I. NAME OF PROCEDURE: MDS Bone Marrow Ficoll

II. PURPOSE:

a. For isolating bone marrow mononuclear cells (BMMNCs) from bone marrow aspirates of study participants with myelodysplastic syndromes (MDS) and idiopathic cytopenias of undetermined significance (ICUS)

III. SCOPE:

a. Starting material is between 5-10 ml of bone marrow aspirate in a green top heparin tube. For information regarding cell processing steps, see SOP for "Sample Receipt". The first 2 x 10^7 cells are to be cryopreserved and all remaining cells are to be used for $CD34^+$ cell enrichment.

IV. EQUIPMENT, REAGENTS AND SUPPLIES:

a. 50 ml sterile conical tubes: Corning Cat. No. 430828
b. 5, 10, and 25 ml sterile pipettes: Corning Cat. Nos. 4487, 4488, and 13-678-11
c. Pipette aid
d. 10X Pharm Lyse solution, 100 ml: Becton Dickinson Cat. No. 555899
e. Lymphocyte Separation Medium (LSM): Ficoll-Paque Plus, GE Healthcare, Cat. NO. 17-1440-03
f. Sterile Phosphate Buffered Saline w/o Ca++ and Mg++ (1X PBS): Corning Cat. No.21040CM or Sigma 59321C
g. 1.8 ml cryovials: Thermo Scientific Cat. No. 375418
h. 190 proof ethanol: University of South Florida Tax-Free Ethyl Alcohol Order Form
i. Isopropanol: Sigma Cat. No. D2650-100ML
j. Kimwipe: Kimberly Clark 34155
k. 22 gauge blunt end needles: Lawson Cat. No. 220950
l. Sterile wooden applicator sticks (Puritan #258072W): Fisher Cat. No. 22-029-641
m. 3 cc syringes: Lawson Cat. No. 110150
n. Sharps containers, 1 qt: Fisher Cat. No. 22-037-970
o. Benchtop centrifuge
p. Mr. Frosty freezing containers: Fisher Cat. No. 15-350-50
q. Medicus Health Blood Tube Rocker (Cat. No. 5265M2): Fisher Cat. No. NC0139535
r. 4°C refrigerator
s. -80°C freezer
t. Cryounit liquid nitrogen

V. PROCEDURE: (NOTE: All techniques to be performed using standard aseptic techniques in a biosafety cabinet)

a. Prepare 1X Pharm Lyse buffer by diluting 10X Pharm Lyse buffer to 1X with sterile H_2O
b. Invert the tube several times to mix and keep at room temperature until needed
c. Clean hood by wiping all work surfaces with 70% alcohol with Kimwipe and replace isopropanol in Mr Frosty if necessary (after every 5 uses)
d. Pipet 15 ml sterile ficoll into two sterile 50 ml conical tubes

i. Only 1 tube of Ficoll is needed if the volume of bone marrow is 5 ml or less

e. Pipet 30 ml sterile 1X PBS into a sterile 50 ml conical tube
f. Unscrew the top from a 50 ml conical tube (to be used for dispensing of the bone marrow)
g. Remove the stopper from the 10 ml green top heparin bone marrow vacutainer tube
h. Use a sterile wooden applicator stick to remove any clots from the tube
i. Attach a 22 gauge blunt end needle to a 3 cc syringe
j. Draw the bone marrow from the vacutainer tube into the syringe, pass it through the needle 3 times to break up the spicules, transfer it to a sterile 50 ml conical tube
k. DISPOSE SYRINGES INTO A SHARPS CONTAINER. SYRINGES ARE NOT TO BE DISPOSED OF IN THE RED BIOHAZARDOUS WASTE TRASH BAGS
l. FICOLL PROCEDURE
    i. ALWAYS DILUTE THE BONE MARROW 1:3 WITH STERILE 1X PBS
    ii. ALWAYS LAYER 20 ML DILUTED BONE MARROW ON TOP OF 15 ML FICOLL
m. Dilute the bone marrow to a final volume of 40 ml (1:3 dilution) with sterile 1X PBS and layer it on top of the ficoll as follows
    i. Use a sterile 10 ml pipet to bring the volume of the bone marrow up to 10 ml with 1x sterile PBS from the stock bottle
    ii. Using the same 10 ml pipet from the previous step, pipet 10 ml PBS from the tube in step e., rinse the vacutainer tube and transfer it to the bone marrow tube
    iii. Using a sterile 25 ml pipet, transfer 16 ml PBS from the tube in step e. to the bone marrow tube and mix well (Total of 36 ml in the bone marrow tube)
        1. It is important to mix well to dilute the red blood cells so they don't clump together with white blood cells and pass through the ficoll gradient
    iv. Using the same pipet from step iii., slowly layer 18 ml diluted bone marrow on top of the ficoll from step d., without mixing the bone marrow with the ficoll
    v. Using the same pipet from step iii., rinse the bone marrow tube with the remaining 4 ml PBS from step eand slowly add 2 ml each to the ficoll tubes from step d.
n. Centrifuge the tubes with brake off at 400 g/30min/19°C/no brake in swinging bucket rotor.
o. After centrifugation collect the enriched cell fraction (interphase) which is below the top plasma layer and above the ficoll layer
p. Transfer the interphase to a new 50 ml conical tube and add 3 volumes of sterile 1X PBS
    i. Sometimes two tubes are needed, depending on how much volume is collected
    ii. To avoid contamination, use a new pipet each time you are pipetting from the bottle of 1X sterile PBS
q. Centrifuge 400g/ 8 min/ 20ºC/ brake 3
r. Remove the supernatant (~ 40 ml) with two sterile 25 ml pipets
s. Use a sterile 5ml pipet to resuspend the cell pellet in 5 ml 1X BD Pharm Lyse, incubate 10 minutes at room temperature on the Blood Tube Rocker
t. Using a sterile 25 ml pipet, add 25 ml sterile 1X PBS to the cells
u. Centrifuge 400g/ 8 min/ 20ºC/ brake 3
v. Remove the supernatant with a sterile 25 ml pipet
w. Use a sterile 25 ml pipet to resuspend the cell pellet in 20ml of sterile 1X PBS.

i. Resuspend smaller cell pellets in less than 20 ml to get an accurate cell count but remember to BRING THE VOLUME UP TO 20 ML before centrifuging

x. Remove 11 µl cells and mix it with 11 µl AOPI staining solution

y. Centrifuge the cells from step w. 400g/ 8 min/20ºC/ brake 3

z. While the cells are centrifuging, count the cells on a Nexcelom A2000 cell counter (See the Cell counting and Viability SOP)

i. The optimal range is 100 – 1,000 cells counted

1. If the number of cells counted is less than 100, centrifuge, resuspend in smaller volume and recount
2. If the number of cells counted is greater than 1,000, dilute the sample and recount

aa. Calculate the total number of cells and allocate them as follows

bb. The first 20 x 10^6 cells are to be frozen

cc. The remaining cells are to be used for $CD34^+$ cell enrichment.

dd. Freeze 5 x 10^6 cells per aliquot with a minimum of 2 aliquots To freeze cells, see the Cryopreservation of Cells SOP

ee. To isolate $CD34^+$ cells, see the $CD34^+$ Enrichment SOP

VI. RESOURCES

VII. DISCUSSION OF LIMITATIONS:

VIII. QUALITY CONTROL:

a. Refer to Bone Marrow Colony Formation Assay Quality Assurance Data Form

b. Refer to Bone Marrow Heparin Tube Clotting_ Quality Assurance Data Form

c. Refer to Bone Marrow Plasma Hemolysis Quality Assurance Data Form

d. Record % viable cells and total number of viable cells based on Nexcelom A2000 cell counter (See the Cell counting and Viability SOP)

I. NAME OF PROCEDURE: MDS Bone Marrow Plasma Collection

II. PURPOSE:

a. For collecting plasma from bone marrow aspirates of study participants with myelodysplastic syndromes (MDS) and idiopathic cytopenias of undetermined significance (ICUS)

III. SCOPE:

a. Starting material is between 5-10 ml of bone marrow aspirate in a green top heparin vacutainer tube and 3 ml bone marrow aspirate in a purple top EDTA vacutainer tube. Anticoagulants distinguish plasma from serum. Plasma comes from blood that is collected in tubes, syringes, or bags containing anticoagulants such as EDTA and heparin. Serum comes from blood that is collected without anticoagulants

IV. EQUIPMENT, REAGENTS AND SUPPLIES:

a. 5 ml sterile pipettes: Corning Cat. No. 4487
b. 2 ml sterile pipettes: Fisher Cat. No. 07-200-572
c. 15 ml conical tubes: Lawson Cat. No. 215960
d.
e. Pipette aid
f. 0.5 ml Matrix tubes, Thermo Scientific No. 3743: Fisher Cat. No. NC1323395
g. 0.5 ml Matrix tube storage boxes, Thermo Scientific No. 4900:
h. Fisher Cat. No. 50-871-150
i. Benchtop centrifuge
j. -80°C freezer

V. PROCEDURE: (NOTE: All techniques to be performed using standard aseptic techniques in a biosafety cabinet)

i. Place the 10 ml green top heparin and 3 ml purple top EDTA vacutainer tubes containing bone marrow into the centrifuge and balance with tubes containing the same volume of water
ii. Centrifuge 600g/ 15'/room temperature with brake off or on lowest possible setting
iii. Pipet the plasma (top layer) from the vacutainer tube without disturbing the interface (middle layer) and dispense the plasma into separate 15 ml conical tubes
iv. Centrifuge 2,000g/ 10'/room temperature with brake off or on lowest possible setting
v. Remove the tubes from the centrifuge and hold them in front of the Specimen Integrity Chart for Hemolysis. Match the color of the plasma to the appropriate color on the chart and record the hemolysis level associated with that color, which ranges from 0- 8gL
vi. Pipet the plasma from each tube without disturbing the pellet and dispense 250 µl of plasma into 0.5 ml Matrix tubes, with a minimum of 2 aliquots and maximum of 10 aliquots. Dispense any remaining plasma into a 1.8 ml cryovial
vii. Store the plasma in Matrix storage boxes in the -80 freezer

1. **KEEP THE TUBES OF PLASMA AT 4ºC until they are stored in the -80ºC freezer**

VI. RESOURCES

a. **Forms**
b. **References to Other Essential SOPs**
c. **Terms, Definitions & Abbreviations**
d. **Other Resources**
e. **Literature**
f. **References**

I. NAME OF PROCEDURE: MDS Bone Marrow Smears

II. PURPOSE:

a. For making bone marrow smears from bone marrow aspirates from study participants with myelodysplastic syndromes (MDS) and idiopathic cytopenias of undetermined significance (ICUS)

III. SCOPE:

a. Starting material is a bone marrow aspirate in a 3ml EDTA tube. Three (3) smears will be made from each sample

IV. EQUIPMENT, REAGENTS AND SUPPLIES:

a. 100 mm diameter watch glass: Cole Parmer Cat. No. 34508-58
b. Pasteur pipets: Fisher Cat. No. 22042816
c. Pipet bulbs: Fisher Cat. No. S32324
d. Frosted microscope slides: Fisher Cat. No. 12-544-2

V. PROCEDURE: (NOTE: All techniques to be performed using standard aseptic techniques in a biosafety cabinet)

a. Pour ~0.5 ml blood from a 5 ml EDTA tube into a watch glass
b. Aspirate a spicule and a tiny amount of blood into a Pasteur pipet and dispense one droplet onto a microscope slide
c. Touch another microscope slide to the droplet of blood and gently smear it across the slide
d. Don't smear to the end of the slide but don't lift the top slide off early. The tricky part is sliding the top slide all the way to the end of the bottom slide and gradually lifting the top slide as it gets to the end of the bottom slide
e. Place the slide in a slide heater for 2 minutes
f. Transport slides to MCC for Wright Giemsa stain and Prussian blue stain (see Histopathology flowchart SOP)

VI. RESOURCES

a. Forms
b. References to Other Essential SOPs
c. Terms, Definitions & Abbreviations
d. Other Resources
e. Literature
f. References

The National Myelodysplastic Syndromes Natural History Study Sponsored by the National Heart, Lung, and Blood Institute in collaboration with the National Cancer Institute NHLBI Contract: HHSN268201400003I	**SOP Number:**	MDS-SOP-072	**Effective Date:**	14 Feb 2022
	CAP Standard:		**Category:**	MDS Sample Processing
	Revision:	2	**Next Review:**	17 Jun 2023

I. NAME OF PROCEDURE: MDS Buccal Cell DNA Extraction

II. PURPOSE:

a. For extracting DNA from buccal cells collected from study participants with myelodysplastic syndromes (MDS) and idiopathic cytopenias of undetermined significance (ICUS)

III. SCOPE:

a. Starting material is buccal cells that were lysed in 400 µl Buffer ATL/20 µl proteinase K according to the Buccal Swab Lysis SOP. There are 2 vials of buccal cell lysate from each participant, one collected from the left cheek and one from the right cheek. The DNA extraction procedure is a modified version of the QiaAmp DNA Investigator Kit protocol for Isolation of Total DNA from Surface and Buccal Swabs

IV. EQUIPMENT, REAGENTS AND SUPPLIES:

a. Thermomixer, heat block, or water bath for 56°C incubation
b. QiaAmp DNA Investigator Kit: Qiagen Cat No. 56504
    i. Kit Contents
        1. QIAamp MinElute Columns
        2. Collection Tubes
        3. Buffer AL
        4. Buffer ATL
        5. Buffer AW1 (concentrate)
        6. Buffer AW2 (concentrate)
        7. Buffer ATE
        8. Proteinase K
        9. Carrier RNA
c. Nanodrop
d. Tapestation
e. Qubit
f. Collection Tubes (2ml): Qiagen catalog number 19201
g. 0.5 ml Matrix tubes: Thermo Scientific catalog number 3743. To be supplied by the MDS Lab
h. 1.5 ml microfuge tubes: USA Scientific catalog number 1415-9700. To be supplied by the MDS Lab

V. PREPARATION OF BUFFERS

Preparing Buffer ATL

Before starting the procedure, check whether precipitate has formed in Buffer ATL. If necessary, dissolve by heating to 70°C with gentle agitation

Preparing Buffer AL

Before starting the procedure, check whether precipitate has formed in Buffer AL. If necessary, dissolve by heating to 70°C with gentle agitation

Preparing Buffer AW1

Add 25 ml ethanol (96–100%) to the bottle containing 19 ml Buffer AW1 concentrate. Tick the check box on the bottle label to indicate that ethanol has been added. Reconstituted Buffer AW1 can be stored at room temperature (15–25°C) for up to 1 year.
Note: Before starting the procedure, mix the reconstituted Buffer AW1 by shaking.

