Standard Operating Procedure (SOP) for DNA/RNA Extraction with Allprep (DNA) and MirVana (Total RNA with Small RNA)

I. SCOPE AND PURPOSE

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 arrays.

For micro RNA analysis, the downstream characterization centers 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 is isolated with the *mir*Vana kit from Applied Biosystems (Life Technologies). The AllPrep kit utilizes the RNeasy prep which excludes small RNAs.

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, and nitrile gloves. Liquid nitrogen and dry ice are extremely cold and may cause 'burns'. Wear cryogenic gloves designed to withstand extremely cold temperatures when handling samples stored in liquid nitrogen and large quantities of dry ice.
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 is an asphyxiate; all work should be conducted in a well-ventilated room.
4. 2-mercaptoethanol (2-ME) is toxic. The 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.
5. Buffer RLT Plus and Buffer AW1 contain a guanidine salt which is not compatible with disinfectants containing bleach.
6. 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.
7. Acid-phenol:chloroform contains phenol, which is a poison and an irritant. Use gloves and personal protective equipment when working with this reagent.

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8. Buffer AW1 contains guanidine thiocyanate. PPE must be used when handling this reagent.

B. Quality Control

1. The incoming tissue samples have a printed label with a 2D barcode and human readable format. The 2D barcode contains the internal LabVantage ID; the human readable has the internal LabVantage ID, CCG BCR barcode, and TSS identifier.
2. Working labels (containing the internal LabVantage ID, CCG BCR barcode, and scannable 2D barcode) are printed from LabVantage and used throughout the extraction process. Labels are printed that match the original LabVantage ID (to follow the subportion) and the corresponding newly created (DNA or RNA) LabVantage IDs. 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 (internal LabVantage ID (DNA or RNA), CCG BCR barcode, TSS identifier, and 2D barcode) 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 subportion 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 BCR-MGL-EQP-1 "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. No aliquot of original specimen, DNA or any other reagent should ever be returned to the original container after sampling.
8. 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.
9. The isolation kit is tested against predetermined specifications to ensure consistent product quality.

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10. All new lots of reagents are tested in parallel with the one in current use before being put into use. All kit components must be quality control tested and used together thereafter. All reagents supplied in a kit must be used only with other reagents in the same kit lot number; reagents with identical lot numbers cannot be used interchangeable between kit lot numbers. Results are recorded on positive control extraction worksheets. All QC results are recorded in the Quality Control notebook.
11. 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.
12. 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: Frozen tissues or control cell line cultured cells.
2. Handling Conditions: Follow standard precautions when handling all tissues or cultured cells. Samples should be stored in liquid nitrogen vapor phase until analytes can be isolated.
3. Sample Preparations: Tissues are prepared by Logistics by cutting a piece (typically 10-30 mg) of frozen tissue and placing into a 2-mL Eppendorf safe-lock tube.
4. Indications for Study: This procedure should be used when DNA and RNA are needed from the same piece of tissue. 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

a. UV visible spectrophotometer
b. Capsule centrifuge
c. Digital dry bath
d. Liquid nitrogen freezer
e. Microcentrifuge
f. Multi-channel and single channel pipettes
g. Qiagen TissueLyser
h. Vortexer

2. Supplies

a. AllPrep DNA/RNA Mini Kit (50) (Qiagen, 80204)

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b. *mir*Vana™ miRNA Isolation Kit (Applied Biosystems, AM1560)
c. Filtered, sterile pipette tips, assorted sizes
d. 1.5 mL Eppendorf tubes (Fisher, #05-408-137)
e. 0.5 mL tubes (Fisher, #05-408-128)
f. 2 mL screw cap tubes (Fisher, 02-707-355)
g. 2 mL SafeLock Eppendorf tubes (Fisher, 022363352)
h. Wet and dry ice
i. Insulating trays for dry ice
j. Personal protective equipment (PPE), including insulated gloves
k. Stainless steel beads, 5 mm (Qiagen, 69989)

3. Reagents

a. 2-mercaptoethanol (2-ME), 100% (Sigma, M3148)
b. Absolute ethanol, molecular grade (Sigma, E7023)
c. Diethylpyrocarbonate (DEPC)-treated water (Invitrogen, 750023)
d. Tris-EDTA Buffer (100X) (Sigma, T9285)
e. Sodium hydroxide, 5M (Sigma, S8263)
f. Reagent DX (Qiagen, 19088)
g. RNase Zap (Ambion, M9780)
h. Water nuclease-free (Sigma, # W4502-6X1L)

Notes: 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.

