Immunovirology Research Network (IVRN)

A network of laboratories across Australia to facilitate strategic clinical immunology and virology research in relation to Australians with HIV or HCV.

IVRN Laboratory Manual

Separation and storage of serum, plasma and PBMCs

Last Updated June 2021

TABLE OF CONTENTS

1. Record keeping and Administration

Accurately recorded details of blood samples processed for the IVRN are critical for subsequent research studies. Please complete the IVRN Blood Processing Workflow Sheet (***Appendix 1***) when specimens are processed.

Record all available specimen details (identifier [ID], 2x2 code, date of birth, study visit number or time point, date/time collected, date/time processed, type and amount of blood received), as well as the laboratory details (Tier One laboratory name, processor's initials) and the study title. The subject ID may be a clinical trial participant number.

Wherever possible, perform whole blood counts in an automated cell counter and record model of cell counter used, the total and differential white cell counts, and the anticoagulant tube. Use of EDTA tubes for whole blood counts is preferred, but another non-liquid anticoagulant may substitute (eg. heparin).

Label cryovials with the following information either using a fine permanent waterproof marker (e.g. Sharpie industrial permanent marker extra fine, or Pentel N50 bullet point), or printed labels.

Lab ID:	eg. UNSW (or if in Lab QAP, Lab Z)
Study:	e.g. IVRN QAP.
Subject identifier:	The subject ID and the 2×2 code (e.g. John Brown = BRJO).
Date of birth:	Record in the following format: _ _. _ _. _ _ dd mm y y
Specific sample type:	PBMC, serum or plasma.
Anticoagulant used:	For plasma samples only record the anticoagulant used in blood collection (e.g. EDTA, LiHep).
Sample volume:	Cell number for PBMCs (e.g. 5M for 5 x 10^6 cells), or volume of serum or plasma. (e.g. 500µl).
Date:	Date of specimen collection (not processing date).

Study visit number or time point may be added if there is sufficient space on the cryovial.

See ***Appendix 2*** for details of specimen shipping to the IVRN Central Specimen Repositories and invoicing for specimen processing.

2. Storage of serum and plasma for virological studies

2.1 Materials

Transfer pipettes (sterile).

Cryogenic vials; high quality grade 1ml, screw cap internal thread w/o skirt (eg. Nunc Cat # 377224).

2.2 Method

1 Serum may be retrieved from any clotted specimen (e.g. SST or Z Serum Clot Activator tubes).

2 Plasma may be retrieved from any anticoagulated whole blood specimen (e.g. EDTA, lithium heparin [LiHep], sodium heparin [NaHep], acid citrate dextran [ACD], sodium citrate tubes).

Note: *Blood for viral load assessment or other quantitative test should not be taken from ACD (liquid anticoagulants dilute the plasma by an unspecified factor). Liquid anticoagulants are acceptable for viral isolation and culture.*

3 Centrifuge at 1000g for 10 min.

4 Transfer 500µl-1000µl plasma/sera to each of the replicate ampoules (or as directed), being careful not to aspirate any cells from the buffy layer. Plasma for viral load measurement must be dispensed in aliquots greater than 1ml. Label tubes with the subject ID and study details as described in section 1, and the sample type (e.g. LiHep plasma, ACD plasma, EDTA plasma, or serum).

