Total RNA Isolation from Human Blood with Qiagen PAXgene™ Tubes

This is a slightly modified version of the protocol that comes with the PAXgene Blood RNA kit (handbook 4/2001, pg 9 – 11 and pg 14 – 15). If you have not isolated RNA before be sure to read Appendix B: General remarks on handling RNA.

Collection of Blood Samples

Blood must be collected in PAXgene Blood RNA Tubes [Qiagen Cat.# 762115 (100 tubes), $600]

1. Collect blood by method of choice into a syringe (no anticoagulants) and *immediately* add 2.5 mls into the PAXgene tube. It is critical to then immediately invert the tube several times to assure complete mixing with the reagents. **NOTE**: Since this is a vacutainer, theoretically the tube will pull the correct volume by simply inserting the needle; however, insure that the injected blood volume is between 2.0 and 2.5 ml, though RNA yields will be the highest at the 2.5 ml volume for which the tubes were designed.
2. Allow the tubes to sit at room temperature for at least 2 hours - but not more than 3 hours, to allow for lysis of the blood cells. Transfer to 4^{o}C for longer storage (up to 4 days) before isolating RNA.

Isolation of RNA from Human Blood

Time Required:

- 3.5 Hr for 24 samples w/ a team of 2 working together

Supplies:

- PAXgene Blood RNA kit – Qiagen Cat.#762134 (50 isolations), $300
  - Buffers
  - RNase-free water
  - Buffer BR1 (Resuspension Buffer)
  - Buffer BR2 (Binding Buffer)
  - Proteinase K
  - Buffer BR3 (Wash Buffer)* contains guanidine isothiocyanate – use appropriate safety measures when handling.
  - Buffer BR4 (Wash Buffer)† supplied as a concentrate. Before using for the first time, add 4 volumes of benzene-free ethanol (96 – 100%) as indicated on the bottle to obtain a working solution.
  - Buffer BR5 (Elution Buffer)
  - Disposables
  - Secondary Hemogard closures
  - PAXgene Blood RNA Spin Columns
  - Processing tubes (2ml)
  - Elution tubes (1.5ml)
- RNase-Free DNase Set – Qiagen Cat.#79254 (50 digestions), $67
- Benzene-free Ethanol (96 - 100%)
- 1.5 ml microcentrifuge tubes
- Sterile, RNase-free pipet tips
- Centrifuge capable of attaining 3000 – 5000 x g equipped with a swing-out rotor and buckets to hold PAXgene Blood RNA Tubes
- Variable-speed microcentrifuge with a rotor for 2ml microcentrifuge tubes (Eppendorf centrifuge model 5415D)

- Variable-speed shaker-incubator (Eppendorf Thermomixer Compact)
- Vortex mixer
- Disposable gloves

Set-up before beginning protocol:

- Pre-heat shaker-incubator to 55°C
- Label tubes:
  - 3 sets of (not supplied) microcentrifuge tubes
  - 1 set of PAXgene columns
  - 1 set of elution tubes
- Set-up 5 sets of processing tubes (1 set not supplied)
- Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the solid DNase I (1500 Kunitz units) in 550 µl 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**.
- 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. Do not refreeze the aliquots after thawing.

Once this isolation protocol is begun, continue through all the steps without any interruptions or hesitations.
All centrifugation steps should be carried out at room temperature (15 - 25°C).

Protocol:

1. Centrifuge the PAXgene blood RNA tubes for 10 min at 3000-5000 x g using a swinging bucket rotor.
   Note: ensure that the blood sample has been incubated for a minimum of 2 h at RT, in order to achieve complete lysis.
2. Remove the supernatant by decanting and blotting excess liquid on bench paper; discard. Add 5 ml of RNase-free water (supplied) to the pellet, and close the tube using a fresh secondary Hemogard closure.
3. Thoroughly resuspend the pellet by vortexing, and centrifuge for 10 min at 3000 – 5000 x g.
4. Remove and discard the *entire* supernatant by decanting Remove drops from the rim of the tube by dabbing the rim onto bench paper followed by wiping the rim with a clean Kimwipe. Residual supernatant will inhibit further processing steps.
5. Add 360 µl Buffer BR1 to the pellet and thoroughly resuspend by vortexing.
6. Transfer the sample, by pipetting, to a 1.5 ml centrifuge tube (not supplied). Add 300 µl Buffer BR2 and 40 µl of Proteinase K. Mix by vortexing and incubate for 10 min at 55°C in a shaker-incubator, or shaking water bath.
   Use of a shaker-incubator with the speed set to maximum is recommended. Do not allow the temperature of the samples to decrease during vortexing. **NOTE**: DO NOT mix buffer BR2 and proteinase K together before adding to the resuspended pellet.
7. Centrifuge for **20 minutes** at maximum speed (at least 10,000 x g) in a microcentrifuge. Increase temperature of shaker-incubator to 65°C.
8. Transfer the supernatant to a fresh 1.5 ml tube (not supplied).
9. Add 350 µl of 100% ethanol. Mix by vortexing. Remove drops from the inside of the tube lid with a pipet tip.

NOTE: Transfer of small amounts of debris remaining in the supernatant will not affect further steps.

10. Apply 700 µl of sample to the PAXgene column sitting in a 2 ml processing tube, and centrifuge for 1 minute at ≥ 8,000 x g (≥10,000 rpm). Place the column in a new 2 ml processing tube, and discard the flow-through and old tube.
11. Apply the remaining sample to the column and repeat the centrifugation. Place the column in a new processing tube, and discard the flow-through and old tube.
12. Apply 350 µl Buffer BR3 to the column and centrifuge for 1 min at ≥ 8,000 x g. Place the column in a new 2 ml processing tube, and discard the flow-through and old processing tube.
13. Add 10 µl DNase I stock solution to 70 µl Buffer RDD for each digestion (example: for 10 samples, add 100 µl DNase I stock solution to 700 µl Buffer RDD). Mix by **gently flicking** the tube, and centrifuge briefly to collect residual liquid from the sides of the tube. **Do not vortex.**
14. Pipet 80 µl DNase I incubation mix directly onto the spin-column membrane, and place on the benchtop (20-30^{o}C) for 15 min.
15. Pipet 350 µl Buffer BR3 into the PAXgene spin column, and centrifuge for 1 min at ≥ x g. Place the column in a new 2 ml processing tube, and discard the flow-through and old tube.
16. Apply 500 µl Buffer BR4 to the column, and repeat centrifugation. Again, place in a new processing tube, discarding the flow-through and old processing tube.
17. Apply another 500 µl Buffer BR4 and centrifuge for ***3*** **min** at >8,000 x g.
18. Place the column in a new 2 ml processing tube (not supplied). Centrifuge again for 1 min to assure removal of residual supernatant.
19. To elute the RNA from the column, transfer the PAXgene column to a 1.5 ml elution tube, and pipet 40 µl Buffer BR5 directly onto the column membrane. Centrifuge for 1 min at ≥ 8,000 x g.
20. Repeat the elution step with another 40 µl of Buffer BR5 and centrifugation.
21. Incubate the eluate for 5 min at 65^{o}C in a heating block or water bath to denature the eluate. Get a bucket of ice. Following incubation, **chill immediately on ice**.
22. Quantitate and qualitate RNA on NanoDrop. Record µg/µl and 260/280 ratio. Typical yields: ~1 µg RNA / 1 ml human blood
23. Aliquot 1.5 µl of 100 ng/µl concentration for BioAnalyzer.
24. Store the eluted RNA at –80^{o}C.

For Agilent Commercial Arrays:
Aliquot of 40-50µl at ~100ng/µl.