Preparing Buffer AW2

Add 30 ml ethanol (96–100%) to the bottle containing 13 ml Buffer AW2 concentrate.
Reconstituted Buffer AW2 can be stored at room temperature (15–25°C) for up to 1 year.
Note: Before starting the procedure, mix the reconstituted Buffer AW2 by shaking.

VI. PROCEDURE:

a. Set the Thermomixer to 56°C
b. Remove 2 vials of lysed buccal cells per participant from the -80°C freezer
    i. Each vial of lysate contains 400 µl Buffer ATL/20 µl proteinase K
    ii. Extract each vial separately through step k.
c. Place the vials of lysed buccal cells in the Thermomixer until thawed (~ 5 minutes)
d. Remove the vials from the Thermomixer and briefly centrifuge the tubes to remove drops from the inside of the lid
e. Preparation of carrier RNA (Note that carrier RNA does not dissolve in Buffer AL. It must first be dissolved in Buffer ATE and then added to Buffer AL)
    -Add 310 µl Buffer ATE to the tube containing 310 µg lyophilized carrier RNA to obtain a solution of 1 µg/µl.
    -Dissolve the carrier RNA thoroughly, divide it into conveniently sized aliquots, and store at –20°C.
    -Do not freeze–thaw the aliquots of carrier RNA more than 3 times.
f. Add 1ul (1 ug) dissolved carrier RNA to 400 µl Buffer AL and mix by pipetting
    i. Scale up and make a cocktail (make more than needed to ensure there is enough cocktail for every sample)
        1. Example: Make enough cocktail for 27 samples when processing 24 samples
        Add 27 µl Carrier RNA to a Sterile 50 ml conical tube
        Add 10.8 ml Buffer AL to the 27µl Carrier RNA and mix the sample by pipetting and swirling the tube
g. Add 401 µl Buffer AL/carrier RNA cocktail from step f. to the lysate, close the lid, and mix by pulse-vortexing for 15 s
    To ensure efficient lysis, it is essential that the sample and Buffer AL are thoroughly mixed to yield a homogeneous solution
h. Place the tube in the thermomixer or heated orbital incubator, and incubate at 70°C with shaking at 900 rpm for 10 min
    If using a heating block or water bath, vortex the tube for 10 s every 3 min to improve lysis
i. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid
j. Add 200 µl ethanol (96–100%), close the lid, and mix thoroughly by pulse vortexing for 15 s.

To ensure efficient binding in step l. it is essential that the sample and ethanol are thoroughly mixed to yield a homogeneous solution

k. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid
NOTE: In step l., the two vials per participant will be combined by transferring the supernatant from both vials to **ONE** QIAamp MinElute column per participant

l. Carefully transfer 700 µl of the supernatant from step k. to the QIAamp MinElute column (in a 2 ml collection tube) without wetting the rim.
Close the lid, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through.
If the lysate has not completely passed through the membrane after centrifugation, centrifuge again at a higher speed until the QIAamp MinElute column is empty

m. Repeat step l. until all of the supernatant from step k. has been passed through the QIAamp MinElute column (3 passes through the column).

n. Place the QIAamp MinElute column in a clean 2 ml collection tube and discard the collection tube containing the flow-through.

o. Carefully open the QIAamp MinElute column and add 500 µl Buffer AW1 without wetting the rim. Close the lid and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute Column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through

p. Carefully open the QIAamp MinElute column and add 700 µl Buffer AW2 without wetting the rim. Close the lid and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through
Contact between the QIAamp MinElute column and the flow-through should be avoided. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains ethanol, coming into contact with the QIAamp MinElute column. Take care when removing the QIAamp MinElute column and collection tube from the rotor, so that flow-through does not come into contact with the QIAamp MinElute column

q. Carefully open the QIAamp MinElute column and add 700 µl of ethanol (96–100%) without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through

r. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min to dry the membrane completely.
This step is necessary, since ethanol carryover into the eluate may interfere with some downstream applications

s. Place the QIAamp MinElute column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the flow-through. Carefully open the lid of the QIAamp MinElute column, and incubate at room temperature (15–25°C) for 30 min

t. Apply 20 µl Buffer ATE to the center of the membrane
Important: Ensure that Buffer ATE is equilibrated to room temperature (15–25°C). Dispense Buffer ATE onto the center of the membrane to ensure complete elution of bound DNA

u. Close the lid and incubate at room temperature (15–25°C) for 10 min. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min

v. Repeat steps s-t with another 20 µl Buffer ATE

w. Quantitate 1.5 µl DNA on the NanoDrop, 3 µl on the Qubit and run 1 µl on the TapeStation

x. Aliquot the DNA as follows

·Always make a minimum of 2 aliquots from every sample

·DNA aliquots should be in a minimum volume of at least 10 µl

·DNA concentration should be 50 ng/µl unless initial concentration < 50 ng/ul

i. Important: Measure the exact DNA volume to ensure the correct quantity in each aliquot

1. If the DNA concentration > 50 ng/µl, dilute the DNA to 50 ng/µl with Buffer ATE
    a. Aliquot 500 ng DNA (10µL) into a maximum of 4 Matrix tubes
    b. Aliquot any extra DNA into a 1.5 ml screw top stock tube and measure the volume
2. If the DNA concentration < 50 ng/µl
    a. Aliquot 500 ng DNA into a maximum of 4 Matrix tubes
    b. Aliquot any extra DNA into a 1.5 ml screw top stock tube and measure the volume
    c. Make 2 equivalent aliquots if less than 1,000 ng DNA and measure the volume

VII. RESOURCES

a. Forms
b. References to Other Essential SOPs
c. Terms, Definitions & Abbreviations
d. Other Resources
e. Literature

I. NAME OF PROCEDURE: MDS Buccal Cell Lysis

II. PURPOSE:

a. For lysing buccal swabs collected from study participants with myelodysplastic syndromes (MDS) and idiopathic cytopenias of undetermined significance (ICUS)

III. SCOPE:

a. This SOP outlines the steps for lysing buccal cells and storing them at -80ºC until they are sent to Moffitt Tissue Core for DNA extraction. Buccal swab lysis is the first step in the DNA extraction protocol and is performed separately to preserve the DNA until it is sent to the Tissue Core for DNA extraction.
b. Two buccal swabs will be shipped overnight to M2Gen at room temperature in separate transport containers. Upon receipt at the CL/B, the buccal cells will be lysed in ATL Lysis Buffer/proteinase-K and stored in a -80°C freezer.

IV. EQUIPMENT, REAGENTS AND SUPPLIES:

a. Eppendorf Thermomixer F1.5: Fisher Cat. No. 05-412-500
b. Eppendorf 5430 centrifuge
c. Buffer ATL, 4 x 50 ml: Qiagen Cat. No. 939011
d. Proteinase K, 2 ml: Qiagen Cat. No. 19131

V. PROCEDURE: (NOTE: All techniques to be performed using standard aseptic techniques in a biosafety cabinet)

a. **NOTE: Use filter tips for all pipetting steps**
b. Set the Thermomixer to 56°C
c. Remove two aliquots of Buffer ATL (400 µl) and Proteinase K (22 µl) from their storage boxes underneath the germline control processing bench and pulse spin them in the Eppendorf 5430 centrifuge
d. Check the Buffer ATL aliquot for precipitation and if needed place it in the 56°C Thermomixer until reconstituted
e. Using separate tips, Pipet 20 µl Proteinase K into each of the Buffer ATL tubes and gently mix with a P1000 pipet
f. Remove the 1st buccal swab from its transport container and place it inside one of the tubes containing Buffer ATL/Proteinase K
g. Snap off the buccal swab handle, close the tube lid, pulse-vortex for 10 seconds in the Eppendorf 5430 centrifuge
h. Repeat steps f and g with the 2nd buccal swab
i. Place the tube in the thermomixer and incubate at 56°C with shaking at 900 rpm for 2 hours

j. Briefly centrifuge the tube to remove drops from the inside of the lid

k. Remove two QIAshredders from their packaging and write the participant ID on the side of the collection tubes

i. For the original cotton swabs: Using an orange Blood Bloc pad, transfer the 1st buccal swab from its tube of lysis buffer to one of the QIAshredders, snap off the handle and close the lid. Repeat for 2nd buccal swab

ii. For the replacement polyester swabs: Open a new package of tweezers, transfer the 1st buccal swab from its tube of lysis buffer to one of the QIAshredders and cut off the QIAshredder lid with the designated pair of scissors. Repeat for 2nd buccal swab with same pair of tweezers. NOTE: IF PROCESSING SWABS FROM MULTIPLE PARTICIPANTS, USE A NEW PAIR OF TWEEZERS FOR EACH PARTICIPANT

l. Centrifuge the QIAshredders for 2 minutes at maximum speed in the Eppendorf 5430 centrifuge

Note: The polyester swabs are centrifuged without the QIAshredder lid because the swab is too long for the QIAshredder

m. Use a P1000 pipet to mix the lysate in the QIAshredder collection tube. Sometimes precipitate forms as a pellet at the bottom of the collection tube. Always look for precipitate and mix it back into solution. Transfer the lysate from the 1st QIAshredder to the tube containing the lysate from the 1st buccal swab. Repeat with the 2nd QIAshredder

n. Log the sample type, date and volume into Lab Vantage

o. Select the sample storage location (Freezer, rack, box, hole) in Lab Vantage

p. Print tube labels from Lab Vantage and affix the labels to the buccal swab tubes

q. Place the two tubes of lysed buccal swabs into their assigned location in a -80 freezer until needed for DNA extraction

VI. RESOURCES

a. Forms

b. References to Other Essential SOPs

c. Terms, Definitions & Abbreviations

d. Other Resources

e. Literature

f. References

I. NAME OF PROCEDURE: MDS CD34 Enrichment Purity Assessment

II. PURPOSE:

a. For determining the purity of CD34 cells enriched from bone marrow aspirates of study participants with myelodysplastic syndromes (MDS) and idiopathic cytopenias of undetermined significance (ICUS)

III. SCOPE:

a. This SOP is for checking the purity of CD34+ and CD34- cells that are sorted using the CD34 Enrichment SOP. Starting material is 10,000 cells each of the CD34+ and CD34- fractions

IV. EQUIPMENT, REAGENTS AND SUPPLIES:

a. Anti-Human CD34-APC antibody: Stem Cell Cat. No. 60013AZ
b. Anti-Human CD45-FITC antibody: Stem Cell Cat. No. 60018FI1
c. BD CompBead anti-Mouse Ig: Fisher Cat. No. BDB552843
d. Propidium Iodide Solution: Miltenyi Cat. No. 130-093-233
e. MACS Buffer: For preparation instructions, see MDS-SOP-025, CD34 Enrichment
f. RoboSep Buffer: Stem Cell Cat. No. 20104
g. FACSCAlibur cytometer: Becton Dickinson
Sterile Phosphate Buffered Saline w/o Ca++ and Mg++ (1X PBS): Lonza Cat. No. 17- 516Q

V. PROCEDURE: (* RoboSep buffer is an acceptable alternative to MACS buffer)

a. Prepare compensation beads
    i. Label two 5 ml polystyrene tubes as indicates and add 100 µl MACS buffer*
        1. APC only
        2. FITC only
    ii. Vigorously vortex the BD CompBeads Negative Control beads and add 1 drop to each tube
    iii. Vigorously vortex the BD CompBeads Anti-Mouse Ig,k beads and add 1 drop to each tube
    iv. Quick spin the tubes in a tabletop centrifuge at 1,500 rpm

b. Label three 5 ml polystyrene tubes as indicated: add 10,000 of the indicated cells
    i. Propidium Iodide only: pre-enriched cells
    ii. Pre-enriched: pre-enriched cells
    iii. $CD34^{+}$: $CD34^{+}$ cells
c. Adjust the volume to 100 µl with MACS buffer*
d. Add 5 µl of the indicated antibodies to the following tubes and mix by agitation
    i. APC only: no antibody
    ii. FITC only: no antibody

iii. **Propidium iodide only: no antibody**

iv. **Pre-enriched: CD34-APC antibody and CD45-FITC antibody**

v. **$CD34^{+}$: CD34-APC antibody and CD45-FITC antibody**

e. **Incubate cells for 20 minutes in 4°C refrigerator and CompBeads at room temperature**
   i. **Place the cells in 4°C refrigerator**
   ii. **Keep the CompBeads at room temperature, protected from light**
f. **Pipet 2ml sterile 1X PBS to each tube, centrifuge 1,500 rpm 5'**
g. **Decant the 1X PBS**
h. **Resuspend the cells in 400 µl MACS buffer***
i. **Add 5 µl Propidium Iodide Solution to all tubes except the APC only and FITC only tubes, mix by agitation**
j. **Acquire samples on the BD FACSCAlibur cytometer**

VI. RESOURCES

a. **Forms**
b. **References to Other Essential SOPs**
   i. **Refer to CD34+ Enriched Cells_Purity Check_ Quality Assurance Data Form**
c. **Terms, Definitions & Abbreviations**
d. **Other Resources**
e. **Literature**
f. **References**

