centrifuge at 400 x *g* for 10 min at 4°C. Completely remove and discard the supernatant. Save the leukocyte pellet.

   The leukocytes will form a pellet after centrifugation. Ensure complete removal of the supernatant. Trace amounts of red blood cells that might remain are eliminated in the following steps.

4. Add Buffer EL to the leukocyte pellet. Use 2 volumes of Buffer EL per volume of whole blood used in step 1. Resuspend cells by vortexing briefly.

   For example, add 10 ml of Buffer EL if 5 ml of whole blood was used in step 1.

5. Centrifuge at 400 x *g* for 10 min at 4°C. Completely remove and discard the supernatant. Save the leukocyte pellet. After centrifuging, heat the centrifuge to 20–25°C if the same centrifuge is to be used in the following centrifugation steps.

   Note: Incomplete removal of the supernatant will inhibit lysis and dilute the lysate, affecting the conditions for binding of RNA to the RNeasy membrane. Both effects may reduce RNA yield.

6. Loosen the leukocyte pellet by flicking the tube. Add the appropriate volume of Buffer RLT (see Table 1). Vortex or pipet to mix.

   If not using healthy blood, refer to the number of leukocytes to determine volume of Buffer RLT required. Buffer RLT disrupts the cells. Before proceeding to the homogenization step, ensure that no cell clumps are visible. Vortex or pipet further to remove any clumps.

   Note: Ensure that β-ME is added to Buffer RLT before use (see "Important notes before starting").

Table 1. Buffer RLT volumes for RNeasy Midi isolation of total cellular RNA from whole blood

Number of leukocytes	Equivalent amount of healthy whole blood (ml)	Buffer RLT (ml)
≤3 x 10^7	≤4.0	2.0
3 x 10^7–1 x 10^8	4.0–10.0	4.0

7. Homogenize the leukocytes using a conventional rotor–stator homogenizer for at least 45 s at maximum speed until the sample is uniformly homogeneous. Alternatively, vortex the sample for 10 s, and pass the lysate at least 5–10 times through an 18- to 20-gauge needle fitted to an RNase-free syringe.

   Note: Incomplete homogenization will lead to significantly reduced yields and can cause clogging of the RNeasy column. Homogenization with rotor–stator homogenizers generally results in higher total RNA yields than with other homogenization methods.

8. Add 1 volume (2.0 ml or 4.0 ml) of 70% ethanol to the homogenized lysate, and mix thoroughly by shaking vigorously. Do not centrifuge.

   If some lysate is lost during homogenization, adjust the volume of ethanol accordingly.

   Note: Visible precipitates may form after the addition of ethanol. Resuspend precipitates completely by vigorous shaking, and proceed immediately to step 9. Insufficient resuspension of precipitates will cause DNA contamination and can lead to impure total RNA.

9. Apply the sample, including any precipitate that may have formed, to an RNeasy Midi column placed in a 15 ml centrifuge tube (supplied). Maximum loading volume is 4.0 ml. Close the tube gently, and centrifuge for 5 min at 3000–5000 x *g*. Discard the flow-through.*

   Reuse the centrifuge tube in step 10.

   If the maximum amount of starting material is used, it may be necessary to increase centrifugation time to 10 min to allow the lysate to completely pass through the column.

   If the volume exceeds 4.0 ml, load aliquots successively onto the RNeasy column and centrifuge as above. Discard the flow-through after each centrifugation step.*

   Optional: QIAGEN offers the RNase-Free DNase Set for convenient on-column DNase digestion during RNA purification. Generally, DNase digestion is not required, because the RNeasy silica-membrane technology efficiently removes 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 RT-PCR analysis with a low-abundant target). If using the RNase-Free DNase Set, follow the steps shown in the *RNeasy Midi/Maxi Handbook* after performing this step.

10. Add 4.0 ml Buffer RW1 to the RNeasy column. Close the centrifuge tube gently, and centrifuge for 5 min at 3000–5000 x *g* to wash the column. Discard the flow-through.*

    Skip this step if performing the optional on-column DNase digestion in the *RNeasy Midi/Maxi Handbook*). Reuse the centrifuge tube in step 11.

11. Add 2.5 ml Buffer RPE to the RNeasy column. Close the centrifuge tube gently, and centrifuge for 2 min at 3000–5000 x *g* to wash the column.

    Reuse the centrifuge tube in step 12. The flow-through does not need to be discarded.

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

* Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach.

12. Add another 2.5 ml Buffer RPE to the RNeasy column. Close the centrifuge tube gently, and centrifuge for 5 min at 3000–5000 x *g* to dry the RNeasy silica-gel membrane.

    It is important to dry the RNeasy membrane, because residual ethanol may interfere with downstream reactions. This centrifugation ensures that no ethanol is carried over during elution.

    Note: After centrifugation, remove the RNeasy column from the centrifuge tube carefully so that the column does not come in contact with the flow-through, because this will result in carryover of ethanol.

13. To elute, transfer the RNeasy column to a new 15 ml collection tube (supplied). Pipet the appropriate volume of RNase-free water (see Table 2) directly onto the RNeasy silica-gel membrane. Close the tube gently. Let it stand for 1 min, and then centrifuge for 3 min at 3000–5000 x *g*.

Table 2. RNase-free water volumes for RNeasy Midi elution

RNeasy column	Expected total RNA yield	RNase-free water
Midi	≤150 µg	150 µl
Midi	150 µg – 1 mg	250 µl

14. Repeat the elution step (step 13) with a second volume of RNase-free water.

    To obtain a higher total RNA concentration, this second elution step may be performed by using the first eluate (from step 13). The yield will be 15–30% less than the yield obtained using a second volume of RNase-free water, but the final concentration will be higher.

Document Revision History

Date	Changes
08/2019	Initial release

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