Plasma and PBMC isolation and cryopreservation from whole blood or buffy coats

Notes

- PBMC = peripheral blood mononuclear cells.

- Principle of separation (source GE Healthcare's booklet Ficoll Paque PLUS 1.077 g/mL): The separation of blood cells is performed by buoyant density centrifugation. The median buoyant densities of human monocytes, lymphocytes and thrombocytes (platelets) are 1.067-1.077, 1.073-1.077 and 1.04–1.08 g/mL, respectively. With the exception of basophils, polymorphonuclear leukocytes (PMNs) have a much greater buoyant density ($\rho > 1.082$ g/ml) than mononuclear cells. Hence, "on centrifugation, cells in the blood sample sediment towards the blood/Ficoll-Paque media interface, where they come in contact with the Ficoll PM400 present in Ficoll-Paque products. Red blood cells are efficiently aggregated by this agent at room temperature. Aggregation increases the rate of sedimentation of the red cells, which rapidly collect as a pellet at the bottom of the tube, where they are well separated from mononuclear cells. Granulocytes also sediment to the bottom of the Ficoll-Paque media layer. This process is facilitated by an increase in their densities caused by contact with the slightly hypertonic Ficoll-Paque media. Thus, on completion of centrifugation, both granulocytes and red blood cells are found at the bottom of the tube, beneath the Ficoll-Paque product. Lymphocytes, monocytes, and platelets are not dense enough to penetrate into the Ficoll Paque PLUS medium layer having a density of 1.077 g/mL. These cells therefore collect as a concentrated band at the interface between the original blood sample and the Ficoll-Paque product. This banding enables the mononuclear cells to be recovered with high yield in a small volume with little mixing with the Ficoll-Paque media. When Ficoll-Paque PREMIUM 1.073 is used (instead of Ficoll-Paque PLUS 1.077 g/mL), some lymphocytes with densities >1.073 will enter the Ficoll-Paque media layer and the resulting cell preparation will be enriched for lower density cells like mesenchymal stromal/stem cells and monocytes. Washing and centrifuging the harvested cells subsequently removes platelets, any contaminating FicollPaque media, and plasma. The resulting cell suspension then contains highly purified, viable lymphocytes, monocytes, mesenchymal stromal/stem cells, and is suitable for further studies."

- Some critical steps are:
    - Perform the isolation and freezing of PBMCs less than 8 hours after the blood collection. Best results are obtained if the procedure is performed less than 2 hours after blood collection.
    - Dilute the blood with PBS prior to overlay: "When erythrocytes in whole blood are aggregated, some lymphocytes are trapped in the clumps and therefore sediment with the erythrocytes. This tendency to trap lymphocytes is reduced by diluting the blood. Dilution gives a better lymphocyte yield and reduces the size of the red cell clumps. Aggregation of erythrocytes is enhanced at higher temperatures (37°C), which consequently decreases the yield of lymphocytes. At lower temperatures (4°C), however, the rate of aggregation is decreased but the time of separation is increased, which also decreases the yield of lymphocytes. A compromise temperature of 18–20 °C gives optimal results" (source: GE Healthcare).
    - Minimize the time that the cells remain in a pellet or in contact with the Ficoll.
    - Wash the cells after the Ficoll.
    - Do not stop during the cell freezing but add slowly the DMSO-containing solution to the cells (in most cases cells are first resuspended in FBS and DMSO-containing solution is then added dropwise). Most labs freeze cells either at 4°C or at room temperature.

- Buffy coats are isolated from whole blood at the blood bank by an initial centrifugation without a density gradient. This initial spin concentrates red blood cells at the bottom and plasma on top, the buffy coat forms the interface and contains most of the leukocytes and platelets from the whole blood (although it still looks like blood). Each unit (~470 mL) of whole blood yields a ~45mL buffy coat. At the Purpan EFS blood bank, the night shift team performs this spin on the day of the blood collection, stores the buffy coat at 20°C and makes it available to the CPTP on the next morning.

- PBMCs are usually frozen right away after the Ficoll isolation and used subsequently in functional assays. However, with functional tests on fresh or frozen cells, a resting period (typically overnight) is often recommended after Ficoll isolation or cell thawing.

