PEAC CENTRAL LABORATORY

SAMPLE PROCESSING MANUAL

VERSION 1.0

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SAMPLE/TUBE LABELLING & LOGGING

All tubes should be labelled with either stickers or permanent marker.

Centre ID codes should be written on each tube in permanent marker and the code should contain the centre ID, year and sample number as shown in the examples below:

e.g.

Birmingham: BHAM2009001

Queen Mary: QMUL2009048

All samples should be logged onto the Tissue Auditor system in the PEAC Biobank storage and also logged into the PEAC Biobank sample book.

Sample information and storage location are also to be logged in the back-up PEAC excel workbook.

PEAC TISSUE PROCESSING & STORAGE

US GUIDED TISSUE BIOPSY

TISSUE - FROZEN

2-6 pieces of tissue*

Cut out 3 individual wells from a 96-well clear flexible PVC microplate

Place 2 pieces of tissue into the bottom of each well, cover with OCT medium and leave for 1 min

Hold well with forceps and freeze with cryospray until OCT turns white (~20secs). Trim round the edge of each well - transfer each well into individual **cryovials**

Long-Term Storage

PEAC Frozen Blocks Box

(-80 C Main & Back-up freezers)

TISSUE - RNA LATER

2-6 pieces of tissue*

Transfer 2 pieces per cryovial

1.0mL RNALater

Leave at 4ºC for 24hrs

Long-Term Storage

PEAC RNALater Box

(Liquid Nitrogen tank, Rack 5, slot A)

TISSUE - FORMALIN

2-6 pieces of tissue*

Transfer tissue wrapped in Whatman blotting paper into a histology cassette(s) & label with PEAC ID No.

Place in cassette pot containing 4% neutral buffered formalin for fixation

When tissue is fixed place cassette(s) into the tissue processor rack and run standard overnight protocol.

Remove cassette(s) & run clean program on tissue processor.

Place cassettes into tissue embedding centre & embed tissue in wax blocks (2 pieces/block)

Allow blocks to harden & either store in **PEAC block store** or sections blocks for H&E/grading

***If only minimum tissue can be retrieved please prioritise RNA and formalin preparations (See Important Information section).If possible process 1 frozen block & store in Main freezer.**

PEAC SYNOVIAL FLUID & URINE PROCESSING & STORAGE

SYNOVIAL FLUID

Processing remaining synovial fluid

1.5-2mL
Synovial fluid – divide between 2 cryovials

Long -Term Storage

PEAC Synovial Fluids & Co-products Box

(-80 C Main & Back-up freezers)

<20mL Syn. Fl.
Centrifuge fluid at 3000rpm for 10min

Divide *XmL* cell-free syn. fluid into cryovials (1mL aliquots)

Add 1mL RNALater to remaining cell pellet & transfer into a cryovial

Long-Term Storage

PEAC Synovial Fluids & Co-products Box

(-80 C Main & Back-up freezers)

>20mL Syn. Fl.
Separate SFMNCs from fluid using standard protocol

Resuspend cells in freezing medium (FM - 80%FCS/20%DMSO) approx. $1x10^6$ cells/100ul FM

Transfer to -80 C (Mr Frosty pot) for min. of 24hrs before transferring to liquid nitrogen

Long-Term Storage

PEAC SFMNCs Box

(Liquid Nitrogen tank, Rack 5, slot E)

URINE SPECIMEN

Transfer **20mL** freshly voided midstream urine into a **FRESH** sterile screw cap universal & label with PEAC ID No.

Long-Term Storage

PEAC Urine Tray

(universal storage)

Back-up freezer only

PEAC BLOOD PROCESSING & STORAGE

METABOLOMICS SERUM

Red (Silica)

Centrifuge at 1500g for 10 min at 20ºC

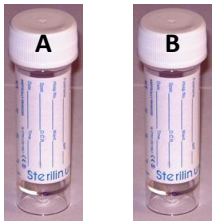
Aliquot approx. **1.0mL** SERUM into 2 cryovials (label with PEAC ID No.)

