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 April 2013

TABLE OF CONTENTS

1. Record keeping and Administration

Accurate and comprehensive records of the details of sample processing are critical for subsequent research projects based on IVRN specimens. The IVRN Blood Processing Workflow Sheet (see Appendix) should be completed at the time of sample processing using the protocols detailed in this Manual.

Upon receipt of a blood sample, record the sample 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 refers to the subject in a clinical trial or similar.

Wherever possible, the anticoagulated whole blood should be analysed in an automated cell counter (record model of cell counter used) and the total and differential white cell counts recorded.

Ensure cryovials are labelled 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 (Nunc Cat # 377224).

2.2 Method

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

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

Note: *Blood collected in EDTA tubes should be processed within 24 hours post collection, if shipped/stored at temperatures between 2 and 25 C, and providing only minimal haemolysis is evident. Blood for viral load assessment or other quantitative test should not be taken in ACD (or other liquid anticoagulants which 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. Tubes are labelled with the appropriate subject ID and study details as described above, 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 IVRN Blood Processing Workflow Sheet in a secure location until shipment.

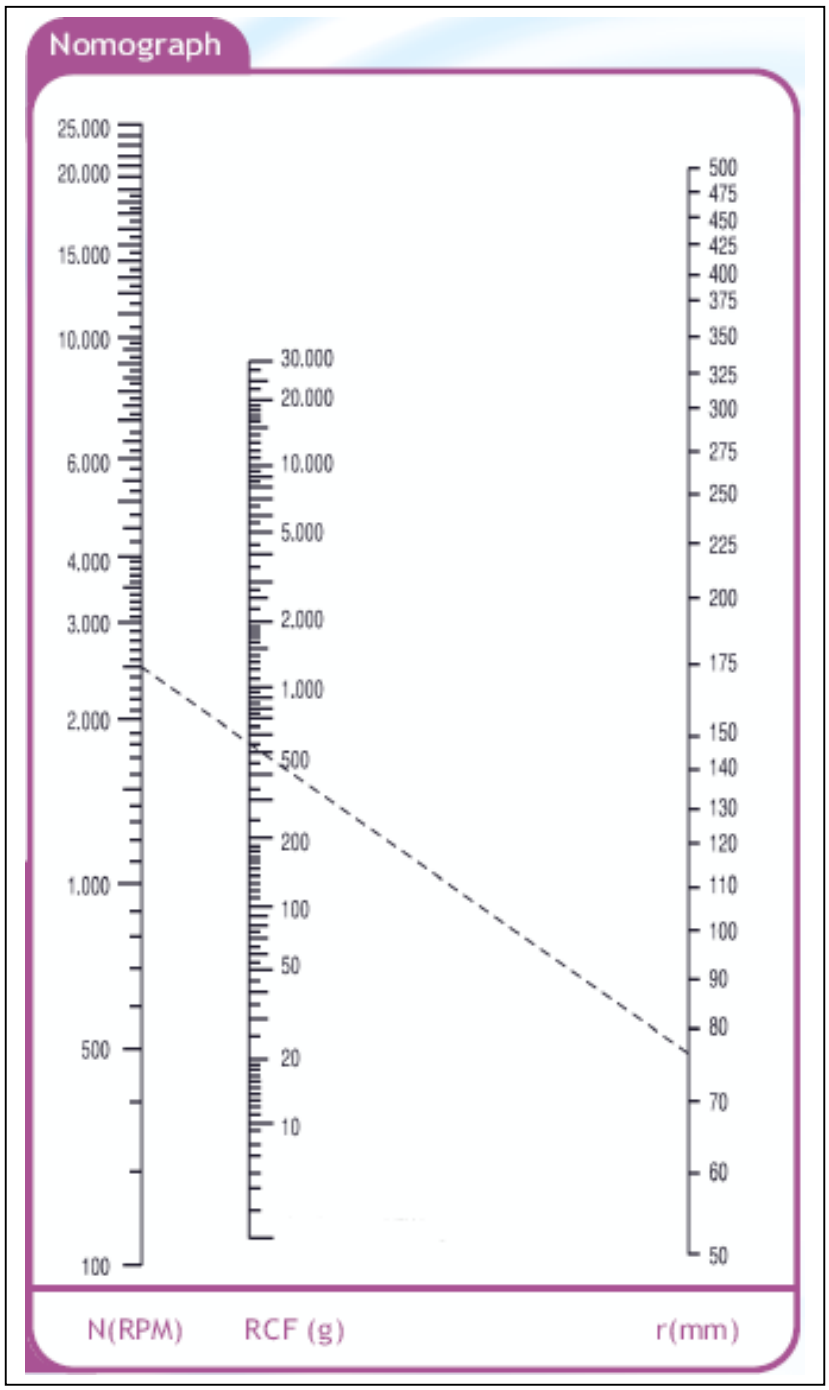
3. PBMC fractionation, cryopreservation and thawing

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 use of your centrifuge, and may require customised settings if the suggested settings in this PBMC manual do not result in the desired yields. It is also important to know the pre-set radius used by the centrifuge to convert RCF (g) into rotor speed (RPM).