I. NAME OF PROCEDURE: CD34 Enrichment

II. PURPOSE:

For isolating $CD34^+$ cells from bone marrow aspirates of study participants with myelodysplastic syndromes (MDS) and idiopathic cytopenias of undetermined significance (ICUS)

III. SCOPE:

This SOP uses the Diamond CD34 Isolation Kit and protocol. Starting material is 5-10ml of bone marrow in a green top heparin vacutainer tube. The first 30 x 10^6 cells are for cryopreservation and the rest are for $CD34^+$ cell enrichment

IV. EQUIPMENT, REAGENTS AND SUPPLIES:

a. 5 ml polystyrene tubes: Fisher Cat. No. 149592A
b. 5, 10, and 25 ml sterile pipettes: Corning Cat. Nos. 4487, 4488, and 13-678-11
c. 15 ml conical tubes: Corning Cat. No. 430790
d. Pipette aid
e. Diamond CD34 Isolation Kit: Miltenyi Cat. No. 130-094-531
f. AutoMACS Rinsing Solution: Miltenyi Cat. No. 130-091-222
g. MACS BSA Stock Solution: Miltenyi Cat. No. 130-091-376
h. MiniMACS Separator: Miltenyi Cat. No. 130-042-102
i. MidiMACS Separator: Miltenyi Cat. No. 130-042-302
j. MACS MultiStand: Miltenyi Cat. No. 130-042-303
k. Pre-Separation Filters, 30 um: Miltenyi Cat. No. 130-041-407
l. LD columns: Miltenyi Cat No. 130-042-901
m. MS columns: Miltenyi Cat. No. 130-042-201
n. Cryovials 1.8 ml: Fisher Cat. No. 12-565-163N
o. 500ml Corning™ Disposable Vacuum Filter/Storage Systems, Fisher Cat. No. 97618
p. Mr. Frosty: Fisher Cat No. 15-350-50

V. PROCEDURE:

<u>Preparing Macs Separation Buffer</u>

The MACS BSA Stock Solution is used for the preparation of a preservative-free MACS Separation Buffer. It contains phosphate buffered saline (PBS) and 10% bovine serum albumin (BSA). When diluted in autoMACS Rinsing Solution an optimal buffer for all MACS Separation obtained.

For the preparation of preservative-free MACS Separation Buffer:

- Empty one bottle MACS BSA Stock Solution into one bottle of autoMACS Rinsing Solution.
- Mix well by inverting the autoMACS Rinsing Solution bottle several times.
- Pour the solution into 500ml sterileVacuum Filter unit and collect the filtered solution in polystyrene storage bottle that comes with the unit.
- Write the date of the preparation on the bottle.
- Store it at 2-8 C protected from light.

A) Magnetic labeling of Lin+ cells

- Work fast, keep cells cold, and use pre-cooled solutions. This will prevent capping of antibodies on the cell surface and non-specific cell labeling.
- Volumes for magnetic labeling given below are for up to 5×10^7 total cells. When working with fewer than 5×10^7 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. if between 5 x 10^7 and 10^8 total cells, use twice the volume of all indicated reagent volumes and total volumes).
- For optimal performance it is important to obtain a single-cell suspension before magnetic labeling. Pass cells through 30 µm nylon mesh (Pre-Separation Filters, # 130-041-407) to remove cell clumps which may clog the column. Moisten filter with buffer before use.
- The recommended incubation temperature is 2–8 °C. Working on ice may require increased incubation times. Higher temperatures and/or longer incubation times may lead to nonspecific cell labeling.

Follow the Ficoll_Bone Marrow SOP through the cell counting step and determine cell number.

1. Pass cells through a 30 µm pre-separation filter to remove cell clumps which may clog the column.
2. Transfer 10,000 cells each into two 5 ml polystyrene tubes (for pre-enrich and PI only samples), bring the volume up to 100 µl with MACS buffer and store in the refrigerator until needed for the flow cytometry purity check. **NOTE: Purity checks are only performed when there are > 1 x10^5 enriched CD34+ cells, but always put aside these two samples in case a purity check is needed**
3. Centrifuge cell suspension at 1,500rpm for 5 minutes. Aspirate supernatant completely.
4. Resuspend cell pellet in 200 µl of Macs separation buffer per 5×10^7 total cells.
    i. NOTE: Scale up volumes per the indicated cell increments throughout the protocol. In this step you would resuspend ≤ 5 x 10^7 cells in 200 µL of Macs separation buffer and 5 x 10^7 - 1 x 10^8 cells in 400 µl of Macs separation buffer, etc.
5. Add 50 µl of Diamond Lin Biotin-Antibody Cocktail per 5×10^7 total cells.
6. Mix well and incubate for 10 minutes in the refrigerator (2−8 °C).
7. Wash cells by adding 2.5 mL of Macs separation buffer per 5×10^7 cells and centrifuge at 1,500rpm for 5 minutes. Aspirate supernatant completely.
8. Resuspend cell pellet in 400 µl of Macs separation buffer per 5×10^7 total cells.
9. Add 100 µl of Anti-Biotin MicroBeads per 5×10^7 total cells.
10. Mix well and incubate for 15 minutes in the refrigerator (2−8 °C).
11. Wash cells by adding 5 ml of Macs separation buffer per 5×10^7 cells. FILTERING THROUGH A PRE-SEPARATION FILTER IS OPTIONAL AND SHOULD ONLY BE PERFORMED IF THERE ARE VISIBLE CELL CLUMPS
12. Centrifuge at 1,500rpm for 5 minutes. Aspirate supernatant completely.
13. Resuspend up to 1 x 10^8 cells in 1,000 µl Macs separation buffer.
    Note: For higher cell numbers, scale up buffer volume accordingly.
14. Proceed to magnetic separation (B).

B) Magnetic separation: Depletion of Lin+ cells

- Always wait until the column reservoir is empty before proceeding to the next step.

Depletion with LD Columns

1. Place LD Column in the magnetic field of a suitable MACS Separator. For details see LD Column data sheet
    - Use one LD column per $1x10^8$ cells of initial starting material and divide cells evenly when using more than 1 column
        - 1×10^8 cells or less = 1 LD column
        - $1 \times 10^8 – 2 \times 10^8$ cells = 2 LD columns.
        - $2 \times 10^8 – 3 \times 10^8$ cells = 3 LD columns, etc.

2. Prepare column by rinsing with 2 mL of Macs separation buffer.
    - Prepare column 5 minutes before use (preparing sooner may result in air bubbles forming in the column and slowing down the flow rate)
3. Place a new 15 ml conical tube under the column.
4. Apply cell suspension onto the column.
5. Collect unlabeled cells that pass through and wash column 3 times with 1 mL of Macs separation buffer. Collect total effluent; this is the unlabeled Lin^- cell fraction. Perform washing steps by adding buffer three times. Only add new buffer when the column reservoir is empty.
6. Proceed to (D) for further enrichment of $CD34^+$ stem cells.

C) Collecting Lin^+ Cells

1. To get Lin^+ cells, add 1 ml of Macs separation buffer to the LD column and elute the cells into a new 15 ml conical tube by firmly pushing the plunger into the column. These are the Lin^+ cells.
2. Centrifuge cell suspension at 1,500rpm for 5 minutes. Aspirate supernatant completely.
3. Resuspend cell pellet in necessary amount of Macs separation buffer and count.
4. Freeze Lin^+ cells as follows:
    - Freeze $< 5 \times 10^7$ cells in 2 equivalent aliquots
    - Freeze $5 \times 10^7 – 1 \times 10^8$ cells in the fewest number of aliquots without going over 2.5×10^7 cells per aliquot
    - Freeze $> 1 \times 10^8$ cells in 4 aliquots of 2.5×10^7 cells, and freeze the remainder of the cells in 1 aliquot

D) Magnetic labeling of $CD34^+$ stem cells

- Volumes for magnetic labeling given below are for an initial starting cell number of up to 5×10^7 cells. For larger initial cell numbers, scale up volumes accordingly.

1. Centrifuge cell suspension at 1,500rpm for 5 minutes. Aspirate supernatant completely.
2. Resuspend cell pellet in 200 µl of Macs separation buffer per 5×10^7 cells of initial starting material
3. Add 50 µl of CD34 Diamond MicroBeads per 5×10^7 cells of initial starting material
4. Mix well and incubate for 30 minutes in the refrigerator (2−8 °C).
5. Wash cells by adding 2.5 mL of Macs separation buffer per 5×10^7 cells of initial starting material. FILTERING THROUGH A PRE-SEPARATION FILTER IS OPTIONAL AND SHOULD ONLY BE PERFORMED IF THERE ARE VISIBLE CELL CLUMPS
6. Centrifuge at 1,500 rpm for 5 minutes. Aspirate supernatant completely.

7. Resuspend cell pellet in 500 µl of Macs separation buffer per 1 x 10^8 total cells.

E) Magnetic separation: Positive selection of $CD34^+$ stem cells

Positive selection with MS Columns

- To achieve highest purities, perform two consecutive column runs.

1. Place MS column (1st of two columns) in MiniMACS separator and attach it to the MACS MultiStand
   - Use one MS column per 2 x 10^8 cells. **If more than 2 x 10^8 cells of initial starting material, subtract the Lin^+ cell count from the initial starting material to determine how many columns to use**
     - 2 x 10^8 cells or less initial starting material = 1 MS column
     - Initial starting material minus Lin^+ cell count = 2 x 10^8 – 4 x 10^8 cells = 2 MS columns
     - Initial starting material minus Lin^+ cell count = 4 x 10^8 – 6 x 10^8 = 3 MS columns

   Example: Initial starting material (5 x 10^8 cells) minus Lin+ cell count (2 x 10^8 cells) = 3 x 10^8 cells. This would require 2 MS columns
2. Place a 15 ml conical tube underneath the column to collect the flow-through
3. Prepare column by rinsing with 500 µl Macs separation buffer. Always wait until the column reservoir is empty before proceeding with the next step
   - Prepare column 5 minutes before use (preparing sooner may result in air bubbles forming in the column and slowing down the flow rate)
4. Apply cell suspension from step D-7 onto the column. Collect flow-through containing unlabeled ($CD34^{minus}$) cells
5. Wash column 3 times with 500 µl Macs separation buffer. Collect unlabeled cells that pass through into the same collection tube from step 4. Only add new buffer when the column reservoir is empty
6. Remove column from separator and place it into a 15 ml conical tube
7. Place a new MS column (2nd of two columns) in MiniMACS separator

   Note: Only 1 column is used in this step, even if multiple columns were used in Step1
8. Place a 15 ml conical tube underneath the column to collect the flow-through
9. Prepare column by rinsing with 500 µl Macs separation buffer. Always wait until the column reservoir is empty before proceeding with the next step
10. Take the collection tube used to collect flow through from the 1st column and place it underneath the 2nd column
11. Pipette 1 ml Macs separation buffer onto the 1st column from Step 1 and elute the cells directly into the 2nd column from Step 7 by firmly pushing the plunger into the column.
12. Repeat Step 11 for each MS column used in Step 1. If two MS columns were used in Step 1, both columns would be eluted into the same column from Step 7
13. Wash column 3 times with 500 µl Macs separation buffer. Only add new buffer when the column reservoir is empty
14. Pipette 1 ml Macs separation buffer onto the 2nd column and elute the cells into a new 15 ml conical tube by firmly pushing the plunger into the column. These are the $CD34^+$ cells.
15. Count the $CD34^+$ and $CD34^{minus}$ cells and transfer 2,000 $CD34^+$ cells to a 5 ml polystyrene tube for purity assessment only when the $CD34^+$ cell count is > 1 x 10^5 cells. Do not perform the purity

assessment when the $CD34^+$ cell count is $< 1 \times 10^5$ cells. **NOTE: Do not use any $CD34^{minus}$ cells for purity assessment.** See CD34 enrichment purity assessment SOP

16. Freeze 2 equivalent aliquots of $CD34^+$ and $CD34^{minus}$ cells
    - If $> 1 \times 10^7$ cells, freeze at least 5×10^6 cells per aliquot
      - 1.3×10^7 cells = 2 aliquots of 6.5×10^6 cells per aliquot
      - 1.5×10^7 cells = 3 aliquots of 5×10^6 cells per aliquot
    - If $> 2 \times 10^7$ cells, freeze 4 aliquots of 5×10^6 cells per aliquot and 1 aliquot containing the remainder of the cells
      - Freeze 1×10^8 cells in 4 aliquots of 5×10^6 cells and 1 aliquot of 8×10^7 cells

IV. RESOURCES

a. Forms
b. References to Other Essential SOPs
c. Terms, Definitions & Abbreviations
d. Other Resources
e. Literature

I. NAME OF PROCEDURE: MDS Cryopreservation of Cells (USE ONLY STERILE MATERIALS FOR ALL PROCEDURES)

II. PURPOSE:

a. For cryopreserving cells isolated from the peripheral blood and bone marrow of study participants with myelodysplastic syndromes (MDS) and idiopathic cytopenias of undetermined significance (ICUS)

III. SCOPE:

a. This cryopreservation process uses a Mr. Frosty freezing container to slowly cool the cells overnight in a -80°C freezer, followed by long-term storage in liquid nitrogen. The following cells will be cryopreserved: peripheral blood mononuclear cells (PBMCs), bone marrow mononuclear cells (BMMNCs), and $CD34^+$ cells enriched from BMMNCs

IV. EQUIPMENT, REAGENTS AND SUPPLIES:

a. 50 ml sterile conical tubes: Corning Cat. No. 430828
b. 5, 10, and 25 ml sterile pipettes: Corning Cat. Nos. 4487, 4488, and 13-678-11
c. Pipette aid
d. Heat-inactivated Fetal Bovine Serum (FBS), 500 ml: Life Technologies Cat No. 10082-147
e. Dimethyl Sulfoxide (DMSO), 100 ml: Sigma Cat No. D2650-100ML
f. 90 proof ethanol: University of South Florida Tax-Free Ethyl Alcohol Order Form
g. Isopropanol: Sigma Cat. No. D2650-100ML
h. Benchtop centrifuge
i. Mr. Frosty freezing containers: Fisher Cat. No. 15-350-50
j. 4°C refrigerator
k. -80°C freezer
l. Cryogenic unit liquid nitrogen
m. A2000 cellometer: Nexcelom Cat. No. A2000

