STANDARD OPERATING PROCEDURE (SOP) FOR DNA/RNA EXTRACTION WITH ALLPREP (DNA) AND MIRVANA (TOTAL RNA WITH SMALL RNA) - MODIFIED LEUKEMIA PROTOCOL

I. SCOPE AND PURPOSE

This SOP is specific to leukemia specimens.

Qiagen AllPrep kits are designed to isolate DNA and total RNA from small quantities of starting material. In addition, they provide a fast and simple method for the preparation of DNA and column purified RNA from human tissues. The purified DNA and RNA are ready for use in standard downstream applications such as DNA amplification and expression array.

A representative sampling of the total RNA content within the tumor tissue homogenate, most notably the low molecular weight species, can be used for micro RNA analysis. Therefore, the flow through from the AllPrep DNA column is taken and the total RNA is isolated with the *mir*Vana kit from Life Technologies.

The *mir*Vana™ miRNA Isolation Kit was designed for purification of RNA suitable for studies of both siRNA and miRNA in natural populations. The kit employs an organic extraction followed by immobilization of RNA on glass-fiber filters to purify total RNA. The *mir*Vana miRNA isolation procedure combines the advantages of organic extraction and solid-phase extraction, while avoiding the disadvantages of both. High yields of ultra-pure, high quality, small RNA molecules can be prepared in less than two hours.

II. PROCEDURE

A. Safety Procedures

1. Wear Personal Protective Equipment (PPE), including a lab coat, goggles or face shield, closed-toe shoes, nitrile gloves and a pair of insulated gloves (when handling LN_2 samples).
2. Bloodborne pathogens can be present in the unfixed frozen tissue (refer to SOP S009, "Bloodborne Pathogen and Exposure Control Plan" found in the BCR Safety Manual). Use all universal precautions.
3. Liquid nitrogen and dry ice are extremely cold and may cause 'burns.' Wear cryogenic gloves designed to withstand extremely cold temperatures.
4. Liquid nitrogen is an asphyxiate; all work should be conducted in a well-ventilated room.
5. 2-mercaptoethanol (2-ME) is toxic. The neat stock solution should be opened in a fume hood only. Solutions containing 1% or less of 2-ME may be used outside of a fume hood. PPE must be used when handling any solution containing 2-ME.
6. Buffer RLT Plus and Buffer AW1 contain a guanidine salt which is not compatible with disinfectants containing bleach. PPE must be used when handling this reagent.

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7. miRNA Wash Solution included in the *mir*Vana kit contains guanidinium thiocyanate, this is a potentially hazardous substance and should be used with appropriate caution.
8. Acid-phenol:chloroform contains phenol, which is a poison and an irritant. Use gloves and personal protective equipment when working with this reagent.

B. QUALITY CONTROL:

1. The incoming tissue samples have a printed label with a 2D barcode and a human readable format.
2. Working labels are printed from LabVantage and used throughout the extraction process. Labels are printed that match the original portion and subportion identifiers and the corresponding newly created DNA or RNA analyte identifiers. Four label colors (Blue, Green, Red, and Yellow) are rotated with each sample. Example: Sample A has blue working labels; Sample B has green working labels, etc.
3. Final storage labels are printed for storage in Matrix 2D barcode tubes.
4. Barcode readers are used throughout the process whenever transferring samples from one tube to another.
5. Samples are tracked in LabVantage. Every portion or analyte is tracked with its position in a freezer or refrigerator. When a technologist processes a sample, LabVantage displays the user name as having custody of that sample until the sample is checked in to a storage location.
6. DNA and RNA analyte stocks are stored as a primary single tube aliquot (when possible) with a smaller secondary aliquot for sample quality control. RNA quality is measured by RIN using Agilent Bioanalyzer (see SOP M002, "RNA Nano Assay") and quantified by Spectrophotometer (see SOP MGL-EQP-6 "BIO-MATE UV-Visible Spectrophotometer"). DNA quality is evaluated for integrity by agarose gel electrophoresis (see SOP M003, "Gel Electrophoresis with the E-gel System"), quantified by PicoGreen Assay (see SOP M017, "Picogreen DNA Quantification (Manual)"), and genotypic identity using SNP loci (see SOP M010, "Tissue Matching by SNP Analysis). Primary stock aliquots should not be subject to numerous freeze thaw cycles.
7. Any deviations from the protocol as written should be documented on extraction worksheets and at the sample level in LabVantage. Protocol deviations that have the potential to compromise analyte quality (pre-analytical variables) should also be documented with an incident report. Pre-analytical variables include, but are not limited to, abnormal sample condition upon arrival (cracked tubes or clotted blood), temperature excursions (in storage freezer or during extraction), abnormal analyte appearance (cloudy or colored analyte elution), and identity failures.
8. The isolation kit is tested against predetermined specifications to ensure consistent product quality.