5 Record the number and type of vials of serum and plasma on the IVRN Blood Processing Workflow Sheet.

6 Store aliquots in a –80 C freezer until shipment.

7 File the IVRN Blood Processing Workflow Sheet in a secure location until shipment.

3. PBMC fractionation, cryopreservation and thawing

3.1 Equipment and Materials

Item	Examples
Centrifuge with graded acceleration/deceleration settings	
Class II laminar flow cabinet	
-80 °C freezer, and liquid or vapour phase nitrogen tank	
Bench top chiller rack, **or** ice water bath and rack	Nalgene Cat # 5116-0032
Centrifugation tubes; 50ml and **10ml**	
Controlled-rate freezing equipment or 'Mr Frosty'or CoolCel.	Nalgene Cat # 5100-0001
Sterile syringes; 20ml	
Sterile mixing cannula	Indoplas Cat # 500-11.012
Sterile disposable pipettes; 10ml	Costar Cat # 4101
Sterile transfer pipettes	Samco Cat # 222-20S
Cryogenic vials; high quality grade, 2ml, internal thread, w/o skirt	Nunc Cat # 377267
Permanent marker; bullet point, alcohol resistant,	Pentel N50, Sharpie
Sterile phosphate buffered saline, Ca++ and Mg++ free (PBS)	
Cell culture medium: RPMI 1640 supplemented with HEPES, penicillin 20 IU/ml, streptomycin 20 μg/ml, L-glutamine 2mM • *Note: glutamine in suspension is unstable, use within 14 days.*	
Lymphocyte separation medium • *Note: different batches of Ficoll-Paque Plus give different lymphocyte yield efficiencies, viability results, and levels of contamination with erythrocytes and granulocytes, as tested by the manufacturer. Request the material specification sheet for every available lot before selection for purchase. Ficoll-Paque Premium is manufactured to the highest GMP standard, and generally gives slightly better lymphocyte yields and purity than Ficoll-Paque Plus (both producst are acceptable for use in IVRN work).*	Ficoll-Paque Plus or Ficoll-Paque Premium (GE Healthcare), or Lymphoprep (Progen)
Dimethyl sulphoxide (DMSO); cell culture grade • *Note: buy small containers and use within 6 months of opening.*	Sigma Hybri-Max Cat # D2650
Foetal calf serum; heat inactivated • *Note: best frozen in 50ml aliquots at -80 °C. It is acceptable to re-freeze in single use sub-aliquots).* • *FCS for the IVRN QAP was screened for complement-mediated anti-human lymphocyte cytotoxicity and for the ability to support the proliferation of PBMCs against a range of stimulants while having low backgrounds in the presence of FCS alone. A similar screen was performed in the ELISPOT assay.* • *FCS selected by each lab for IVRN clinical trials and other non-QAP PBMC collection should likewise provide low background control proliferation of PBMC when tested in the LPA, or low background IFN-γ producing cells in the ELISPOT assay. At least 2 samples of FCS should be screened for selection from a suitable supplier (eg. Thermo Trace, JRH (CSL), or other suppliers of Australian sourced serum). A sufficient quantity of the chosen batch of FCS should be reserved to last the duration of the FCS shelf life (batch chosen should have an expiry date of at least 3 years).*	

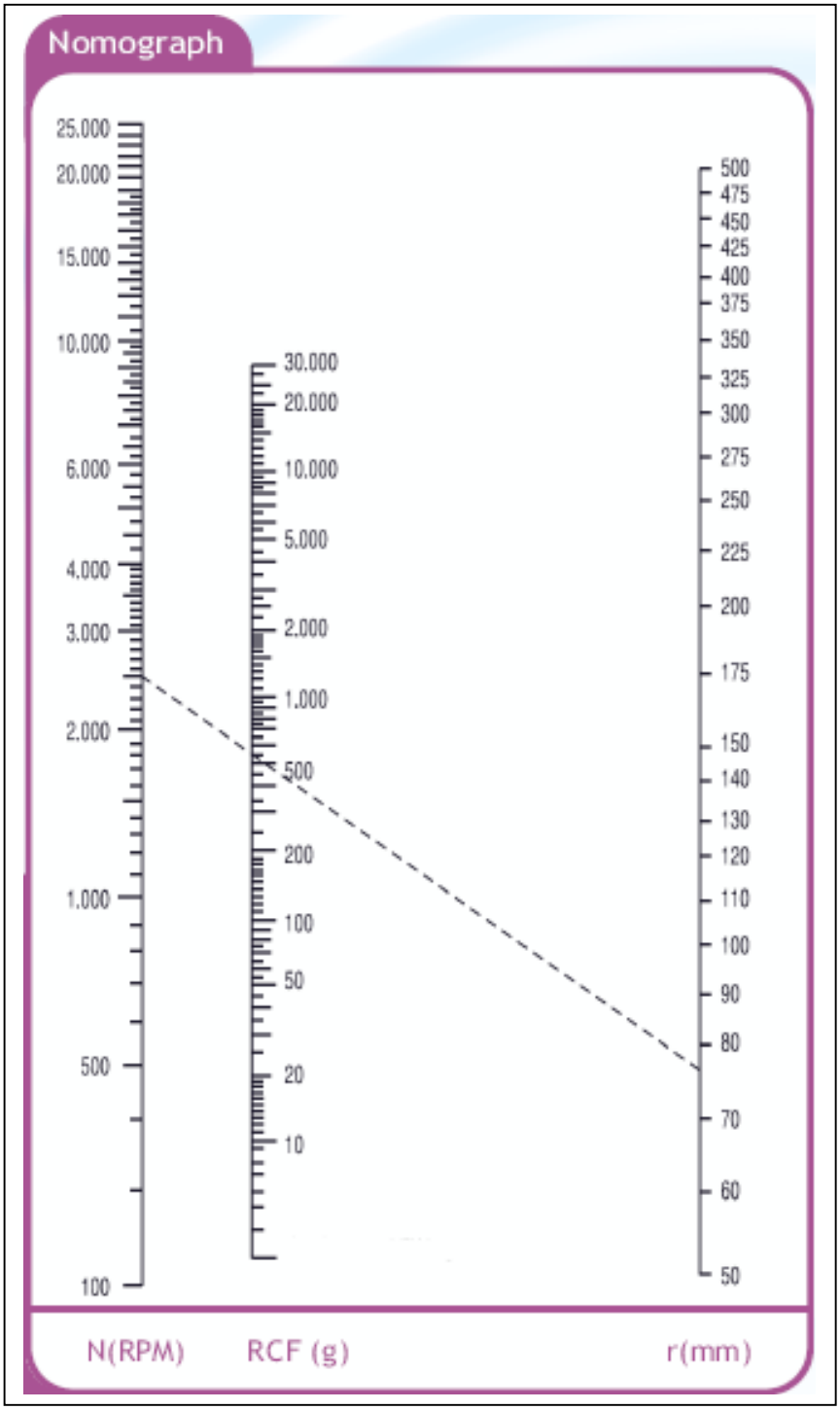
3.2 Establish correct settings for your centrifuge

The ability to harvest sufficient PBMC and to avoid significant losses during wash steps depends on correct centrifuge use. Modern centrifuges automatically convert between RCF (g) and rotor speed (RPM) according to the radius from the centrifuge spindle to the bottom of the specimen tube for each rotor. Alternatively, conversion between RCF and RPM can be performed manually using the nomogram shown at the right, by simply drawing a straight line from the rotor radius value to your known RCF, and the resulting RPM is the value intersected by the line. RCF can also be calculated using the following formula:

$RCF = 1.118 \times 10^{-5} \times \text{radius (cm)} \times RPM^2$

The radius for many lab centrifuges is 18cm to the bottom of the tube, and centrifuge speeds suggested in this manual are based on this standard size; eg, spin PBMC on Ficoll at 700g / 2000rpm.

The centrifuge brake setting is the other crucial parameter that greatly influences yield and purity. Rapid braking may cause internal turbulence during centrifuge slow down, causing cells to be washed off the pellet into the supernatant. This problem is more pronounced with wider centrifuge tubes, or when the tube is half full. Old centrifuges with a simple on-off brake setting should always have the brake off. Centrifuges with a high/low/off brake setting range may also have to be used in the off mode if the low brake speed is too fast. Centrifuges with a 1 to 10 scale of braking should be used with the brake set on 3 or less. The brake should always be off for the Ficoll spin.



How to customise your centrifuge for optimal use? If your lab purchases a new centrifuge, it is recommended that after establishing the settings, a direct comparison between old and new centrifuges be conducted using duplicate specimens. Carefully observe the specimen after spinning to ensure the desired result was achieved, and make adjustments where needed. Consider the following examples:

1. Have all cells been deposited as a buffy coat, or are some remaining in the plasma? If not sure, aspirate a small specimen of plasma near the bottom and examine in a haemacytometer. If cells are present, spin at a higher speed or for longer, and reduce brake force.

2. Is the cell pellet smaller than expected? Are there any signs of cells washed off into the supernatant? Aspirate a small supernatant specimen, and if cells are present, consider reducing the brake or turning the brake off. Perhaps increase centrifuge speed, or spin in a narrow tube (10 or 15 ml) instead of a 50ml tube, and ensure the tube is full so as to reduce inner turbulence during braking.

Always visually examine the supernatant before discarding so as to avoid cell losses, and be prepared to customise your centrifuge settings in order to achieve the desired result.

3.3 Separation of PBMC by density gradient centrifugation

Sterile procedures should be used throughout this method, and all work with open specimens should be performed inside a Class II biosafety cabinet.