V. PROCEDURE: (NOTE: All techniques to be performed using standard aseptic techniques in a biosafety cabinet)

a. Perform cell counts (See Cell Counting and Viability SOP) and determine the number of cells and aliquots to be cryopreserved based on the following workflows
   i Peripheral blood workflow- PBMCs:
      1. Freeze at least 5 x 10^6 cells per aliquot with a minimum of 2 aliquots and a maximum of 10 aliquots
      2. 5 x 10^7 cells = 10 aliquots of 5 x 10^6 cells
      3. > 5 x 10^7 cells = 4 aliquots of 5 x 10^6 cells and divide the rest of the cells evenly into 6 aliquots
   ii Bone marrow workflow- BMMNCs:
      1. Freeze the first 20 x 10^6 cells in 4 aliquots of 5 x 10^6 cells/aliquot.
      2. If less than 20 x 10^6 cells, freeze at least 5 x 10^6 cells per aliquot with a minimum of 2 aliquots

iii Bone marrow workflow-$CD34^+$ cells enriched from BMMNCs:
1. Freeze 2 equal aliquots

iv Bone marrow workflow-Lin+ cells from the CD34+ enrichment:
1. Freeze 2 equal aliquots

v Bone marrow workflow-$CD34^-$ cells from the $CD34^+$ enrichment:
1. Freeze 2 equal aliquots

b. Centrifuge the cells to be frozen at 400 g for 8 minutes
c. Prepare freezing media consisting of 90% FBS/10% DMSO and mix. Prepare an extra 1 ml for each sample
   i. Example: 6 ml freezing media = 0.6 ml DMSO and 5.4 ml FBS
d. Resuspend the cell pellet in 1 ml freezing media (90% FBS/10% DMSO) per aliquot to be frozen
e. Aliquot 1 ml cells into 1.8 ml cryovials, transfer to a room temperature Mr. Frosty, and place the Mr. Frosty in a -80°C freezer in M2Gen Freezer 1 on bottom shelf.
   Note: Replace the isopropanol in the Mr Frostys after every 5 uses
f. Transfer the cryovials into a liquid nitrogen cryogenic unit the next day.

VI. RESOURCES

a. Forms
b. References to Other Essential SOPs
c. Terms, Definitions & Abbreviations
d. Other Resources
e. Literature
f. References

I. NAME OF PROCEDURE: MDS Eyebrow Hair DNA Extraction

II. PURPOSE:

a. For extracting DNA from eyebrow hairs plucked from study participants with myelodysplastic syndromes (MDS) and idiopathic cytopenias of undetermined significance (ICUS)

III. SCOPE:

a. Starting material is eyebrow hairs that were lysed according to the Eyebrow Hair Lysis SOP. The DNA extraction procedure is a modified version of the QiaAmp DNA Investigator Kit protocol for Isolation of Total DNA from Nail Clippings and Hair

IV. EQUIPMENT, REAGENTS AND SUPPLIES:

a. Thermomixer, heat block, or water bath for 56°C incubation
b. QiaAmp DNA Investigator Kit: Qiagen Cat No. 56504
    i. Kit Contents
        1. QIAamp MinElute Columns
        2. Collection Tubes
        3. Buffer AL
        4. Buffer ATL
        5. Buffer AW1 (concentrate)
        6. Buffer AW2 (concentrate)
        7. Buffer ATE
        8. Proteinase K
        9. Carrier RNA
c. Nanodrop
d. Qubit
e. Collection Tubes (2ml): Qiagen catalog number 19201
f. 0.5 ml Matrix tubes: Thermo Scientific catalog number 3743. To be supplied by the MDS Lab
g. 1.5 ml microfuge tubes: USA Scientific catalog number 1415-9700. To be supplied by the MDS Lab

V. PREPARATION OF BUFFERS

Preparing Buffer ATL

Before starting the procedure, check whether precipitate has formed in Buffer ATL. If necessary, dissolve by heating to 70°C with gentle agitation

Preparing Buffer AL

Before starting the procedure, check whether precipitate has formed in Buffer AL. If necessary, dissolve by heating to 70°C with gentle agitation

Preparing Buffer AW1

Add 25 ml ethanol (96–100%) to the bottle containing 19 ml Buffer AW1 concentrate. Tick the check box on the bottle label to indicate that ethanol has been added. Reconstituted Buffer AW1 can be stored at room temperature (15–25°C) for up to 1 year.
Note: Before starting the procedure, mix the reconstituted Buffer AW1 by shaking.

Preparing Buffer AW2

Add 30 ml ethanol (96–100%) to the bottle containing 13 ml Buffer AW2 concentrate.
Reconstituted Buffer AW2 can be stored at room temperature (15–25°C) for up to 1 year.
Note: Before starting the procedure, mix the reconstituted Buffer AW2 by shaking.

VI. PROCEDURE:

a. Set the Thermomixer to 56°C
b. Remove a vial(s) of lysed eyebrow hairs from the -80°C freezer
    i. Each vial of lysate contains 300 µl Buffer ATL/20 µl proteinase K/20 µl DTT
c. Place the vial of lysed eyebrow hairs in the Thermomixer until thawed (~ 5 minutes)
d. Remove the vial from the Thermomixer and briefly centrifuge the tube to remove drops from the inside of the lid
e. Preparation of carrier RNA (Note that carrier RNA does not dissolve in Buffer AL. It must first be dissolved in Buffer ATE and then added to Buffer AL)
    -Add 310 µl Buffer ATE to the tube containing 310 µg lyophilized carrier RNA to obtain a solution of 1 µg/µl.
    -Dissolve the carrier RNA thoroughly, divide it into conveniently sized aliquots, and store at –20°C.
    -Do not freeze–thaw the aliquots of carrier RNA more than 3 times.
f. Add 1ul (1 ug) dissolved carrier RNA to 300 µl Buffer AL
    i. Scale up and make a cocktail (make more than needed to ensure there is enough cocktail for every sample)
        1. Example: Make enough cocktail for 27 samples when processing 24 samples
        Add 27 µl Carrier RNA to a Sterile 50 ml conical tube
        Add 8.1 ml Buffer AL to the 27µl Carrier RNA and mix the sample by pipetting and swirling the tube
g. Add 301 µl Buffer AL/carrier RNA cocktail to the lysate, close the lid, and mix by pulse-vortexing for 10 s
    To ensure efficient lysis, it is essential that the sample and Buffer AL are thoroughly mixed to yield a homogeneous solution
h. Place the tube in the thermomixer or heated orbital incubator, and incubate at 70°C with shaking at 900 rpm for 10 min
    If using a heating block or water bath, vortex the tube for 10 s every 3 min to improve lysis
i. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid
j. Add 150 µl of ethanol (96–100%), close the lid, and mix thoroughly by pulse vortexing for 15 s.

To ensure efficient binding in step l. it is essential that the sample and ethanol are thoroughly mixed to yield a homogeneous solution

k. Briefly centrifuge the 1.5 ml tube to remove drops from the inside of the lid

l. Carefully transfer the supernatant from step k. to the QIAamp MinElute column (in a 2 ml collection tube) without wetting the rim

m. Close the lid, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through
If the lysate has not completely passed through the membrane after centrifugation, centrifuge again at a higher speed until the QIAamp MinElute column is empty

n. Carefully open the QIAamp MinElute column and add 500 µl Buffer AW1 without wetting the rim. Close the lid and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute Column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through

o. Carefully open the QIAamp MinElute column and add 700 µl Buffer AW2 without wetting the rim. Close the lid and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through
Contact between the QIAamp MinElute column and the flow-through should be avoided. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains ethanol, coming into contact with the QIAamp MinElute column. Take care when removing the QIAamp MinElute column and collection tube from the rotor, so that flow-through does not come into contact with the QIAamp MinElute column

p. Carefully open the QIAamp MinElute column and add 700 µl of ethanol (96–100%) without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp MinElute column in a clean 2 ml collection tube, and discard the collection tube containing the flow-through

q. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min to dry the membrane completely.
This step is necessary, since ethanol carryover into the eluate may interfere with some downstream applications

r. Place the QIAamp MinElute column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the flow-through. Carefully open the lid of the QIAamp MinElute column, and incubate at room temperature (15–25°C) for 30 min

s. Apply 20 µl Buffer ATE to the center of the membrane
Important: Ensure that Buffer ATE is equilibrated to room temperature (15–25°C). Dispense Buffer ATE onto the center of the membrane to ensure complete elution of bound DNA

t. Close the lid and incubate at room temperature (15–25°C) for 10 min. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min

u. Repeat steps s-t with another 20 µl Buffer ATE

v. Quantitate 1.5 µl DNA on the NanoDrop, 3 µl on the Qubit and run 1 µl on the TapeStation

w. Aliquot the DNA as follows
·Always make a minimum of 2 aliquots from every sample
·DNA aliquots should be in a minimum volume of at least 10 µl
·DNA concentration should be 50 ng/µl unless initial concentration < 50 ng/ul
    i. Important: Measure the exact DNA volume to ensure the correct quantity in each aliquot

1. If the DNA concentration > 50 ng/µl, dilute the DNA to 50 ng/µl with Buffer ATE
    a. Aliquot 500 ng DNA (10µL) into a maximum of 4 Matrix tubes
    b. Aliquot any extra DNA into a 1.5 ml screw top stock tube and measure the volume
2. If the DNA concentration < 50 ng/µl
    a. Aliquot 500 ng DNA into a maximum of 4 Matrix tubes
    b. Aliquot any extra DNA into a 1.5 ml screw top stock tube and measure the volume
    c. Make 2 equivalent aliquots if less than 1,000 ng DNA and measure the volume

VII. RESOURCES

a. Forms
b. References to Other Essential SOPs
    i. Refer to Eyebrow Hair_ DNA Quality Assurance Data Form
c. Terms, Definitions & Abbreviations
d. Other Resources
e. Literature
f. References

I. **NAME OF PROCEDURE:** MDS Eyebrow Hair Lysis

II. PURPOSE:

a. For lysing eyebrow hairs collected from study participants with myelodysplastic syndromes (MDS) and idiopathic cytopenias of undetermined significance (ICUS)

III. SCOPE:

a. Eyebrow hair samples will be collected and stored in batches for DNA extraction. Eyebrow hair lysis is the first step in the DNA extraction protocol and is performed separately to preserve the DNA during storage. 12 eyebrow hairs will be shipped overnight to M2Gen at room temperature in a 1.5 ml microfuge tube, lysed in ATL Lysis Buffer/proteinase-K upon arrival, and stored in a -80°C freezer until used for DNA extraction. The DNA extraction procedure is a modified version of the QiaAmp DNA Investigator Kit protocol for Isolation of Total DNA from Nail Clippings and Hair

IV. EQUIPMENT, REAGENTS AND SUPPLIES:

a. Eppendorf Thermomixer F1.5: Fisher Cat. No. 05-412-500
b. proteinase K, 2 ml: Qiagen Cat No. 19131
c. Dithiothreitol (DTT), 1 g: Sigma Cat. No. D0632-1G
d. Buffer ATL, 4 x 50 ml: Qiagen Cat No. 939011

V. PROCEDURE:

a. Set the Thermomixer to 56°C
b. Remove 1 aliquot of 20 µl 1 M DTT from the -20 freezer and let thaw at room temperature
c. Centrifuge the microfuge tube of eyebrow hairs in a microfuge at full speed for 1 minute to pellet the eyebrow hairs to the bottom of the tube
d. Briefly centrifuge 1 aliquot of 300 µl Buffer ATL, 20 µl proteinase K, and 20 µl 1 M DTT per sample
e. Add 20 µl proteinase K and 20 µl 1 M DTT to 300 µl Buffer ATL , mix by pipetting and transfer the solution to the tube of eyebrow hairs
f. Close the lid and mix by pulse-vortexing for 10 s
g. Briefly centrifuge the tube to remove drops from the inside of the lid and make sure all eyebrow hairs are submerged in lysis buffer
h. Place the tube in the thermomixer and incubate at 56°C with shaking at 900 rpm for 2 hours
i. Briefly centrifuge the tube to remove drops from the inside of the lid
j. Place the tube of lysed eyebrow hairs into a -80 freezer until needed for DNA extraction

VI. RESOURCES

a. Forms
b. References to Other Essential SOPs
    i. Refer to Eyebrow Hair_ DNA Quality Assurance Data Form
c. Terms, Definitions & Abbreviations

I. NAME OF PROCEDURE: FlexStar 1-5ml Frozen Whole Blood SOP

II. PURPOSE:

a. Automated purification of DNA from 10 ml PAXgene Blood DNA tubes on the FlexStar

III. SCOPE:

a. Each 10 ml PAXgene DNA tube will be split in half and each half will be extracted independently on the FlexStar

b. The 2 extractions from III-a will have their own NanoDrop and Qubit quantifications, TapeStation readings, and aliquots.

c. Do **NOT** combine the DNA from the 2 extractions in III-a.

IV. EQUIPMENT, REAGENTS AND SUPPLIES:

a. Buffer ATE (20 ml), Qiagen cat. no. 1067929

V. PROCEDURE:

Labeling the Sample tubes

- Place labels on the middle back left side of each input and output sample tube.