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9. All new lots of reagents are tested in parallel with the one in current use before being put into clinical use. Results are recorded on positive control extraction worksheets.
10. At each step in the DNA isolation, the supernatant or pellet that should not contain the DNA is retained until after isolation and quantitation is completed.
11. RNA is extremely susceptible to degradation by ribonucleases that are ubiquitous in the environment. To ensure preservation of target RNA or RNA probes, special precautions are needed.
    a. Bench space is wiped down at the beginning of each extraction session with RNase Zap. Pipettes are wiped down with RNase Zap once a week or as needed.
    b. Gloves should always be worn throughout the process and should be changed frequently.
    c. Only sterile, disposable plasticware and pipettes that are dedicated strictly to RNA work should be used to prevent cross-contamination with RNases from shared equipment.
    d. Containers and tubes with samples must be kept covered when possible during the entire procedure to ensure they remain dust- and RNase-free.

C. Specimen Information

1. Type: Liquid AML tumor samples provided by the Tissue Source Site.
2. Handling Conditions: Follow standard precautions when handling all tissues or cultured cells. Samples should be stored in liquid nitrogen until analytes can be isolated.
3. Indications for Study: This procedure should be used when DNA and RNA are needed from the same liquid tumor sample. DNA is isolated from the AllPrep DNA column and Total RNA including small RNAs is derived from the *mir*Vana isolation kit.

D. Required equipment, supplies, and reagents

1. Equipment

UV visible spectrophotometer
Capsule centrifuge
Digital dry bath
Liquid nitrogen freezer
-20^{o}C freezer
Microcentrifuge
Multi-channel and single channel pipettes
Refrigerator
Qiagen TissueLyser
Vortex
Water bath
Cytospin (if applicable)
Microscope (if applicable)
Serological pipette

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2. Supplies

Allprep DNA/RNA mini Kit (Qiagen 80204)
*mir*Vana™ miRNA Isolation Kit (Applied Biosystems, Cat# AM1560)
Filtered, sterile pipette tips, assorted sizes
1.5 mL Eppendorf tubes
0.5 ml tubes (Fisher, #05-408-128)
2 mL screw cap tubes (Fisher, Cat# 02-707-355)
2 mL SafeLock Eppendorf tubes (Fisher, Cat#022363352)
Wet and dry ice
Insulating trays for dry ice
Personal protective equipment (PPE), including insulated gloves
Stainless steel beads, 5 mm (Qiagen, Cat# 69989)
Hemacytometer
Coverslips
Cytofunnels (ThermoScientific, #5991040) (if applicable)
Cytoslides (ThermoScientific, #5991051) (if applicable)
15mL tubes (Fisher, #50-869-570)
Filtered serological pipets assorted sizes