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

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- 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.

E. Reagent Preparation (including storage conditions):

1. 2-mercaptoethanol (2-ME) – must be added to Buffer RLT Plus before use (final 1% 2-ME). Add 450 µL of 2-ME to an unopened stock bottle of Buffer RLT Plus (45ml). Buffer RLT Plus is stable at room temperature for 1 month after the 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 in the refrigerator, but warm to room temperature before use.
5. An aliquot of DEPC water needs to be heated to 95ºC for the elution step.
6. To prepare 50 mM NaOH: dilute 10 mL of stock 5M NaOH with 990 mL deionized water. This reagent may be stored at room temperature for up to one year.
7. 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.

F. Homogenization

1. Remove tissues from liquid nitrogen storage and place in dry ice.
2. Add 600 µL Buffer RLT Plus containing 1% 2-ME to each 2 mL safe lock Eppendorf tube containing 25-30 mg of tissue and immediately place in a rack at room temperature.
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 TissueLyser Adapter Set (see MGL-EQP-21: Qiagen TissueLyser II), making certain the machine is balanced, operate for 2 minutes at 20 Hz.

   Note: Prepare a maximum of eight tubes and begin homogenization in under five (5) minutes from thawing to minimize RNA degradation. Excursions above five minutes should be noted on the isolation worksheet.
5. Disassemble the adapter set. Remove tubes from adapter and observe for homogenization.
6. If samples are not completely homogenized, then rotate the rack of tubes so that the tubes nearest the TissueLyser are now outermost and reassemble the adapter set. Rearranging the tubes ensures uniform disruption and homogenization.
7. Operate the TissueLyser for another 1 minute at 20 Hz.
8.
9. If samples are still not completely homogenized, operate for another 1 minute at 20 Hz. The duration of disruption and homogenization depends on the tissue being processed. If processing fiber-rich tissues, then complete disruption and homogenization may not be possible. Homogenization should not be attempted for more than 4 minutes total at 20 Hz, as this may result in shearing the nucleic acids.
10. Remove tubes from TissueLyser.
11. 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; discard in biohazard waste.
12. Centrifuge the homogenate at room temperature for 3 minutes at maximum speed (16,100 x g).

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13. 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.
14. Close the lid gently, and centrifuge at room temperature for 30 seconds at 8,000 x g.
15. Place the AllPrep DNA spin column into a new, 2 mL collection tube. At this point, the DNA isolation can continue with step II.H.1 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 by proceeding with step II.G.1.

G. Total RNA purification using the mirVana 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. Place mixture on ice for 10 minutes. 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). 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.
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, mix thoroughly by vortexing and spin briefly to collect. For example, for a 600 µL aqueous phase, add 750 µL of 100% ethanol.
10. For each sample, place a mirVana RNA 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 larger samples, apply the mixture in successive applications to the same filter.
12. Centrifuge at room temperature for 15 seconds at 10,000 x g. Warning: Spinning faster than this may damage the filters.
13. Discard the flow-through, and repeat steps 11 and 12 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 at room temperature 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 (room temperature).
16. Discard the flow-through from the collection tubes, and replace the filter cartridge into the same collection tube and repeat step II.G.15.
17. 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.
18. Transfer the filter cartridge into a fresh collection tube (provided with kit). Apply 100 µL preheated (95º C) DEPC water to the center of the filter, and close the cap. Centrifuge at room temperature for 20-30 seconds at 10,000 x g to recover the RNA.
19. If multiple columns were required for a sample, combine all eluates into a single tube.
20. Place all samples on wet ice immediately and proceed to RNA quantification step before freezing.

