Centre of Research Excellence in Pulmonary Fibrosis

Australasian Interstitial Lung Disease Registry (AILDR) Biobanking – Standard Operating Procedure
Blood, BAL and Tissue Collection, Processing and Storage

Blood samples

A) Blood sample collection

1. Collect four tubes (5ml lithium heparin tubes) of blood.

B) Isolation of PBMC

1. Collect 100% plasma (within 2 hours of collection)
    - Spin collection tubes at 1000g for 10 mins
    - Remove plasma and mix blood with an equal volume of RPMI, proceed to PBMC isolation
    - Note - if plasma is not immediately removed, mix whole blood with an equal volume of RPMI and invert carefully to mix
    - Store blood at room temp until use (it can be stored for up to 24hrs)
2. Underlay the lymphoprep
    - Warm the RPMI and lymphoprep
    - Add lymphoprep to the tubes
        - For small blood volumes use I5ml tubes with 2.5ml lymphoprep and 5ml blood/RPMI
        - For larger blood volumes use 50ml tubes with 10ml lymphoprep and up to 30ml blood/RPMI
        - Using a mixing cannula attached to a syringe underlay the lymphoprep into the blood/RPMI tubes, the lymphoprep with form a layer below the blood/RPMI.
        - Spin at 800g (approx 1350RPM) for 20min at room temp with NO brake
3. Harvest plasma if necessary (remember this is 50% plasma)
4. Using a disposable transfer pipette, collect PBMC from the interface and wash in RPMI + 2% heat-inactivated FCS
5. Spin approx 500g (1350 RPM) for 10 mins with brake on.
6. Pool tubes and wash in RPMI + 2% heat-inactivated FCS
7. Spin 500g for 7 mins
8. Pool tubes and wash in RPMI + 2% heat-inactivated FCS
9. Spin 500g for 7 mins
10. Resuspend in 0.5-5ml RPMI + 2% heat-inactivated FCS
11. Count cells using white cell counting fluid which stains white blood cell nuclei blue
12. Either use cells immediately or cryopreserve for later use

C) Cryopreservation of PBMC

1. Calculate how many vials and at what cell concentration are to be frozen
    - Cells are generally frozen down at 10×10^6 -50×10^6 /vial
2. Resuspend your cells the appropriate volume of RPMI + 2% heat-inactivated FCS
    - For every cryovial of cells you will need 0.5ml media, ie resuspend your cells in 2ml if freezing into 4 vials
    - Keep cells on ice
3. Slowly add an equal volume of freezing mixture (15% DMSO in FCS) dropwise to the cell suspension using a disposable transfer pipette, mixing after each addition.
4. Keep cells on ice and aliquot into cryovials (it is best to add freezing medium to the total cell volume and then aliquot into individual vials)
5. Ensure cryovials are appropriately labelled and place in a freezing container (Mr Frosty) and place into a -80°C freezer
6. Do not move for at least 4 hours
7. Move cells into liquid nitrogen the following day

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BAL samples

A) BAL sample collection

1. At diagnostic bronchoscopy, 20-50ml of BAL fluid is collected in a specimen jar.

B) Processing of BAL cells

1. Wash BAL sample by centrifuging for 10 minutes at 800g.
2. Store supernatant at – 80°C.

C) Storage of BAL cells

1. Cells resuspended in 10 mL of media (RPMI/10% FCS) and counted using haemocytometer (0.5 to 5 million cells expected).
2. From 0.5 to $5x10^6$ cells resuspended in 0.5 mL of media and combined with 0.5 mL of freezing media (100% FCS + 15% DMSO) in Cryotube.
3. Cells placed in freezing vessel (Mr Frosty) overnight at -80°C.
4. Cells transferred to Liquid Nitrogen for long term storage.

