Material

The PAXgene Blood RNA Kit (Qiagen, cat. no. 762134) is used for the isolation of cellular RNA from 2.5 ml samples of human whole blood, collected in PAXgene Blood RNA Tubes (cat. no. 762125).

Centrifuge with swing-out rotor, minimum speed 3000g, buckets and adapters for 12 ml centrifuge tubes; or centrifuge with fixed-angle rotor suitable for 12 ml centrifuge tubes.

Set of variable pipettes: 10, 200, 1000 µl; other pipettes in the 2-10 ml range.

Methods

A. Handling of PAXgene RNA Spin Columns

Owing to the sensitivity of nucleic acid amplification technologies, the following precautions are necessary when handling PAXgene RNA spin columns to avoid cross-contamination:

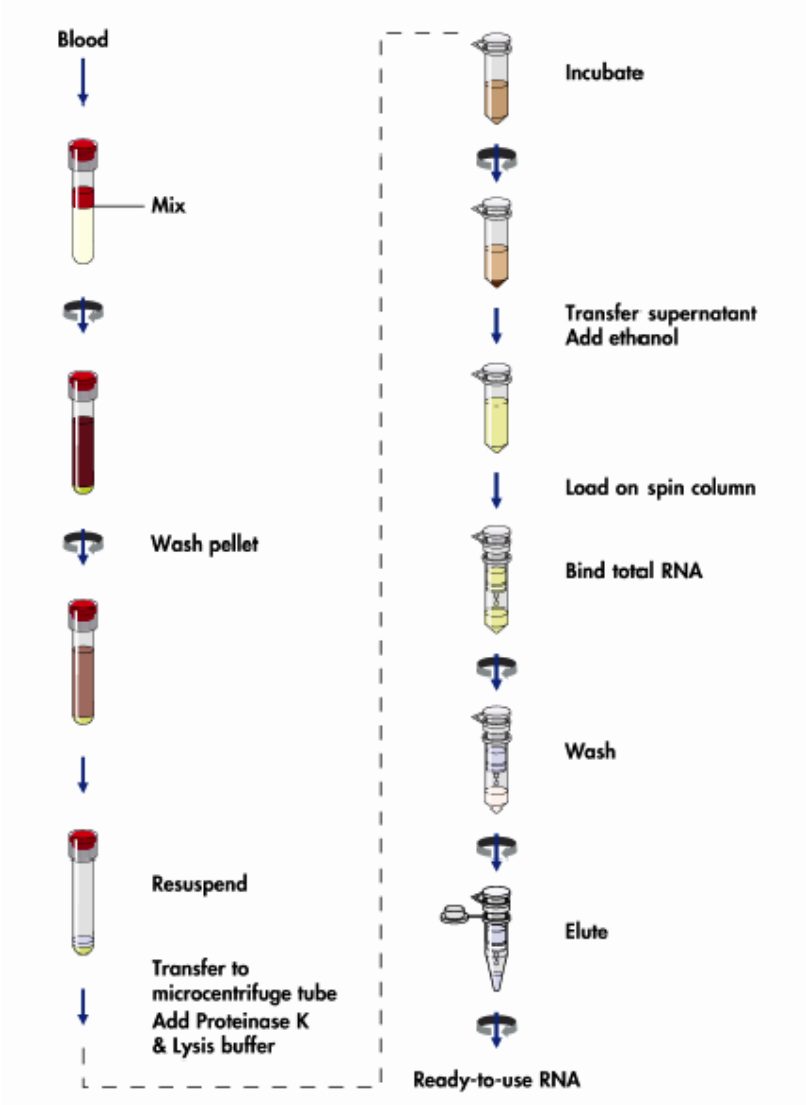
1. Carefully pipet the sample onto the PAXgene column, without moistening the rim of the column.
2. Use of aerosol-barrier pipet tips is recommended.
3. Avoid touching the PAXgene membrane with the pipet tip.
4. After all vortexing steps, to avoid cross-contamination, briefly centrifuging the microcentrifuge tubes is recommended to remove drops from the inside of the lid.
5. Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.
6. Close the PAXgene column before placing it in the microcentrifuge. Centrifuge as described.
7. Note that the flow-through discarded after centrifugation steps may contain hazardous substances and should be disposed appropriately.
8. Open only one PAXgene column at a time, and take care to avoid generating aerosols.
9. For efficient parallel processing of multiple samples, fill a rack with processing tubes to which the PAXgene columns can be transferred after centrifugation. Discard the used processing tubes containing flow-through, and place the new processing tubes containing PAXgene columns directly in the microcentrifuge.