H. Genomic DNA Extraction

1. Add 500 µL Buffer AW1 to the AllPrep DNA spin column (prepared in 2.F.15). Close the lid gently and centrifuge for 1 minute at 14,000 x g at room temperature. Transfer the column to a clean 2mL 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 at room temperature to wash the spin column membrane.
3. Place AllPrep DNA spin column in the previously prepared 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 at room temperature 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. Do not store in the refrigerator for more than one week.

I. Quantification and Normalization of RNA Samples

1. Measure and record the volume of the RNA from the extraction procedure.
2. Prepare a set of 0.5 mL tubes with the unique RNA sample identifier. Add 98 µL of 50 mM sodium hydroxide to each tube.
3. Add 2 µL of the concentrated stock RNA sample to the sodium hydroxide. Vortex for at least 5 seconds to ensure that the diluted sample is well mixed and briefly spin down. Read and record the absorbance for 260, 280 and 320 nm in a spectrophotometer using a quartz cuvette.

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4. 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 (see MGL-EQP-23: DNA Centrifugal Concentrators) with no heat.
5. After diluting or concentrating samples, repeat step 3 to confirm that the sample is within the target concentration range. NOTE: The starting volume of RNA must be known to complete all calculations.
6. Once the samples are at target concentration, transfer the liquid to a labeled matrix tube (Primary sample aliquot) for final storage. Create a 2.5 µL aliquot for subsequent sample quality control assay (see SOP M002, "RNA Nano Assay").

J. Quantification and Normalization of DNA Samples - Refer to SOP M017 for DNA quantification and normalization by PicoGreen.

K. Sample Storage

1. RNA samples should be stored in a liquid nitrogen freezer.
2. DNA samples should be stored in a -80ºC freezer.

L. Sample Calculations

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

Example:

((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

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

Example:

((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

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III. REFERENCES

A. Allprep DNA/RNA Mini Kit Handbook (November 2005)
B. mirVana miRNA Isolation Kit Handbook (2011)
C. BCR-REF-001, "BCR Acronym List"
D. BCR-SOP-M002, "RNA Nano Assay"
E. BCR-SOP M003, "Gel Electrophoresis with the E-gel System
F. BCR-SOP M010 "Tissue Matching by SNP Analysis"
G. BCR-M017, "Picogreen DNA Quantification Manual"
H. BCR-SOP-S009, "Bloodborne Pathogen and Exposure Control Plan"
I. SOP MGL-EQP-6 "BIO-MATE UV-Visible Spectrophotometer"

IV. COMPREHENSIVE REVISION HISTORY

A. Changes made in Version 4, Effective Date 4/27/2016
1. Made title not all capitalize
2. Updated equipment SOP number for BioMate UV Spec.
3. Specimen Information - added that tissue pieces are "typically" 10 -30 mg. and that procedure is used when "specimen" DNA and RNA are needed from the same piece of tissue.
4. All centrifugation steps are completed at room temperature.
5. Procedure 13. Corrected numbers or steps to repeat for washing (11 and 12)
6. Procedure 20. specified that purified RNA is put on "wet" ice "Immediately" after eluted.
7. H.1. Specified step where DNA column is taken.
8. Added reference for DNA concentrators.
9. Added NOTE that "Starting volume of RNA must be known to complete all calculations.

B. Changes made in Version 3, Effective Date 09/04/2014
1. In sections II.F.8. and II.G. 16., clarified the steps to follow
2. In section II. F.15., updated the steps to follow

C. Changes made in Version 2, Effective Date 09/02/2014
1. New format used
2. Removed any reference to TCGA
3. Removed any reference to concentration range
4. Removed the step of adjusting volume with RLT before the sample goes through the DNA spin column.
5. Removed the reference to creating an aliquot for SNP and Gel Electrophoresis
6. Removed the reference to pooling samples and re-extracting samples.

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

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Signatures

Approved By: Signature on file

Julie Gastier-Foster, PhD, FACMG
Principal Investigator

Date: Date on file