

VASCULITIS IRELAND NETWORK REGISTRY AND BIOBANK

Chief Investigator: Professor Mark Little

STANDARD OPERATING PROCEDURE

SOP No:

SAMPLE PROCESSING LAB HANDBOOK

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CONTROLLED DOCUMENT				
The user of the SOP must read the following thoroughly before proceeding to undertake the methods described. Under no circumstances are these instructions to be amended or altered in any way other than by the author/authorised personnel.				

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1. Standard Operating Procedure: Sample Processing

1.1. Receipt of the samples at laboratory:

It is the responsibility of the biobank technician or research nurse carrying out this procedure to ensure that all steps are completed competently and safely.

1. Research laboratory personnel/research nurses will be trained in the receipt and inspection of samples on arrival at the laboratory. All specimens will be inspected upon arrival, verifying that the correct specimen was received and that the information on the sample form, specimen collection container and sample identification, match each other. During this process of verification, appropriate handling temperature will be maintained.
2. If the integrity of the sample container is compromised, the proper amount of sample is not present, or the sample containers are not adequate this will be documented in the appropriate section, in the sample data section of the Case Report Form.
3. When the sample arrives in the biobank it must be registered and recorded in accordance with local laboratory procedure. The date and time that the specimen is received will be recorded in the Case Report Form.

1.2. Notes on Centrifugation and the importance of setting the centrifuge to G or RCF and not RPM

It is important that care is taken to ensure that you are spinning all samples at G or RCF centrifuge setting and not RPM- The biobank technician will show all processors how to do this during their training but if there are any doubts please ask!

1.4. Processing of urine for Metabolomics

1. A red capped 50ml tube containing approximately 15ml of urine will be received on ice.
2. Spin the tube for 10 minutes at 2000G at 4 degrees.
3. While taking care not to disturb the pellet, transfer the supernatant into 4x1ml cryovials with blue caps and 1x5ml cryovial with white cap and blue insert.
4. Handwrite Main Study ID, Urine M, and Visit Date on each tube.
5. Place the aliquots at -80 as soon as possible (see notes below on voiding to freezing time*).

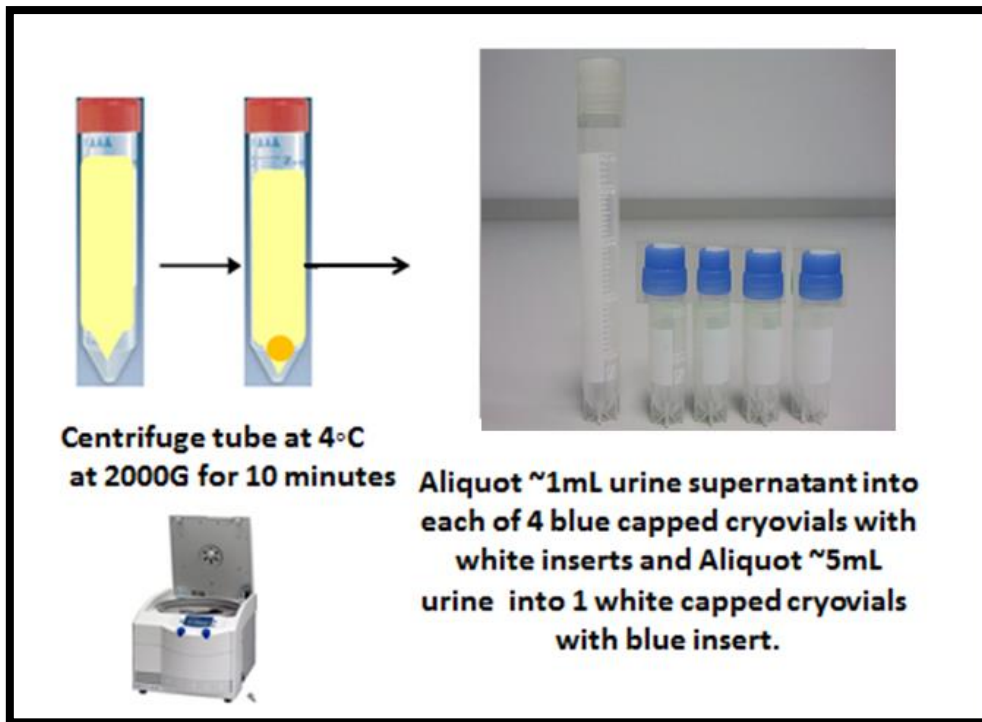


Figure: Processing and Aliquoting of Urine for Metabolomics

* The voiding to freezing time for all samples should ideally be a maximum of 2-3 hours. All sample collection and freezing times are recorded. If, on occasion, the voiding to freezing time extends over 3 hours, the samples are still valuable for many types of analysis; this timing may not be crucial for some purposes however it is important to let the Biobank Technician know if this time is consistently extending to over 4 hours and never within 3 hours. This would indicate that there may be a problem with local clinic timings or processing that would have to be solved. The biobank technician will be monitoring the arm to freezing times at each site to ensure that there is no problem and assessing if changes need to be made in this regard.