3. Reagents

2-mercaptoethanol, 100% (Sigma M3148-100ml)
Absolute ethanol, molecular grade (Sigma, E7023)
Diethylpyrocarbonate (DEPC)-treated water (Invitrogen, 750023)
Tris-EDTA Buffer - 100X (Sigma T9285, 100ml)
Sodium hydroxide – 5M (Sigma S8263, 150 mL)
Reagent DX (Qiagen 19088, 1mL)
Hanks Balanced Salt Solution (HBSS) (Sigma, #H8264)
Dulbecco Modified Eagle Medium (DMEM) (Sigma, #D5796)
0.4% Trypan Blue (Sigma, Catalog# T8154)
Fetal Bovine Serum (FBS) (Sigma, #F2442)

It is possible to substitute disposable materials and certain equipment from other vendors as long as they are the equivalent of the item described above.

In the event that a reagent or disposable item either becomes contaminated or is even suspected to be contaminated, it must be discarded.

E. REAGENT PREPARTION (INCLUDING STORAGE CONDITIONS):

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1. 2-mercaptoethanol (2-ME) – must be added to Buffer RLT Plus before use (final 1% 2-ME). Buffer RLT Plus is stable at room temperature (15-25°C) for 1 month after addition of 2-ME.
2. Buffer AW1 and Buffer AW2 are each supplied as a concentrate. Before using for the first time, add the appropriate volume of ethanol (96-100%), as indicated on the bottle, to obtain a working solution. Add 25 mL EtOH to an unopened bottle of Buffer AW1 to obtain a 44 mL total volume. Add 30 mL EtOH to an unopened bottle of Buffer AW2 to obtain a 43 mL total volume Buffer AW1 and Buffer AW2 are stable for 1 year at room temperature.
3. Add 21 mL 100% ethanol to miRNA Wash Solution 1 before use. Add 41 mL 100% ethanol to miRNA Wash Solution 2/3 to obtain a working solution.
4. miRNA Wash Solution can be stored at room temp for up to 1 month. For longer term storage, store at 4ºC, but warm to room temperature before use.
5. An aliquot of DEPC water needs to be heated to 95ºC for elution step.
6. Defrosting Media is 80% DMEM and 20% FBS. A 12 mL aliquot is necessary for each sample. To make 12 mL stock, add 9.6 mL of DMEM and 2.4 mL of FBS.
7. Cytospin Media is 95% DMEM and 5% FBS. For a 30 mL stock add 28.5 mL of DMEM and 1.5 mL of FBS.
8. To prepare 50 mM NaOH: dilute 10 mL of stock 5 M NaOH with 990 mL deionized water. This reagent may be stored at room temperature for up to one year.
9. 0.1X TE is made by diluting a stock solution of 100X TE. Add 1 mL of 100XTE to 999 mL of deionized water. This reagent may be store at room temperature for up to one year.

III. PROCEDURE-STEPWISE:

A. Thawing

1. Place 12 mL of defrosting media in a 15 mL conical tube and place in 37°C water bath.
2. Remove the specimen vial from the freezer and place immediately in the 37°C water bath. Constantly shake the vial until the sample is completely thawed. Make sure the cap does not get immersed in water to avoid contamination. It is very important to thaw cells quickly, but gently.
3. Once completely thawed, spray ethanol on tube, wipe off residual ethanol and open the vial. Drop in a few drops of warm defrosting media. Using a sterile pipette, immediately take out all the cells and gently mix them into the defrosting media. Place the tip of the dispensing pipette into the defrosting media and gently move it up and down while dispensing the cell suspension. Do not drop cells through the air or create too many air bubbles while mixing.
4. Centrifuge at 1000 rpm for 8 minutes at room temperature. Pour off supernatant and use finger to tap loose cell pellet in the residual supernatant. Bring volume up to **2.5 mL** with HBSS and take 10 µL of the cell suspension for counting.

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5. Place cell suspension into ice when not in use.