Long-Term Storage

PEAC Blood Co-products Box

(-80 C Main & Back-up freezers)

WHOLE BLOOD RNA LATER



2.0mL whole blood in 2 universals containing 5.0mL *RNALater (7mL total vol.)*

A

Divide in to 5 cryovials (1-1.5mL per aliquot) & label with PEAC ID No.

Long-Term Storage

PEAC Whole Blood RNALater Box

(-80 C Main freezer only)

B

Long-Term Storage

PEAC Blood Tray universal storage

Back-up freezer only

SERUM

Gold (Silica)

Centrifuge at 1500g for 10 min at 20ºC

Aliquot approx. **1.0mL** SERUM into 2 cryovials (label with PEAC ID No.)

Long-Term Storage

PEAC Blood Co-products Box

(-80 C Main & Back-up freezers)

BUFFY COAT & PLASMA

Green (Heparin)

Centrifuge at 1500g for 10 min at 20ºC

Remove white layer (buffy coat) & transfer to cryovial containing 1mL RLT lysis buffer.

Add beta-ME to the buffy coat (1ul per 100ul vol.) & split sample between 2 cryovials

Aliquot approx. **1.0mL** PLASMA into 2 cryovials (label with PEAC ID No.)

PEAC Blood Co-products Box (-80 C Main & Back-up freezers)

PBMNCs

Yellow (ACD) x2

Separate PBMNCs from blood using standard protocol

Resuspend cells in freezing medium (FM - 80%FCS/20%DMSO) approx. $1x10^6$ cells/100ul FM

Max. Of 10^7 cells/cryovial

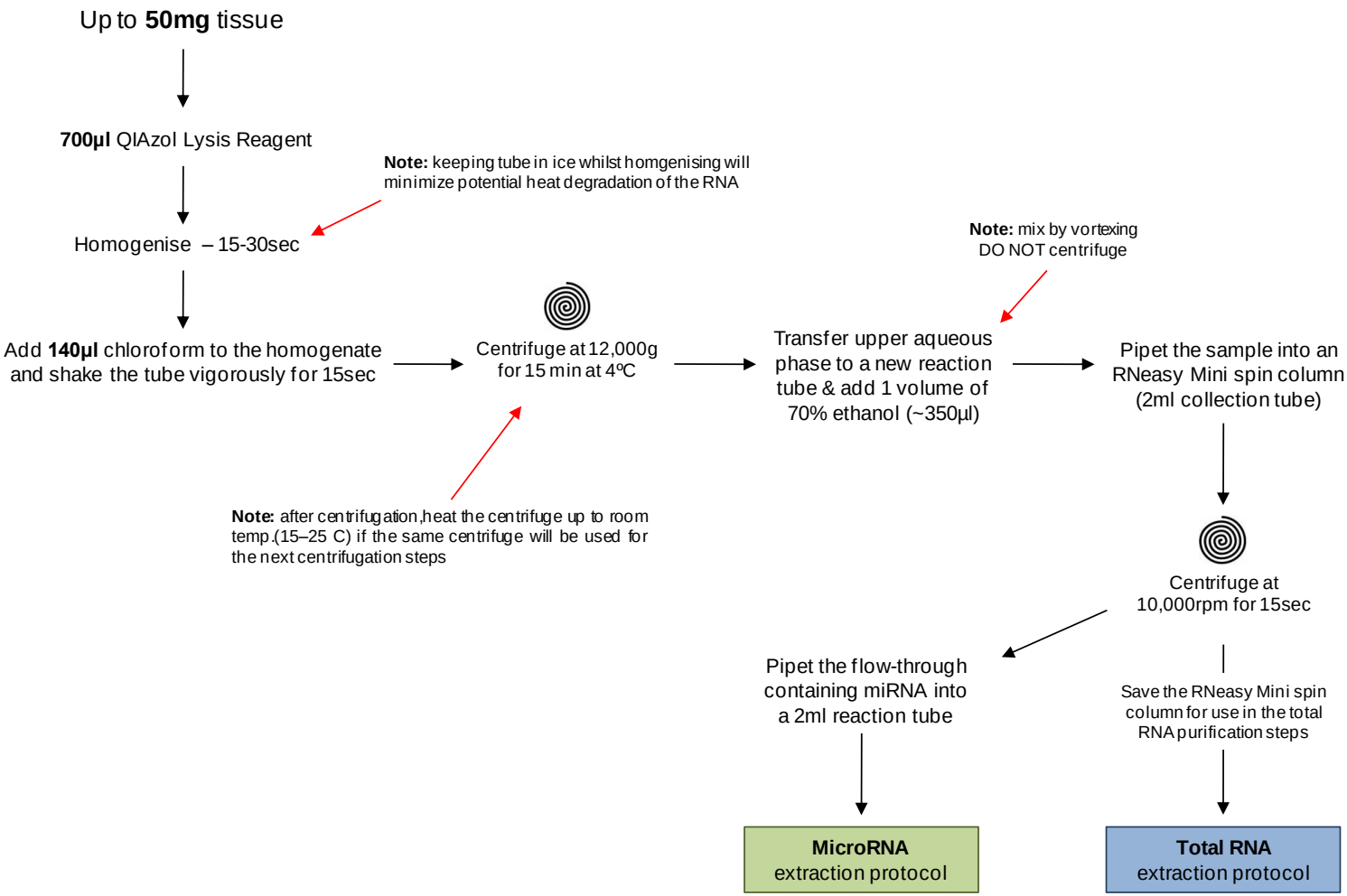
Transfer to -80 C (Mr Frosty pot) for min. of 24hrs before transferring to liquid nitrogen

Long-Term Storage

PEAC PBMNCs Box

(Liquid Nitrogen tank, Rack 5, slot C)

Purification of Total & MicroRNA from RNALater stored Tissues (Qiagen miRNeasy Method)



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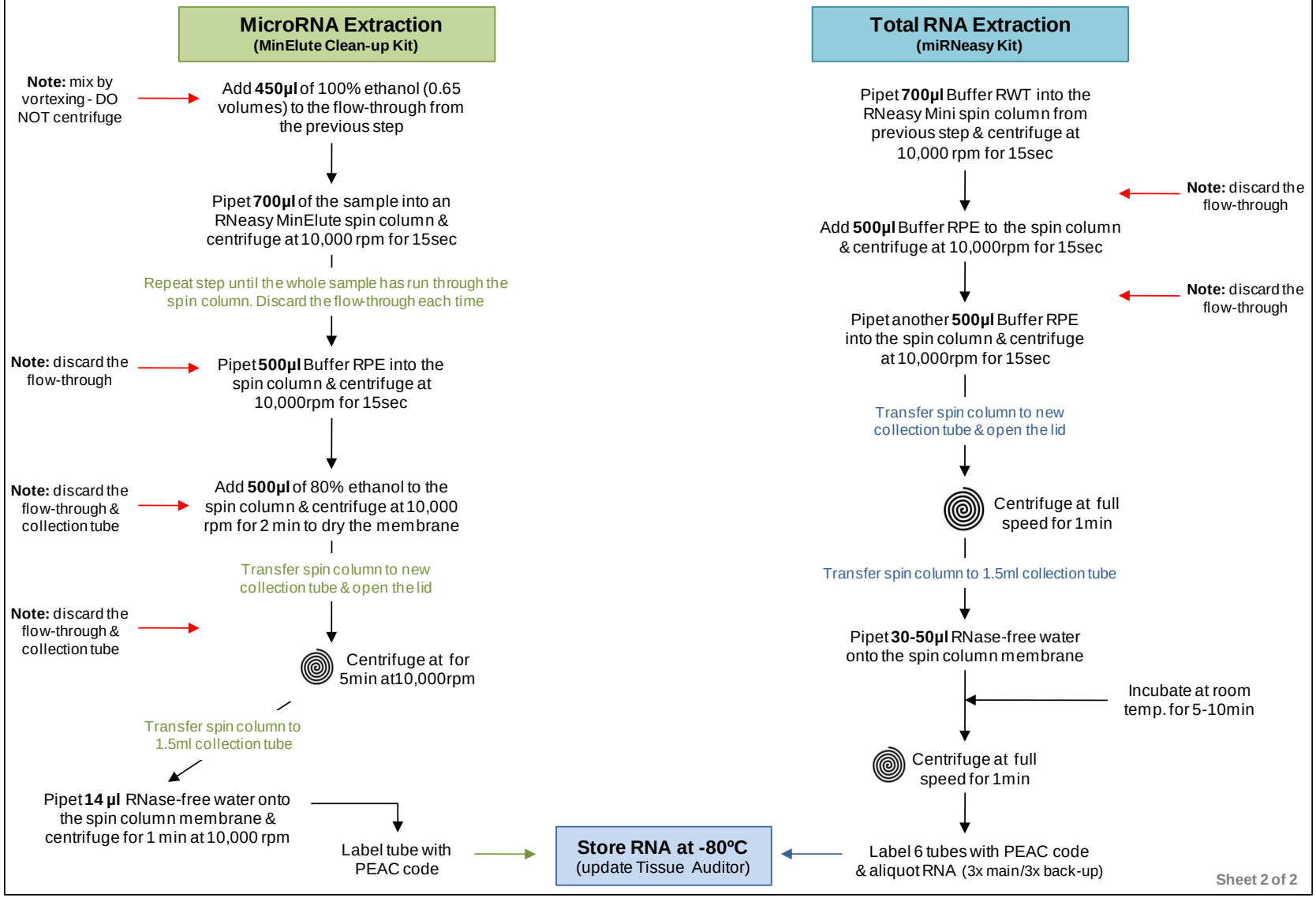
Purification of Total & MicroRNA from RNALater stored Tissues (Qiagen miRNeasy Method)



