QIAGEN Supplementary Protocol

Isolation of Peripheral Blood Mononuclear Cells (PBMC) and Purification of Total RNA from PBMC Using the RNeasy® Micro or Mini Kit

This protocol describes how to isolate PBMC from human whole blood. Once purified, the protocol describes how to purify RNA from PBMC using the RNeasy Micro Kit or the RNeasy Mini Kit. The purification procedure requires use of either the RNeasy Micro Kit or the RNeasy Mini Kit.

IMPORTANT: Please read the handbook supplied with the RNeasy Micro Kit or the RNeasy Mini Kit, paying careful attention to the "Safety Information" and "Important Notes" sections, before beginning this procedure. Both kits are intended for molecular biology applications. These products are not intended 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 Micro Kit (cat. no. 74004) or RNeasy Mini Kit (cat. no. 74104)
- QIAshredder (cat. no. 79654)
- RNase-free DNase Set (cat. no. 79254) This is included in the RNeasy Micro Kit, but must be ordered separately when using the RNeasy Mini Kit
- Ficoll-Paque® PLUS density-gradient medium (GE Healthcare, cat. no. 17-1440-02)
- 96–100% and 70% ethanol (for use with both kits) and 80% ethanol (required only if using the RNeasy Micro Kit). Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone
- S-Monovette® Lithium-Heparin (7.5ml) (Sarstedt, cat. no. 01.1608)
- Gibco® RPMI 1640 Medium (Life Technologies, cat. no. 22409-015)
- Balanced Salt Medium (see "Things to do before starting")
- β-mercaptoethanol (β-ME; commercially available solutions are usually 14.3 M) or 2 M dithiothreitol (DTT)
- Trypan blue
- Sterile, RNase-free pipet tips

- Disposable gloves
- Microcentrifuge (with rotor for 2 ml tubes)
- 50 ml centrifuge tubes
- Centrifuge with a swinging bucket rotor for 50 ml centrifuge tubes
- Liquid nitrogen

Important points before starting

- If working with RNA for the first time, read Appendix A in the *RNeasy Mini Handbook* or *RNeasy Micro Handbook*.
- If using the RNeasy Mini or Micro Kit for the first time, read the "Important Notes" section of the relevant kit handbook.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature (15–25°C).
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. See "Safety Information" in the *RNeasy Micro Handbook* or *RNeasy Mini Handbook*.

Things to do before starting

- Prepare the Balanced Salt Medium. First, prepare stock solutions A and B (Tables 1 and 2). Then, mix 1 volume of Solution A with 9 volumes Solution B. Balanced Salt Medium should be freshly prepared each week.

Table1. Balanced Salt Medium: Stock solution A

Component	Concentration (g/liter)
Anhydrous D-glucose	1.0
$CaCl_2 \times 2H_2O$	0.0074
MgCl2 × 6H2O	0.1992
KCl	0.4026
Tris	17.565

Dissolve all components in approximately 950 ml distilled water. Add 10 M HCl until pH is 7.6 before adjusting the volume to 1 liter.

Table2. Balanced Salt Medium: Stock solution B

Component	Concentration (g/liter)
NaCl	8.19

- For isolation of RNA from PBMC, add β-mercaptoethanol (β-ME) to Buffer RLT before use. Add 10 µl β-ME per 1 ml Buffer RLT. Dispense in a fume hood and wear appropriate protective clothing. Buffer RLT containing β-ME can be stored at room temperature (15–25°C) for up to 1 month. Alternatively, add 20 µl of 2 M dithiothreitol (DTT) per 1 ml Buffer RLT. The stock solution of 2 M DTT in water should be prepared fresh, or frozen in single-use aliquots. Buffer RLT 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 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- If performing the optional on-column DNase digestion, prepare DNase I stock solution as described below.
- Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the lyophilized DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. 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.
- For long-term storage of DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.

Procedure

Isolation of PBMC

The blood fraction that contains PBMC also includes all blood cells with a round nucleus (e.g., lymphocytes, monocytes, and macrophages). One method of obtaining PBMC is density-gradient centrifugation. Here, the use of a commercially available centrifugation medium (Ficoll-Paque PLUS) for isolation of PBMC is described.

1. **Collect 7.5 ml of blood into lithium-heparin tubes.**
2. **Mix 6 ml Li-heparin–treated blood and 6 ml Balanced Salt Medium in a 50 ml centrifuge tube and invert gently 8–10 times.**
3. **Add 9 ml Ficoll-Paque PLUS to a 50 ml centrifuge tube. Then, layer the diluted blood sample over the Ficoll-Paque PLUS.**
4. **Centrifuge the tubes in a swing-out rotor for 30–40 min at 400 x *g* at room temperature (18–25°C).**

   Note: Make sure the brake is off for this centrifugation step.

5. **Using a pipet, carefully remove and discard the clear, top plasma-containing layer. Take care not to disturb the white interface layer, which contains the PBMC.**
6. **Using a clean pipet, carefully transfer the white interface layer to a new 50 ml centrifuge tube.**
7. **Add at least 3 volumes Balanced Salt Medium and suspend the PBMC by gently inverting the tube 4–5 times.**
8. **Centrifuge the tubes in a swing-out rotor for 10–15 min at 350 x *g* at room temperature (18–25°C). Carefully remove and discard the supernatant.**
9. **To wash the cells, add 10 ml Balanced Salt Medium to each tube and invert 5 times.**
10. **Centrifuge tubes in a swing-out rotor for 10–15 min at 350 x *g* at room temperature (18–25°C). Carefully remove and discard the supernatant. Repeat this step.**
11. **Resuspend cells in RPMI 1640 containing 10% fetal bovine serum. Adjust the PBMC concentration to 1 x 10^5 – 1 x 10^6 or >1 x 10^6 – 1 x 10^7 viable cells/ml with standard trypan blue exclusion.**
12. **Centrifuge PBMC for a further 10–15 min at 350 x *g* and remove and discard the supernatant. The PBMC pellet can either be snap frozen in liquid nitrogen and stored at –80°C for later RNA purification or used directly in an RNA purification procedure using the RNeasy Micro Kit (<1 x 10^6 cells) or RNeasy Mini Kit (1 x 10^6 – 1 x 10^7 cells).**

Total RNA purification

Differences in the protocols are denoted by the terms "(Micro)" for the RNeasy Micro Kit and "(Mini)" for the RNeasy Mini Kit.