1 **Note:** If blood for PBMC preparation is received in multiple tubes, buffy coats can be pooled before PBMC separation. Process PBMC from different anticoagulant tubes separately. In most situations, proceed according to option (a) below:

(a) Centrifuge whole blood at 1000g (2200rpm) for 10 minutes at room temperature. Remove plasma to within 5mm from the buffy coat (store plasma if required at 4 × 1.5ml aliquots), then transfer buffy coats to a 50ml centrifugation tube (approx. 15ml of buffy coat from up to 5 blood tubes from a single donor can be pooled into one 50ml tube) and dilute with 15ml PBS. Mix well.

Note: for small blood samples (single tube), and if plasma is not required for storage, follow step 1b.

…or…

(b) To minimize cell losses from small blood samples, pour whole blood into centrifuge tube and add residual blood washed from collection tube with PBS, and mix well.

Note: PBS is preferable to RPMI, because lack of calcium and magnesium in PBS may minimise cell clumping and aid cell separation.

2 If separating blood in 50ml tubes, use a syringe and sterile mixing cannula to layer 15ml of Ficoll beneath the diluted buffy coat. A fresh cannula must be used for each pooled patient sample. Be careful not to mix the layers, by ensuring Ficoll flows out slowly, thereby maintaining a clearly defined interface between Ficoll and blood.

Note: Diluted blood can be overlayed onto the Ficoll with a 10ml pipette, again being careful not to mix the layers.

3 Centrifuge the tubes at 700g (1800rpm) for 20 minutes at 20°C, with the brake off.

4 Use a sterile transfer pipette to remove the lymphocyte bands from replicate tubes from each patient sample and pool into a sterile 50ml centrifuge tube. Avoid removing any white platelet plaque attached to the side of the centrifuge tube adjacent to the PBMC layer - these are sticky

activated platelets, and may cause unwanted cell clumping and possible losses. Minimise the amount of Ficoll layer harvested. Fill tube with PBS.

Note: Dilute the harvested PBMC with at least the same volume of PBS to avoid cell losses in the following spin step.

5 Centrifuge tubes at 400g (1400rpm) for 10 minutes, with brake on lowest setting.

Note: A significant proportion of the platelets harvested with PBMC may remain suspended in the supernatant and should be discarded. If the PBMC pellet is smaller than expected, remove a drop of supernatant and examine in a haemocytometer to ensure all PBMC have been spun down, before pouring off the supernatant. Also note that a significant number of cells can be lost from the pellet because of turbulence generated in the tube during centrifuge slow down if spinning a 50ml tube that is not full. Spin smaller volumes in 10 or 15ml tubes.

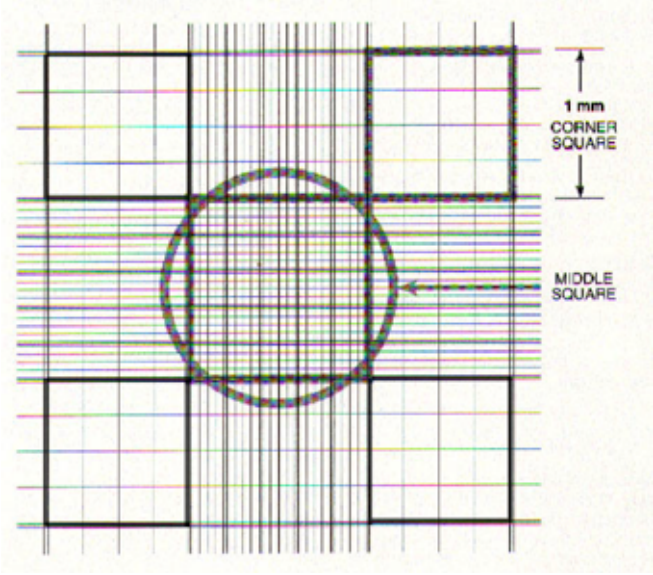
6 Discard supernatant and resuspend cell pellet in 10ml RPMI or PBS, and remove a small sample for counting.

Note: It is advisable to leave the PBMC in RPMI with 5-10% FCS if the freezing step is delayed by more than 1 hour, to keep the cells viable in a full nutrient medium.

