DNA/RNA Co-isolation with Qiagen AllPrep Kit (DNA/RNA) and mirVana (Total & Small RNAs)

This protocol applies to: Clear Cell Sarcoma of the Kidney (CCSK), Neuroblastoma (NBL; after 2013 only), Osteosarcoma (OS), Rhabdoid Tumors of the Kidney (RT), and Wilms Tumor (WT)

The protocol herein describes the procedures used by Nationwide Children's Hospital to process disease tissues for RNA and/or DNA subsequently used for characterization in the NCI's TARGET initiative. All nucleic acid samples used in TARGET projects were quality tested for consistency using picogreen quantification and SSTR genotyping methods, regardless of where the nucleic acid was originally extracted.

I. PRINCIPLE

Qiagen AllPrep kits are designed to isolate DNA and total RNA from small quantities of starting material; however, MGL currently only uses the DNA isolation portion of the kits. In addition, they provide a fast and simple method for the preparation of DNA from human tissues. The purified DNA is ready for use in standard downstream applications such as DNA amplification and expression arrays.

For microRNA (miRNA) analysis, some investigators may require a representative sampling of the total RNA content within the tumor tissue homogenate, most notably the low molecular weight species. Therefore, the flow through from the AllPrep DNA column is taken and the total RNA with small RNAs are isolated with the *mir*Vana kit from Applied Biosystems.

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.

II. SPECIMEN

A. Type

Tumor or normal tissue (snap frozen or OCT-embedded is preferred, fresh is acceptable, scrolls cannot be used), frozen ficolled white blood cells from peripheral blood or bone marrow.

B. Handling Conditions

Standard precautions must be followed when handling all tumor and normal tissue. Tissue samples must be stored in a -80°C freezer prior to isolation. Ficolled white blood cells are stored in liquid nitrogen freezers.

C. Sample Preparation

Tissue is obtained from the BPC, in a 2 mL Safe Lock tube. The weight of tissue requested is 30 mg. Ficolled blood or bone marrow specimens should be thawed according to the Thawing Cyropreserved Specimens procedure, REF-9.

D. Indications for Study

DNA and total RNA isolated from tissue and frozen ficolled white blood cells are to be used for Children's Oncology Group (COG) related research.

III. REQUIRED EQUIPMENT, SUPPLIES AND REAGENTS

A. Equipment

Centrifuge, Capsule
Pipetters - Adjustable, 1-10 μL, 5-20 μL, 20-200 μL, 100-1000 μL
Vortex Mixer
TissueLyser (120V, 50/60 Hz) (Qiagen, catalog# 85210)
TissueLyser Adapter sets (2 X 24)
TEC label printer
Digital dry bath

B. Supplies

AllPrep Kit (Qiagen, catalog# 80204)
mirVana™ miRNA Isolation Kit (Applied Biosystems, catalog# AM1560)
Filtered, sterile pipette tips, assorted sizes
1.5 mL Eppendorf tubes
2 mL screw cap tubes (Fisher, catalog# 02-707-355)
2 mL SafeLock Eppendorf tubes (Fisher, catalog# 022363352)
Wet and dry ice
Forceps
Insulating trays for dry ice
Personal protective equipment
Insulated gloves
Stainless steel beads, 5 mm (Qiagen, catalog# 69989)

C. Reagents

2-mercaptoethanol, 100% (Sigma, catalog# M3148-100 mL)
Absolute ethanol, molecular grade (Sigma, catalog# E7023)
Diethylpyrocarbonate (DEPC)-treated water (Invitrogen, catalog# 750023)

Tris-EDTA Buffer (1X) (Sigma catalog# T9285, 100 mL)
Sodium hydroxide (Sigma catalog# S8263, 150 mL)
Reagent DX (Qiagen catalog# 19088, 1 mL)

IV. WARNINGS / PRECAUTIONS

Use Standard Precautions when handling all body fluids, tissues and cell cultures.

Products and disposable materials used need to be RNase-free, and handled only with gloved hands in order to prevent contamination with skin RNases. 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.

All reagents must be made with RNase-free materials and chemicals, and containers and tubes with samples must be kept covered when possible during the entire procedure to ensure they remain dust-free and RNase-free.