1. **Disrupt the cells by adding Buffer RLT (supplemented with β-ME or DTT).**

   For pelleted cells, loosen the cell pellet thoroughly by flicking the tube. Add the appropriate volume of Buffer RLT (see Table 3) and vortex or pipet to mix.

   Note: Incomplete loosening of the cell pellet may lead to inefficient lysis and reduced RNA yields.

Table 3. Amount of Buffer RLT required for cell disruption

Number of pelleted cells	Volume of Buffer RLT (µl)	Kit
<1 x 10^5	75	RNeasy Micro Kit
1 x 10^5 – 1 x 10^6	350	RNeasy Micro Kit
1 x 10^6 – 5 x 10^6	350	RNeasy Mini Kit
5 x 10^6 – 1 x 10^7	600	RNeasy Mini Kit

2. **Homogenize the lysate by pipetting the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuge for 2 min at full speed.**

3. **Add 1 volume of 70% ethanol to the homogenized lysate, and mix well by pipetting. Do not centrifuge!**

   Note: The volume of lysate may be less than 350 µl (Micro) or 600 µl (Mini) due to loss during homogenization.

   Note: When purifying RNA from isolated cells, precipitates may be visible after addition of ethanol. This does not affect the procedure.

4. **Transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy MinElute® spin column (Micro) or RNeasy Mini spin column (Mini) placed in a 2 ml collection tube (supplied). 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 6.**

   If the sample volume exceeds 700 µl, centrifuge aliquots in the same RNeasy MinElute spin column (Micro) or RNeasy Mini spin column (Mini). Discard the flow-through after each centrifugation.

   Note: Flow-through contains Buffer RLT and is not compatible with disinfectants containing bleach. See "Safety Information" in the relevant kit handbook.

   If performing the optional on-column DNase digestion, follow steps 5–8. If omitting DNase digestion, continue with step 9.

5. **Add 350 µl Buffer RW1 to the RNeasy MinElute spin column (Micro) or RNeasy Mini spin column (Mini). Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 8.**

6. **Add 10 µl DNase I stock solution (see "Things to do before starting") to 70 µl Buffer RDD. Mix by gently inverting the tube, and centrifuge briefly to collect residual liquid from the sides of the tube. Buffer RDD is supplied with the RNase-Free DNase Set.**

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

7. **Add the DNase I incubation mix (80 µl) directly to the RNeasy MinElute spin column (Micro) or RNeasy Mini spin column (Mini) membrane, and leave at room temperature (20–30°C) for 15 min.**

   Note: Be sure to add the DNase I incubation mix directly to the membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

8. **Add 350 µl Buffer RW1 to the RNeasy MinElute spin column (Micro) or RNeasy Mini spin column (Mini). Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through. Continue with the first Buffer RPE wash step (step 10), reusing the collection tube.**

   Note: Flow-through contains Buffer RLT and is not compatible with disinfectants containing bleach. See "Safety Information" in the relevant kit handbook.

9. **Only if the optional DNase I digest has not been performed: Add 350 µl Buffer RW1 to the RNeasy MinElute spin column (Micro) or 700 µl Buffer RW1 to the RNeasy Mini spin column (Mini). Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.**

   Note: Flow-through contains Buffer RLT and is not compatible with disinfectants containing bleach. See "Safety Information" in the relevant kit handbook.

   Note: After centrifugation, carefully remove the spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely. Reuse the collection tube in step 10.

10. **Add 500 µl Buffer RPE to the RNeasy MinElute spin column (Micro) or RNeasy Mini spin column (Mini). Close the lid gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through. If using the RNeasy Micro Kit, proceed with step 11. If using the RNeasy Mini Kit repeat step 10 and then proceed to step 12. Reuse the collection tube for both kits.**

    Note: Flow-through contains Buffer RLT and is not compatible with disinfectants containing bleach. See "Safety Information" in the relevant kit handbook.

11. **RNeasy Micro Kit only: Add 500 µl 80% ethanol 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. 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.

12. **Optional: Place the RNeasy MinElute spin column (Micro) or RNeasy Mini spin column (Mini) 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 5 min (Micro) or 1 min (Mini).**

    This step eliminates any possible carryover of Buffer RPE or if residual flow-through remains on the outside of the spin columns.

13. **Place the RNeasy MinElute spin column (Micro) or RNeasy Mini spin column (Mini) in a new 1.5 ml collection tube (supplied). Add 14 µl (Micro) or 30–50 µl (Mini) RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute the RNA.**

    Note: As little as 10 µl RNase-free water can be used for elution from the RNeasy MinElute column (Micro) if a higher RNA concentration is required. However, the yield will be reduced by approximately 20%. Do not elute with less than 10 µl RNase-free water, as the spin column membrane will not be sufficiently hydrated.

    Note: If the expected RNA yield is >30 µg from the RNeasy Mini procedure, repeat this step using another 30–50 µl RNase-free water, or using the eluate (if high RNA concentration is required). If using the eluate, 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.

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