7 Count cells in an automated haematology analyser if available, or use a haemocytometer.

Prepare an appropriate dilution of cells to count in a routinely calibrated automated cell counter; eg undiluted if your counter samples only 10-20µl, or dilute if >100µl is sampled. Sensitive multi-parameter machine,s eg Cell-Dyn models, give more reliable results than 3- or 5-cell Sysmex cell counter models. Record total PBMC as the sum of lymphocytes and monocytes. If the monocyte population is too small cannot be determined, record only the lymphocyte count as the PBMC population. Do not use the white cell count because any contaminating granulocytes present with the PBMC will be included, and will dead when thawed for assessment and therefore reduce the recovery. If your cell counter cannot differentiate between white blood cell sub-populations, then it is advisable to use a haemocytometer.

If using a haemocytometer, count at least 50-100 cells per 1mm grid for the count to be representative. Therefore, depending on the number of PBMC harvested (look at the size of the cell pellet), dilute 10µl of cell suspension in a suitable volume of PBS, and then dilute 1:1 in Trypan Blue. Evenly mix then load the haemocytometer to fill the area under the cover slip. Make sure the haemocytometer chamber depth is 0.1mm, and use the reusable cover slips provided with the haemocytometer. Allow the cell suspension to settle in the haemocytometer for 30 seconds before counting. Count the 4 large corner quadrants (see diagram). Viable PBMCs will be clear; non-viable PBMCs will be blue. Do not count large cells of granulocyte appearance as being PBMC (these do not survive cryopreservation), so as to not over estimate the PBMC content. Include cells that touch either the top line or left vertical perimeter line of any corner square. Do NOT count any cells that touch either the bottom line or right vertical perimeter line of any corner square.



8 Calculate total PBMC recovered:

=(lymphocytes + monocytes) cells/ml × dilution factor× sample volume (ml)

or for haemocytometer cell counts:

=(average counts/quadrant) ×10^4 × dilution factor (medium and Trypan Blue dilutions) × sample volume (ml)

3.4 Cryopreservation of cells

1 PBMCs are prepared and counted as described above.

2 Prepare an appropriate number of cryovials according to the PBMC yield (total cells ranging from 5 - 10 ×10^6 PBMCs/vial). Please ensure that each aliquot of PBMC from a single patient specimen has the same number of PBMC.

3 Ensure cryovials are labelled as described in section 1.

4 After labelling, chill cryovials in a lab top cooler rack (or ice water bath) to 4°C before use.

5 Prepare freezing medium, containing 10% DMSO & 20% FBS in RPMI. Chill to 4°C before use. Freezing medium is best made fresh, or can be stored at 4°C for up to 1 week.

6 Centrifuge cells at 400g (1400rpm) for 10 min, discard all supernatant, and resuspend the cell pellet directly in an appropriate amount of freezing medium to ensure a final PBMC concentration of 5-10 x 10^6 cells/ ml per ampoule, and immediately dispense into the chilled cryovials, 1ml per vial. Ensure that the quantity of cell suspension is dispensed accurately into each vial. Cap ampoules and keep chilled until placing in the cryo device (DMSO is toxic to cells at room temp).

7 Initiate cryopreservation using either a mechanical controlled rate freezer, or a "Mr Frosty" or CoolCel device placed in a -80°C freezer.

<u>Mechanical controlled rate freezing method:</u>

1 The cryovials are placed in a rack in a controlled rate freezer held at 4°C.

2 Set an appropriate temperature gradient; e.g. reduce the temperature by -1°C/minute until -25°C followed by a second cooling phase of -5°C/minute until -100°C is attained.

3 Cryovials should be removed and stored in nitrogen vapour or liquid as soon as the freezing run is complete.

<u>"Mr Frosty" freezing method:</u>

1 Fill the Mr Frosty unit with isopropyl alcohol to the specified level, and chill to 4°C before use (the Mr Frosty filled with alcohol should be stored at room temperature when not in use). Replace the isopropyl alcohol after the 5th freezing cycle, because water is gradually absorbed by the alcohol, which alters the freezing rate.

2 Place cryovials in the 'Mr Frosty' unit and transfer into a -80°C freezer, for a minimum of 4 hrs, or overnight, before storing in a nitrogen tank.

<u>CoolCel freezing method:</u>

Keep the device at 4°C ready for use. Insert chilled ampoules and transfer device immediately into a -80°C freezer for a minimum of 4 hours or overnight, before transferring specimens to nitrogen.

3.5 Notes on handling frozen ampoules

It is essential that frozen PBMC are moved from the -80°C freezer to cryogenic temperatures (liquid or vapour phase N2 tanks) within 24hrs (or over the weekend as a maximum allowed time).