B. Cell Counting

1. Approximate a dilution ratio (cell suspension: 0.4% Trypan blue) that is adequate for counting 10-100 cells per square on a hemacytometer.
2. Load 10 µL of the cell suspension mixture into one side of a hemacytometer with a proper cover slip. Do not overload.
3. Count the number of live cells and then dead cells in one large corner square (divided into 16 smaller ones). Repeat with another corner square. If the cells are too numerous to count, they can be further diluted with Trypan blue.
   a. Be sure to note the ratio of cell suspension to Trypan blue used so that the dilution factor is known for the calculation of the cell concentration.
   b. If the cell counts in the two large squares are highly disparate, count additional squares and use the average.
4. Rinse the hemacytometer with isopropanol and wipe with a Kimwipe between each use. Ensure that the isopropanol is dry before adding a new sample, as residual alcohol may kill viable cells.
5. Enter data into LabVantage to get the cell volume needed for cytospin slides.

C. Cytospin Slides

1. Print 2 slide labels from LabVantage using the "Histology Label" label, Molecular Printer2 printer and label the cytoslides. Place the slides into a cytoclip with the cytofunnel correctly positioned and place the whole unit into the barrel. Be sure the barrel is balanced.
2. Put the LabVantage specified volume of cell suspension for 70,000 cells depending on the cell concentration into two labeled 0.5 mL Eppendorf tubes.
3. Add cytospin media to the cell suspension so that the final volume is 150 µL and mix by pipetting.
4. Load the entire sample into its corresponding cytofunnel. Secure the lid on the barrel, and place barrel back into the cytocentrifuge.
5. Centrifuge at 750 rpm for 3 minutes with medium acceleration on the ThermoShandon Cytospin 4 centrifuge.
6. Place the slides in a histology transport box and deliver to histology for staining.

D. Cell Prep

1. Centrifuge cell suspension for 7 minutes at 400g at 4°C.
2. Pipette off supernatant and keep cell pellets on ice until ready for homogenization.

E. Homogenization

1. Add 600 µL Buffer RLT Plus containing 1% 2-ME to the cell pellet and transfer to a 2 mL safe lock Eppendorf tube and immediately place in a rack at room temperature.
2. Add 3 µL Reagent DX and one 5 mm stainless steel bead to each tube.

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3. Place the tubes (up to 48) in the TissueLyser Adapter Set, making certain the machine is balanced, operate for 30 seconds at 20 Hz.
   NOTE: Prepare a maximum of eight tubes per laboratory technician and begin homogenization in under five (5) minutes to minimize RNA degradation. Excursions above five minutes should be noted on the isolation worksheet.
4. Disassemble the adapter set. Remove tubes from adapter and observe for homogenization.
5. If samples are not completely homogenized, rotate the rack of tubes so that the tubes nearest the TissueLyser are now outer most and reassemble the adapter set. Rearranging the tubes ensures uniform disruption and homogenization.
6. Operate for another 30 seconds at 20 Hz.
7. Repeat step 4.
8. If samples are still not completely homogenized, operate for another 30 sec at 20 Hz.
9. Remove tubes from TissueLyser.
10. Spin tubes down briefly in microcentrifuge and transfer homogenate to a clean, labeled 1.5 mL Eppendorf tube. Do not reuse the stainless steel beads.
11. Centrifuge the homogenate for 3 min at maximum speed (16,100 x g).
12. Carefully remove the supernatant from each sample by pipetting, and transfer it to the AllPrep DNA spin column placed in a 2 mL collection tube (supplied in the AllPrep kit). Avoid aspirating any solids or debris.
13. Close the lid gently, and centrifuge for 30 seconds at 8000 x g.
14. Place the AllPrep DNA spin column into a new, 2 mL collection tube. At this point, the DNA isolation can continue with step G1, or the columns can be stored in the refrigerator for up to 18 hours for later isolation. Use the flow-through for the RNA purification and proceed with step F1.

