Biomarkers: Isolating RNA from PAXgene Tubes

All reagents, water, and test tubes can be found in the PAXgene Kit located at the RNA Bench.

1. Day before experiment, take tubes out of –80C and leave over night in 4'C. Day of experiment, leave samples on the bench until RT, 1-2 hours. **RNA yield will be greatly reduced if colder than RT.** (Tubes should first be frozen at -80'C before processing.)
2. Make 4 barcodes for each patient.
3. Note volume of blood for each tube on the back of this sheet.
4. Centrifuge the PAXgene tubes for 10 minutes at 3900g, 23°C using a swing-out rotor. Program 24. *PreAnalytiX allows for range of speeds 3000g-5000g.*
5. Remove the supernatant by decanting. Add 5 ml of RNase-free water to the pellet, and close the tube using a fresh secondary Hemoguard closure.
6. Thoroughly resuspend the pellet by vortexing, and centrifuge for 10 minutes at 3900g, 23°C. Remove and discard the entire supernatant. Incomplete removal of the supernatant will inhibit lysis and dilute the lysate, which will affect conditions for binding RNA to the PAXgene column.
7. Add 360µl Buffer BR1 to the pellet. Thoroughly resuspend the pellet by vortexing. Pipet the sample into a 1.5 ml microcentrifuge tube

Move to the fume hood for steps 8-20.

8. Add 300 µl Buffer BR2 and 40 µl Proteinase K (pipet up and down to get all Proteinase K out of the pipet tip). Mix by vortexing, and incubate for 10 minutes at 55°C, 1400 rpm, using a shaker-incubator.
9. Centrifuge for 10 minutes at 14,000 rpm in Eppendorf 5417C. Transfer supernatant to a fresh 1.5 ml microcentrifuge tube (tube usually contains the 350 ul EtOH for the next step). AVOID transfer of small debris remaining in supernatant after centrifugation; this *will affect* the yield.
10. Add 350 µl 100% ethanol (*Located in flammable liquids box under hood).* Mix by vortexing; also can centrifuge briefly (1-2 sec; <1000g) to remove drops from the inside of the tube. Do not exceed more than a 2 second spin; this may result in pelleting of the nucleic acid.
11. Apply 700 µl sample to the PAXgene column sitting in a 2 ml processing tube (pipet sample up and down while in 1.5 ml tube to remove any RNA from walls). Also, add any precipitate to the column that may have formed due to the addition of EtOH.
12. Centrifuge for 1 minute at 9500rpm in Eppendorf 5417. Discard the flow-through and reuse the collection tube. *Discard flow through in Buffer BR2 Hazardous Waste (Located in hood).*
13. Apply the remaining sample to the PAXgene column, and centrifuge for 1 minute at 9500rpm in Eppendorf 5417. Place the PAXgene column in a new 2 ml processing tube, and discard the old processing tube containing flow-through. *Discard flow through in Buffer BR2 Hazardous Waste.*
14. Pipet 350 µl Buffer BR3 into a PAXgene column. Centrifuge for 1 minute at 9500rpm in Eppendorf 5417. Discard the flow-through and reuse processing tube. *Discard flow through in Buffer BR3 Hazardous Waste (Located in hood).*
15. From RNase-free DNase Kit, add 10 µl DNase I stock solution to 70µl Buffer RDD to a separated test tube (*Kit located in 4'C refrigerator).* Mix by gently flicking the tube, and centrifuge briefly to collect residual liquid from the sides of the tube. Do not vortex DNase I, only gently flick the tube to mix, since DNase is sensitive to physical denaturation. *Ususally 40 ul of DNase is aliquoted into 0.5 ml tubes and stored at –20'C. 280 ul of RDD is then added to this aliquot, which is enough solution for one patient.*
16. Pipet the DNase I incubation mix (80 µl) *directly onto* the spin-column membrane, and place on the benchtop (20-30°C) for 30 minutes.
17. Pipet 350 µl Buffer BR3 onto the PAXgene spin column, and centrifuge for 1 minute at 9500rpm in Eppendorf 5417. Discard the flow-through and reuse the collection tube. *Discard flow through in Buffer BR3 Hazardous Waste.*

18. Apply 500 µl Buffer BR4 to the PAXgene column, and centrifuge for 1 minute at 9500rpm in Eppendorf 5417. Place the PAXgene column in a new 2 ml processing tube, and discard the old processing tube containing flow-through. ***Ensure BR4 is diluted with ethanol. ** Discard flow through in Buffer BR3 Hazardous Waste*.
19. Add another 500 µl Buffer BR4 to the PAXgene column. Centrifuge for 3 minutes at 11500 rpm in Eppendorf 5417 to dry the PAXgene column membrane.
20. Discard the tube containing the flow-through, and place the PAXgene column on a new 2 ml processing tube. Centrifuge for 1 minute at 11500 rpm in Eppendorf 5417. *Discard flow through in Buffer BR3 Hazardous Waste*.

At RNAse-free bench:

21. To elute, discard the tube containing the flow-through, transfer the PAXgene column to a 1.5 ml elution tube, and pipet 45 µl Buffer BR5 directly onto the PAXgene column membrane. Incubate one minute before centrifuging for 1 minute at 9500rpm in Eppendorf 5417.
22. Repeat elution step 21 (45 µl Buffer BR5) using the same 1.5 ml elution tube.
23. Pool eluates from all four columns giving a total volume of 360 ul.
24. Heat the eluate at 65C for 5 minutes, 1400rpm in shaker incubator, then return sample directly onto ice.
25. Take 4 microliters for OD via Agilent BioAnalyzer. Store in Agilent Prep box in –80C until 12 samples are collected for analysis. Place barcode on tube.
26. If needed, bring volume up to 200 microliters with DEPC. Add 0.1 volumes of 3 M sodium acetate, pH 5.2, and 2.0 volumes of 100% ethanol to the pooled sample. (For 360 µl sample, add 36 µl NaAc and 720 µl EtOH.) Place barcode on tube.
27. Mix and incubate the sample on dry ice for at least 1/2 hour, to precipitate the RNA. Usually we incubate for at least 1 hour, or several days at –80°C due to convenience.
28. Place one of the two remaining barcodes on the Patient Sample Data binder and the other at the bottom of this page.
29. Remove two of North Shore's barcodes for the PAXgene tubes and place one at the bottom of this page and one in the Patient Sample Data binder.
30. Put aliquot in Agilent Prep Box located in –80'C. Place RNA in RNA boxes in –80'C.
31. **Update RNA Box Maps.**

Patient #:						
New Bar Code:						
Paxgene 1 ml:						
Paxgene 2 ml:						
Paxgene 3 ml:						
Paxgene 4 ml:						
RNA Storage Box:						

N.S. Bar Codes: