**RNA, BLD, BMA, Manual Extraction Procedure**

1. **PRINCIPLE:**
   1. Isolation of RNA from specimens is necessary for some assays in Molecular Genomic Pathology Laboratory. Multiple techniques are available for the isolation of RNA and these techniques result in products of varying purity and quality. The quality of the RNA produced is highly dependent on the starting material as well as the method of preparation. The QIAamp RNA blood mini kit is used for the isolation of RNA from blood and bone marrow aspirates.
   2. The QIAamp RNA blood mini kit allows total RNA preparation by combining the selective binding properties of a silica-gel-based membrane with the speed of microspin technology. A specialized high salt buffer system allows RNA longer than 200 bases to bind the QIAamp membrane.
   3. In the QIAamp RNA blood purification procedure, erythrocytes are selectively lysed, and leukocytes are recovered by centrifugation. The leukocytes are then lysed using highly denaturing conditions which immediately inactivate RNases to allow the isolation of intact RNA. After homogenization of the lysate by a brief spin through a QIAshredder spin column, ethanol is added to adjust binding condition and the sample is applied to a QIAamp RNA blood mini spin column. Total RNA is bound to the silica-gel membrane during a brief spin in a microcentrifuge. Contaminants are washed out and total RNA is eluted in 30ul or more of water for direct use in any downstream application. Since the QIAamp RNA mini blood procedure relies on intact leukocytes, frozen blood cannot be used.
   4. The QIAamp RNA blood mini kit enriches for RNA fragments larger than 200 nucleotides in length. Small RNA’s such as 5.8s RNA, 5s RNA, and tRNA (approximately 160, 120, and 70-90 nucleotides in length, respectively), which make up 15-20% of the total RNA, do not bind quantitatively under the conditions used. Thus, the size distribution of RNA isolated with the QIAamp RNA blood procedure is comparable to RNA isolated by centrifugation through a CsCI cushion, where small RNAs do not sediment efficiently. The RNA is then ready to be used in cDNA synthesis and Reverse Transcription-PCR.
2. **SAMPLES:**
   1. RNA can be isolated from whole blood and bone marrow aspirate.
   2. Refer to individual test procedures for acceptable specimen types.
3. **MAJOR EQUIPMENT:**
   1. Eppendorf 5430R High-Speed Refrigerated Centrifuge
   2. Eppendorf 5415D Digital Micro-Centrifuge
4. **REAGENTS:**
   1. 2-mercaptoethanol. Store at room temperature under hood in flammable cabinet.
   2. Ethanol (100%). Store at room temperature in flammable cabinet.
   3. Proteinase K. Stored at -20°C in 20ul aliquots. Qiagen DNA Mini Kit #51106.
   4. Qiagen QIAamp RNA Blood Mini Kit. Store at room temperature.
      1. EL Buffer
      2. RWI Buffer
      3. RPE buffer – this is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96-100%) to obtain a working solution and mark bottle with date of dilution.
      4. RLT buffer
         1. This may form a precipitate upon storage. If necessary, warm to redissolve.
         2. 2-mercaptoethanol must be added right before use, add 10ul per 1ml of RLT.
   5. RNase free water. Store at room temperature.
5. **PROCEDURE:**
   1. **Aliquot your sample: Aliquot Action**
      1. Log into Soft Molecular.
      2. Open Extractions by using the Extractions tile on the dashboard.
         1. **Note:** If the RLT step is completed and the specimen is stored in the freezer overnight, the RNA Extraction tech will find the sample pending for the RNA Extraction BLD, BMA Manual action. **Prior to starting an RNA extraction, always check if any samples are pending at the RNA Extraction BLD, BMA Manual action.**
      3. Highlight the Aliquot branch in the action tree.
      4. Highlight the Barcode# field. Scan the SoftLab specimen label and press **Enter** on the keyboard. Repeat this step for all applicable specimens.
      5. If a DNA test is also attached, select **ALQ** protocol for the DNA test in the child level dropdown for the correct patient sample.
      6. Select **Save**.
   2. **Check Soft Lab Cell Count Action**
      1. Check WBC count in Soft Lab and write the value on the SoftLab specimen label for each patient sample.
         1. If a WBC count was not performed: Write ‘0’ on the SoftLab specimen label for each applicable patient sample.
      2. Highlight the Check SoftLab Cell Count branch in the action tree.
      3. Highlight the Barcode# field. Scan the SoftLab specimen label and press **Enter** on the keyboard. Repeat this step for all applicable specimens.
         1. **NOTE: If a patient sample has both DNA and RNA ordered on the same specimen, highlight the sample by selecting the pin icon. Do not scan the SoftLab specimen label for this order type.**
      4. Enter the WBC count previously written on the patient sample SoftLab specimen label into the WBC Count (10e9/L) field.
      5. Select **Save**.
   3. **RNA Lysis and Incubate Action**
      1. Highlight the RNA Lysis and Incubate branch in the action tree.
      2. Mark all applicable samples using the **push-pin** icon.
      3. Enter a final elution volume of 50 in the Prod Vol column.
      4. Select the Spec/Tube Reagents field and in the dropdown, scan the appropriate TQC reagent label to add the QIAamp RNA Blood Mini Kit lot number to each specimen.
      5. Mark the **Completed** checkbox.
      6. Print Soft Molecular specimen label.
         1. Select **Print** tab.
         2. Click the **Print Label** button.
         3. Select the ‘**…**’ button.
         4. Select **OK** to save before print.
         5. Select the correct printer and verify the Template field is set to **SPEC LBL V1**.
         6. Select **Print**.
      7. Select **Back** in the Extractions window.
      8. Exit Soft Molecular application.
   4. **RNA Lysis and Incubate** 
      1. All pipettors and surfaces should be wiped down with RNase-Away Reagent.
      2. For BCR-ABL lot-to-lot control preparation, refer to Appendix A: RNA QC Control Preparation.
      3. For bone marrow aspirates, use up to 1.5ml of sample with 600ul of Buffer RLT and 6ul of 2-mercaptoethanol.
      4. For whole blood, the WBC count is entered in the WBC Count (10e9/L) field in Soft Molecular.
         1. Use an appropriate amount of whole blood. Up to 3ml of whole blood can be processed. If blood with elevated numbers of leukocytes is used, reduce the amount appropriately using Table 1 below as a guide. Also see step 10.

