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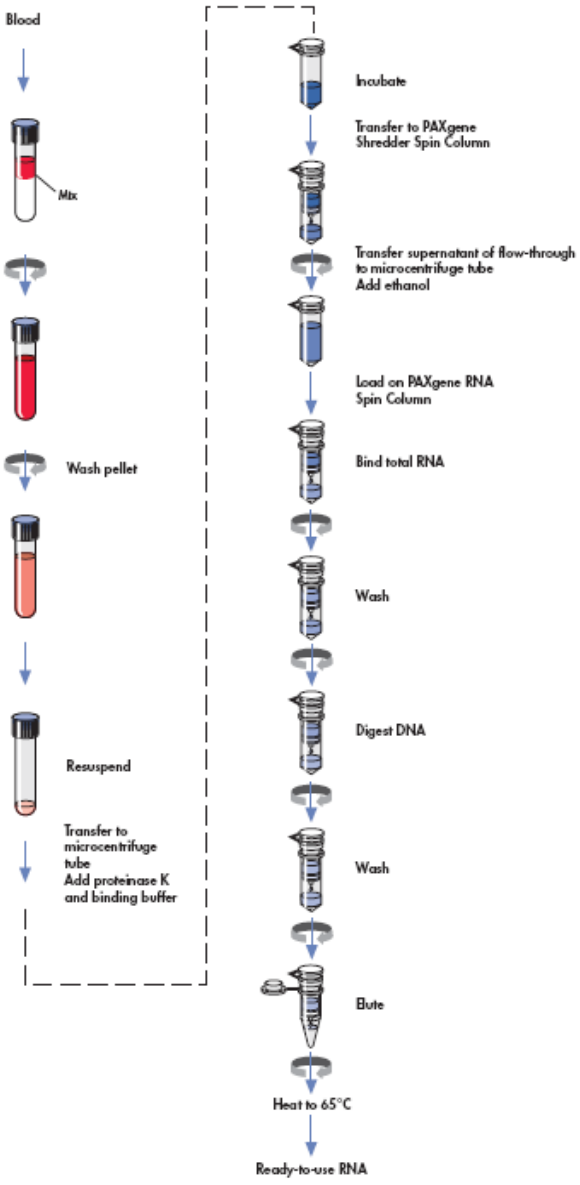
PAXgene Blood RNA Kit

Procedure Flow Chart



Step1: Blood collection protocol

1. Ensure that the PAXgene Blood RNA Tube (B) is at room temperature (18°C-25°C) prior to use and properly labeled with patient identification.

2. If the PAXgene Blood RNA Tube is the only tube to be drawn, a small amount of blood should be drawn into a “Discard Tube” prior to drawing blood into the PAXgene Blood RNA Tube. Otherwise, the PAXgene Blood RNA Tube should be the last tube drawn in the phlebotomy procedure.

3. Hold the PAXgene Blood RNA Tube vertically, below the blood donor's arm during blood collection. Allow at least 10 seconds for a complete blood draw to take place. Ensure that the blood has stopped flowing into the tube before removing the tube from the holder.

4. Gently invert the PAXgene Blood RNA Tube 8 to 10 times.Store the PAXgene Blood RNA Tube upright at room temperature (18°C-22ºC) or 4ºC.
PAXgene Blood RNA Tubes are intended for the collection of whole blood and stabilization of cellular RNA for up to 3 days at 18–25°C or up to 5 days at 2–8°C. Currently available data shows stabilization of cellular RNA for at least 6 months at –20°C or –70°C (**horizontally**) .

Step2: Things to prepare before starting

1. Ensure that the PAXgene Blood RNA Tubes are incubated for at least 2 hours at room temperature after blood collection to ensure complete lysis of blood cells.

2. Incubation of the PAXgene Blood RNA Tube overnight may increase yields.

3. Set the incubator temperature to 55°C.

4. Prepare the extraction area as RNase free zone (See Appendix II). Use the RNAzap wipe the whole working surface and pipettors.

