PBL Isolation and Freezing Procedure

Purpose and Scope:

The purpose of this procedure is to separate Peripheral Blood Leukocytes (PBL, as synonym for PBMC, Peripheral Blood Mononuclear Cells or lymphocytes and monocytes) from whole blood with the final aim to cryopreserve the isolated cells to perform a Telomere Analysis Technology (TAT) assay. The most widely used and robust method to separate mononuclear cells from whole blood is the centrifugation on a discontinuous density gradient.

List of Reagents

DMSO(Dimethyl Sulfoxide)
PBS (Phosphate Buffered Saline)
Histopaque-1077
Trypan Blue 0.4%
2-Propanol (Isopropanol)
HI-NCS(Heat-inactivate New-born Calf Serum)
Household Bleach or 5% Sodium Hypochlorite

List of Tools/Equipment

Benchtop centrifuge with swinging bucket rotor. With buckets for 15 mL tubes.
Laminar flow hood (class 2 biosafety hood/cabinet)
Aspiration pump for liquid waste (optional)
Thermostatic water bath, range 37º-60ºC
-80°C Freezer
-20°C Freezer
2-8°C Refrigerator
Automated Cell Counter Countess®
When an optical microscope capable of a magnification of 100-400x is available, the automatic cell counter can be replaced by a Hemacytometer
Automatic pipette pump or pipette bulb
"Mr. Frosty®" Cryo 1°C Freezing Container
Either variable-volume or fixed-volume automatic pipettes for 10 µL and 1000 µl
Laboratory permanent marker

List of Disposables

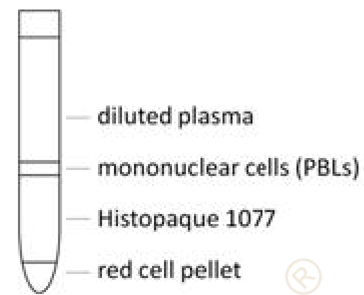
15 mL Conical centrifuge tubes
12 mL Leucosep™ tube (optional)
Universal Containers, 30 mL
Tube racks
Sterile Serological pipette, 10 mL
Transfer pipettes, single wrapped sterile
1-200 µl pipette tips, racked and sterile, fit to the automatic 10 µL pipette
1 mL pipette tips, racked and sterile, fit to the automatic 1000 µL pipettor
1.5 mL Eppendorf tubes

Cryogenic tubes, 1.8 mL
BD Vacutainer Sodium or Lithium Heparin tube (4 mL)
BD Vacutainer Sodium or Lithium Heparin tube (6 mL)

Procedure Description

1. Bring reagents (Histopaque, PBS and HI-NCS) to room temperature.
2. Gently mix the blood in the two Vacutainer tubes (10 ml in total). Bring and equilibrate at room temperature if needed. It is critical that PBL must be isolated from the whole blood within 24h of the blood draw.
3. Prepare a universal container per blood sample. Label with the permanent marker on the outside and load with 10 mL PBS at room temperature.
Note. The volume of PBS is matched to that of blood, with the intention of preparing a 1:1 dilution.
4. Remove the caps/plugs from the Vacutainer tubes from a sample and pour blood into appropriately labelled PBS-containing universal containers.
5. Close the container and gently mix by inversion.
6. Label two 15 mL centrifuge tubes with the corresponding sample ID and pipette 5 mL of room temperature Histopaque in each of them.
Note 1. Take Histopaque out of the refrigerator and let it equilibrate at room temperature at least 1 hour before use protected from light.
Note 2. Leucosep™ tubes with porous barrier can be used here alternatively. They should be loaded with 3 mL of Histopaque as per manufacturer's instructions. In this case, 9 ml of starting blood should be used rather than 10 ml and 3 Leucosep™ tubes are required per sample (each tube will be filled with 3 mL Histopaque + 6 ml diluted blood (as described in 3)) .
7. Using a 10 mL sterile pipette carefully layer 10 mL of the diluted blood sample over the dispensed Histopaque. Avoid disturbing the Histopaque surface as much as possible during the process. Carefully layer the first ~3-4 mL of sample by letting it pour down the tube very slowly on the inner tube wall. The rest of sample may be poured a bit faster as long as the interphase with the Histopaque does not suffer major disturbances.
Note. Smooth oscillations at the Histopaque surface when pouring are normal. Avoid jets of diluted blood dipping into it. Disturbances here associate to fuzzy separations. If a sample has not overlaid the Histopaque neatly, consider recovering it out of the tube with a pipette, leaving behind the disturbed region, and loading it into a new labelled tube loaded with fresh density medium.
8. Carefully load the two conical tubes in opposite positions in the centrifuge buckets without disturbing the layers.
9. Centrifuge at 400 x g for 30 minutes at room temperature. Verify centrifuge setting. The centrifuge brake must be turned OFF for the separation to be clean and to maximize retrieval of the PBL.
10. After spinning, carefully remove the buckets from the centrifuge and place them inside the laminar flow.
11. Carefully remove the 15 mL conical tubes from the buckets and place in rack.