Important notes before starting

1. Buffer BR4 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.
2. After collection of the blood sample, it is important to incubate the PAXgene Blood RNA Tube for at least 2 hours at room temperature, in order to ensure complete lysis. Incubation of the PAXgene Blood RNA Tube overnight may increase yields slightly in some cases. Storage of the PAXgene Blood RNA Tube at room temperature or 4°C for 5 days is possible.

3. The use of a shaker–incubator is strongly recommended for use in step 5. Set the temperature of the shaker–incubator to 55°C. If a shaker–incubator is not available, use a heating block or water bath heated to 55°C.
4. Heat a heating block or water bath to 65°C for use in step 15.
5. All centrifugation steps should be carried out at room temperature (15–25°C).

Simplified flowchart for RNA isolation from blood



Protocol (steps 1-9: ~50 min)

1. Centrifuge the PAXgene Blood RNA Tube for 10 min at 3000–5000 x g using a swing-out rotor (e.g., Beckman Allegra 25; 4000 g)
2. Remove the supernatant by decanting or pipetting. Add 5 ml RNase-free water to the pellet, and close the tube using a fresh secondary Hemogard closure. If the supernatant is decanted, dry the rim of the tube with a clean paper towel.
3. Thoroughly resuspend the pellet by vortexing, and centrifuge as above. Remove and discard the entire supernatant. Small debris remaining in the supernatant after vortexing but before centrifugation will not affect the procedure.

   Note: Incomplete removal of the supernatant will inhibit lysis and dilute the lysate which will affect the conditions for binding RNA to the PAXgene membrane.
4. Thoroughly resuspend the pellet in 360 µl Buffer BR1 by vortexing
5. Pipet the sample into a microcentrifuge tube (Eppi). Add 300 µl Buffer BR2 and 40 µl Proteinase K. Mix by vortexing, and incubate for 10 min at 55°C using a shaker–incubator, heating block, or water bath. Use of a shaker–incubator with the speed set to maximum is recommended. If a heating block or water bath is used, vortex each sample once during the incubation. Do not allow the temperature of the sample to decrease during vortexing.

   Note: Do not mix Buffer BR2 and Proteinase K together before adding them to the sample.
6. Centrifuge for 3 min at maximum speed in a microcentrifuge. Transfer the supernatant to a fresh Eppi. Transfer of small debris remaining in the supernatant after centrifugation at full speed will not affect the procedure.
7. Add 350 µl 100% ethanol. Mix by vortexing, and centrifuge briefly (1–2 s; ≤ 1000 x g) to remove drops from the inside of the tube lid.

   Note: The length of the centrifugation must not exceed 1–2 s, as this may result in pelleting of nucleic acids and reduced yields of total RNA.
8. Apply 700 µl sample to the PAXgene column sitting in a 2 ml processing tube, and centrifuge for 1 min at ≥ 8000 x g (10,000 rpm). Place the PAXgene column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.
9. Apply the remaining sample to the PAXgene column, and centrifuge for 1 min at ≥ 8000 x g (10,000 rpm). Place the PAXgene column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.

On-column DNase digestion during RNA purification can be performed with the QIAGEN RNase-Free DNase Set (cat. no. 79254). If using the RNase-Free DNase Set, follow the steps as described below (A-E). If not, continue with step 10 and finally perform DNase I digestion after step 15 as described in the final chapter “RNA clean-up with RNase-free Dnase I and Phenol/ chloro-form extraction”.

1. Prepare DNase I stock solution when using the RNase-Free DNase Set for the first time. Dissolve the solid DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. Take care that no DNase I is lost when opening the vial. Mix gently by inverting the tube. **Do not vortex.**

2. 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.

(Steps A-E: ~30 min)

A. Add 350 µl Buffer BR3 to the PAXgene spin column. Centrifuge for 1 min at ≥ 8000 x g (10,000 rpm). Either discard the flow-through, or transfer the PAXgene spin column to a new processing tube (not supplied) and discard the old processing tube containing the flow-through.

B. Add 10 µl DNase I stock solution (see above) to 70 µl Buffer RDD. Mix by gently flicking 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 flicking the tube. Do not vortex.

C. Add the DNase I incubation mix (80 µl) directly onto the PAXgene spin column membrane, and incubate at room temperature for 15 min.