Equipment, reagents and reagent preparation

- PBS without calcium and magnesium. Keep and use at room temperature.
- Density gradient media suitable for human blood cells: e.g. Ficoll-Paque PLUS (density 1,077 g/mL, GE Healthcare Pharmacia,) or Pancoll human density 1,077 g/mL (PanBiotech ref P04-60500).
  Do not use Ficoll-Paque PREMIUM density 1,073 g/mL (gives greater enrichment in monocytes or mesenchymal stem cells) or 1.084 g/mL (adapted for rodent PBMC).
- DMSO Grade culture (e.g. Sigma ref D2650). After opening, undiluted DMSO is stable at room temperature (15 to 30°C) when protected from light and moisture, for 6 months. Reagent may be aliquoted in small amounts to help preserve sterility. Label aliquots with "DMSO," the date

opened/aliquoted, the expiration date (six months from opening) and tech initials. Protect aliquots from light.

- Heat-inactivated FBS thawed and stored at 2 to 8°C (stable for one calendar month).
- NALGENE® Mr. Frosty, 1°C/minute cryo-freezing container (can hold 18 cryovials). Mr Frosty requires isopropanol that needs to be replaced once a month (in a specific waste bottle) and level must be checked after each freeze-thaw cycle. Alternatively, some dry cryopreservation modules can be used such as the StrataCooler CryoLite preservation module (32 cryovial holder from Agilent Technologies) that cools down at a controlled rate of 0.4–0.6°C/minute. The freezing boxes must be left to dry between uses and cooled down at 4°C overnight before the PBMC isolation.
- 2 mL cryotubes (e.g. Biosigma CL2ARBIPS).

Plasma isolation from buffy coats or whole blood without loss of PBMC

1. Store blood tubes at room temperature prior to the isolation of PBMCs. All steps below are performed at room temperature.
2. Mix the blood by gently inverting the blood collection tubes or bag 6-8 times and wipe off the blood tubes or buffy coat bags with 70% ethanol
3. Transfer the blood tubes or the buffy coat in a single recipient
4. Transfer 10 mL blood into a 15 mL Falcon tube and spin at 400 *xg* for 10 min (**1400 rpm** on Heraeus Meafuge 1.0R)

    1.1 Transfer plasma (do not touch the interface!) to a labeled new tube for a second centrifugation at 800 x*g* for 10 min (**2000 rpm on Heraeus Megafuge 1.0R** or **3000 rpm on Eppendorf centrifuge**) to remove any cellular debris. Note: for the EFS biobank we perform the 2nd spin only for the fraction that will be used for the CMV and toxoplasmosis serology (i.e. 0.7 mL to obtain 0.5 mL plasma).

    1.2 Add PBS to the non-plasma part to bring back the blood to its original volume, add it back the whole blood and proceed to PBMC isolation

PBMC isolation from buffy coats or whole blood by Density Gradient Cell Separation

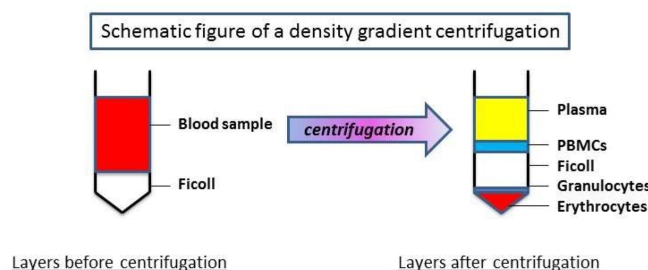
1. Dilute blood with equal amount of PBS
2. Mix gently the blood and PBS

    2.1 Invert the Ficoll-Paque PLUS or Pancoll density 1.077 g/mL (optimized for human cells as explained above). Shake the bottle several times to ensure thorough mixing

    2.2 Add Ficoll-Paque at the bottom of a new tube without touching the side of the tube since the reagent is toxic. Use 20 mL Ficoll-Paque PLUS for 30 mL diluted blood in a 50 mL conical tube

    2.3 Gently overlay the Ficoll with the diluted blood

    1.3 Gradient centrifugation: Centrifuge at **20°C** 760 x*g* for 20 min (**1900 rpm** on Heraeus Megafuge 1.0R) with the **brakes OFF** since the deceleration disrupts the density gradient. If the centrifuge starts shaking, stop immediately but gently; reweigh and balance the tubes



3. PBMC collection and washing **3 washes (blood draws) or 5 washes (buffy coats)**

   3.1 Remove as much plasma layer as possible

   3.2 Collect the mononuclear cells from the plasma/Ficoll interface with a disposable transfer pipet. Transfer the cells from each Ficoll tube into a new sterile conical tube. **Do not pool cells yet**. Fill each tube PBS QSP 40 mL

   3.3 Wash 1: Spin cells at 400 x*g* (**1400 rpm** on Heraeus Megafuge 1.0R) for **12 min**. Do not forget to switch on again the centrifuge brakes