The radius of the rotor is usually measured from the spindle out to the bottom of the centrifuge tube, and the centrifuge uses this distance to convert between RCF and RPM. Conversion between RCF and RPM can be done 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. Alternatively, RCF can 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. An appropriate speed for purifying PBMC on Ficoll is 2000rpm, which is approx 800g at the bottom of the tube. However, the RCF will only be 650g at the Ficoll interface (r = 15cm). Note that different manufacturers of density gradient media recommend different RCF in their protocols, because the radius is either based on the distance to the middle of the centrifuge tube (eg. Ficoll) or to the bottom of the tube (eg. Histopaque). Therefore, to avoid ambiguity, the IVRN Lab Manual has standardized all radius measurements to the bottom of the tube (which appears to be the measurement used by most centrifuge software when converting between RPM and g), and the suggested RCF is also given in RPM speeds, based on an 18cm radius.

The centrifuge brake setting is the other crucial parameter that greatly influences yield and purity. Rapid braking may cause rotational turbulence inside the tube 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 2 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. In addition, it is also recommended that you 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.

Always examine the supernatant (visually at least) 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

Method:

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 different anticoagulant tubes, after removal of plasma for storage, buffy coats can be pooled before PBMC separation with the exception of EDTA blood (PBMC from EDTA blood may be of poorer quality than PBMC from other anticoagulants, unless blood is processed soon after collection). Process PBMC from EDTA blood 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 (15ml of buffy coat from approx. 5 blood tubes from a single donor can be pooled into one 50ml tube) and dilute with 15ml of serum-free medium or PBS. Mix well.

Note: for small whole blood sample volumes (<20ml), and assuming plasma is not required for storage, follow step 1b ONLY.

…or…

(b) To minimize cell losses from small blood samples (<20ml), pour whole blood into 50ml centrifuge tube, wash out residual blood from collection tubes with PBS or medium into the centrifuge tube, and top up with PBS or medium to twice original blood volume. Mix well.

Note: PBS is an acceptable alternative to RPMI (serum free), and it is thought that the lack of calcium and magnesium in PBS may reduce cell clumping and aid cell separation.

2 Using a syringe and sterile mixing cannula, layer approximately 15ml of Ficoll beneath the buffy coat and medium mixture. 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: An acceptable alternative is to overlay the blood mixture onto the Ficoll with a 10ml pipette, again being careful not to mix the layers.

3 Centrifuge the tubes at 700g (1950rpm) for 20 minutes at 20°C, with the brake off.

4 Using sterile transfer pipettes, remove the lymphocyte bands from each of the samples for each subject and place into sterile, labelled 50ml centrifugation tubes. Do not remove the white platelet plaque attached to the side of the centrifuge tube adjacent to the PBMC layer - these are sticky activated platelets, and inclusion with PBMC may cause unwanted cell clumping, and possible losses. Minimise the amount of Ficoll layer harvested. Add RPMI (serum free) or PBS to bring the total volume of the tubes up to 50ml.

Note: At least the same volume of medium as the harvested PBMC solution should be added to achieve a sufficient dilution of Ficoll, or some cells may be lost at the following step.

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

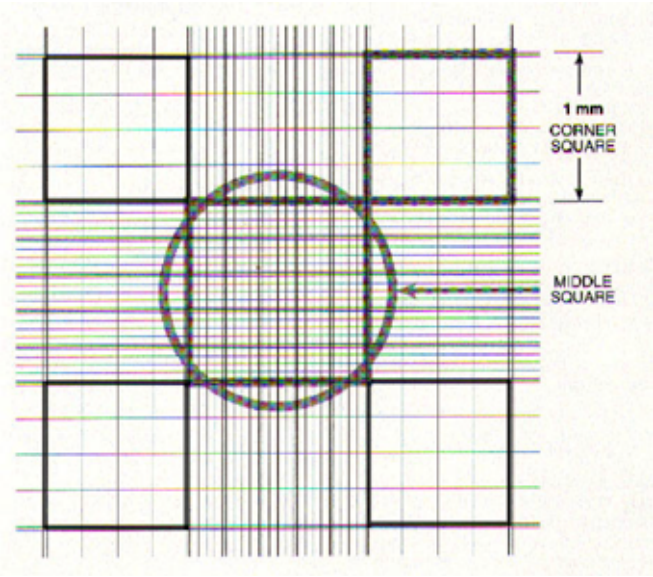
Note: A significant proportion of the platelets harvested with the PBMC may remain in the medium and should be discarded. If the PBMC pellet is smaller than expected, check the supernatant (before pouring off) to make sure some PBMC have not remained in suspension. Also note that a significant number of cells can be lost from the pellet during turbulence generated in the tube during centrifuge slow down if spinning a 50ml tube that is not full. This problem is reduced when using a narrow tube (10ml or 15ml) – accordingly smaller volume samples may be best centrifuged in 10 or 15ml tubes.