F. Total RNA purification using the *mir*Vana kit

1. Transfer the flow-through from each DNA column into a separate labeled 2 mL screw cap tube. Adjust the volume of each sample to 600 µL with Buffer RLT Plus containing 1% 2-ME.
2. Add 60 µL (1/10 volume) of miRNA Homogenate Additive to each flow-through, and mix well by vortexing or inverting the tube several times.
3. Leave the mixtures on ice for 10 min. Co-isolation for the DNA could be completed during this incubation.
4. Add 600 µL of Acid-phenol:chloroform to each flow through (volume equal to the lysate volume before addition of the miRNA Homogenate Additive). Be sure to withdraw from the bottom phase in the bottle of Acid-phenol:chloroform, because the upper phase consists of an aqueous buffer.
5. Vortex for 30-60 seconds to mix.

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6. Centrifuge for 10 minutes at 10,000 x g at room temperature to separate the aqueous and organic phases. After centrifugation, the interphase should be compact; if it is not, repeat the centrifugation.
7. Begin heating DEPC treated water for elution now.
8. Carefully remove the aqueous (upper) phase without disturbing the lower phase or interphase layers, and transfer it to a fresh labeled 1.5 mL tube. Note the volume removed.
9. Add 1.25 volumes of room temperature 100% ethanol to each aqueous phase and mix thoroughly by vortexing.
10. For each sample, place a filter cartridge into one of the collection tubes supplied.
11. Pipet each lysate/ethanol mixture onto a filter cartridge. Up to 700 µL can be applied to a filter cartridge at a time. For samples larger than this, apply the mixture in successive applications to the same filter.
12. Centrifuge for 15 seconds at 10,000 x g. **Warning:** Spinning faster than this may damage the filters.
13. Discard the flow-through, and repeat until all of the lysate/ethanol mixture is through the filter. Reuse the collection tube for the washing steps.
14. Apply 700 µL miRNA Wash Solution 1 (working solution mixed with ethanol) to each filter cartridge and centrifuge for 5-10 seconds at 10,000 x g. Discard the flow-through from the collection tubes, and replace the filter cartridge into the same collection tube.
15. Apply 500 µL Wash Solution 2/3 (working solution mixed with ethanol) and centrifuge the filter cartridge for 5-10 seconds at 10,000 x g. Discard the flow-through from the collection tubes, and replace the filter cartridge into the same collection tube. Repeat one time for a total of 2 washes with Wash Solution 2/3.
16. After discarding the flow-through from the last wash, replace the filter cartridge in the same collection tube and spin the assembly at 10,000 x g for 2 minutes to remove residual fluid from the filter.
17. Transfer the filter cartridge into a fresh collection tube (provided with the kit). Apply 100 µL preheated (95º C) DEPC water to the center of the filter, and close the cap. Spin for 20-30 seconds at 10,000 x g to recover the RNA.
18. If multiple columns were required for a sample, combine all eluates into a single tube.
19. Place all samples on ice and proceed to RNA quantification step before freezing.

G. Genomic DNA Extraction

1. Add 500 µL µl Buffer AW1 to the AllPrep DNA spin column. Close the lid gently and centrifuge for 1 minute at 14,000 x g. Transfer the column to a clean 2 mL collection tube.
2. Add 500 µL Buffer AW2 to the AllPrep DNA spin column. Close the lid gently and centrifuge for 2 minutes at 14,000 x g to wash the spin column membrane.

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3. Place one AllPrep DNA spin column per sample in the previously labeled 1.5 mL collection tube. Add 100 µL 0.1X TE (10 mM Tris: 1 mM EDTA, pH 8.0) directly to the spin column membrane and close the lid. Incubate at room temperature for 1 minute and then centrifuge for 1 minute at 14,000 x g to elute the DNA.
4. If multiple columns were required for a sample, combine all eluent into a single tube with a single channel pipette. Discard the empty tubes.
5. Check the samples into the designated PicoGreen plate in LabVantage and place in the corresponding PicoGreen plate in the refrigerator. The physical sample should be accompanied by an empty labeled matrix tube with final storage label for final transfer by the technician completing the picogreen quantification. Proceed to DNA quantification and normalization steps after multiple DNA samples are ready for quantification.