Step3: RNA extraction step

1. Centrifuge the PAXgene Blood RNA Tube for 10 minutes at 3000–5000 x g using a swing-out rotor.

2. Remove the supernatant by decanting or pipetting. Add 4 ml RNase-free water to the pellet, and close the tube using a fresh secondary Hemogard closure.

3. Vortex until the pellet is visibly dissolved, and centrifuge for 10 minutes at 3000–5000 x *g* using a swing-out rotor. Remove and discard the entire supernatant.
Note: Incomplete removal of the supernatant will inhibit lysis and dilute the lysate, and therefore affect the conditions for binding RNA to the PAXgene membrane.

4. Add 350 µl Buffer BR1, and vortex until the pellet is visibly dissolved.

5. Pipet the sample into a 1.5 ml microcentrifuge tube. Add 300 µl Buffer BR2 and 40 µl proteinase K. Mix by vortexing for 5 seconds, and incubate for 10 minutes at 55°C using a shaker–incubator at 400–1400 rpm. After incubation, set the temperature of the shaker–incubator to 65°C (for step 20).

Note: Do not mix Buffer BR2 and proteinase K together before adding them to the sample.

6. Pipet the lysate directly into a PAXgene Shredder spin column (lilac) placed in a 2 ml processing tube, and centrifuge for 3 minutes at maximum speed (but not to exceed 20,000 x *g*).
   Note: Carefully pipet the lysate into the spin column and visually check that the lysate is completely transferred to the spin column.To prevent damage to columns and tubes, do not exceed 20,000 x *g*.

7. Carefully transfer the entire supernatant of the flow-through fraction to a fresh 1.5 ml microcentrifuge tube without disturbing the pellet in the processing tube.

8. Add 350 µl ethanol (96–100%, purity grade p.a.). Mix by vortexing, and centrifuge briefly (1–2 seconds at 500–1000 x *g*) to remove drops from the inside of the tube lid.
   Note: The length of the centrifugation **must not exceed 1–2 seconds,** as this may result in pelleting of nucleic acids and reduced yields of total RNA.

9. Pipet 700 µl sample into the PAXgene RNA spin column (red) placed in a 2 ml processing tube, and centrifuge for 1 minute at 8000–20,000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.

10. Pipet the remaining sample into the PAXgene RNA spin column, and centrifuge for 1 minute at 8000–20,000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.
    Note: Carefully pipet the sample into the spin column and visually check that the sample is completely transferred to the spin column.

11. Pipet 350 µl Buffer BR3 into the PAXgene RNA spin column. Centrifuge for 1 minute at 8000–20,000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.

12. Add 10 µl DNase I stock solution(In -20°C freezer) to 70 µl Buffer RDD in a 1.5 ml microcentrifuge tube. Mix by gently flicking the tube, and centrifuge briefly to collect residual liquid from the sides of the tube.
    Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently flicking the tube. Do not vortex.

13. Pipet the DNase I incubation mix (80 µl) **directly onto the PAXgene RNA spin column membrane**, and place on the benchtop (20–30°C) for 15 minutes.
    Note: Ensure that the DNase I incubation mix is placed directly onto the membrane. DNase digestion will be incomplete if part of the mix is applied to and remains on the walls or the O-ring of the spin column.

14. Pipet 350 µl Buffer BR3 into the PAXgene RNA spin column, and centrifuge for 1 minute at 8000–20,000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.

15. Pipet 500 µl Buffer BR4 to the PAXgene RNA spin column, and centrifuge for 1 minute at 8000–20,000 x *g*. Place the spin column in a new 2 ml processing tube, and discard the old processing tube containing flow-through.

16. Add another 500 µl Buffer BR4 to the PAXgene RNA spin column. Centrifuge for 3 minutes at 8000–20,000 x *g*.

17. Discard the tube containing the flow-through, and place the PAXgene RNA spin column in a new 2 ml processing tube. Centrifuge for 1 minute at 8000–20,000 x *g*.