DENSITY SEPARATION OF WHOLE BLOOD MONONUCLEOCYTES (PBMNC)

Reagents required

ACD tube(s) – approx. 8ml whole blood/ tube

Note: 8mL whole blood should yield approx. 107 cells.

Lymphoprep solution (500ml) – density 1.077g/ml

Density Separation Procedure

- Aliquot 16mL lymphoprep solution into a 50mL falcon tube(s) – use at a ratio of 2:1 (2mL Lymphoprep: 1mL blood).
- Slowly and gently layer 8mL blood onto the lymphoprep solution – take care not to mix.
- Centrifuge falcon tube(s) for 20min at 2,000rpm (20ºC/room temp.).
- Label fresh falcon tube(s) and carefully remove the white layer of cells from the preparation – transfer the cells to the new falcon tube(s).
- Add a wash solution (PBS/BSA/EDTA) to the cells and make up to a volume of 10mL.
- Gently mix the cell solution and count cell number using a haemocytometer.

  Note: use approx. 7ul of cell solution for counting and count the total no. of cells in 16 squares (x10 magnification).

  Calculate total no. of cells in the 10mL solution = *N* cells x 10^4 x 10(mL).
- Wash the cells by centrifuging the cell solution for 5min at 2,000rpm (20ºC/room temp.).
- Carefully remove the wash solution leaving the cell pellet at the bottom of the falcon tube(s)
- Resuspend the pellet in *Xul/mL* freezing medium – 80% FCS & 20% DMSO.

  Note: freeze cells down at approx. 10^7 cells/1mL freezing medium.
- Transfer to Mr Frosty storage pot at -80C for a minimum of 24hours.
- Transfer cells to liquid nitrogen for long-term storage.