Keep ampoules in dry ice when transferring frozen ampoules between the -80°C freezer to the N2 tank, or in preparation to ship frozen cells to another lab. Never use wet ice to transfer frozen PBMC specimens.

3.6 Thawing cryopreserved cells

Unlike freezing, when it is important that cells are cooled slowly, optimal recovery of viable cells is obtained when cells are rapidly thawed. Medium is usually added slowly for gradual osmotic diffusion of DMSO out of the cell to minimise cell damage, however rapid dilution may also be effective. For an evidence-based discussion on acceptable variations to the procedure, please see *Honge, et al, 2017*.

1 Transfer cryovials from the nitrogen tank on dry ice.

2 Pre-label centrifuge tubes and prepare all media before thawing.

3 Thaw using a water bath or warm tap water in a beaker at 37-42°C (Note: 42°C enables rapid thawing without overheating the contents). Electronic water baths are a notorious source of contamination, and require regular disinfection. Ensure the cryovial lid is on tight and hold upright in the bath with the cap out of the water to minimise possible contamination with water. Shake vial gently while thawing until ice crystal is approximately 5mm dia.

4 Sterilise the surface of the ampoule with 70% ethanol or an alcohol wipe before opening. Pour the contents into a sterile centrifuge tube. Rinse ampoule with approx. 1ml FBS (37°C) to wash out remaining cell suspension, and add to the tube.

5 Add pre-warmed medium (RPMI/10% FBS; 37°C) in a drop-wise manner until approximately 5ml has been added, then fill the tube. Mix and centrifuge at 300g for 10 minutes.

6 **Optional DNase I treatment for long-term frozen PBMC:** If PBMC were frozen for >10 years, or if cell clumps were present when similar specimens were thawed, the best option is to briefly enzymatically digest sticky cell-free DNA (*Garcia-Pineres, et al, 2006*). Otherwise proceed to step 7.

- Prepare DNase: Stemcell Technologies (Cat# 07900)- 0.1mg/ml final conc. Dilute a 1ml ampoule to 10ml in RPMI (or a medium that contains Ca^{2+}/Mg^{2+} to activate DNase).
- Resuspend PBMC pellet in 500µl diluted DNase, and incubate 15 minutes at room temperature.
- After incubation, add 10-15mlml RPMI/10% FBS, mix and centrifuge.

7 Discard the supernatant, and wash another time with RPMI/10% FBS.

8 Discard the supernatant and resuspend in 2-5ml medium for recovery/viability counting.

3.7 Procedures that may reduce PBMC quality

1 Separate PBMC within 8 hours of blood collection; avoid EDTA tubes unless used for immediate PBMC separation.

2 Do not chill whole blood before fractionation; keep at ambient (22^{o}C) lab temperature.

2 Avoid prolonged contact with Ficoll; harvest PBMCs and wash ASAP after centrifuge stops.

3 Do not leave centrifuged cells as a compressed pellet for any time longer than necessary; resuspend cells in medium before taking a break.

4 Do not vortex cell pellets; flick the tube to loosen cell pellet or resuspend by gentle pipetting.

5 Do not leave cells in serum free medium for more than 1hr; add 5-10% FCS if processing other specimens before batch freezing.

6 Avoid exposure to DMSO medium at ambient temperature. When freezing, resuspend cell pellets in cold DMSO medium, dispense into cold cryovials, then place directly in cold (4°C) "Mr Frosty" or pre-chilled controlled rate freezer.

4. Suggested reading

Bull, M., Lee, D., Stucky, J., Chiu, Y.L., Rubin, A., Horton, H., MeElrath, M.J. Defining blood processing parameters for optimal detection of cryopreserved antigen-specific responses for HIV vaccine trails. J Immunol Methods 2007, 322: 57-69.

Disis, M.L., dela Rosa, C., Goodell, V., Kuan, L.Y., Chang, J.C., Kuus-Reichel, K., Clay, T.M., Lyerly, H.K., Bhatia, S., Ghanekar, S.A., Maino, V.C. and Maecker, H.T. Maximizing the retention of antigen specific lymphocyte function after cryopreservation. J Immunol Methods 2006, 308, 13-18.

Carcia-Pineres, A.J., Hildesheim, A., Williams, M., Trivett, M., Stobel, S., Pinto, L.A. DNAse treatment following thawing of cryopreserved PBMC is a procedure suitable for lymphocyte functional studies. J Immunol Methods 2006, 313: 209-213.