Table 1

|  |  |  |  |
| --- | --- | --- | --- |
| White blood cell count (X10⁹/L) | Whole blood (ml) | Buffer RLT (ul) | 2-mercaptoethanol  (ul) |
| <3.5 | 1.5 | 350 | 3.5 |
| 3.5-15 | 1.5 | 600 | 6 |
| 15-35 | 1 | 600 | 6 |
| >35 | 0.5 | 600 | 6 |

* + 1. Mix 1 volume of whole blood with 5 volumes of Buffer EL in a 50ml sterile conical centrifuge tube.

**NOTE 1**: If white blood cell count is exceptionally low, see director for use of up to 3ml of blood.

**NOTE 2**: Centrifuge spin times may need to be adjusted if the centrifuge starts counting down before the appropriate speed has been reached.

**NOTE 3**: For optimal results and efficient mixing, the volume of the mixture (Blood + Buffer EL) should not exceed ¾ of the volume of the tube.

* + 1. Incubate for 10-15 minutes on ice. Mix by vortexing briefly 2 times during incubation. The cloudy suspension becomes translucent during incubation, indicating lysis of erythrocytes. If necessary, incubation time may be extended to 20 minutes.
    2. During the 10–15-minute incubation, turn on the Eppendorf 5430R High-Speed Refrigerated Centrifuge to ensure it will reach 4°C prior to step 8.
    3. Centrifuge at 400 x g or 1000 rpm for 10 minutes at 4°C. Completely remove and discard supernatant. Leukocytes will form a pellet after centrifugation. Ensure complete removal of supernatant without disturbing the pellet. Trace amounts of erythrocytes that give the pellet a red tint will be eliminated in the following wash step.
    4. Add Buffer EL to the cell pellet: Use 2 volumes of Buffer EL per volume of whole blood used in step 3. For example, add 2ml of Buffer EL per 1ml of whole blood used in step 3. Resuspend cells by vortexing briefly.
    5. Centrifuge at 400 x g for 10 minutes at 4°C and completely remove and discard supernatant.
    6. **NOTE 1**: Incomplete removal of the supernatant will interfere with lysis and subsequent binding of RNA to the QIAamp RNA column, resulting in a low yield. Pellet may have a pinkish-red tint, but the pellet should not be red. If it is red, repeat step 5 and incubate on ice for 20 minutes before centrifugation.

**NOTE 2**: After erythrocyte lysis, all steps of the RNA Blood Mini protocol should be performed at room temperature (20-25°C) as quickly as possible.