DENSITY SEPARATION OF SYNOVIAL FLUID MONONUCLEOCYTES (SFMNC)

Reagents required

Hanks Balanced salt Solution (HBSS), Sigma, H4385 (100ml)

Baxter Sterile Water for Irrigation (1000ml)

Lymphoprep solution (500ml) – density 1.077g/ml

Density Separation Procedure

- Dilute HBSS solution in 100ml fresh Baxter water (remove 50ml water and replace with 50ml HBSS)
- Dilute the Synovial fluid sample (falcon tube/universal) in diluted HBSS at a ratio of 1:5 (20ml SF:80ml HBSS)
- Wash the cells (SF/HBSS) by centrifuging at 2,100rpm for 10min at 20°C (always remember to ensure the centrifuge is well balanced)
- Whilst cells are washing prepare the lymphoprep: label universals and aliquot 8mls of lymphoprep into each tube (also aliquot some HBSS solution into a separate universal for further use)
- Remove supernatant from the centrifuged samples leaving the cell pellet at the bottom of the tube
- Add 2mls of HBSS solution onto cell pellets and leave at room temperature for 5-10min to soak
- Gently disaggregate the cells using a Gilson so that no cell clumps are visible
- Add more HBSS to the resuspended cells to a volume of approximately 16mls
- Gently layer the resuspended cells onto the lymphoprep – release the cell suspension so as not to mix the layers. The cell suspension should sit on top of the lymphoprep solution. Mixing of layers will ruin the whole preparation.
- Transfer the universals to the centrifuge and spin at 1,800rpm for 20min at 20°C (always remember to ensure the centrifuge is well balanced)
- Remove tubes from the centrifuge and remove the cell layer at the interface (pale yellow layer) containing SFMNCs and transfer into a fresh 50ml falcon tube. This layer may also appear opaque. Use a Pasteur pipette for this step and ensure no supernatant is collected.

- Fill each falcon tube to 50mls with HBSS solution and wash the cells by centrifuging at 2,000rpm for 10min at 4°C (always remember to ensure the centrifuge is well balanced)
- Remove the supernatant and resuspend the cell pellet in 2mls HBSS solution. Top up each falcon with 20-30mls of HBSS solution. Gently mix sample to further resuspend the cells.
- Wash again by centrifuging at 1,300rpm for 10min at 4°C (always remember to ensure the centrifuge is well balanced)
- Repeat previous 2 steps for final wash: remove the supernatant and resuspend the cell pellet in 2mls HBSS solution. Top up each falcon with 20-30mls of HBSS solution. Gently mix sample to further resuspend the cells.
- Wash again by centrifuging at 1,300rpm for 10min at 4°C (always remember to ensure the centrifuge is well balanced)
- After the final wash remove the supernatant leaving the cell pellet and some residue HBSS solution at the bottom of the tube.
- Add an equal volume (approx. 200µl) of freezing medium (80%FCS/20%DMSO) to the cell pellet/residue HBSS and resuspend cells by gently mix with a Gilson pipette.
- Transfer the MNC cell suspension to a cryovial and store in the Mr Frosty container at -80°C for 1-7 days?
- Remove cryovials and transfer to a designated tube box for long-term storage at -80°C (or in LN tank)

Stabilization of RNA in Whole Blood & RNA Extraction

Whole Blood Stabilisation in RNAlater

a. Mix blood sample by gently inverting the collection tube several times.

b. Add 300–500 µL anticoagulated blood to 1.3 mL RNAlater in a 2 mL microfuge tube (not included in the kit). Mix thoroughly by inverting the tube several times.

Once a sample is mixed with RNAlater, it can be stored for up to 3 days at ambient temperature. Storing RNAlater treated samples for longer periods at ambient temperature will result in a gradual decrease in RNA yield and quality.

Samples mixed with RNAlater can also be stored at –20°C for long-term storage.

A Initial RNA Extraction Steps

1. Samples in RNAlater Solution: centrifuge and remove the supernatant

a. Centrifuge sample for 1 min in a microcentrifuge. The blood cells and plasma proteins will form a large brown or reddish-brown pellet which may smear upward along the side of the tube, and the supernatant may be pale pink, brown, or colorless (but it is often turbid).

b. Remove and discard the supernatant by aspiration or pouring.