Honge, B.L, Petersen, M.S, Olesen, R., Moller, B.K, Erikstrup, C. Optimizing recovery of frozen human peripheral blood mononuclear cells for flow cytometry. Plos One, Nov 1 2017.

Olson WC, Smolkin ME, Farris EM, Fink RJ, Czarkowski AR, Fink JH, Chianese-Bullock KA, Slingluff CL Jr. (2011) Shipping blood to a central laboratory in multicenter clinical trials: effect of ambient temperature on specimen temperature, and effects of temperature on mononuclear cell yield, viability and immunologic function. J Transl Med. 2011 Mar 8;9:26

Tree, T.I., Roep, B.O. and Peakman, M. Enhancing the sensitivity of assays to detect T cell reactivity: the effect of cell separation and cryopreservation media. Ann N Y Acad Sci 2004, 1037, 26-32.

Appendix1: IVRN Blood Processing Workflow Sheet

Tier One Lab ID _____

Processor's Initials _____

1. Record sample details
   N.B in 2 × 2 first two letters of surname then first two letters of given name

2. Study title:

3. Record tube type and amount of blood received
   e.g. **LiHep** blood **9ml** received, **EDTA** blood **4ml** received

   _____ blood _____ mL received

   _____ blood _____ mL received

   _____ blood _____ mL received

4. Record whole blood sample results

Model of cell counter used _____

5. Separate serum and plasma & record number and type
   e.g. **4 LiHep plasma, 3 SST serum**

6. Ficoll blood and wash

Ficoll product details: _____

7. Resuspend PBMC and record PBMC result

8. Suspend PBMC in freezing media at concentration of 5-10 × 10^6cells/ml and aliquot into 2ml cryovials

FCS Source:	**Lot Number:** .
Batch Number:	**Expiry Date:** .

9. Label **ALL** vials with **Tier One Lab ID, Study name, Subject ID, 2 × 2, Date of Birth, anticoagulant** (e.g. LiHep plasma), **Cell number** (PBMCs) **or Sample volume** (serum or plasma), **Date specimen collected.**

10. Record # vials and concentration PBMC/vial

PBMCs

Cells/vial x 10^6 _____ # vials _____

Sample details

Sample ID _____ Surname _____ Firstname _____

2 × 2 Code _____

dd.mm.yy Date of Birth _____ Study Visit No. or Timepoint _____

Date Collected _____ Time Collected _____

Date Processed _____ Time Processed _____

Whole blood sample results

WCC _____ 10^6/mL

neutrophils _____ 10^6/mL _____ %

lymphocytes _____ 10^6/mL _____ %

monocytes _____ 10^6/mL _____ %

Serum

# vials	type

Plasma

# vials	type

PBMC results

WCC _____ 10^6/mL

neutrophils _____ 10^6/mL _____ %

lymphocytes _____ 10^6/mL _____ %

monocytes _____ 10^6/mL _____ %

Appendix 2 Shipment of processed specimens to IVRN Central Specimen Laboratories and invoicing

Below are a series of steps to follow when ready to ship specimens to the Central Specimen Repositories (CSRs) at UNSW (Sydney) and at NRL (Melbourne).

Record Keeping

Prepare a simple list of the specimens being sent at the time of shipment, including ID number, 2x2 code, sampling time-points for each subject and total number of specimen vials, and send this list with the shipment.

Shipping

Labs in WA, SA and Vic should ship specimens to the NRL CSR, while labs in QLD and NSW should ship specimens to the UNSW CSR. Lab staff should contact the IVRN Project Coordinator at UNSW when they are ready to ship specimens. In addition, please send copies of the IVRN Blood Processing Workflow Sheets and any paperwork that arrived with the original blood samples (e.g. request forms) with the shipment.

Invoicing

Invoice IVRN (via the Westmead Institute for Medical Research) at the time of shipment to the CSRs, and send a copy of this invoice and a copy of the specimen list to the IVRN Project Coordinator at UNSW.

Invoicing details are as follows:
ABN: 54 145 482 051
Westmead Institute for Medical Research
PO Box 412 WESTMEAD NSW 2145 Australia

Contact: Joanne Reidy
Phone: +612 8627 3004

Email: joanne.reidy@sydney.edu.au