   Note: Ensure that the DNase I incubation mix is added directly to the PAXgene spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

D. Add 350 µl Buffer BR3 to the PAXgene spin column. Centrifuge for 1 min at ≥ 8000 x g (10,000 rpm). Place the PAXgene spin column in a new 2 ml processing tube, and discard the old processing tube containing the flow-through.

E. Skip step 10 and continue with step 11.

(Steps 10-15: ~20 min)

10. Apply 700 µl Buffer BR3 to the PAXgene column, and centrifuge for 1 min at ≥ 8000 x g (10,000 rpm). Place the PAXgene column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.
11. Apply 500 µl Buffer BR4 (previously completed by addition of 4 vol. ethanol) to the PAXgene column, and centrifuge for 1 min at ≥ 8000 x g (10,000 rpm). Place the PAXgene column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.
12. Add another 500 µl Buffer BR4 to the PAXgene column. Centrifuge for 3 min at maximum speed to dry the PAXgene column membrane. Continue directly with step 13, or to eliminate any chance of possible Buffer BR4 carryover, first carry out the optional step described below. After this washing step, the silica-gel membrane may be light or dark brown in color. This does not influence the quality of the RNA isolated, and has no effect on any downstream applications.

Note: Residual Buffer BR4 in the eluate may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, leading to the flow-through, containing Buffer BR4, contacting the PAXgene column. Flow-through may also come into contact with the PAXgene column when the column and processing tube are removed from the rotor. In these cases, the optional centrifugation step described below should be performed.

(OPTIONAL) Discard the tube containing the flow-through, and place the PAXgene column in a new 2 ml processing tube (not supplied). Centrifuge for 1 min at full speed.

13. To elute, discard the tube containing the flow-through, transfer the PAXgene column to anEppi, and pipet 40 µl Buffer BR5 directly onto the PAXgene column membrane. Centrifuge for 1 min at ≥ 8000 x g (10,000 rpm). It is important to wet the whole membrane with Buffer BR5 in order to achieve maximum elution efficiency. Elution Buffer BR5 will not interfere with downstream applications.
14. Repeat the elution step (step 13) as described, using 40 µl Buffer BR5.
15. Incubate the eluate for 5 min at 65°C in a heating block or water bath. Following incubation, chill immediately on ice and store RNA sample at –80°C.

Denaturation of the eluate may be essential for maximum efficiency in some downstream applications, such as RT-PCR, other amplification reactions, or cDNA synthesis. It is not necessary to denature samples more than once, and samples remain denatured after freezing and thawing.

Determine RNA concentration and purity as recommended in the chapter “RNA quality and quantity control”.

B. RNA clean-up with RNase-free DNase I and phenol/chloroform extraction

Material

RNase inhibitor (e.g., Fermentas, 40 u/µl, cat. no. EO0311)

RNase-free DNase I (e.g. Amersham, 10 u/µl, cat. no.27-0514-01)

Phenol/cloroform/isoamyl alcohol (25:24:1; e.g., Roth, cat. no.)

Polyacryl-Carrier (Molecular Research Center Inc., cat. no. PC 152; distributed by Fermentas)

3 M Sodium Acetate pH 5.2; RNase-free, autoclaved

Ethanol, abs., p.a.

RNAse-free H_2O (e.g. autoclaved in the presence of 0.1% Diethylpyrocarbonat, DEPC)

75% ethanol, p.a. (ethanol, abs., pa., diluted with DEPC-treated H_2O)

Procedure (Steps 16-22: ~60 min)

16. To 80 µl RNA solution, add 1 µl RNase inhibitor and 1 µl RNase-free DNase I. Incubate at 37°C for 30 min.

17. Add 80 µl phenol/chloroform mix and vortex thoroughly.
18. Centrifuge with vmax for 5 min at RT.
19. Transfer the aqueous upper phase to a new Eppi and add 8 µl (1/10 vol.) 3M Sodium acetate, pH5.2, 1 µl Polyacryl-Carrier and 220 µl (2.5 vol.) ethanol, abs.
20. Mix and centrifuge with vmax for 5 min at RT.
21. Remove the supernatant completely, wash RNA precipitate with 200 µl 75% ethanol and centrifuge for 1 min.
22. Remove the supernatant completely, air dry the RNA precipitate and disolve in 80 µl RNAse-free H_2O. Store the RNA at –80°C.

Determine RNA concentration and purity as recommended in the chapter “RNA quality and quantity control”.

Version	Tracking of changes	Name	Date