Loading of Samples into the Computer

- Turn on and log into the computer.
- Click on FS samples Icon and sign in.
- Click on the sample tab then select “input sample tracking information”.
- Click on the “Rack ID” tab and scan the “Sample Input Rack” barcode, followed by the Sample tubes barcodes.
- Select the “DNA Tube Rack” tab and repeat for the DNA Input tubes.
- Click on the “Sample tube rack” tab and scan/ type in the sample ID’s. Click “OK” once finish.
    - The Samples ID’s will self-populate into the “DNA Tube Rack” tab.

Things to do before starting

- **Preparation of protease working solution:**

(1) Hydrate the lyophilized QIAGEN Protease in 7 ml reagent FG3 to make a QIAGEN Protease stock solution.

(2) Prepare a QIAGEN Protease working solution by diluting QIAGEN Protease stock solution 1/10 with high-quality distilled water by mixing 3.5 ml QIAGEN Protease stock solution and 31.5 ml high-quality distilled water in a 50 ml centrifuge tube.

(3) Attach the 50 ml centrifuge tube containing the protease working solution to reagent site 2 on the FLEXSTAR PLUS.

- Preparation the samples:

Thaw and equilibrate the whole blood samples to room temperature (15–25ºC). The samples should be thawed quickly in a 37ºC waterbath with mild agitation.

- Mixing the sample using a pipette then transfer 5mL of samples into F*lexstar 5-hole tube unit* from left to right. If the volume of a sample needs to be adjusted, add the appropriate volume of PBS.
    - Take into account that the machine needs to have an even number of samples and tube unit to stay balanced during the run.
- If any blood sample the number of cells in the sample is low, add 40ul of Glycogen Solution (20 mg/mL) into the corresponding output tube. (Optional)

Running the Samples

- Place the "Sample Input Rack" and "DNA Output Rack" into the machine.
- Check to ensure that all centrifuge buckets are in the centrifuge.
- Turn on the power breaker, surge protector, and machine screen.
- Select "FG Blood Frozen" and confirm that the centrifuge buckets are in the machine.
- Select "Prime Reagent", then "Init" system check, then lines "1, 2, 3, 4, 5 and 8".
- Set the amount to 7.5mL, press enter, and run the program twice.
- Process back and select "Automatic Run"
- Click ok on "Tube type 5"
- Enter sample number in the "Number of Samples" section.
- Ensure the number of tubes correlates with "Number of tubes"
- Ensure that "Parameter File No." is set to "1"
- Change "Dispersing Select" to circulate and then click okay.
- If a communication errors, makes sure the computer is on and the file is been set to Run.
- Ensure the reagent volume is sufficient, select Init, then check, then start.

Elusion and centrifuging of Samples

- Once the run is finish turn off both power supplies and remove the "Sample Input Racks" and "DNA Output Rack".
    - Dispose of the "Sample Input Tubes"
- Use pipette to remove residual 70% ethanol from each sample well, let sample tubes air dry under BSC for 10 minutes before hydration.
- Hydrate the "DNA Output Tubes" with the desired amount of elusion buffer, place clean "Tube Caps" onto of "DNA Output Tubes", then heat at 65°C for 1-2 hours in a water bath.
    - **Important:** Use Buffer ATE to reconstitute the DNA. For ordering information, see Section I**V. EQUIPMENT, REAGENTS AND SUPPLIES**
- Remove DNA Samples from water bath and allow cooling to room temperature.
- Once DNA samples have cooled, place and balance them inside of the centrifuge, then turn the machine back on.
- Selected any protocol, and then proceed to the "Maintenance".
    - Use the password 741953 to gain access.

- Select Centrifuge, 3000rpm, 1 minute, and then start. Once finished select “Table” and “open”.
- Turn off the machine and remove the DNA samples.

DNA Quantification

- **Important:** Do **NOT** combine DNA. Each of the 2 extractions from each 10 ml PAXgene tube will have its own NanoDrop and Qubit quantifications, and TapeStation readings

DNA aliquots

- **Important:** Do **NOT** combine DNA. Each of the 2 extractions from each 10 ml PAXgene tube will have its own aliquots

- **Important**: Measure the exact sample volume to ensure the correct quantity in each aliquot
- **Note:** Always make a minimum of 2 aliquots from every sample
- **Note:** DNA aliquots should be in a minimum volume of at least 10 µl (This has priority over concentration being 50 ng/ul)

- **Note:** DNA concentration should be 50 ng/µl unless initial concentration < 50 ng/ul or volume < 10 µl and needs to be brought up to 10 µl
- **Aliquot as follows in Step 1, 2, or 3**

1. If the DNA concentration > 50 ng/µl, dilute the DNA to 50 ng/µl with Buffer ATE
    - Aliquot 500 ng DNA (10µl) into a maximum of 4 Matrix tubes
    - Aliquot any extra DNA into a 1.5 ml screw top stock tube (use Matrix tube if < 500 ng DNA) and measure the volume. If necessary, bring the volume up to 10 µl and calculate the new concentration

      Note: DNA for the stock tube does not need to be diluted to 50 ng/µl, but it can be if convenient
2. If the DNA concentration < 50 ng/µl
    - Aliquot 500 ng DNA into a maximum of 4 Matrix tubes
    - Aliquot any extra DNA into a 1.5 ml screw top stock tube (use Matrix tube if < 500 ng DNA) and measure the volume. If necessary, bring the volume up to 10 µl and calculate the new concentration
3. Make 2 equivalent aliquots in Matrix tubes if less than 1,000 ng DNA

VI. REFERENCES

a. **References to Other Essential SOPs**
b. **Forms**
c. **Terms, Definitions & Abbreviations**
d. **Other Resources**
e. **Literature References**

I. NAME OF PROCEDURE: Making Cell Pellets from Whole Bone Marrow EDTA Tubes

II. PURPOSE:

a. To make cell pellets from bone marrow aspirates from MDS study participants

III. SCOPE:

a. Starting material is bone marrow aspirate in a 3ml EDTA tube. Cell pellets are made after bone marrow smears are made and plasma is collected from the 3 ml EDTA tube. The pellets are flash frozen in liquid nitrogen and stored in the -80 freezer. One pellet will be used for DNA extraction and remaining pellets will remain frozen until otherwise indicated by the Executive Committee.

IV. EQUIPMENT, REAGENTS AND SUPPLIES:

a. 10X Pharm Lyse solution, 100 ml: Becton Dickinson Cat. No. 555899
b. 50 ml sterile conical tubes: Corning Cat. No. 430828
c. 5, 10, and 25 ml sterile pipettes: Corning Cat. Nos. 4487, 4488, and 13-678-11
d. Sterile Phosphate Buffered Saline w/o Ca++ and Mg++ (1X PBS): Corning Cat. No.21040CM or Sigma 59321C
e. Medicus Health Blood Tube Rocker (Cat. No. 5265M2): Fisher Cat. No. NC0139535
f. 3 ml syringes: Moffitt Stores, Lawson # 110150
g. 22 gauge blunt end needles: Moffitt Stores, Lawson # 220950
h. Sharps containers, 1 qt: Fisher Cat. No. 22-037-970
i. Liquid Nitrogen flask
j. 4°C refrigerator
k. -80°C freezer
l. Sterile Water: Fisher Cat. No. MT25055CV

V. PROCEDURE: (NOTE: All techniques to be performed using standard aseptic techniques in a biosafety cabinet)

a. Clean hood by wiping all work surfaces with 70% alcohol with Kimwipe
b. Fill a flask with Liquid Nitrogen, close the lid, and set aside until needed
c. Prepare 1X Pharm Lyse buffer by diluting 10X Pharm Lyse buffer to 1X with sterile H_2O
    i. Invert the tube several times to mix and keep at room temperature until needed
d. Use a transfer pipette to remove approximately 0.25-0.5 ml bone marrow from the EDTA tube and set it aside for smears
e. Measure the amount of bone marrow remaining in the EDTA tube after removing some for making bone marrow smears but before collecting plasma
    i. Measure the volume of bone marrow by holding the bone marrow EDTA tube against an empty reference EDTA tube with the volume marked in 0.5 ml increments
f. Collect the plasma from the bone marrow tube (See 'MDS Bone Marrow Plasma Collection' SOP) before proceeding with the lysis of the bone marrow
g. Pass the bone marrow through a 22 gauge blunt end needle three times and transfer to a sterile 50 ml conical tube

h. DISPOSE SYRINGES INTO A SHARPS CONTAINER. SYRINGES ARE NOT TO BE DISPOSED OF IN THE RED BIOHAZARDOUS WASTE TRASH BAGS
i. Using a sterile pipet, dilute the bone marrow in 1X sterile Pharm Lyse buffer so the final volume is 10X the volume of the bone marrow BEFORE collecting plasma
    i. Example:
    ii. EDTA tube volume = 3.5 ml
    iii. 0.5 ml removed for smears
    iv. 3.5 ml - 0.5 ml = 3 ml bone marrow to be lysed
    v. 3 ml x 10 = final lysis volume of 30 ml
    vi. Add 27 ml lysis buffer to the 3 ml bone marrow
j. Mix by inverting the tube until the blood is dissolved in the lysis buffer
k. Rock the sample for 15 minutes on the blood tube rocker
l. Centrifuge for 5 minutes at 1,500 rpm
m. Using a sterile pipet remove the supernatant without disturbing the cell pellet
n. Using a sterile pipet, resuspend the pellet in 12 ml 1X Pharm Lyse buffer
o. Rock the sample for 10 minutes on the blood tube rocker
p. Using a sterile pipet, add 12 ml 1X sterile PBS to the sample, close lid, mix by inversion
q. Centrifuge for 5 minutes at 1,500 rpm
r. Using a sterile pipet remove the supernatant without disturbing the cell pellet
s. Using a sterile pipet, resuspend the pellet in 25 ml 1X Sterile PBS
t. Centrifuge for 5 minutes at 1,500 rpm
u. Using a sterile pipet remove the supernatant without disturbing the cell pellet
v. Using a sterile pipet, resuspend the pellet in 10 ml 1X Sterile PBS
    i. Resuspend smaller cell pellets in less than 10 ml to get an accurate cell count but remember to bring the volume up to 10 ml before centrifugation
w. Remove 11µl cells and mix with 11 ul AOPI staining solution
x. Count the cells on a Nexcelom A2000 cell counter (See the Cell Counting and Viability SOP)
    i. The optimal range is 100 – 1,000 cells counted
        1. If the number of cells counted is less than 100, centrifuge, resuspend in smaller volume and recount
        2. If the number of cells counted is greater than 1,000, dilute the sample and recount
y. Centrifuge the 10 ml bone marrow tube from step v for 5 minutes at 1,500 rpm
z. Using a sterile pipet remove the supernatant without disturbing the cell pellet
aa. Calculate how many aliquots are to be made
    NOTE: Always freeze a minimum of 2 equivalent aliquots per sample
    i. Freeze < 2×10^6 cells in 2 equivalent aliquots
    ii. Freeze 2×10^6 – 3×10^7 cells in 6 equivalent aliquots and freeze all remaining cells in 1 tube

    Example 1: 2×10^6 total cells = 6 aliquots at 3.33×10^5 cells
    Example 2: 3×10^7 total cells = 6 aliquots at 5×10^6 cells
    Example 3: 3.1×10^7 cells = 6 aliquots at 5×10^6 cells and 1 tube at 1×10^6 cells
    Example 4: 1×10^8 cells = 6 aliquots at 5×10^6 cells and 1 tube at 7×10^7 cells

bb. Resuspend the cell pellet in 0.7 ml 1X Sterile PBS per aliquot to be made and transfer 0.7 ml cells into DNase/RNase free microfuge tubes
    i. Example: If freezing 1 x 10^6 cells (2 aliquots x 5 x 10^5 cells), resuspend the cell pellet in 1.4 ml 1X Sterile PBS and aliquot 0.7 ml into two DNase/RNase free microfuge tubes

cc. Centrifuge for 5 minutes at 1,500 rpm
dd. Completely remove the supernatant from each aliquot without disturbing the cell pellet
ee. Close the tube lids
ff. Place the tubes in a floating Styrofoam tube holder
gg. Drop the tubes into the flask of liquid nitrogen to flash freeze the pellets
hh. Immediately transfer the tubes from the liquid nitrogen flask to their assigned locations in the -80 freezer

VI. RESOURCES

a. Forms
b. References to Other Essential SOPs
c. Terms, Definitions & Abbreviations
d. Other Resources
e. Literature
f. References

I. NAME OF PROCEDURE: MDS Peripheral Blood Ficoll

II. PURPOSE:

a. For isolating peripheral blood mononuclear cells (PBMCs) from the peripheral blood of study participants with myelodysplastic syndromes (MDS) and idiopathic cytopenias of undetermined significance (ICUS)

III. SCOPE:

a. Starting material is 20 ml peripheral blood in two 10 ml green top heparin vacutainer tubes. Cells are cryopreserved in 5 x 10^6 cell aliquots

IV. EQUIPMENT, REAGENTS AND SUPPLIES:

a. 50 ml sterile conical tubes: Corning Cat. No. 430828
b. 5, 10, and 25 ml sterile pipettes: Corning Cat. Nos. 4487, 4488, and 13-678-11
c. Pipette aid
d. 10X Pharm Lyse solution, 100 ml: Becton Dickinson Cat. No. 555899
e. Lymphocyte Separation Medium (LSM): Ficoll-Paque Plus, GE Healthcare, Cat. NO. 17-1440-03
f. Sterile Phosphate Buffered Saline w/o Ca++ and Mg++ (1X PBS). Corning Cat. No.21040CM or Sigma 59321C
g. 1.8 ml cryovials: Thermo Scientific Cat. No. 375418
h. 190 proof ethanol: University of South Florida Tax-Free Ethyl Alcohol Order Form
i. Isopropanol: Sigma Cat. No. D2650-100ML
j. Kimwipe: Kimberly Clark 34155
k. Benchtop centrifuge
l. Mr. Frosty freezing containers: Fisher Cat. No. 15-350-50
m. Medicus Health Blood Tube Rocker (Cat. No. 5265M2): Fisher Cat. No. NC0139535
n. 4°C refrigerator
o. -80°C freezer
p. Cryounit

