STANDARD OPERATING PROCEDURE (SOP) FOR DNA/RNA Extraction with AllPrep (DNA) and mirVana (Total RNA with small RNA) 3 Column-Modified Melanoma Protocol

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.

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.

This protocol has specifically been prepared to reduce melanin associated with RNA isolated from melanoma tumor samples. Melanin is a naturally occurring cation exchange material known to intercalate with DNA and crosslink with RNA under oxidative conditions (White, 1958; Geng, 2010; Wilkins, 2006). Removal of melanin from RNA derivatives is important as melanin interferes with mRNA/miRNA analysis by reversibly binding to DNA Polymerase and inhibiting RT-PCR (Eckhart, 2000). Furthermore, melanin complicates quantitative approaches for determining RNA concentration by interfering with UV absorbance and fluorescence emissions.

This protocol varies from the standard AllPrep *mir*Vana protocol in that it includes an overnight incubation of the mirVana eluted RNA with the cationic detergent cetyl-trimethylammonium bromide (CTAB)-urea. CTAB is widely used to separate proteins and polysaccharides from nucleic acids, as it selectively forms complexes with anionic nucleic acids (Lagonigro, 2004). This results in solution where charged nucleic acids can be purified from neutral proteins and polysaccharides. Urea is included in the solution as it has been found to enhance the specificity of the reaction (Lagonigro, 2004). In this protocol, melanotic RNA is complexed with CTAB-urea to facilitate isolation of charged RNA from the insoluble uncharged melanin.

II. PROCEDURE

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A. Safety precautions

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 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.
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.
8. CTAB solubility: 0.3 g/100 mL dH_2O at 20°C, up to 10% by heating to 65°C.

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
2. Working labels are printed from LabVantage and used throughout the extraction process. Labels are printed that match the original portion and subportion LabVantage ID 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 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 (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 (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 (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.
10. 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.
11. The CTAB-UREA solution is made fresh with each extraction. This solution must be used on a control melanoma sample once a week when any melanoma extraction is completed. The control melanoma samples are highly pigmented samples, therefore after using the CTAB-UREA one must document the change in color of the flow-through from Day 1 and Day 2 of the procedure. Due to the limited number of control melanoma samples, it is not necessary to run a control each time an extraction is performed.
12. At each step in the RNA and DNA isolation, the supernatant or pellet that should not contain the RNA or DNA is retained until after isolation and quantitation is completed.
13. 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:

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1. Type: Frozen Melanoma tumor or adjacent normal tissues.
2. Handling Conditions: Follow standard precautions when handling all tissues. Samples should be stored in liquid nitrogen until analytes can be isolated.
3. Sample Preparations: Tissues are prepared by Logistics by cutting 25-30 mg pieces of frozen tissue and placing into a 2-mL Eppendorf lock tube.
4. Indications for Study: This procedure should be used when DNA and RNA are needed from the same piece melanoma 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

UV visible spectrophotometer
Capsule centrifuge
Digital dry bath
Liquid nitrogen freezer
Freezer (-20^{o}C)
Microcentrifuge, Eppendorf 5415 R
Multi-channel and single channel pipettes
Refrigerator (4^{o}C)
Qiagen TissueLyser
Vortex
Water bath

2. Supplies

AllPrep DNA/RNA Mini Kit (Qiagen 80204)
*mir*Vana™ miRNA Isolation Kit (Ambion, Cat# AM1560)
Filtered, sterile pipette tips, assorted sizes
1.5 mL Eppendorf tubes (Fisher, 05-408-137)
0.5ml 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)

3. Reagents

2-mercaptoethanol (2-ME), 100% (Sigma M3148-100 mL)
Absolute ethanol, molecular grade (Sigma, E7023)
Diethylpyrocarbonate (DEPC)-treated water (Invitrogen, 750023)
Tris-EDTA Buffer (1X) (Sigma T9285, 100 mL)
Sodium hydroxide (Sigma S8263, 150 mL)
Reagent DX (Qiagen 19088, 1 mL)

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RNase ZAP (Ambion, M9780)
5M NaCL (Ambion AM9760G, 100mL)
CTAB, Hexadecyltrimethylammonium bromide, BioUltra, >=99.0%, (Sigma 52365-50G, 50g)
Urea, Ultrapure MB grade (Ambion AM9902, 1kg)
0.5M EDTA pH 8.0 (Ambion AM9260G, 100mL)
1M Tris:Cl pH 7.0 (Ambion AM9850G, 100mL)
Water, Molecular Biology Reagent, DNase/RNase Free (Sigma W4502, 1L)

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.