1.5. Processing of EDTA Plasma Sample for Proteomics and DNA Extraction.

1. The EDTA tube for plasma and DNA (or BD P100 tube if indicated) is received on ice and should remain on ice/at 4°C throughout processing.
2. **The arm to freezing time should ideally be a maximum of 2 hours.** All sample collection and freezing times are recorded, and this enables the researchers to request only samples with an arm to freezing time of 2 hours if this is what is necessary for their analysis. This proteomics sample is one of the most prone to degradation and so it is imperative that the sample is processed and frozen as quickly as possible.

3. Centrifuge the plasma tube at 2000G for 10 minutes at 4°C. Record the time of spinning in the Sample Data Section of the Case Report Form. This spin can be done at the same time as the serum spin, D3.7 below.
4. The Blood will separate into 3 layers: a clear, upper plasma layer; a lower red blood cell layer; and a thin interface containing the white blood cells (buffy coat) as indicated in Figure 13 below.
5. Using a plastic transfer pipette, collect the plasma (clear liquid) from the EDTA tube for plasma, being very careful not to disturb the white blood cell layer and transfer it into a 15ml red capped tube.
6. Label this tube with Main study ID
7. Centrifuge the 15ml red-capped tube containing the plasma again at 2000g for 10 minutes to remove all platelets.
8. Remove the 15ml tube from the centrifuge after the 2nd spin is complete and using a P1000 pipette, -distribute the plasma among 3 cryovials with white lids and purple inserts.
9. Label each cryovial with the Main study ID, Plasma and Date of Visit.
10. Transfer the tubes to a -80°C Freezer for storage. Record the time of freezing in the sample details section of the Case Report Form. Also Record that 2nd spin has been done as illustrated below:

SERUM DETAILS		PLASMA DETAILS	
TIME OF COLLECTION:		TIME OF EDTA TUBE COLLECTION:	
TIME OF SPINNING:		TIME OF SPINNING:	
TIME OF FREEZING:		TIME OF FREEZING:	
Number of 2ml cryovials frozen:		Number of 2ml cryovials frozen:	
Volume stored in each cryovial:		Volume stored in each cryovial:	
Centrifuge setting for Blood Spins (please circle)		Protease Inhibitor added:	Yes/ NO- Please Circle
1500G	2000G	Number vials have PI:	
Plasma had 2 spins?:	Yes/ No - Please Circle	Number vials don't have PI:	
DNA DETAILS		EDTA TUBE PBMC- PREP DETAILS	

Record what setting centrifuge was set at for blood spins here

Record if Plasma had second spin here

Record how many Vials of plasma had PI added/not added here

11. When the plasma has been aliquoted **do not discard the EDTA tube**. Label it with the Main Study ID, Date of Visit and EDTA DNA on the tube and store at -80 for DNA extraction. The biobank technician will arrange for DNA extraction to be carried out later and aliquots of normalised DNA will be barcoded and labelled and stored at - 20°C.