18. Discard the tube containing the flow-through. Place the PAXgene RNA spin column in a 1.5 ml microcentrifuge tube, and pipet 40 µl Buffer BR5 directly ontothe PAXgene RNA spin column membrane. Centrifuge for 1 minute at 8000–20,000 x *g* to elute the RNA.
    Note: It is important to wet the entire membrane with Buffer BR5 in order to achieve maximum elution efficiency.

19. Repeat the elution step (step 18) as described, using 40 µl Buffer BR5 and the same microcentrifuge tube.
20.
21. Remove the column. Incubate the eluate for 5 minutes at 65°C in the shaker–incubator (from step 5) without shaking. After incubation, chill immediately on ice.
    Note: This incubation at 65°C denatures the RNA for downstream applications. Do not exceed the incubation time or temperature.

22. **If the RNA samples will not be used immediately, store at –20°C or –70°C.** Since the RNA remains denatured after repeated freezing and thawing, it is not necessary to repeat the incubation at 65°C.

Step 4: Prepare RNA before use or quantification

1. Thaw RNA samples on ice or 4°C before use, thaw as slow as possible.

Appendix I. RNA quantification and qualification

There is two ways to measure the RNA quantity and quality:

A. The way we use

1. Clean cuvette by both ddH2O and alcohol.
2. Press the "**RNA**" key on the spectrophotometer
3. Press "**DILUTE**" as 5 + 95 (means 5 ul samples and 95ul ddH2O)
4. Add 100ul ddH2O to the cuvette, use KIMWIP clean the cuvette surface, then put in the well, press "**BLANK**".
5. After the null reading, empty the cuvette, add 95ul H2O and 5ul thawn RNA sample, mix well. Put in the well, then press" **SAMPLE**"
6. Reading of concentration and quality score will show on the screen. Take note for everything.

B. The way suggested in PAXgene kit handbook

1. Quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (*A*260) in a spectrophotometer. To ensure significance, readings should be in the linear range of the spectrophotometer. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per ml (*A*260 = 1 fi 44 µg/ml). This relation is valid only for measurements in 10 mM Tris·Cl,* pH 7.5. Therefore, if it is necessary to dilute the RNA sample, this should be done in 10 mM Tris·Cl. As discussed below (see "Purity of RNA"), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

When measuring RNA samples, be certain that cuvettes are RNase-free. Use the buffer in which the RNA is diluted to zero the spectrophotometer, and make sure to add the same volume of Buffer BR5 as the volume of eluted RNA to be diluted.

An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 80 ml
Dilution = 10 ml of RNA sample + 140 ml 10 mM Tris·Cl, pH 7.5 (1/15 dilution)

Measure absorbance of diluted sample in a cuvette (RNase-free).
*A*260 = 0.3

Concentration of RNA sample = 44 x *A*260 x dilution factor
= 44 x 0.3 x 15
= 198 mg/ml

Total yield = concentration x volume of sample in milliliters
= 198 mg/ml x 0.08 ml
= 15.8 mg RNA

2. Purity of RNA

The ratio of the readings at 260 nm and 280 nm (*A*260/*A*280) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein. However, the *A*260/*A*280 ratio is influenced considerably by pH. Lower pH results in a lower *A*260/*A*280 ratio and reduced sensitivity to protein contamination.* For accurate values, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5. Pure RNA has an *A*260/*A*280 ratio of 1.8–2.2 in 10 mM Tris·Cl, pH 7.5. Always calibrate the spectrophotometer with the same solution.

Appendix II. RNA Handling

1. Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to destroy RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the purification procedure. In order to create and maintain an RNase-free environment, precautions must be taken during pretreatment and use of disposable and nondisposable vessels and solutions while working with RNA.

2. General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep purified RNA on ice when aliquots are pipetted for downstream applications.

Protocols for removing RNase-contamination from glassware and solutions can be found in general molecular biology guides, such as Sambrook, J. and Russell, D. W. (2001) *Molecular Cloning: A Laboratory Manual*, 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.