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). 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. Buffer AW1 and Buffer AW2 are stable for one year.
3. Add 21 mL 100% ethanol to miRNA Wash Solution 1 before use. Add 41 mL 100% ethanol to miRNA Wash Solution 2/3.
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. Store Elution Solution at -20ºC, 4ºC or room temp.
6. An aliquot of DEPC water needs to be heated to 95ºC for elution step.
7. 10 M Urea solution: Make at least 1 day prior to homogenizing the specimen(s): 30.035 g Urea, bring up to 50 mL with RNAse-free dH_20; Sterile filter 0.2 μM; store at room temperature. Heat to 37°C to re-solubilize. It is stable up to 6 months.
8. 10% CTAB solution: Make at least 1 day prior to homogenizing the specimen(s): 5.0 g CTAB, bring up to 50 mL with RNAse-free dH_20, heat to 37°C to dissolve, sterile filter 0.2 μM store at room temperature; stable up to 6 months. Heat to 65°C to re-solubilize.
9. CTAB-UREA solution (50 mM Tris-HCl pH 7.0, 1% CTAB, 4 M Urea, 1mM EDTA): Make fresh same day. Add together in sterile conical tube: 1.25 mL 1 M Tris-HCl pH 7.0; 2.50 mL 10% CTAB; 10.00 mL 10 M Urea; 0.05 mL 0.5 M EDTA and 11.20 mL RNAse-free water to obtain a final volume of 25 mL working solution. Keep at 25°C -30°C (to avoid precipitation) until just before use. It is stable less than 3 days.

F. Homogenization

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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, making certain the machine is balanced, and operate for 2 min 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.
5. Disassemble the adapter set. Remove tubes from adapter and observe for homogenization.
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 min at 20 Hz.
8. Repeat step 5.
9. If samples are still not completely homogenized, operate for another 1 min at 20 Hz. 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 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.
12. Centrifuge the homogenate for 3 min at maximum speed (16,100 x g).
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 for 30 seconds at 8000 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 I1, 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.

G. 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.

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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.
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 to 95°C.
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. Spin tubes down briefly in microcentrifuge.
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.
16. Discard the flow-through from the collection tubes, replace the filter cartridge into the same collection tube, and repeat step 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 the kit). Apply 100 µL preheated (95ºC) 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.
19. Bring the volume up to 100 µL with DEPC water.

H. CTAB-urea separation of RNA from Melanin

1. Set the filter cartridge aside and add 32.5 µL 5 M NaCl to the eluent and mix gently by inversion.
2. Add 400 µL CTAB-UREA (4 volumes) to the sample and mix gently by inversion.

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3. Incubate sample overnight at 4°C; slow rotation may help the detergent break up and capture the melanin. Make sure cap is tight. Place Parafilm around cap to keep it sealed.
4. The next day, retrieve the sample from the refrigerator and add 53 µL miRNA Homogenate Additive. Mix well by vortexing or inverting the tubes several times.
5. Incubate the mixtures on ice for 10 min.
6. Pipet each 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.
7. Begin heating DEPC-Treated water to 95°C.
8. Centrifuge for 15 seconds at 10,000 x g. **Warning:** Spinning faster than this may damage the filters.
9. Discard the flow-through, and repeat until all of the mixture is through the filter. Reuse the collection tube for the washing steps.
10. 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.
11. 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.
12. Discard the flow-through from the collection tubes, replace the filter cartridge into the same collection tube, and repeat step 11.
13. 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.
14. Transfer the filter cartridge into a fresh collection tube (provided with the kit). Apply 100 µL preheated (95ºC) 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.
15. Remove the filter cartridges and place the samples on ice before proceeding to the RNA quantification step.

I. Genomic DNA Extraction

1. 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. 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.
3. Place one AllPrep DNA spin column per sample in the previously prepared 1.5 mL collection tube. Add 100 µL 0.1 X 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.

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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 in the designated PicoGreen plate in LabVantage and place in the corresponding PicoGreen plate in the refrigerator (4°C). 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.

J. 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. Using a single channel micro-pipette, add 2 µL of the concentrated stock 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 the absorbance for 260, 280 and 320nm in a spectrophotometer using a quartz cuvette.
4. Desired sample concentration may vary among projects.
    a. If the concentration is above the expected 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 shown below in Example 2. This should be accomplished with the use of a speed vac with no heat.
5. After diluting or concentrating samples, repeat step 3 to confirm that the sample is within the target concentration range.
6. Once the samples are at target concentration, transfer the liquid to a labeled matrix tube (Primary sample aliquot) for final storage. Create a 2.5uL aliquot for subsequent sample quality control assay (SOP M002, "RNA Nano Assay").

K. Quantification and Normalization of DNA Samples

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

L. Sample Storage

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

M. Sample Calculations

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

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= ((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

III. REFERENCES

A. Allprep DNA/RNA Mini Kit Handbook (November 2005)
B. mirVana miRNA Isolation Kit Handbook (2011)
C. Lagonigro M, De Cecco L, Carninci P, Di Stasi D,Ranzani T, Rodolfo M, and Gariboldi M. CTAB–Urea Method Purifies RNA from Melanin for cDNA Microarray Analysis. Pigment Cell Research 2004; 17:3: 312–315.
D. Eckhart L, Bach J, Ban J, Tschachler E. Melanin Binds Reversibly to Thermostable DNA Polymerase and Inhibits Its Activity. Biochemical and Biophysical Research Communications 271, 726–730 (2000)

IV. COMPREHENSIVE REVISION HISTORY

A. Changes made in Version 2, Effective Date 12/31/2014
1. Updated format
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.
7. Added a reference section

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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