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

V. REAGENT PREPARATION (INCLUDING STORAGE CONDITIONS)

A. 2-mercaptoethanol (2-ME) – must be added to Buffer RLT Plus before use (final 1% 2-ME); record in the QC book or in-lab documentation sheets. Buffer RLT Plus is stable at room temperature for 1 month after addition of 2-ME.

B. 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; record in the QC book or in-lab documentation sheets. Buffer AW1 and Buffer AW2 are stable for 6 months at room temperature.

C. Add 21 mL 100% ethanol to miRNA Wash Solution 1 before use. Add 41 mL 100% ethanol to miRNA Wash Solution 2/3.

D. miRNA Wash Solutions (both 1 and 2/3) can be stored at room temp for up to 1 month. For longer term storage, store at 4°C, but warm to room temperature before use.

E. An aliquot of DEPC water needs to be heated in a 95°C in a dry bath for elution step.

VI. QUALITY CONTROL

A. Specimen identification is assured though each phase of handling by assignment of a specific color of label to each sample. A colored label is placed on each tube and column used throughout the procedure. All tubes are labeled with two identifiers (e.g. USI# and BPC#) and if a matching normal tissue is processed with the tumor then “normal” or “tumor” is included on the label.

B. All new lots of reagents will be tested in parallel with the one in current use before a new lot is put into use. The lot numbers and date of QC are recorded in the QC log book.

C. The tubes from each step are labeled with identifiers and the step and kept until QC has been performed to ensure that DNA and RNA were obtained.

VII. PROCEDURE – STEPWISE

A. Homogenization of Tissue

1. Remove tissues from storage and bury in dry ice immediately.
2. Add 600 µL Buffer RLT Plus containing 1% 2-ME to each 2 mL safe lock Eppendorf tube containing tissue.
3. Add 3 µL Reagent DX and one 5 mm stainless steel bead to each tube.
4. Place the tubes (up to 48) in the TissueLyzer Adapter Set. Making sure the machine is balanced, operate for 2 minutes at 20 Hz.
   Note: Prepare a maximum of eight tubes per laboratory technologist and begin homogenization in under five (5) minutes to minimize RNA degradation.
5. Disassemble the adapter set. Remove tubes from adapter and observe for homogenization; the sample should appear smooth with no visible chunks.
6. If samples are not completely homogenized, rotate the rack of tubes so that the tubes nearest the TissueLyzer are now outer most and reassemble the adapter set. Rearranging the tubes ensures uniform disruption and homogenization.
7. Operate for another 1 minute at 20 Hz.
8. Repeat steps 6 – 7 to observe homogenization.
9. If samples are still not completely homogenized, operate for another 1 minute at 20 Hz for a total of 4 minutes. The duration of disruption and homogenization depends on the tissue being processed. If processing fiber-rich tissues, complete disruption and homogenization may sometimes not be possible.
10. Remove tubes from TissueLyzer.

11. Spin tubes down briefly in microcentrifuge and pipette the homogenate to a clean, labeled 1.5 mL Eppendorf tube. Do not reuse the stainless steel bead – dispose beads in a biohazard bin.
12. Centrifuge the homogenate for 1 minute at maximum speed (16,100 x g) with brake.
13. Carefully remove the supernatant from each sample by pipetting, and transfer into a clean, labeled 1.5 mL Eppendorf tube. Avoid aspirating any solids or debris.
14. Pipette the homogenate (600 mL) into the AllPrep DNA spin column placed in a labeled 2 mL collection tube (supplied in the AllPrep kit). Close the lid gently and centrifuge for 30 seconds at 8,000 x g with brake.
15. Place the AllPrep DNA spin column into a new 2 mL collection tube and store at 4°C for later use in the DNA purification. DNA may be stored on the column for up to 18 hours at 4°C. Proceed immediately by using the flow-through for RNA purification.