V. PROCEDURE: (NOTE: All techniques to be performed using standard aseptic techniques in a biosafety cabinet)

i. ALWAYS LAYER 20 ML DILUTED PERIPHERAL BLOOD ON TOP OF 15 ML FICOLL
ii. ALWAYS DILUTE THE PERIPHERAL BLOOD 1:1 WITH STERILE 1X PBS OR DILUTE IT MORE TO MAINTAIN THE CORRECT RATIO OF 20 ML DILUTED PERIPHERAL BLOOD PER 15 ML FICOLL
    1. 20 ML PERIPHERAL BLOOD: 20 ML PBS
    2. 17 ML PERIPHERAL BLOOD: 23 ML PBS

b. Prepare 1X Pharm Lyse buffer by diluting 10X Pharm Lyse buffer to 1X with sterile H_2O
c. Invert the tube several times to mix and keep at room temperature until needed
d. Clean hood by wiping all work surfaces with 70% alcohol with Kimwipe and replace isopropanol in Mr Frosty if necessary (after every 5 uses)

e. Pipet 15 ml sterile ficoll into two sterile 50 ml conical tubes
    i. Only 1 tube of Ficoll is needed if the original volume of peripheral blood is 10 ml or less
f. Pipet 20 ml sterile 1X PBS into a sterile 50 ml conical tube
g. Unscrew the top from one empty 50 ml conical tube
h. Remove the stopper from the two 10 ml green top heparin peripheral blood vacutainer tubes
i. Dilute the peripheral blood to a final volume of 40 ml (1:1 dilution) with sterile 1X PBS and layer it on top of the ficoll as follows (If the original volume of peripheral blood is 10 ml or less, dilute it to a final volume of 20 ml)
    i. Use a sterile 10 ml pipet to bring the volume of both peripheral blood tubes up to 10 ml with 1X sterile PBS from the stock bottle
    ii. Using the same pipet from step i, transfer the peripheral blood from each vacutainer tube into the 50 ml conical tube from step g.
    iii. Using the same 10 ml pipet from the previous step, rinse each vacutainer tube with 8 ml PBS from the tube in step f. and transfer it to the peripheral blood tube from step g
    iv. Using a sterile 25 ml pipet, mix the diluted peripheral blood well (It is important to mix well to dilute the red blood cells so they don't clump together with white blood cells and pass them through the ficoll gradient) and slowly layer 18 ml diluted peripheral blood on top of the ficoll in each tube from step e. without mixing the peripheral blood with the ficoll
    v. Using the same pipet from step iv, rinse the peripheral blood tube from step g with the remaining 4 ml PBS from step f and slowly add 2 ml each to the ficoll tubes from step e
j. Centrifuge the tubes with brake off at 400 g/30min/19°C in swinging bucket rotor.
k. After centrifugation collect the enriched cell fraction (interphase) which is below the top plasma layer and above the ficoll layer.
l. Transfer an equal volume of the interphase from both tubes into two 50 ml conical tubes.
m. Use a sterile 25 ml pipet to add 3 volumes (~30 ml) of 1X sterile PBS to each tube containing interphase. To avoid contamination, use a new pipet each time you are pipetting from the bottle of 1X sterile PBS.
n. Centrifuge 400g/8 min/20ºC/brake 3.
o. Remove the supernatant with ~~a~~sterile 25 ml pipets.Don't re-use the same pipet when it is full, use as many new pipets as necessary to remove the supernatant from both tubes
p. Using a sterile 5 ml pipet, add 5 ml 1X BD Pharm Lyse to one of the pellets, mix by pipetting up and down, transfer the cells to the second tube containing a pellet, mix by pipetting up and down, incubate 10 minutes on the rocker in the Biosafety Cabinet
q. Using a sterile 25 ml pipet, add 25 ml sterile 1X PBS to each tube.
r. Centrifuge 400g/ 8 min/ 20ºC/ brake 3. Remove the supernatant with a sterile 25 ml pipet.
s. Use a sterile 25 ml pipet to resuspend the cell pellet in 20ml of sterile 1X PBS.
    i. Resuspend smaller cell pellets in less than 20 ml to get an accurate cell count but remember to BRING THE VOLUME UP TO 20 ML before centrifuging.
t. Remove 11 µl cells and mix it with 11 µl AOPI staining solution.
u. Count the cells on a Nexcelom A2000 cell counter (See the Cell Counting and Viability SOP.)
    i. The optimal range is 100 – 1,000 cells counted.

1. If the number of cells counted is less than 100, centrifuge, resuspend in smaller volume and recount
2. If the number of cells counted is greater than 1,000, dilute the sample and recount

v. Calculate the total number of cells and allocate them as follows:
   i. Freeze at least 5 x 10^6 cells per aliquot with a minimum of 2 aliquots and a maximum of 10 aliquots
      1. 5 x 10^7 cells = 10 aliquots of 5 x 10^6 cells
      2. > 5 x 10^7 cells = 4 aliquots of 5 x 10^6 cells and divide the rest of the cells evenly into 6 aliquots

w. Make freezing media (90% FBS/10% DMSO), refer to the Cryopreservation of Cells SOP
x. Centrifuge the tube from step s. 400g/8 min/20°C/brake 3
y. To freeze cells, see the Cryopreservation of Cells SOP.

VI. RESOURCES

a. Forms
b. References to Other Essential SOPs
   i. Peripheral Blood Heparin Tube_Clotting_ Quality Assurance Data Form
c. Terms, Definitions & Abbreviations
d. Other Resources
e. Literature
f. References

NAME OF PROCEDURE: MDS RNA Extraction from Cryopreserved CD34$^+$ Cells

I. PURPOSE:

a. For RNA extraction from CD34$^+$ cells enriched from bone marrow of study participants enrolled in the National MDS Natural History Study

II. SCOPE:

a. Starting material is cryogenic preserved CD34$^+$ cells stored in liquid nitrogen. The CD34$^+$ cells were enriched from ficolled bone marrow. The RNA extraction procedure uses the Qiagen RNeasy Micro Kit protocol for Purification of Total RNA from Animal and Human Cells

III. EQUIPMENT, REAGENTS AND SUPPLIES:

a. Water bath for 37°C incubation
b. RNeasy Micro Kit (50): Qiagen Cat No. 74004
    i. Kit Contents
        1. RNeasy MinElute Spin Columns
        2. Collection Tubes
        3. Buffer RLT
        4. Buffer RW1
        5. Buffer RPE (concentrate)
        6. RNase-Free Water
        7. Carrier RNA, poly-A
        8. RNase-Free DNase Set
            a. RNase-Free DNase I (lyophilized)
            b. Buffer RDD
            c. RNase-Free Water
c. 14.3 M β-mercaptoethanol (β-ME)
d. 70% ethanol
e. Invitrogen UltraPure DNase/RNase-Free Distilled Water (Fisher Scientific Cat. No. 10-977-015)
f. 96- 100% ethanol
g. Microcentrifuge (with rotor for 2 ml tubes)
h. Vortexer

i. Collection Tubes (2ml): Qiagen catalog number 19201
j. Thermo Scientific Matrix tubes. To be supplied by the MDS Lab
k. TOPMed aliquot Matrix tubes. To be supplied by the MDS Lab
l. TruPoint filter pipet tips, Certified RNase, DNase, Human DNA, Pyrogen-FREE (Alkali Scientific)

Things to do before starting

Preparing Buffer RPE

Buffer RPE is supplied as a concentrate. Before using for the first time, add

4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

Preparing 80% ethanol
Before using the kit for the first time, prepare 80% ethanol by mixing 24 ml ethanol (96–100%) and 6 ml RNase-free water (supplied).

Preparing 70% ethanol
The procedure also requires 70% ethanol, which can be prepared by diluting ethanol (96–100%) with Invitrogen UltraPure DNase/RNase-Free Distilled Water (not supplied).

Buffer RLT may form a precipitate during storage. If necessary, redissolve by warming, and then place at room temperature.

Preparing DNase I stock solution
Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the lyophilized DNase I (1500 Kunitz units) in 550 µl of the RNasefree water provided. **Do not vortex.**

IV. **PROCEDURE:** Qiagen RNeasy Micro Kit protocol for Purification of Total RNA from Animal and Human Cells

Note: Process a maximum of 10 samples. Because RNA is chemically unstable and more susceptible to degradation than DNA, complete the entire procedure in 1 day and freeze the RNA in a -80°C freezer. DO NOT store the RNA overnight at room temperature or in the refrigerator.

a. Set a water bath to 37°C

b. Remove up to ten $CD34^+$ cell cryogenic vials from the cryogenic unit, place them on dry ice, and transport them to a 37°C water bath. Thaw all samples in the 37°C water bath at the same time. Thawing time is less than 5 minutes, until last ice crystal disappears.

c. Pellet the cells by centrifuging for 5 min at 3000 x g at 4C (pre-chilled centrifuge) in a centrifuge tube (not supplied). After centrifuging, place samples on wet ice. Carefully remove all supernatant by aspiration, and proceed to step d.

Note: Incomplete removal of cell-culture medium will inhibit lysis and dilute the lysate, affecting the conditions for binding of RNA to the RNeasy MinElute membrane. Both effects may reduce RNA yield.

d. Disrupt the cells by adding Buffer RLT.

Note: Add β-mercaptoethanol (β-ME) to Buffer RLT before use. Add 10 µl β-ME per 1 ml Buffer RLT

For pelleted cells, loosen the cell pellet thoroughly by flicking the tube. Add 350 µl Buffer RLT. **(if processing ≤1 x 10^5 cells, add 75 µl Buffer RLT instead)** Vortex for 10-15 seconds to homogenize the sample. and proceed to step e.

Note: Incomplete loosening of the cell pellet may lead to inefficient lysis and reduced RNA yields.

e. Add 1 volume of 70% ethanol to the lysate and mix well by pipetting. Do not centrifuge. Proceed immediately to step f.

Note: When purifying RNA from certain cell lines, precipitates may be visible after addition of ethanol. This does not affect the procedure.

f. Transfer the sample, including any precipitate that may have formed, to an RNeasy MinElute spin column placed in a 2 ml collection tube (supplied). Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow- through.

g. Add 350 µl Buffer RW1 to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.

Reuse the collection tube in step j.

h. Add 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube.

Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex.

i. Add the DNase I incubation mix (80 µl) directly to the RNeasy MinElute spin column membrane, and place on the benchtop (20–30°C) for 15 min.

Note: Be sure to add the DNase I incubation mix directly to the RNeasy MinElute spin column membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the spin column.

j. Add 350 µl Buffer RW1 to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through and collection tube.

k. Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Add 500 µl Buffer RPE to the spin column. Close the lid gently, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through.

Reuse the collection tube in step l.

Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Things to do before starting").

l. Add 500 µl of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently, and centrifuge for 2 min at ≥8000 x g (≥10,000 rpm) to wash the spin column membrane. Discard the flow-through and collection tube.

Prepare the 80% ethanol with ethanol (96–100%) and the RNase-free water supplied with the kit.

Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

m. Place the RNeasy MinElute spin column in a new 2 ml collection tube (supplied). Open the lid of the spin column and let it sit for 10 minutes to let any residual ethanol evaporate from the column and column ring.

It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Leaving the lids open for 10 minutes ensures that no ethanol is carried over during RNA elution.

n. Close the lid of the spin column and centrifuge at full speed for 5 min. Discard the flow-through and collection tube

o. Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 18 µl RNase-free water directly to the center of the spin column membrane and let it incubate for 1 minute. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.

The dead volume of the RNeasy MinElute spin column is ~ 2 µl: elution with18 µl RNase-free water results in ~ 16 µl eluate.

p. Use 2 µl RNA for Qubit High Sensitivity Assay
Note: Qubit concentration is the only measurement being taken. Do not use NanoDrop and TapeStation

q. Determine RNA quantity and prepare and aliquot the samples as follows:
Note: Make derivatives in LabVantage for the TOPMed aliquot Matrix tubes. **DO NOT** print and affix LV Sample ID labels to the TOPMed aliquot Matrix tubes. On the Tissue Core Experimental Worksheet, enter both the LV Sample ID and TOPMed Aliquot tube ID for each sample.

Tissue Core Experimental Worksheet

Study ID	LV Parent ID	LV Sample ID (created for documentation of the TOPMed Aliquot tube in LV)	TOPMed Aliquot tube ID (TOPMed tubes)	Aliquot Vol (ul)	Source Material (instead of "Amount")	Final RNA Volume	Nanodrop Quantity (ug)	Original Concentration (ng/uL)	260	280	260/280 ratio	260/230 ratio	RIN	ssRNA Final Quantity (ug)	ssRNA Aliquot Concentration ng/ul	DIN	dsDNA Final Quantity(ug)	dsDNA Final Concentration (ng/ul)	Quantification Method	Entry Date	Experiment	Comments	ssRNA Original Volume (uL)	ssRNA Original Concentration (ng/ul)	ssRNA Current Quantity for LV (ug)
MDS-10047	07S16021049		0317865847	54.0	Cryopreserved CD34+ cells	54.0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.308	5.71	N/A	N/A	N/A	Qubit HS				14.2	21.70	0.308
MDS-10330	07S17035879		0317866639	54.0	Cryopreserved CD34+ cells	54.0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.719	13.31	N/A	N/A	N/A	Qubit HS				14.5	49.60	0.719
MDS-10410	07S18008830		0317865142	14.0	Cryopreserved CD34+ cells	14.0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.179	12.80	N/A	N/A	N/A	Qubit HS				14	12.80	0.179

Tissue Core Experimental Worksheet Data Dictionary

Aliquot Vol (ul) = the volume of RNA in each aliquot

Final RNA Volume = Aliquot Vol (ul) because there is only 1 aliquot

ssRNA Final Quantity (ug) = the quantity (µg) of the entire sample

ssRNA Aliquot Concentration (ng/µl) = the concentration (ng/µl) of each aliquot (Calculated as follows: ssRNA Current Quantity for LV / Aliquot Vol *1000)

ssRNA Current Quantity for LV (µg) = the quantity (µg) of RNA in each aliquot

i. Measure the volume of each sample and calculate the RNA quantity

Note: For steps ii - iv below, the empty TOPMed aliquot Matrix tubes will be provided in a TOPMed Matrix tube rack which is to be kept at room temperature. Each TOPMed aliquot Matrix tube has a unique ID and is assigned to a specific sample, which is listed on the **MDS Service Request Form** (See example form below). After aliquoting the RNA into the TOPMed aliquot Matrix tubes, **DO NOT** put them back into the TOPMed Matrix tube rack. Put all RNA aliquoted into TOPMed aliquot Matrix tubes into a **NEW** Matrix tube rack and store it in the -80° freezer.