Four layers should now be visible (top to down):
- Plasma
- Mononuclear cell layer (turbid, whitish)
- Histopaque (transparent, colorless)
- Red blood cell pellet



12. Remove the upper plasma layer with either an aspiration pump or a 10 mL pipette just until ~1 cm above PBL layer, and discard. Avoid disturbances to the cellular layer.
13. Draw the cloudy interface (PBL layer) with a pipette and transfer into appropriately labelled 15 mL conical tubes (2 tubes per original blood sample). Recover as much of the PBL layer as possible without taking plasma or Histopaque.
Note. The collected cell suspension should be approximately 3 ± 1 mL per tube.
14. Wash 1. Add 10 mL of PBS per sample tube, balance tubes if necessary with PBS, close and gently mix by inversion.
Note. Inclusion of 0.3 mL of HI-NCS per tube can improve cell recovery.
15. Load tubes into centrifuge buckets and centrifuge at 250 x g for 10 minutes at room temperature. (Use normal acceleration and deceleration rates).
16. Place buckets back into laminar flow. Remove tubes and place in rack.
17. Decant the supernatant carefully, removing all of the fluid, taking care not to disturb the cell pellet. Discard the supernatant directly into liquid waste flask.
Note. If the pellet is sufficiently tight (which depends on variables such as tube size, volume and centrifuge settings) inverting the tube directly on the waste flask leaves behind a small amount of PBS by the cell pellet, sufficient to allow its re-suspension.
18. Wash 2. Add 10 mL of PBS to the centrifuge tube, close, mix gently by inversion and centrifuge for 10 minutes at 250 x g at room temperature.
19. Discard the supernatant directly into liquid waste flask and add 5 mL fresh PBS on each pellet and resuspend gently. Ensure homogeneous resuspension by pipetting up and down if necessary. Pool both 5-mL volumes of the same sample in one of its original 15 mL tubes to make 10 mL of a single cell suspension.
19.1. Transfer 10 µL of the suspension to a separate Eppendorf tube with a pipette to perform a white cell count.
Note 1. From each sample there were two tubes in step 18, their pellets are re-suspended each in 5 mL PBS and collected into a single tube in step 19. A small aliquot is taken to measure cell concentration.
Note 2. An haemocytometer or an automatic cell counter such as Countess?can be alternatively used. Stain with Trypan Blue and count live cells by following manufacturer instructions.
20. Measure the cell concentration from the aliquot of step 19.1 and record the result as "number of cells x 10^6/ mL" in the aliquot.
21. Calculate the amount of cells available by multiplying this concentration value by "10 mL" (the main volume from which the aliquot was taken). Overall "Number of cells available" = "Number of cells x 10^6/ mL" x 10 mL.

22. Calculate the volume of freezing medium to prepare according to the following formula:
Volume (mL)= "Number of cells available"/ 10^7
Note 1. The target concentration in which the cells are intended to be stored is 10 million cells/ mL. The minimum number of cryovials intended to be prepared for each sample is four.
Note 2. A minimum of 4 million cells should be recovered. Therefore, a minimum of 100 µl per cryovial should be loaded.
Note 3. Have ready and precooled an amount of freezing medium at least equal to the volume calculated here. If using a thawed aliquot mix by inversion before use.
Note 4. Freezing medium preparation. For 10 mL of freezing medium add 1 mL of DMSO to 9 mL of HI-NCS, in order to prepare a solution of 90% serum and 10% DMSO.
23. Label cryotubes. Chill empty cryotubes and Nalgene "Mr. Frosty" container on ice for at least 10 minutes prior to cell freezing.
Note. Four cryotubes are required per sample.
24. Centrifuge the cells again for 10 minutes at 250 x g at room temperature. After the centrifugation decant supernatant discarding to waste as before. Put the tube in upright position and aspirate residual supernatant if necessary.
Note. There should be no more than ~2-3 mm of leftover liquid on top of the cell pellet. Just enough for a comfortable cell resuspension but small enough as to not dilute freezing medium significantly in the next step. An automatic pipette is a convenient means to remove some excess of supernatant.
24.1. Resuspend pellet in the limited residual supernatant by tapping gently with fingers.
25. Add the volume calculated in step 22 of precooled freezing medium. Add it dropwise while mixing at the same time. Once added, ensure a homogeneous suspension without clumps by pipetting it up and down smoothly for a few times. Keep on ice until ready to proceed.
26. Divide the volume calculated in step 22 by 4 that is the minimum number of aliquots intended to be prepared.
27. Aliquot the volume calculated in step 26 into four labeled cryotubes and close tightly. Residual amounts can be discarded as liquid waste.
28. Immediately transfer the cryotubes to the precooled Nalgene "Mr. Frosty" container and place in a -80°C freezer for 16 to 24 hours.
Note 1. Follow manufacturer instructions to use the "Mr. Frosty" container. Fill with isopropanol and put in a fridge at 4° one day in advance or for a few hours before starting with this protocol.
Note 2. It is important that this container is not at below zero temperature when the tubes are placed inside, as the purpose is to provide a gradual and even freezing from 4°C to -80°C. Once the cryotubes are cooled to the target temperature (e.g. next day), they should be transferred to a longer term storage box and "Mr. Frosty" should be warmed up ready for next use.
29. Frozen PBL can be kept at -80° for not longer than 10 days. Longer-term storage requires liquid nitrogen instead.