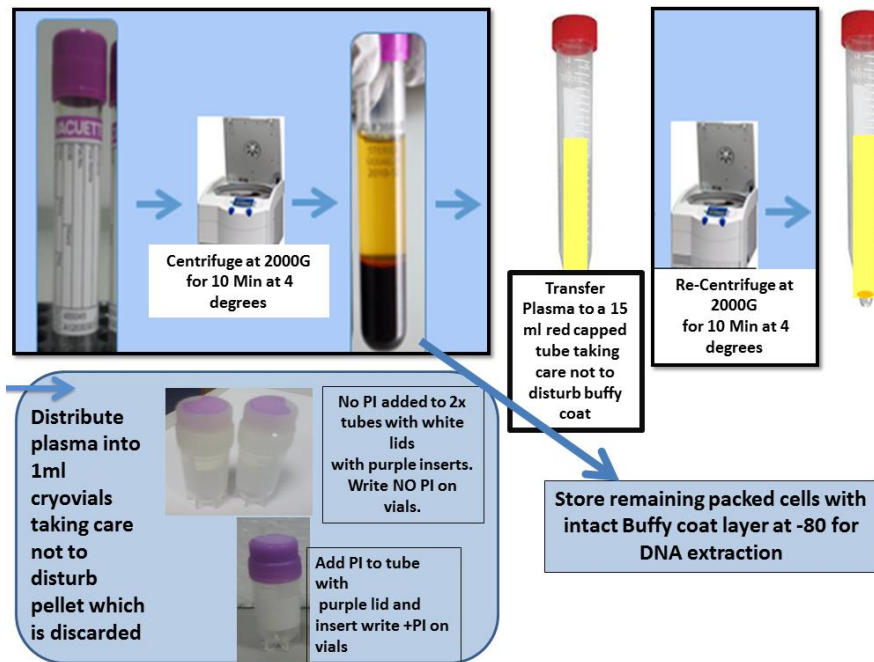


Figure: Processing and Aliquoting of Plasma Sample and storage of EDTA DNA sample for DNA Extraction (PI is no longer added to plasma tubes).

1.6. Processing of Serum

1. The red capped serum tube is received at room temperature and should remain at room temperature throughout processing apart from the 10-minute spin at 4 degrees.
2. Centrifuge the serum tube at 2000G for 10 minutes at 4°C. Remove from Centrifuge once spin is over and place back at room temperature.
3. Record the time of spinning in the Sample Data Section of the Case Report Form.
4. The Blood will separate into 2 layers: a clear, upper serum layer; a lower red blood cell layer.
5. Using a plastic transfer pipette, collect the serum (clear liquid) from the serum tube, being careful not to disturb the red cell clot.
6. Distribute the serum among 3 x 1ml cryovials and cap with yellow lids.
7. Label each cryovial with the barcoded label marked 'Serum'
8. Transfer the tubes immediately to a -80°C Freezer for storage.
9. Record the time of freezing in the Sample details section of the Case Report Form.

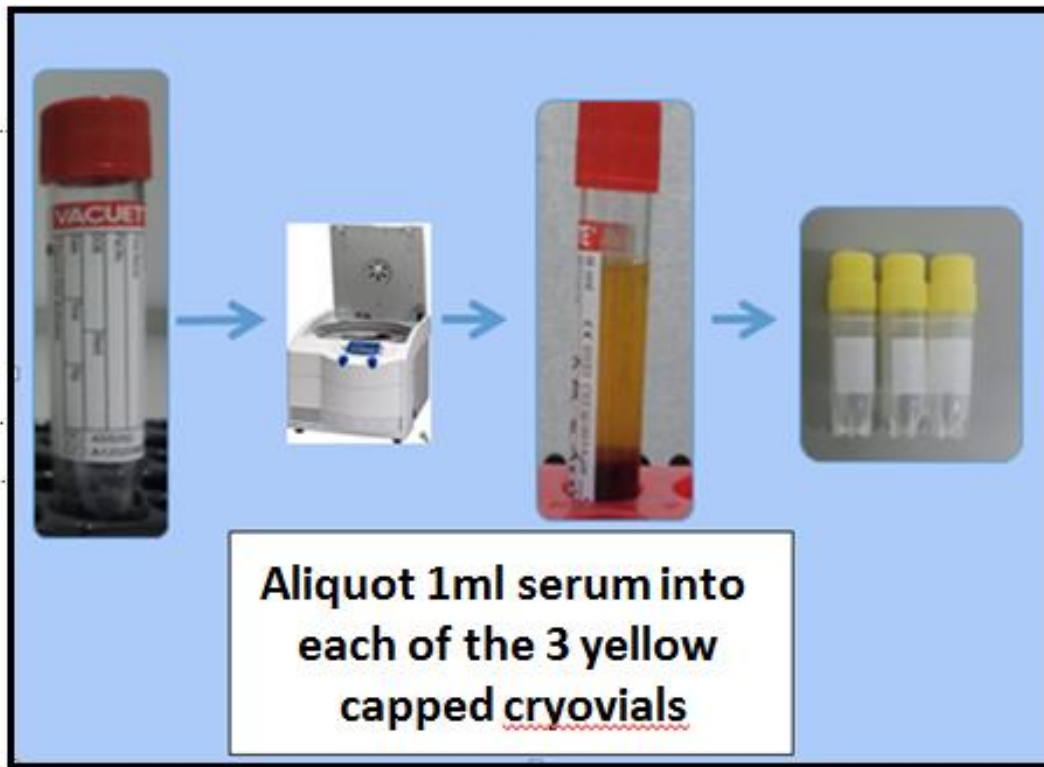


Figure: Processing and Aliquoting of serum sample

1.7. Processing of PaxGene tube for RNA analysis.

1. The PaxGene sample is received **at room temperature** and should be kept upright at all times.
2. Ensure tube is labelled with the appropriate barcoded label. Hold specimen at room temperature for a minimum of 2 hours before storage.