Sample Number	Sample Name	Sample ID	Sample Description	Instructions	Qty Current	Qty Unit	Sample storage	Extraction Method	Extraction Protocol	Resuspension Buffer	TOPMed Matrix Rack	TOPMed Aliquot Matrix Tube ID	Tube position in Matrix Rack. Please do not rearrange
1	MDS-10047	07S16021049	Cryopreserved CD34+ cells	Perform on-column DNase digestion(Steps 7-8). Reconstitute RNA in RNase-free water	0.82	x 10^6	cryogenic unit	RNeasy Micro Kit	MDS SOP_RNA extraction from CD34+ cells	RNase-free water	MTR-07668	0317865847	A:1
2	MDS-10330	07S17035879	Cryopreserved CD34+ cells		0.685	x 10^6	cryogenic unit	RNeasy Micro Kit	MDS SOP_RNA extraction from CD34+ cells	RNase-free water	MTR-07668	0317866639	B:1
3	MDS-10410	07S18008830	Cryopreserved CD34+ cells		1.065	x 10^6	cryogenic unit	RNeasy Micro Kit	MDS SOP_RNA extraction from CD34+ cells	RNase-free water	MTR-07668	0317865142	C:1
4	MDS-10498	07S18021493	Cryopreserved CD34+ cells		0.985	x 10^6	cryogenic unit	RNeasy Micro Kit	MDS SOP_RNA extraction from CD34+ cells	RNase-free water	MTR-07668	0317865907	D:1
5	MDS-10585	07S18032202	Cryopreserved CD34+ cells		0.656	x 10^6	cryogenic unit	RNeasy Micro Kit	MDS SOP_RNA extraction from CD34+ cells	RNase-free water	MTR-07668	0317865891	E:1
6	MDS-10614	07S19000124	Cryopreserved CD34+ cells		0.5	x 10^6	cryogenic unit	RNeasy Micro Kit	MDS SOP_RNA extraction from CD34+ cells	RNase-free water	MTR-07668	0317867606	F:1
7	MDS-10679	07S19009639	Cryopreserved CD34+ cells		0.772	x 10^6	cryogenic unit	RNeasy Micro Kit	MDS SOP_RNA extraction from CD34+ cells	RNase-free water	MTR-07668	0330654261	G:1
8	MDS-11096	07S20019870	Cryopreserved CD34+ cells		9.86	x 10^5	cryogenic unit	RNeasy Micro Kit	MDS SOP_RNA extraction from CD34+ cells	RNase-free water	MTR-07668	0317865766	H:1
9	MDS-11137	07S20025757	Cryopreserved CD34+ cells		5.7	x 10^5	cryogenic unit	RNeasy Micro Kit	MDS SOP_RNA extraction from CD34+ cells	RNase-free water	MTR-07668	0317867691	A:2
10	MDS-11183	07S20032330	Cryopreserved CD34+ cells		7.05	x 10^5	cryogenic unit	RNeasy Micro Kit	MDS SOP_RNA extraction from CD34+ cells	RNase-free water	MTR-07668	0317867725	B:2

ii. If <200 ng RNA, aliquot the entire sample into the TOPMed aliquot Matrix tube assigned to that sample, which is listed on the MDS Service Request Form (**DO NOT** adjust the sample volume to 54 µl with RNase-free water)

1. RNA will be extracted from a 2nd aliquot of cryopreserved CD34+ cells for all samples with <200 ng RNA in the 1st aliquot. To be performed in a later experiment (see step V.)

For an example, see the aliquot for MDS-10410 in the Tissue Core Experimental Worksheet (see example worksheet on p. 5)

iii. If > 200 ng RNA, **adjust the volume of the sample to 54 µl with RNase-free water** (because minimum volume needs to be 50 µl), recalculate the sample concentration for the Tissue Core Experimental Worksheet (see example worksheet on page 5), aliquot it all into the TOPMed aliquot Matrix tube assigned to that sample, which is listed on the MDS Service Request Form.

For an example, see the aliquots for MDS-10047 and MDS-10330 in the Tissue Core Experimental Worksheet (see example worksheet on page 5)

V. RNA EXTRACTION FROM A 2nd ALIQUOT OF CRYOPRESERVED CD34+ CELLS FROM SAMPLES THAT YIELDED <200 ng RNA FROM THE 1st ALIQUOT

a. Follow steps a – p in this SOP

b. Prepare and aliquot the samples as follows:

Note: Make derivatives for these samples in LabVantage and enter the LV aliquot sample ID on the Tissue Core Experimental Worksheet, even though the RNA is being dispensed directly into the frozen TOPMed barcoded Matrix tube containing the 1st aliquot of RNA

i. Measure the volume of the fresh RNA

ii. Add RNase-free water to the fresh RNA samples so the combined volume of the fresh and frozen RNA will be 54 µl (because minimum volume needs to be 50 µl), and recalculate the combined concentration and quantity of the fresh and frozen RNA for the Tissue Core Experimental Worksheet (The volume (ul) and quantity (ug) of the frozen RNA will be on the MDS Service Request Form. See step iii for combining the fresh sample with the frozen aliquot

MDS Service Request Form

Sample Number	Sample Name	Sample ID	Sample Description	Instructions	Qty Current	Qty Unit	Sample storage	Extraction Method	Extraction Protocol	Resuspension Buffer	Frozen TOPMed Aliquot Matrix Tube ID	Frozen TOPMed Aliquot Volume (ul)	Frozen TOPMed Aliquot Quantity (ug)
1	MDS-10410	07S18008831	Cryopreserved CD34+ cells	Perform on-column DNase digestion(Steps 7-8). Reconstitute RNA in RNase-free water	1.065	x 10^6	cryogenic unit	RNeasy Micro Kit	MDS SOP_RNA extraction from CD34+ cells	RNase-free water	0317865142	14	0.179

Tissue Core Experimental Worksheet

Study ID	LV Parent ID	Fresh LV Aliquot sample ID (Thermo Scientific tubes)	Frozen TOPMed Aliquot tube ID (TOPMed tubes)	Aliquot Vol (ul)	Source Material (instead of "Amount")	Final RNA Volume	Nanodrop Quantity (ug)	Original Concentration (ng/uL)	260	280	260/280 ratio	260/230 ratio	RIN	ssRNA Final Quantity (ug)	ssRNA Aliquot Concentration ng/ul	DIN	dsDNA Final Quantity(ug)	dsDNA Final Concentration (ng/ul)	Quantification Method	Entry Date	Experiment	Comments	ssRNA Original Volume frozen RNA (ul)	ssRNA Original Quantity frozen RNA (ug)	ssRNA Original Volume fresh RNA (ul)	ssRNA Original Concentration fresh RNA (ng/ulul)	ssRNA Original Quantity fresh RNA (ug)	ssRNA Current Quantity for LV (ug)
MDS-10410	07S18008831	Created in LV (no physical tube)	0317865142	54.0	Cryopreserved CD34+ cells	54.0	N/A	N/A	N/A	N/A	N/A	N/A	N/A	0.465	8.61	N/A	N/A	N/A	Qubit HS				14	0.179	14.3	20	0.286	0.465

a. Example:
   i. Fresh RNA volume = 14.3 µl
   ii. Frozen RNA volume = 14 µl
   iii. Add 25.7 µl RNase-free water to the fresh aliquot

iii. Dispense the fresh RNA into the frozen TOPMed aliquot Matrix tube containing the 1st aliquot of RNA from each sample

1. Remove a frozen RNA aliquot from the -80°C freezer **ONE ALIQUOT AT A TIME** and place it on dry ice (Do one tube at a time so the RNA does not thaw). The TOPMed aliquot Matrix tube ID for each of the frozen aliquots will be listed on the MDS Service Request Form

Sample Number	Sample Name	Sample ID	Sample Description	Instructions	Qty Current	Qty Unit	Sample storage	Extraction Method	Extraction Protocol	Resuspension Buffer	Frozen TOPMed Aliquot Matrix Tube ID	Frozen TOPMed Aliquot Volume (ul)	Frozen TOPMed Aliquot Quantity (ug)
1	MDS-10410	07S18008831	Cryopreserved CD34+ cells	Perform on-column DNase digestion(Steps 7-8). Reconstitute RNA in RNase-free water	1.065	x 10^6	cryogenic unit	RNeasy Micro Kit	MDS SOP_RNA extraction from CD34+ cells	RNase-free water	0317865142	14	0.179

2. Remove the lid from the frozen RNA aliquot and dispense the fresh RNA directly on top of the frozen RNA, being careful the fresh RNA is laying on top of the frozen RNA and not scattered on the sides of the tube

3. Put the lid back on the tube, immediately put the RNA back into the -80°C freezer, remove the next frozen aliquot, repeat steps 2 -3 until finished

I. NAME OF PROCEDURE: Serum collection from peripheral blood

II. PURPOSE:

a. **To collect serum from the peripheral blood of study participants with myelodysplastic syndromes (MDS) and idiopathic cytopenias of undetermined significance (ICUS)**

III. SCOPE:

a. **Starting material is 3 ml of peripheral blood in a red top vacutainer tube containing no anticoagulants. Anticoagulants distinguish serum from plasma. Serum comes from blood that is collected without anticoagulants. Plasma comes from blood that is collected in tubes, syringes, or bags containing anticoagulants such as EDTA and heparin.**

IV. EQUIPMENT, REAGENTS AND SUPPLIES:

a. **5 ml sterile pipettes: Corning Cat. No. 4487**
b. **2 ml sterile pipettes: Fisher Cat. No. 07-200-572**
c. **15 ml conical tubes: Lawson Cat. No. 215960**
d. **Pipette aid**
e. **0.5 ml Matrix tubes, Thermo Scientific No. 3743: Fisher Cat. No. 50-871-135**
f. **0.5 ml Matrix tube storage boxes, Thermo Scientific No. 4900:**
g. **Fisher Cat. No. 50-871-150**
h. **Benchtop centrifuge**
i. **-80°C freezer**

V. PROCEDURE: (NOTE: All techniques to be performed using standard aseptic techniques in a biosafety cabinet)

a. **Place the 3 ml red top vacutainer tube containing peripheral blood into the centrifuge and balance with a 5 ml FACSFlow tube containing the same volume of water**
b. **Centrifuge 2,000g/ 20'/RT with the brake off or on lowest possible setting**
c. **Pipet the serum (top layer) from the vacutainer tube without disturbing the interface (middle layer)and dispense the serum into an empty 15 ml conical tube**
d. **Centrifuge 2000g/10'/RT with the brake off or on lowest possible setting**
e. **Remove the tube from the centrifuge and hold it in front of the Specimen Integrity Chart for Hemolysis. Match the color of the serum to the appropriate color on the chart and record the hemolysis level associated with that color, which ranges from 0- 8gL**
f. **Pipet the serum (top layer) from the 15 ml conical tube without disturbing the interface (middle layer) and dispense 250 µl plasma into 0.5 ml Matrix tubes, with a minimum of 2 aliquots and maximum of 10 aliquots. Dispense any remaining serum into a 1.8 ml cryovial**
g. **Store the plasma in Matrix storage boxes in the -80 freezer**
    i. **KEEP THE TUBES OF SERUM AT 4ºC UNTIL THEY ARE STORED IN THE -80ºC FREEZER**

VI. RESOURCES

a. **Forms**
b. **References to Other Essential SOPs**
    i. **Refer to Peripheral Blood Serum Hemolysis Quality Assurance Data Form**
c. **Terms, Definitions & Abbreviations**
d. **Other Resources**
e. **Literature**
f. **References**

I. NAME OF PROCEDURE: MDS Thawing Cryopreserved Cells

II. PURPOSE:

a. For thawing cryopreserved cells isolated from the peripheral blood and bone marrow of patients that do not have MDS

III. SCOPE:

a. These non-MDS samples are used to QC the procedure used to process and cryopreserve MDS study samples
b. The samples will be thawed in matching pairs (1 peripheral blood and 1 bone marrow vial per case)
c. Flow cytometry will be performed on all samples for the purpose of measuring viability of the different cell lineages (lymphocytes, monocytes, granulocytes, etc.)