* + 1. Add 2-mercaptoethanol to RLT. Add Buffer RLT to pelleted leukocytes according to Table 1 above.
    2. Cell lysates (in buffer RLT) can be stored at -70°C. To process frozen lysates, thaw and incubate for 10 minutes at room temperature to ensure that all salts have dissolved.
  1. **RNA, BLD, BMA Manual Extraction Action** 
     1. Log into Soft Molecular.
     2. Open Extractions by using the Extractions tile on the dashboard.
     3. Highlight the RNA, BLD, BMA Manual Extraction branch in the action tree.
     4. Highlight the Barcode# field. Scan the SoftLab specimen label and press **Enter** on the keyboard. Repeat this step for all applicable specimens.
        1. **NOTE: If a patient sample has both DNA and RNA ordered on the same specimen: highlight the sample by selecting the pin icon. Do not scan the SoftLab specimen label for this order type.**
     5. Scan the Soft Molecular specimen label.
     6. Press **Enter** on the keyboard.
     7. Navigate to the **Print** tab and click the **Print Product Labels** dropdown menu. Select the ‘**…**’ button that appears.
     8. Select **OK** when asked to save before print.
     9. In the Print Product Labels window, verify the correct printer and product label template is selected (PROD LBL V1). Change the Number of copies to 4. Click **Print**.
     10. Mark the **Completed** checkbox and select **Save**.
     11. Select **Back** in the Extractions window.
     12. Exit Soft Molecular application.
  2. **RNA, BLD, BMA Manual Extraction** 
     1. Transfer lysate to a thawed pre-aliquoted tube of Proteinase K (20ul). Vortex to mix, and then quick spin.
     2. Incubate at 55°C for 15 minutes, vortexing frequently, about 4 times throughout 15 minutes.
     3. After the 15 minutes, quick spin to collect contents.
     4. Pipette lysate directly into a QIAshredder spin column sitting in a 2ml collection tube. Centrifuge for 2 minutes in microcentrifuge at maximum speed to homogenize. Discard QIAshredder column and save homogenized lysate.
     5. Add 1 volume (350ul or 600ul) of 70% ethanol to the homogenized lysate and mix by pipetting 10 times. Do not centrifuge.

**NOTE**: A precipitate may form after the addition of ethanol. This will not affect the QIAamp RNA procedure.

* + 1. Apply sample, including any precipitate which may have formed, to the QIAamp RNA mini spin column and centrifuge at full speed for 15-30 seconds at ≥8000 x g (≥13,000 rpm in the microfuge). If using ‘short’ spin button, add 5 seconds to spin time to accommodate ramp time for all steps. Maximum loading volume is 700ul.
    2. If the volume of the sample exceeds 700ul, load aliquots successively onto the QIAamp RNA column and centrifuge as above. Collect the flow-through and run it through the column a second time if cell count is low. Then, discard the flow-through and reuse the collection tube in step 7

**NOTE: Flow-through contains Buffer RLT and is, therefore, not compatible with bleach. Pour it into the chemical waste bottle on the sample prep bench.**

* + 1. Apply 700ul of Buffer RW1 to QIAamp RNA column and centrifuge at full speed for 15-30 seconds at ≥8000 x g (≥13,000 rpm speed in the microfuge) to wash.

**NOTE: Flow-through contains Buffer RW1 and is, therefore, not compatible with bleach. Pour it into the chemical waste bottle on the sample prep bench.**

* + 1. Place QIAamp RNA column in a new 2ml collection tube (supplied). Pipette 500ul of Buffer RPE into the QIAamp RNA column and centrifuge at full speed for 15-30 seconds at ≥8000 x g (≥13,000 rpm speed in the microfuge).

Discard flow-through and collection tube.

* + 1. **NOTE**: Ensure ethanol is added to Buffer RPE by checking for a dilution date (See section ‘III. REAGENTS’ above).
    2. Place QIAamp RNA column in a new 2ml collection tube (supplied). Pipette 500ul of Buffer RPE into the QIAamp RNA column and centrifuge for 3 minutes at maximum speed to dry the QIAamp RNA membrane.

**NOTE**: It is important to dry the QIAamp membrane, since residual ethanol may interfere with subsequent reactions. This 3-minute spin ensures that no ethanol is carried over during elution.