H. Quantification and Normalization of RNA Samples

1. Prepare a set of 0.5 mL tubes with the appropriate LabVantage labels. Add 98 µL of 50 mM sodium hydroxide to each tube.
2. Using a single channel micro-pipette, add 2 µL of the RNA stock sample to the sodium hydroxide. Vortex for at least 5 seconds to ensure that the diluted sample is well mixed and spin briefly. Read the absorbances for 260, 280 and 320nm in a spectrophotometer using a quartz cuvette.
3. Desired sample concentration may vary among projects.
   a. If the concentration is above the desired concentration use the known volumes and concentrations to calculate the amount of DEPC-treated water to add to the sample to yield a final desired concentration as shown below in Example 1.
   b. If the concentration of the RNA sample is less than the desired concentration, use the known volumes and concentrations to calculate the amount to concentrate the sample to the desired volume as shown below in Example 2. This should be accomplished by the use of a speed vac with no heat.
4. After diluting or concentrating samples, repeat step 2 to confirm that the sample is within the target concentration range.
5. Once the samples are at target concentration, transfer the liquid to a labeled matrix tube (Primary sample aliquot). Create a second aliquot for subsequent sample quality control assay (see SOP M002, "RNA Nano Assay").

I. Quantification and Normalization of DNA Samples

1. Refer to SOP M017 for DNA quantification and normalization by PicoGreen.

J. Sample Storage

1. RNA samples should be stored in a liquid nitrogen freezer.
2. DNA samples should be stored in a -80^{o}C freezer.

K. Sample calculations

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Example 1: Samples with concentrations > 0.165 µg/µL need to be diluted using:

Normalization to 0.165 µg/µL
((current concentration/desired concentration) x current volume) – current volume
= ((0.21 µg/µL/0.165 µg/µL) x 18 µL) – 18 µL
= 4.9 µL (Volume of diluent to add)

Final sample volume = 22.9 µL

Example 2: Samples with concentrations < 0.165 µg/µL need to be concentrated using:

Normalization to 0.165 µg/µL
((current concentration/desired concentration) x current volume) – current volume
= ((0.08 µg/µL/0.165 µg/µL) x 18 µL) – 18 µL
= - 9.3 µL (Volume to be removed during speedvac concentration)

Final sample volume = 8.7 µL

IV. REFERENCES

A. Allprep DNA/RNA Mini Kit Handbook (November 2005)
B. mirVana miRNA Isolation Kit Handbook (2011)
C. BCR SOP S009, "Bloodborne Pathogen and Exposure Control Plan"
D. BCR SOP M002 "RNA Nano Assay"
E. SOP MGL-EQP-6 "BIO-MATE UV-Visible Spectophotometer"
F. BCR SOP M003 "Gel Electrophoresis with the E-Gel System"
G. BCR SOP M017 "PicoGreen DNA Quantification (Manual)"
H. BCR SOP M010 "Tissue Matching by SNP Analysis"

V. COMPREHENSIVE REVISION HISTORY

A. Changes made in Version 2, Effective Date 12/31/2014

1. Updated formatting
2. Removed any reference to TCGA
3. Removed any reference to concentration range
4. Removed the reference to creating an aliquot for SNP and Gel Electrophoresis
5. Removed the reference to pooling samples and re-extracting samples.
6. Updated reagents and vendor catalog numbers
7. Updated reference section
8. Removed the step of adjusting volume with RLT before the sample goes through the DNA spin column.

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9. Indicated that this SOP is specific to leukemia
10. Removed specifics of barcodes

B. Version 1, Effective Date 9/14/2012 - New

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Signatures

Approved By: **Signature on file** **Date:** **Date on file**

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