   ⚠ Remove carefully the supernatant (the pellet is loose because of red blood cells and platelets) by pipetting off the supernatant without touching the pellet. Suspend the cell pellet by gentle pipetting in a low volume of PBS with a p5-10mL (**do not use a p1000 tip**)

   3.4 Wash 2: PBS QSP 30 mL and spin cells at 400 x*g* (**1400 rpm** on Heraeus Megafuge 1.0R) for **10 min**

   3.5 Wash 3: Pool tubes, PBS QSP 30 mL, and spin cells at 400 x*g* (**1400 rpm** on Heraeus Megafuge 1.0R) for **10 min**

   3.6 Wash 4: PBS QSP 30 mL and for increased removal of platelets, centrifugation at low speed i.e. 200 x*g* (**1000 rpm** on Heraeus Megafuge 1.0R) for **10 min**

   3.7 Cell count and wash 5: for buffy coats, suspend pellet thoroughly in 10 mL PBS (pipet app. 10 times) add 40 mL PBS, count cells in duplicate, and spin cells at 400 x*g* (**1400 rpm** on Heraeus Megafuge 1.0R) for **10 min**

4. Analysis of PBMC count and viability (blood draws: after the 1st wash; buffy coats: after the 4th wash) with the **Cellometer 2000** (linearity: $1x10^6$ to $10x10^6$ cells/mL)

   4.1 Bring cells at approximately $3\text{-}6x10^6$ cells/ml (max 10.10^6 cells/ml), knowing that each mL of fresh blood will give a rough average of $1x10^6$ PBMCs or that a buffy coat contains 200-400 million PBMCs

   4.2 Ex PBMC from buffy coats into 50mL PBS

   4.3 Ex PBMC from 30mL fresh blood into 5mL PBS

   4.4 Principle: Cells are stained with acridine orange, AO, and propidium iodide, PI. AO is a nuclear staining (nucleic acid binding) dye permeable to both live and dead cells. It stains all nucleated cells to generate green fluorescence. PI can only enter dead cells with compromised membranes. PI stains all dead nucleated cells to generate red fluorescence. Cells stained with both AO and PI fluoresce red due to quenching, so all live nucleated cells fluoresce green and all dead nucleated cells fluoresce red

   4.5 Protocol: Remove the 2 plastic covers of the counting slide (each slide has 2 counting chambers). Mix 20 microL cells with 20 microL AO/PI. Mix 5 times and load 20 microL into the chamber. Adjust the focus (perform an image preview) and read immediately and only once the cell staining (perform a cell count using the "immune cells, high RBC" program). Record the live cell concentration and the percentage of live cells

PBMC freezing (below 4°C freezing protocol), example for 2.10^7 cells/cryovial

1. Thaw an aliquot of FBS and allow cooling at 2 to 8°

2. Put the NALGENE® Mr. Frosty or StrataCooler freezing boxes at 2 to 8°C for a few hours.
   With NALGENE® Mr. Frosty, check the date of replacement of isopropanol (monthly change)

3. Prepare the 20% DMSO/FBS mixture and allow cooling at 2 to 8°. The mixture can be stored at 2 to 8°C for **1 working day**.

4. Hand-label the cryotubes or print cryolabels (e.g. Protocol /Participant code/PBMC/date yyyy-mm-dd).

5. Perform the steps below on **an ice pan** and do not stop during the procedure:

    - Suspend PBMCs in cold FBS at $4x10^7$cells/mL: first add 0.5 to 1 mL FBS, mix/detach the cells by gentle pipetting with a p5 mL (do not use narrow p1000 tips) and then add the remaining volume of FBS.

    - Add the same volume of cold FBS 20% DMSO dropwise SLOWLY **1 drop per second** while swaying the tube to gently mix the cell suspension (final solution: FBS 10% DMSO).

    - Mix gently 5 times by tube inversion.

    - Using the p5 mL pipette, aliquot 1 mL cell suspension to each cryotube, firmly close the lid and put the tubes into the NALGENE® Mr. Frosty or StrataCooler freezing boxes

    - If freezing boxes are not available, the following "low technology" method may work:

        - Place the cryovials in a Styrofoam rack (e.g., racks for 15 ml conical tubes).

        - Place a second Styrofoam container of the same type over the first one and tape the two containers together.

        - Insert into a plastic bag leaving some air in the bag before taping it closed.

6. Place the freezing boxes or the Styrofoam container immediately into a -80°C freezer for 12 to 24 h (max 4 days), then transfer the cryovials into the liquid nitrogen tank.

7. Avoid any temperature increase during the transfer in the nitrogen tank and, in general, prior to the thawing of the cells.