(If it is not suitable due to time constraints to maintain the Paxgene tube at room temperature for two hours it may be stored at room temperature for up to 72hours before placing at -20. However, **the PaxGene tube must be stored at room temperature for a minimum of 2 hours before freezing at -20.**)

The PaxGene tube should remain at -20 at the local site until subsequent transfer to the central biobank for long-term storage at -80.

3. The time and date of freezing at -20 is recorded on the sample details section of the Case Report form.

1.8. Processing of 2 x EDTA tubes for PBMC preparation.

1. Adjust temperature of "Mr Frosty" to 4°C.
2. Transfer the blood sample (~9ml) from each EDTA tube into a 50ml tube. Wash the residual blood from each EDTA tube with 9ml of PBS (GIBCO Invitrogen catalogue

number: 14190-094) and add to the new tube to ensure maximum sample collection and 1:1 dilution of blood with PBS.

3. In two additional tubes pipette 9ml of lymphoprep (Axis Shield catalogue: 1114545).
4. Slowly pipette the 18mls of diluted blood sample into each tube containing lymphoprep (2:1 ratio of diluted blood to lymphoprep). The blood should sit as a layer above the lymphoprep.
5. Centrifuge the sample at 400g for 25 minutes at room temperature with 0 Acceleration and 0 Brake.
6. Remove sample from centrifuge and discard top layer which contains plasma, taking care not to disrupt the buffy coat layer directly beneath the plasma.
7. Hold a Pasteur pipette above the buffy coat layer and gently pipette out the cells into an additional tube. Circle the tube with the pipette to remove all cells.
8. Add PBS to fill the tube containing the cells. Centrifuge at 800g for 5 minutes.
9. Remove the supernatant and resuspend cell pellet in 50ml PBS.
10. Centrifuge at 400g for 10 minutes.
11. Remove supernatant and resuspend cell pellet in media (Complete RPMI – see recipe below).
12. Count the cells using a haemocytometer according to the following formula: NO. OF CELLS IN ONE LARGE SQUARE X DILUTION FACTOR X 10,000 = Number of cells/ml
13. Place cells on ice or in fridge for a few minutes to cool.
14. Dilute cells with media to give a concentration of $\sim 10 \times 10^6$ cells/ml
15. Label required number of cryovials (will depend on number of cells obtained) Main Study ID, date, No. of cells per vial and processor initials
16. Add 500 μ l of cold cells ($\sim 5 \times 10^6$ cells) to cryovials on ice. Quickly add 500 μ l of cold freezing mix (see recipe below)
17. Place in “Mr Frosty” and store in a -80°C freezer. NOTE: Freezing media contains DMSO which is toxic to cells at ambient temperature, so once added to cells tubes should be transferred to -80°C freezer with minimum delay.
18. The time and date of freezing is recorded on the sample details section of the Case Report form.
19. Next day, transfer cells from “Mr Frosty” to normal freezer box and eventually to liquid nitrogen for long term storage.

A. Complete RPMI media recipe:

- 500ml RPMI media (GIBCO Invitrogen catalogue number: 61870)
- 5ml Pen/Strep (GIBCO Invitrogen catalogue number: 15140-026)
- 5ml Fungizone (GIBCO Invitrogen catalogue number: 15290-122)
- 50ml FCS (Biosera-from Labtech catalogue number: FB1001/500)

B. Cold freezing mix recipe:

80% FCS (Biosera-from Labtech catalogue number: FB1001/500)

20%DMSO Dimethyl sulfoxide (Sigma Aldrich Catalogue number: D8418)