• When aspirating the supernatant, be sure to thoroughly remove all of the fluid, including the portion directly above the cell pellet, which may be more turbid, and which may contain some white particulate matter. Note, this material is not the “buffy coat” fraction seen in untreated whole blood after centrifugation.

• If the supernatant is removed by pouring, tap the rim of the inverted tube gently against a paper towel to remove all residual fluid.

• Remove any fluid from inside the tube cap.

2. Lyse blood cells in 800 µL Lysis Solution and 50 µL Sodium Acetate Solution

a. If the blood sample was not stored in RNAlater Solution, mix by gently inverting the collection tube several times.

b. Add 800 µL Lysis Solution and 50 µL Sodium Acetate Solution to 300–500 µL anticoagulated whole blood in a 2 mL microfuge tube, or to the cell pellet from RNAlater-stabilized samples.

c. Vortex vigorously to lyse the blood cells. Invert the tube to be sure the solution is homogenous. Samples that were stabilized in RNAlater will require more vigorous vortexing to resuspend and lyse the cells.

3. Extract with 500 µL Acid-Phenol: Chloroform

a. Withdraw 500 µL of Acid-Phenol:Chloroform from beneath the overlying layer of aqueous buffer, add it to the cell lysate, and shake vigorously or vortex for 30 sec.

Note: If addition of 500 µL of Acid-Phenol:Chloroform would cause the tube to be too full to permit adequate mixing, you can use as little as 250 µL of Acid-Phenol:Chloroform.

b. Store the mixture at room temp for 5 min.

c. Centrifuge at room temp for 1 min to separate the aqueous and organic phases. The aqueous phase may appear cloudy or clear after centrifugation.

4. Recover the aqueous phase in a fresh 2 mL tube

Transfer the aqueous (upper) phase containing the RNA to a new 2 mL tube (not provided in the kit). If samples were split for the Acid-Phenol:Chloroform extraction, collect the aqueous phases into a single tube. Typically the aqueous phase volume is ~1–1.2 mL.

Avoid transferring the colored material from the organic (lower) phase, which contains heme and proteins. Discard the lower phase. Note: If the aqueous phase volume is less than 800 µL, see section III.B. Difficulty Recovering Aqueous Phase after Organic Extraction on page 10.

5. Add 600 µL of 100% ethanol to each sample

To each tube of aqueous phase recovered after the Acid-Phenol: Chloroform extraction, add 600 µL (~one-half volume) of 100% ethanol, and vortex briefly but thoroughly. If desired, the tube may then be centrifuged very briefly (~1 sec) to collect the fluid from around the lid of the tube.

B Final RNA Extraction Steps

Notes: Before you start a. Add 56 mL 100% ethanol to Wash Solution 2/3.

• Add 56 mL of 100% ethanol to the bottle labeled Wash Solution 2/3 Concentrate, and mix well.

• Store at room temp after the ethanol has been added if the Wash Solution will be used within 1 month. Store at 4°C for longer times, but warm to room temp before use.

b. Heat an aliquot of the Elution Solution to ~75°C in an RNase-free tube. Typically each sample is eluted in ~100 µL, but additional Elution Solution may be heated to allow for evaporation.

c. Assemble and label the plastic ware.

• For each sample, place a Filter Cartridge into one of the Collection Tubes supplied in the kit. Label the lid of the resulting Filter Cartridge assembly.

• Label a second Collection Tube for each sample; these will be used to elute the RNA from the glass fiber filter.

1. Pass the sample through a Filter Cartridge ~700 µL at a time

a. Apply ~700 µL of the sample (aqueous phase mixed with ethanol from step II.B.5) to a Filter Cartridge assembly, and centrifuge for ~5–10 sec to pass the liquid through the filter. Discard the flow-through from the Collection Tube, and replace the Filter Cartridge into the same Collection Tube.

b. Load the next ~700 µL of sample onto the filter. Spin as before to pass the sample through the filter and discard the flow-through. Repeat to filter the remaining sample.