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Lung tissue (including Cryobiopsy)

Materials

1. Sterile dissection equipment – Forceps, scissors, scalpel.
2. Sterile specimen pots.
3 Yellow top 5mL tubes (Techno Plas).
4 RNAlater (Invitrogen).
5 10% neutral buffered formalin
6 OCT fluid
7 RPMI (plus 2% FCS and Penicillin-Streptomycin-Glutamine (PSG)) and serum free RPMI
8 Collagenase/dispase (Sigma cat 11097113001) is resuspended in 10ml sterile PBS and frozen in 1ml aliquots.
9 Digestion buffer recipe: 100mls of serum free RPMI, 2 vials of DNase 1 (Sigma cat SLBZ5105), 1 x 1ml vial of collagenase/dispase. Keep warm in water bath (37^{o}C) until ready to use.
10 Red cell lysis buffer (RCLB): For 500ml of 10x – 40.2g ammonium chloride, 4.2g sodium bicarbonate, 1ml of 0.5M EDTA. Filter buffer and dilute with ultrapure water as required.
11 Freezing media: sterile filtered, heat inactivated FCS with 15% DMSO
12 3 pieces of sterile stainless-steel mesh (cut into 130mm squares, Sefar - 260um)
13 General disposables including; sterile transfer pipettes, paper towel, ethanol, sterile cell culture dishes and 50ml screw cap tubes.

Large Equipment

1. Biosafety cabinet
2. CO_2 incubator
3. Refrigerator
4. -80°C freezer

A) Sample collection

1. Lung tissue sample availability will widely vary by site. Samples may include lung biopsies, cryobiopsies or transplant specimens. Available sample volume and site research priorities will determine the processing and storage approach.
2. Note the time at which the lung tissue was removed from the patient, and the time at which it was handed off to the scientist. Take the lung tissue back to the laboratory.

B) General sample processing

1. Divide available tissue sample in a Biosafety cabinet.

C) Specific sample processing and storage of lung tissue

Options, dependent on available tissue volume	Processing	Storage
RNAlater	1. Cut one small piece of tissue (minimum 0.1cm^3, dependant on available volume of tissue) and transfer the tissue strips into an "RNAlater" yellow top tube. 2. Add RNAlater to the "RNAlater" yellow top tube, enough to submerge the tissue, and place in the fridge.	After 24 hours, remove the RNAlater from the 3 tubes in the fridge and transfer to the -80°C freezer.
Formalin fixed paraffin embedded tissue	1. Cut one small piece of tissue (~0.3-0.5cm^3, dependant on available volume of tissue) and transfer into specimen jar containing 10% neutral buffered formalin (fixative volume at least 5-10 times tissue volume). 2. After 24 hrs fixation process for paraffin embedding.	Store embedded tissue in paraffin blocks at room temperature in the dark.
Frozen tissue section	1. Cut one small piece of tissue	Place in freezer.

	(~0.3-0.5cm^3, dependant on available volume of tissue) and place in small weigh boat, cover with OCT and then cover with second weigh boat and seal with tape, label.	
Single cell suspension	1. Mince the tissue as fine as possible with sterile scissors and place in 50ml sterile tube with 33ml of the digestion buffer. 2. Place the tube into Rotator and incubate at 37^{o}C for 1 hour. 3. After incubation mash the tissue through stainless-steel mesh with hard end of 10ml syringe in a sterile petri dish, using extra RPMI (plus 2% FCS and PSG) to wash the dissociated cells through the mesh. 4. Then filter all digest wash through a 70um tube top filter. Centrifuge at 800g for 8mins and resuspend in 15ml of RCLB, incubate for 10mins at room temperature. 5. Fill tube with RPMI (plus 2% FCS and PSG) and centrifuge as above. 6. Discard supernatant and resuspend in 5ml of RPMI (plus 2% FCS and PSG). 7. Perform a cell count and then freeze cells across 10 cryovials. Using a 1:1 ratio of the above media and freezing media.	Place tubes in Mr Frosty's (freezing container) in the -80 overnight and then transfer to liquid nitrogen the following day.
Snap frozen tissue	1. Cut one small piece of tissue (~0.2-0.3cm^3, dependant on available volume of tissue). 2. Place 1 piece each in empty 1.5 ml Eppendorf tubes. 3. Label and immediately place in in rack submerged in liquid nitrogen for 4 hours.	After 4 hours, move to -80°C.