6 Discard supernatant and resuspend cell pellet in 10ml RPMI or PBS, and remove a small sample for counting. Top up volume to 50ml and leave cells in suspension until ready to freeze.

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

7 Count cells and assess viability using Trypan blue as follows:

Subject to the number of cells harvested (e.g. from 5 - 10 ml ACD blood tubes), perform an initial 1:5 or 1:10 dilution of the cell pellet in medium, followed by a 1:1 dilution in Trypan Blue (omit the initial dilution in medium if only 1 or 2 blood 8-10ml collection tubes were processed). Load the haemocytometer with the Trypan/cell mixture to fill the area under the cover slip. Make sure the haemocytometer chamber depth is 0.1mm, and use the cover slip that is specific for the haemocytometer. Allow the cell suspension to settle in the haemocytometer for at least 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.



Notes: Alternatively, prepare an appropriate dilution of cells for use in an automated cell counter. Automated cell counters may reduce operator error in using a haemocytometer only if the machine is regularly (daily) calibrated, as is the case with a large facility. Sensitive multi-parameter counting machines like the Cell-Dyn 3200 give more reliable results than 3- or 5-cell Sysmex cell counter models. The haemocytometer remains the best option for labs processing less than 10 specimens per day (see detailed summary of automated cell counting in the report from the 5th SD QAP).

For the Cell-Dyn 3200 counter, record PBMC as being the sum of lymphocytes and monocytes. For a Sysmex counter, record only the lymphocyte count as the PBMC population. If your cell counter cannot differentiate between white blood cell sub-populations, then you must count with a haemacytometer. Otherwise, if a significant number of contaminating granulocytes are present with the PBMC, these will be included in the total count, and will reduce the yield when thawed for assessment.

8 For haemocytometer cell counts, record the total PBMC yield for each specimen according to the following formula:

(average counts/quadrant) $\times 10^4$ × dilution factor (ie. medium and Trypan dilutions) × sample volume (ml)

9 Take the cell suspension from item 6 (above) to the cryopreservation protocol (below).

3.4 Cryopreservation of cells

It is possible to successfully freeze and retrieve viable cells following controlled freezing in a –80°C freezer or in the vapour phase of liquid nitrogen. The best results, however, are obtained by using a controlled-rate freezer that allows cells to be cooled against a defined gradient. Methods for both systems are presented.

Method:

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 5×10^6 to 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, with a minimum of 20% FCS in medium, or 10% DMSO in pure FCS. Chill to 4°C before use (Tree et al., 2004). Freezing medium is best made fresh, however aliquots may be frozen, and thawed freezing medium 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, and immediately dispense into the chilled cryovials, at 1ml per vial. Ensure that the quantity of cell suspension is dispensed accurately into each vial.

Keep resuspended cells in an ice water bath or a chilled rack, since DMSO is toxic to cells at room temp. Wipe vials dry when removing from a water bath to the freezing unit.

7 Initiate cryopreservation using either a controlled rate freezer or a "Mr Frosty", CoolCel, or similar unit.

Controlled rate freezing method:

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 further phase of -5°C/minute until -100°C is attained.

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

"Mr Frosty" freezing method:

1 Controlled freezing rates of approx. 1°C/min can be achieved in a -80°C freezer by placing cryovials in a small chamber insulated by an outer chamber filled with isopropyl alcohol (e.g. the Mr Frosty unit, which takes up to 18 ampoules).

2 The Mr Frosty unit is filled with isopropyl alcohol up to the specified level indicated on the unit, and chilled at 4°C (or stored at room temperature when not in use). Filling to the specified level is essential if the precise freezing rate is to be achieved. Replace the isopropyl alcohol after the 5th freezing cycle (because water is absorbed by the alcohol, which alters the freezing rate).

3 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 liquid nitrogen.

CoolCel freezing method:

Proceed according to methods for the Mr Frosty device, above. Keep the device at 4°C ready for use. Isopropyl alcohol is not required.

To maintain a high standard of cryopreservation, the IVRN recommends that all Tier 1 labs processing PBMC for the IVRN use either a mechanical controlled rate freezer, or Mr Frosty or similar semi-controlled rate freezer devices. Use of insulated specimen racks in the -80°C freezer is not accurate, and not acceptable for IVRN studies.