2. Wash filter with 700 µL Wash Solution 1

a. Apply 700 µL Wash Solution 1 to the Filter Cartridge assembly and centrifuge for ~5–10 sec to pass the solution through the filter. Discard the flow-through from the Collection Tube, and replace the Filter Cartridge into the same Collection Tube.

3. Wash filter with 2 x 700 µL Wash Solution 2/3

a. Apply 700 µL Wash Solution 2/3 (working solution mixed with ethanol) to the Filter Cartridge assembly and centrifuge for ~5–10 sec to pass the solution through the filter. Discard the flow-through from the Collection Tube, and replace the Filter Cartridge into the same Collection Tube.

b. Repeat with a second 700 µL aliquot of Wash Solution 2/3.

c. After discarding the flow-through from the last wash, replace the Filter Cartridge in the same Collection Tube and spin the assembly for 1 min to remove residual fluid from the filter.

4. Elute RNA with 2 x ~50 µL preheated Elution Solution

a. Transfer the Filter Cartridge into a labeled Collection Tube (provided with the kit). Apply ~50 µL Elution Solution (preheated to ~75°C) to the center of the filter, and close the cap. Leave the assembly at room temp for ~20 sec, then spin for ~20–30 sec at maximum speed to recover the RNA.

b. Repeat with a second ~50 µL of Elution Solution, collecting the RNA into the same tube. Spin for 1 min to recover all of the Elution Solution in the Collection Tube.

c. Store the eluted RNA at –20°C or below.

(Optional) DNase I Treatment

1. Add 20X DNase Buffer and DNase I to the RNA

Add 1 /20th volume 20X DNase Buffer and 1 µL DNase I (8 U/µL) to the eluted RNA and mix gently but thoroughly.For example if the RNA was eluted in 100 µL Elution Solution, add 5µL 20X DNase Buffer and 1 µL DNase I.

2. Incubate 30 min at 37°C

3. Add 20% volume of DNase Inactivation Reagent, mix thoroughly, and leave at room temp for 2 min.

Use a volume of DNase Inactivation Reagent equal to 20% of the volume of RNA treated. For example if 100 µL of RNA is treated with DNase, add 20 µL of DNase Inactivation Reagent.

Vortex the tube briefly to thoroughly mix the DNase Inactivation reagent with the RNA, and store the sample at room temp for 2 min. Flick or vortex the tube once or twice during this period to resuspend the DNase Inactivation Reagent.

4. Pellet the DNase Inactivation Reagent and transfer the RNA to a new tube

Centrifuge the sample for 1 min in a microfuge to pellet the DNase Inactivation Reagent and then transfer the RNA solution to a new RNase-free tube (not supplied with the kit).

QUANTIFICATION OF RNA & DNA

NanoDrop® ND-1000 Spectrophotometer ($A_{260}/_{280}$)

- Access the N-1000 V3 1.0 software
- Select parameter - Nucleic Acids
- Zero the system by placing 1ul of nuclease-free water onto the sensor, close the lid and click OK. A reading of NaN is required to proceed
- Open the lid and wipe residue water off of the sensor with soft lint-free tissue
- Select the nucleic acid to be quantified – RNA/DNA (double stranded - ds)
- Blank the system by placing 1ul of nuclease-free water onto the sensor, close the lid and click on BLANK. A value of 0.0 means to system is ready to use
- Open the lid and wipe residue water off of the sensor with soft lint-free tissue
- To quantify the samples place 1ul of RNA/DNA onto the sensor, close the lid and click on MEASURE
- A reading is given at the bottom right-hand of the screen in ng/ul
- Open the lid and wipe the sample residue off of the sensor with soft lint-free tissue
- Repeat this procedure for each sample (wiping the sensor clean in-between sample measurements)
- When finished clean the sensor wipe by re-blanking the system with 1ul of nuclease-free water. Wipe the sensor clean
- An image of each measurement can be printed (click PRINT SCREEN) onto stickers or saved (go to File' – 'Save Window' – 'Name' your image – 'Save')
- Select SHOW REPORT to access a list of all samples quantified. This report can be saved