1.9. Sample Processing Summary

Serum	Transport at room temp. Centrifuge at 4°C at 2000G for 10 minutes	Aliquot ~1mL plasma into each of 3 yellow capped cryovials.	Label Main Study ID, Sample Type, Visit Date	Store in Patient Encounter bag in -80°C Freezer
EDTA: Plasma & DNA	Transport on ice. Centrifuge at 4°C at 2000G for 10 minutes. Remove plasma taking care not to disturb buffy coat to a new 15ml tube. Respin at 2000G for 10 minutes at 4 degrees. Remove plasma with P1000 pipette.	Aliquot final (spun twice) ~1mL plasma 3x white capped cryovials with purple inserts	Label with Main Study ID, Sample Type, Visit Date Ensure remaining tube with packed cells including intact buffy coat is labelled with Main Study ID, Sample Type, Visit Date	Store all in Patient Encounter bag in -80°C Freezer
PaxGene tube for RNA	No Initial Processing. Transport at room temp.	Ensure tube is labelled with the appropriate barcoded label. (Pax-RNA)	N.B. Hold specimen at room temperature (18°C-25°C) for a minimum of two hours before storage.	Store in open plastic rack in -20°C Freezer for 24hours and then transfer to -80°C. NB: DO NOT STORE IN POLYSTYRENE RACK
2x EDTA tube for PBMC prep.	No Initial Processing. Transport at room temp. Record time of collection on tubes.	Ensure tubes are labelled with Main Study ID, Sample Type, Visit Dat	N.B. Hold specimen at room temperature until transfer to a scientist trained in PBMC preparation.	
Urine for metabolomics	Transport on ice. Centrifuge tube at 4°C at 2000G for 10 minutes	Aliquot ~1mL urine into each of 4 blue capped cryovials with blue inserts and Aliquot ~5mL urine into 1 white capped cryovials with blue insert.	Main Study ID, Sample Type, Visit Date	Store in Patient Encounter bag in -80°C Freezer

Table: Sample Processing Summary Table-Quick Reference Guide

2. Standard Operating Procedure: Sample Transportation after processing at local sites

Samples will be stored at their local hospital sites until intermittent transport to the RKD Biobank

- Samples will be transported on dry ice to ensure against freeze/thaw to maintain the integrity of the samples.
- Samples will be transported in bulk.
- The biobank technician will liaise with the local Research Nurse at the relevant sites to arrange sample pick-up.
- No samples are to be removed from the -80C freezer until the time of pick-up.
- A log of samples being transported should be provided with the specimens at the time of collection.

3. Standard Operating Procedure: Sample Storage at the VINE Biobank

- The specimens should reach suitable storage at the -80C/-20C freezer as soon as possible.
- All samples are barcoded at the Biobank.
- All samples stored will be checked in using the central biobank inventory system.
- All sample data are entered onto the inventory database.
- If there are any issues with the samples or data, the Biobank technician will feed this back to the research nurse to gain clarity.
- If any aliquots are removed from the freezer, they must be checked out in the data management system.
- Under normal circumstances, biobank staff only are permitted to access to the Registry samples.

4. S.O.P. Emergency Response in case of main Biobank Freezer malfunction.

The Biobank Freezers are monitored by the XILTRIX monitoring system. This system sends a text message and email to Mark Little and nominated research staff in the event of a power loss or if the temperature rises to above -60 degrees.

TCD freezers are no longer supported by CO2 backup.

Decant -80 Freezers are situated in the IMM Room 0.38 in the Basement. There are also some decant -30 Freezers in Room 0.44 in the basement.

At times further decant freezers may be temporarily situated on the corridor of the first floor by Freezer room 2.17 and there are at times a -80 Freezer empty or some space available in the Biobank Freezer room 1.35 Ground Floor.

If you come across a freezer malfunctioning at any of the sites, please inform a member of the biobank team and take a record of the highest temperature that the freezer reaches while containing the samples. Please ensure you are aware of the location of any decant freezers at the site you work at by communicating with your lab manager.

5. References

- Safety, Health and Welfare at work Act (Biological Agents) 1994.
- WHO Guidance on regulations for the transport of Infectious Substances.
- Molecular Medicine Ireland Guidelines for Standardised Biobanking. First Edition 2010.

6. Sample Collection and Processing Consumables

Item Description	Catalogue Number	Supplier
9ml EDTA tubes (pack of 100)	455/045	Cruinn Diagnostics
9ml serum tubes	455092	Cruinn Diagnostics
2.5ml Pax gene RNA tubes	762165	BD
BD Vacutainer Safety Lok blood collection set 21g Needle 7" tubing, Pre Attached Holder (Pack of 25)	368654	BD
2ml cryopure tube external thread yellow (1 box of 500 vials)	72.379.004	Sarstedt Ltd.
Violet colour coded inserts- cryotubes	65.386.007	Sarstedt Ltd.
Green colour coded inserts- cryotubes	65.386.005	Sarstedt Ltd.
Yellow colour coded inserts- cryotubes	65.386.004	Sarstedt Ltd.
Blue 1.8ml cryopure tubes (pack of 500 vials)	72.379.006	Sarstedt Ltd.
Transfer pipettes	86.1171.001	Sarstedt Ltd.
50 ml PP Skirted Sterile Base tube with assembled screwcap	62.559.001	Sarstedt Ltd.
15ml PP Conical sterile Base tube with assembled screwcap	62.554.002	Sarstedt Ltd.
100ml urine collection tube	75.562.105	Sarstedt Ltd.

Table: Sample collection and processing consumables