3.5 General 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 recommended time).

When transferring frozen ampoules between the -80°C freezer to the N2 tank, or in preparation to ship frozen cells to another lab, ampoules in the Mr Frosty, in racks, or in any other container, must be kept on dry ice to maintain cryogenic temperatures. An appearance of being frozen does not guarantee

viability of the cells in the specimen; dry ice temperature is the minimum acceptable standard (i.e the highest allowable temperature) for acceptable maintenance of cell viability.

NOTE: never use wet ice to transfer frozen PBMC specimens, not even briefly.

3.6 Thawing of cryopreserved cells

Unlike freezing, when it is important that cells are cooled slowly, the best yield and viability is obtained when cells are thawed relatively quickly. Cells being thawed are most vulnerable to damage at 0°C, when the sample is half frozen and half thawed. At this point the osmotic pressure across the cell membrane is such that damage may occur. Slow drop-wise addition of medium to dilute the DMSO may also minimise cell damage by gradual osmotic diffusion of DMSO out of the cell.

Method:

1 Remove cryovials from liquid nitrogen and place in an Esky containing dry ice (leave for a few minutes to allow trapped liquid nitrogen to slowly escape the vial).

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

3 Warm water bath or add fresh water into beaker at 42°C (this temperature provides the fastest thawing without overheating the contents). Note that electronic water baths are a notorious source of contamination, and a fresh warm water bath in a beaker is a better option.

4 Hold cryovials upright in the water bath and agitate rapidly until remaining ice crystal has a diameter of approximately 5mm. Avoid getting the cap wet (to prevent contamination at the seal).

5 Swab the cryovial with 70% ethanol, allowing excess to evaporate before opening the ampoule. Pour the contents into a labelled sterile 20ml centrifuge tube. Rinse ampoule with approx. 1ml FCS (37°C) to wash out remaining cell suspension, and add to 20ml tube.

6 Add pre-warmed medium (RPMI + 10% FCS; 37°C) in a drop-wise manner until approximately 10ml has been added. Add additional medium to 20ml total.

Notes: Slow addition of medium is required to avoid undue stress on the cell resulting from rapid dilution of DMSO and associated osmotic potential across the cell membrane.

A decent wash in at least 20ml is good to dilute out the DMSO initially, but a second wash should also be viewed as essential if DMSO is to be sufficiently removed.

Addition of cold medium to the thawed cells may reduce viability (Disis et al., 2006) and should be avoided.

7 Mix and centrifuge at 200g for 10 minutes. Discard the supernatant and resuspend in another 10ml of medium, transfer to a 10ml or 15ml tube, and centrifuge again.

8 Discard the supernatant and resuspend in 2ml assay medium for counting.

9 Count cells and check viability as described above.

10 Results should be entered into the IVRN Blood Processing Workflow Sheet (see Appendix)

3.7 Things you should never do with PBMCs

1 Do not chill whole blood; keep at ambient (22^{o}C) lab temperature (Olson *et al*, 2011).

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 or violently shake cell pellets; resuspend by gentle pipetting.

5 Do not leave cells in serum free medium for more than 1-2hr; add 5-10% FCS if waiting for other cells to be ready before bulk 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 (Tree et al., 2004).

4. References

The methods used by the IVRN are based on best practice and common sense, these being tried and tested lab procedures. There are many references stating the use of specific methods, however there is considerable divergence between these reports despite generally acceptable outcomes. References to studies comparing variations on a method are surprisingly rare. Below are examples of such studies.

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. (2006) Maximizing the retention of antigen specific lymphocyte function after cryopreservation. J Immunol Methods 308, 13-18.

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

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

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.

ACH²

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

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		**Plasma**	
# vials	type	# vials	type
# vials	type	# 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 that IVRN Tier One lab staff should follow when they are ready to ship specimens to the Central Specimen Repositories (CSRs) at UNSW (Sydney) and at NRL (Melbourne) with regard to the shipment and invoicing of processed specimens.

Record Keeping

Lab staff should prepare a simple list of the specimens being sent at the time of shipment (including ID number, 2x2 code, sampling timepoints for each subject and total number of specimen vials). This list should accompany the shipment.

Shipping

Labs in WA, SA and Vic should ship specimens to the NRL CSR, while Tier One 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, copies of the IVRN Blood Processing Workflow Sheets and any paperwork that arrived with the original blood samples (e.g. request forms) should be sent with the shipment.

Invoicing

Lab staff should invoice IVRN (via the Burnet Institute) at the time of shipment to the CSRs, and should also 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: 49 007 349 984
Macfarlane **Burnet Institute** for Medical Research and Public Health
85 Commercial Rd Melbourne Vic 3004 Australia

Contact: Alison Greenway
Phone: +61 3 9282 2111
Fax: +61 3 9282 2100