* + 1. Discard the flow-through and centrifuge the column in a collection tube at full speed for 1 minute. This helps eliminate the chance of possible RPE carryover.
    2. Following the spin, remove the QIAamp RNA column from the collection tube carefully so the column does not contact the flow-through, as this will result in carry over of ethanol.
    3. Transfer QIAamp RNA column to a new 1.5ml collection tube (supplied) and pipette 50ul of RNase-free water directly onto the QIAamp RNA membrane.
    4. Centrifuge for 1 minute at ≥8000 x g (≥13,000 rpm speed in the microfuge) to elute. Save the spin column and the 1.5ml collection tube.
    5. Pass the flow-through from above over the same spin column by pipetting it back onto the spin column.
    6. Centrifuge at full speed for 1 minute.
  1. **Refer to the NanoDrop 2000 Instrument Procedure to measure the nucleic acid concentration of each patient sample.**
  2. **RNA Quantitation Action**
     1. Log into Soft Molecular.
     2. Open Extractions by using the Extraction tile on the dashboard.
     3. Highlight the RNA Quantitation branch on the action tree.
     4. Select **Tools** tab followed by **Import** to import Nanodrop results.
     5. Choose file location using the ‘**…**’ button next to the Directory field and confirm by clicking **OK**.
     6. Select **RNA** from the dropdown in the Template field.
     7. Choose file name using the ‘**…**’ button next to the File Name field and confirm by clicking **Open**.
     8. Select **Import**.
     9. In the Import Finished window, select **OK**.
     10. Close the Import from Excel window using the ‘**X**’.
     11. Highlight the Barcode# field, scan the product label, and select **Enter** on the keyboard. Repeat this step for all applicable specimens. Select **Save**.
     12. Select **Back** in the Extractions window.
     13. Exit Soft Molecular application.
     14. Store RNA at -80°C.

1. **ADDITIONAL INFORMATION:**
   1. **Handling of QIAamp spin columns:**
      1. Owing to the sensitivity of nucleicacid amplification technologies, the following precautions are necessary when handling QIAamp spin columns to avoid cross-contamination between sample preparations.
      2. Carefully apply the sample or solution to the QIAamp spin column. Pipette the sample into the QIAamp column without wetting the rim of the column.
      3. Change pipette tips between all liquid transfer steps. Use aerosol-barrier tips.
      4. Avoid touching the QIAamp membrane with the pipette tip.
      5. After all pulse vortexing steps, briefly centrifuge 1.5ml microcentrifuge tubes to remove drops from the inside of the lid.
      6. Wear gloves throughout the procedure. In case of contact between gloves and sample, change gloves immediately.
   2. **Centrifugation:**
      1. Close the QIAamp spin column before placing it in the microcentrifuge.
      2. Remove the QIAamp spin column and collection tube from the microcentrifuge. Place the QIAamp spin column in a new collection tube. Discard the filtrate and the old collection tube.
      3. Open only one QIAamp spin column at a time, taking care to avoid generating any aerosols.
      4. For efficient parallel processing of multiple samples, it is recommended to fill a rack with collection tubes to which the QIAamp spin columns can be transferred after centrifugation. Used collection tubes containing the filtrate can be discarded, and the new collection tubes containing the QIAamp spin columns can be placed directly in the microcentrifuge.
      5. QIAamp spin columns will fit into most standard 1.5 or 2 ml microcentrifuge tubes. Additional 2ml collection tubes are available separately.
      6. Centrifugation of QIAamp spin columns may be performed at 6000 x g (8000 rpm) to limit centrifuge noise. Centrifugation at full speed will not affect RNA yield. Centrifugation at lower speeds for lysate loading and the first wash step is also acceptable, provided that the complete solution is transferred through the membrane. At the second wash step, centrifugation at full speed is strongly recommended.
      7. All centrifugation steps are carried out at room temperature.
2. **ATTACHMENTS**:
   1. RNA, BLD, BMA, Manual Extraction Procedure Appendix A: RNA QC Control Preparation
3. **REFERENCES:**
   1. QIAamp DNA Blood Mini Handbook fourth edition 2015.
   2. QIAamp RNA blood mini Kit Handbook, second edition April 2010
4. **REVISIONS:**
   1. 3/20/2018: Additional information added concerning centrifugation spin times starting after the spin rate is achieved and new chart for specimens with low/high white blood cell counts. Formatting also corrected.
   2. 7/3/2019: Modified protocol to increase RNA yield, including addition of Proteinase K, centrifugation after final RPE, elution volume, and passing flow-through into the spin column.
   3. 1/16/2020: Updated with new procedure name, steps for Soft Molecular and updated footer with new lab name.
   4. 10/5/2020: Clarified RNA lysis steps and instructions for processing frozen lysate.
   5. 3/6/2022: Added reference to Appendix A and clarified additional steps.