IV. EQUIPMENT, REAGENTS AND SUPPLIES: (NOTE: All techniques to be performed using standard aseptic techniques in a biosafety cabinet)

a. 50 ml sterile conical tubes: Corning Cat. No. 430828
b. 5, 10, and 25 ml sterile pipettes: Corning Cat. Nos. 4487, 4488, and 13-678-11
c. Pipette aid
d. RPMI media (Life Technologies), 1000 ml: Fisher Cat No. 11875085
e. Benchtop centrifuge
f. 4°C refrigerator
g. -80°C freezer
h. Heat Inactivated FBS: ThermoFisher Cat. No. 10082-147
i. A2000 cellometer: Nexcelom Cat. No. A2000
j. Anti-Human CD34-APC antibody: Stem Cell Cat. No. 60013AZ
k. Anti-Human CD45-FITC antibody: Stem Cell Cat. No. 60018FI1
l. Propidium Iodide Viability Staining Solution: Miltenyi Cat. No. 130-093-233
m. Anti-Human CD3-APC antibody: Stem Cell Cat. No. 60011FL.1
n. RoboSep Buffer: Stem Cell Cat. No. 20104

V. PROCEDURE: Thawing cryopreserved cells

a. NOTE: All techniques to be performed using standard aseptic techniques in a biosafety cabinet
b. Clean hood by wiping all work surfaces with 70% alcohol with Kimwipe
c. Prepare 10 ml of thawing media per tube to be thawed (RPMI+ 10% FBS) by mixing 5 ml 100% FBS with 45 ml RPMI media
d. Warm the thawing media to 37ºC by placing in a 37ºC water bath
e. Fill a flask with liquid nitrogen to be used for keeping the frozen cells cold until they are thawed
f. Remove 2 vials of cells (1ml) from the cryogenic unit and immediately place it into the 37ºC water bath. Use liquid nitrogen to transport the cells to the water bath.
    i. DO NOT THAW MORE THAN 2 VIALS OF CELLS AT THE SAME TIME (because it is important to quickly proceed to the step of reconstituting the cells in RPMI media)

g. Remove the thawing media from the water bath, wipe it off , spray it with 70% ethanol and wipe off the ethanol
h. Place the thawing media in the hood and label two sterile 50 ml conical tubes with the sample IDs
i. Remove the vials of cells from the water bath
    i. It is important to remove the vial of cells from the water bath immediately after they are thawed or when there is a small piece of frozen material remaining
    ii. Work quickly after removing the cells from the water bath
j. Spray the vials of cells with 70% ethanol, wipe off the ethanol and place the cells in the hood
k. Open one vial of cells and use a sterile 5 ml pipet to transfer them to the sterile 50 ml conical tube
l. Pipet 9 ml RPMI media with a sterile pipet, rinse the empty cryovial, and slowly pipet (~ 1 ml per second) the 9 ml of RPMI down the side of the tube into the 1 ml of cells
    i. It is important for rejuvenation of the cells that the RPMI is added slowly
m. Repeat steps j-l with the 2nd vial of cells
n. If thawing > 1 x 10^6 cells, gently mix the cells and proceed to cell counting (See Cell Counting and Viability SOP). If thawing < 1 x 10^6 cells, let the cells rest for 10 minutes, transfer to 15 ml conical tube, centrifuge 1,500 rpm 5 minutes, resuspend in 1 ml RPMI, count the cells

VI. PROCEDURE: Flow cytometry

a. Bone Marrow Mononuclear Cells (BMMNCs) will be stained with 5 ul CD34 APC and 5 ul CD45 FITC antibodies
b. Peripheral Blood Mononuclear Cells (PBMCs) will be stained with 5 ul CD3 APC and 5 ul CD45 FITC antibodies
c. Label 5 ml polystyrene tubes for the following samples
    i. Compensation tubes
        1. CD3 APC only (use compensation beads)
        2. CD45 FITC only (use compensation beads)
        3. PI only (use BMMNCs)
    ii. Sample tubes
        1. CD3APC /CD45 FITC/PI for PBMC samples
d. CD34 APC/CD34 FITC/ PI for BMMNC samples
e. Transfer 1 x 10^5 cells to each tube requiring cells
f. Add 1 ml RoboSep buffer
g. Centrifuge 1,500 rpm for 5 minutes
h. Decant the supernatant into the sink
i. Repeat steps f-h
j. Add 5 µl CD3 APC and 5 µl CD45 FITC to the tubes containing PBMCs
k. Add 5 µl CD34 APC and 5 µl CD45 FITC to the tubes containing BMMNCs
l. Mix by shaking the tubes and incubate 4ºC for 20 minutes
m. Prepare compensation beads
    i. Pipet 100 µl RoboSep buffer into the compensation tubes
    ii. Vortex the negative and positive compensation beads and add 1 drop of each to each compensation tube

iii. Add 10 µl CD3 APC to the 'CD3 APC only' tube
iv. Add 15 µl CD45 FITC to the 'CD45 FITC only' tube
v. Mix by shaking the tubes and incubate room temperature for 20 minutes

n. Add 2 ml RoboSep buffer to each tube, centrifuge 1,500 rpm for 5 minutes, decant
o. Resuspend each sample in 400 µl RoboSep buffer
p. Add 5 µl PI to each sample EXCEPT THE TUBES CONTAINING COMPENSATION BEADS and incubate in the dark for 5 minutes at room temperature
q. Acquire the samples on the FACSCalibur

VII. RESOURCES

a. Forms
b. References to Other Essential SOPs
c. Terms, Definitions & Abbreviations
d. Other Resources
e. Literature
f. References

The National Myelodysplastic Syndromes Natural History Study Sponsored by the National Heart, Lung, and Blood Institute in collaboration with the National Cancer Institute NHLBI Contract: HHSN268201400003I	**SOP Number:**	MDS-SOP-073	**Effective Date:**	12 Apr 2019
	CAP Standard:		**Category:**	MDS Sample Processing
	Revision:	2	**Next Review:**	13 June 2020

NAME OF PROCEDURE: MDS Whole Bone Marrow Cell Pellet DNA Extraction

I. PURPOSE:

a. For extracting DNA from whole bone marrow cell pellets collected from study participants enrolled on the National MDS Natural History Study

II. SCOPE:

a. Starting material is up to 5 x 10^6 cells flash-frozen in liquid nitrogen and stored in a -80ºC freezer. The cell pellets were processed according to the MDS Making Cell Pellets from Bone Marrow EDTA Tube SOP. The DNA extraction procedure is a modified version of the QiaAmp DNAMini Kit protocol for DNA Purification from Blood or Body Fluids

III. EQUIPMENT, REAGENTS AND SUPPLIES:

a. Thermomixer, heat block, or water bath for 56°C incubation
b. QIAamp DNA Mini Kit: Qiagen Cat No. 51304
    i. Kit Contents
        1. QIAamp Mini Spin Columns
        2. Collection Tubes
        3. Buffer AL
        4. Buffer ATL
        5. Buffer AW1 (concentrate)
        6. Buffer AW2 (concentrate)
        7. Buffer AE
        8. Proteinase K
c. Nanodrop
d. Tapestation
e. Qubit
f. Buffer ATE (20 ml): Qiagen catalog number 1067929
g. 1X Dulbecco's PBS, without Calcium and Magnesium (6 x 1L), Corning # 21040CM: VWR catalog number 45000-448. To be supplied by the MDS Lab
h. Collection Tubes (2ml): Qiagen catalog number 19201
i. Proteinase-K (Included in the DNA Mini Kit): Qiagen catalog number 19131
j. 0.5 ml Matrix tubes: Thermo Scientific catalog number 3743. To be supplied by the MDS Lab
k. 1.5 ml microfuge tubes: USA Scientific catalog number 1415-9700. To be supplied by the MDS Lab

IV. PREPARATION OF BUFFERS

Preparing Buffer AL

Before starting the procedure, check whether precipitate has formed in Buffer AL. If necessary, dissolve by heating to 70°C with gentle agitation

Preparing Buffer AW1

Add 25 ml ethanol (96–100%) to the bottle containing 19 ml Buffer AW1 concentrate. Tick the check box on the bottle label to indicate that ethanol has been added. Reconstituted Buffer AW1 can be stored at room temperature (15–25°C) for up to 1 year.
Note: Before starting the procedure, mix the reconstituted Buffer AW1 by shaking.

Preparing Buffer AW2

Add 30 ml ethanol (96–100%) to the bottle containing 13 ml Buffer AW2 concentrate.
Reconstituted Buffer AW2 can be stored at room temperature (15–25°C) for up to 1 year.
Note: Before starting the procedure, mix the reconstituted Buffer AW2 by shaking.

V. **PROCEDURE:** (Important changes have been highlighted)

a. Set the Thermomixer to 56°C
b. Remove cell pellet tubes from the -80°C freezer
c. Add 200 µl PBS without Calcium and Magnesium to all samples
    i. The MDS Lab will supply the PBS
d. Add 20 µl proteinase K to all samples and mix by vortexing
    i. It is important to ensure proper mixing because proteinase K is not compatible with Buffer AL in the next step
e. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid
f. Add 200 µl Buffer AL to the sample. Mix by pulse-vortexing for 15 s.
    i. Note: Do not add proteinase K directly to Buffer AL.
g. Place the 1.5 ml tube in a thermomixer or heated orbital incubator, and incubate at 56°C for 30 minutes, shaking at 900 rpm
h. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid
i. Add 200 µl ethanol (96–100%) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid
j. Carefully apply the mixture from step i to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate
    i. If the lysate has not completely passed through the column after centrifugation, centrifuge again at max speed until the column is empty
k. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate
l. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.
m. Place the QIAamp Mini spin column in a clean 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 3 min.

n. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube from step m. Carefully open the lid of the QIAamp Mini spin column, and incubate at room temperature (15–25°C) for 30 min. to dry the column

o. Add Buffer ATE to the center of the column
   i. DO NOT USE BUFFER AE WHICH IS SUPPLIED WITH THE DNA MINI KIT. ORDER BUFFER ATE SEPARATELY. SEE EQUIPMENT, REAGENTS AND SUPPLIES FOR ORDERING INFORMATION
   ii. Refer to the Sample Submission form for the volume of Buffer ATE to add to each column. Either 50 µl or 200 µl depending on the number of cells in each sample
      1. Elute < 2 x 10^6 cells from the column with 50 µl Buffer ATE
      2. Elute > 2 x 10^6 cells from the column with 200 µl Buffer ATE

p. Close the lid and incubate at room temperature (15–25°C) for 10 min. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min

q. Remove the Buffer ATE from the 1.5 ml microcentrifuge tube and re-apply it to the column
   i. Re-elute with the same Buffer ATE from step o. Do not add new Buffer ATE

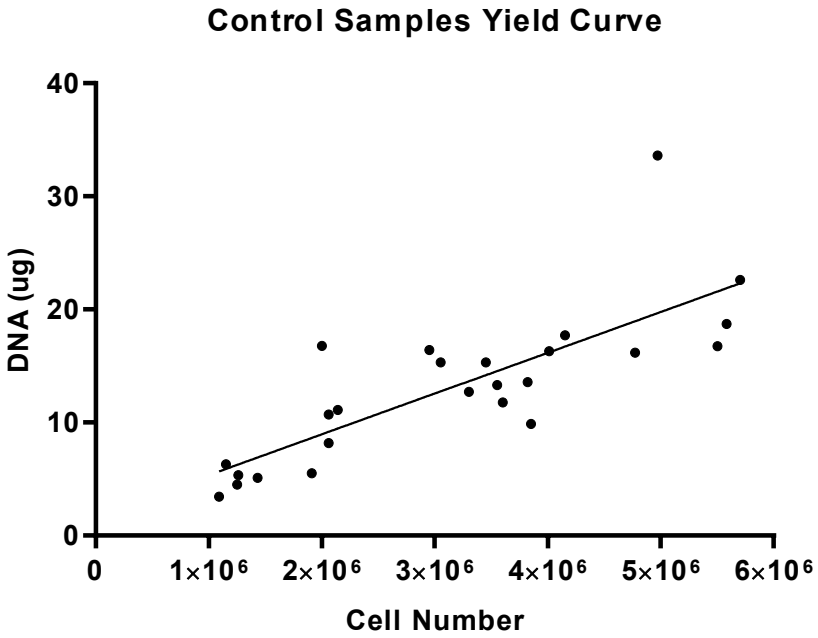
r. Close the lid and incubate at room temperature (15–25°C) for 10 min. Centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min

s. Quantitate 1.5 µl DNA on the nanodrop, 3 µl on the Qubit and run 1 µl on the Tapestation

t. Aliquot the DNA as follows (MDS Lab will supply the Matrix tubes and 1.5 ml screw top tubes)
   ·Always make a minimum of 2 aliquots from every sample
   ·DNA aliquots should be in a minimum volume of at least 10 µl (This has priority over concentration being 50 ng/ul)
   ·DNA concentration should be 50 ng/µl unless initial concentration < 50 ng/ul or volume < 10 µl and needs to be brought up to 10 µl
   i. Important: Measure the exact sample volume to ensure the correct quantity in each aliquot
      1. If the DNA concentration > 50 ng/µl, dilute the DNA to 50 ng/µl with Buffer ATE
         a. Aliquot 500 ng DNA (10µl) into a maximum of 4 Matrix tubes
         b. Aliquot any extra DNA into a 1.5 ml screw top stock tube (use Matrix tube if < 500 ng DNA) and measure the volume. If necessary, bring the volume up to 10 µl and calculate the new concentration
      2. If the DNA concentration < 50 ng/µl
         a. Aliquot 500 ng DNA into a maximum of 4 Matrix tubes
         b. Aliquot any extra DNA into a 1.5 ml screw top stock tube (use Matrix tube if < 500 ng DNA) and measure the volume. If necessary, bring the volume up to 10 µl and calculate the new concentration
         c. Make 2 equivalent aliquots in Matrix tubes if less than 1,000 ng DNA
      3. CONTROL DNA SAMPLE ALIQUOTING ONLY
         a. Make 2 equivalent aliquots in 1.5 ml screw top stock tubes

VI. RESOURCES

a. Forms
b. References to Other Essential SOPs
c. Terms, Definitions & Abbreviations
d. Other Resources- Cell pellet extraction optimization study, see below chart for correlation of staring cell material and DNA yield
e. Literature
f. References



DNA was extracted from 25 control whole bone marrow cell pellets ranging from $1x10^6$ to $5x10^6$ cells using the 'Whole Bone Marrow Cell Pellet DNA Extraction' SOP. Cell number is plotted vs DNA yield and shows a linear correlation between cell number and DNA yield. 5 x 10^6 cells is the maximum amount to use for DNA extraction using this SOP