B. Homogenization of Ficolled Cells

1. Follow the procedure for Thawing Cryopreserved Cells (MGL-REF-9). Add 600 µL of Buffer RLT Plus containing 1% 2-ME to the 15 mL conical tube containing the cells. Transfer all of this sample to a labeled 2mL safe lock Eppendorf tube.
2. Add 3 µL Reagent DX and one 5 mm stainless steel bead to each tube.
3. Place the tubes (up to 48) in the TissueLyzer Adapter Set. Making sure the machine is balanced, operate for 30 seconds at 20 Hz.
4. Remove tubes from TissueLyzer.
5. Spin down tubes briefly in microcentrifuge and transfer homogenate to a clean, labeled 1.5 mL Eppendorf tube. Do not reuse the stainless steel beads – dispose of beads in a biohazard bin.
6. Centrifuge the homogenate for 3 minutes at maximum speed (16,100 x g) with brake applied.
7. Carefully remove the supernatant from each sample by pipetting and transfer it to the AllPrep DNA spin column placed in a 2 mL labeled collection tube (supplied in the AllPrep kit). Avoid aspirating solids or debris. Close the lid gently and centrifuge for 30 seconds at 8,000 x g.
8. Place the AllPrep DNA spin column into a new, labeled 2 mL collection tube and store at 4°C for later use in the DNA purification. DNA may be stored on the column up to 18 hours at 4°C. Use the flow-through for the RNA purification.

C. 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 briefly or inverting the tube several times.
3. Leave the mixtures on wet ice for 10 minutes.
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 to prevent evaporation.
5. Vortex for 30-60 seconds to mix.
6. Centrifuge for 10 minutes at 10,000 x g with brake at room temperature to separate the aqueous and organic phases. After centrifugation there should be 3 phases, and the interphase should be compact; if it is not, repeat the centrifugation.
7. Carefully remove the aqueous (upper) phase without disturbing the lower phase or interphase layers, and transfer it to a fresh labeled 2 mL tube. Estimate and note the volume removed by the pipette volume.
8. Add 1.25 volumes of room temperature 100% ethanol to each aqueous phase and mix thoroughly by vortexing.
9. For each sample, place a *mir*Vana filter cartridge into a clean, labeled collection tube.
10. 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.
11. Centrifuge for 15 seconds at 10,000 x g with brake applied at room temperature. Warning: Spinning faster than this may damage the filters.
12. 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 (13 and 14)
13. 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 with brake applied at room temperature. Discard the flow-through from the collection tubes, and replace the filter cartridge into the same collection tube.
14. 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 at room temperature with brake. Discard the flow-through from the collection tubes, and replace the filter cartridge into the same collection tube.
15. Repeat step 14 once more.
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 at room temperature with brake to remove residual fluid from the filter.

17. Transfer the filter cartridge into a fresh, labeled collection tube (provided with the kit). Apply 100 µL preheated (95°C) DEPC water to the center of the filter, and close the cap. Spin for 20-30 seconds at 10,000 x g at room temperature with brake to recover the RNA.
18. Place all samples on ice and proceed to RNA quantification step before freezing (refer to GEN-2, Quantitation of Nucleic Acids)

D. Genomic DNA Extraction

1. Retrieve the AllPrep DNA spin column from the refrigerator.
2. Add 500 µL Buffer AW1 to the AllPrep DNA spin column. Close the lid gently and centrifuge for 1 minute at 14,000 x g at room temperature with brake. Discard the flow-through.
3. 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 at room temperature with brake. Discard the tube containing the flow through.
4. Place one AllPrep DNA spin column per sample in a labelled 1.5 mL collection tube. Add 100 µL 0.1X TE 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 at room temperature with brake to elute the DNA.

E. Quantiation of Nucleic Acids

DNA: Refer to the SOP for DNA quantification and normalization by PicoGreen.

RNA: Read the absorbance for 260, 280 and 320nm in a spectrophotometer using a quartz cuvette.

VIII. INTERPRETATION / ANALYSIS / DOCUMENTATION

A. Enter the following information into the appropriate TARGET spreadsheet: TARGET ID, BPC#, COG#, sample volume, sample concentration (ng/µL), yield (µg), and any necessary additional QC and comments.

B. For RNA QC refer to the RNA Nano Assay SOP

C. For DNA QC refer to the Gel Electrophoresis with the E-Gel System and Identifiler SSTR SOPs.

IX. REFERENCES

1. QIAGEN AllPrep Kit Handbook
2. *mir*Vana miRNA Isolation Kit Handbook