Immune monitoring platform *Centre Physiopathologie de Toulouse-Purpan (CPTP)*	***PBMC isolation from buffy coats or whole blood*** ***PBMC cryopreservation***	cptp Instituts thématiques Inserm Institut national de la santé et de la recherche médicale

Notes

- 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.
  - Minimize the time that the cells remain in a pellet or in contact with the Ficoll.
  - Wash the cells carefully 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.
- The expected PBMC yields from whole blood for healthy populations are (source: HIV AIDS Network coordination PBMC processing SOP):

Population	Mononuclear Cell Yield Range (cells/mL blood)
Adult	(0.8 to 3.2) x 10^6
Pediatric—less than 6 months	(3 to 10) x 10^6
Pediatric—6 mo. to 2 years	(2 to 9) x 10^6
Pediatric—2 to 5 years	(1 to 6) x 10^6
Pediatric—more than 5 years	(0.8 to 4) x 10^6
Pediatric—Unknown age	(1 to 10) x 10^6

- 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 (~550 mL) of whole blood yields a ~60-45 mL buffy coat, which contains ~200-800*10^6 mononuclear cells. The Purpan EFS blood bank 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 day.
- PBMCs are usually frozen right away after the Ficoll isolation and used subsequently in functional assays. However, for 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

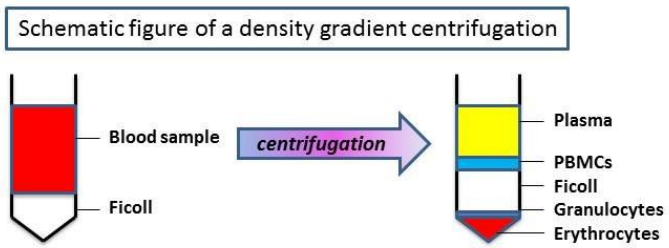
- PBS without calcium and magnesium. Keep and use at room temperature (15 to 30°C) or non-complemented RPMI-1640 (may increase PBMC viability).
- Blood cell density gradient media: e.g. Ficoll-PAQUE Plus (GE Healthcare Pharmacia, endotoxin tested ref 17-1440-02) or Pancoll human density 1,077 g/l (PanBiotech ref P04-60500).
- 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 should be stored at ambient temperature (15-30°C) between uses. The isopropanol level must be correct and the isopropanol must be completely replaced after the fifth freeze-thaw cycle. 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).

What parameters are being tested in 2015?

- The use of dry StrataCooler (Agilent Technology: does not need isopropanol and can hold 30 cryovials) instead of NALGENE® Mr. Frosty.
- PBMC freezing at room temperature instead of 4°C.

PBMC isolation from buffy coats or whole blood

1. Store blood tubes at room temperature prior to the isolation of PBMCs. Measure the usable whole blood volume within 0.5 mL. All steps below are performed at room temperature.
2. Blood dilution:
   - 2.1 Remix the blood by gently inverting the blood collection tube 6-8 times.
   - 2.2 Wipe off the blood tubes or buffy coat bags with 70% ethanol.
   - 2.3 Transfer the blood tubes or the buffy coat in a single recipient that can contain at least twice its volume.
   - 2.4 Dilute the blood with equal amount of PBS. Non-complemented RPMI can be used in lieu of PBS in order to increase cell viability.
   - 2.5 Mix gently the blood and PBS.
3. Density Gradient Cell Separation:
   - 3.1 Add Ficoll PAQUE or Pancoll at the bottom of a new tube without touching the side of the tube since the reagent is toxic.
     - 3.1.1 Use 4 mL Ficoll PAQUE or Pancoll for 6 to 10 mL diluted blood in a 15 mL conical tube.
     - 3.1.2 Use 20 mL Ficoll PAQUE or Pancoll for 30 to 35 mL diluted blood in a 50 mL conical tube.
   - 3.2 Gently overlay the Ficoll with the diluted blood. Allow the diluted blood to flow down the side of the tube and pool on top of the density gradient media surface without breaking surface plane (see scheme below).
   - 3.3 Gradient centrifugation:
     - 3.3.1 **Buffy coats**: Centrifuge at room temperature at 760 x*g* (1900 rpm on Heraeus Megafuge 1.0R) for 20 min 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.
     - 3.3.2 **Whole blood**: Centrifuge at room temperature at 350 x*g* (1300 rpm on Heraeus Megafuge 1.0R) for 20 min with the **brakes OFF.**



4. PBMC collection and washes
   - 4.1 ⚠ **Thorough washing of buffy coats is important in order to reduce platelet contamination. With whole blood, 2 to 3 washes are generally sufficient**.
   - 4.2 Label new sterile conical tube(s), same number and same volumes as for the density gradient cell separation, which will contain the PBMCs.
   - 4.3 Remove as much plasma layer as possible.
   - 4.4 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**. While collecting the cells, be sure to aspirate plasma only and as little Ficoll as possible (typically 2 mL from a 15 mL tube and 8 mL from a 50 mL tube). Fill each tube with PBS.

4.5 **WASH 1 (buffy coats and whole blood)**: Spin cells 350 x*g* (1300 rpm on Heraeus Megafuge 1.0R) for 8 min (do not forget to switch on again the centrifuge brakes). ⚠ Remove carefully the supernatant (the pellet is loose because of red blood cells) by tilting the tube and pipetting off the supernatant without touching the pellet. Suspend the cell pellet by gentle pipetting in 1 mL PBS and fill the tube with PBS.

4.6 **WASH 2 (buffy coats and whole blood)**: Centrifuge at 350 x*g* (1300 rpm on Heraeus Megafuge 1.0R) for 8 min at room temperature. Pipet off the supernatant, suspend the cell pellet by gentle pipetting in 1 mL PBS and fill the tube with PBS.

4.7 **WASH 3 (buffy coats)**: Centrifuge at 350 x*g* (1300 rpm on Heraeus Megafuge 1.0R) for 8 min at room temperature. Pipet off the supernatant, suspend the cell pellet by gentle pipetting in 1 mL PBS PBS and fill the tube with PBS. At this step, tubes from the same donor can be pooled 2 by 2.

4.8 **WASH 3 (whole blood) or WASH 4 (buffy coats) with platelet removal**: For removal of platelets, centrifuge at low speed i.e. 200 x*g* (1000 rpm on on Heraeus Megafuge 1.0R) for 10 min with the centrifuge brakes on. Do not perform this step if the blood volume was low.

5. Perform analysis of PBMC count and viability:

5.1 After the last wash, pipet off the supernatant and loosen the pellet by adding 0.5 to 1 mL PBS and gently resuspend cells with the 1 mL pipet**.** Add PBS to bring cells at approximately $5x10^6$ cells/ml (max 10.10^6 cells/ml), knowing that each mL of blood will give a rough average of $1.5x10^6$ PBMCs or that a buffy coat contains 200 million to 1 billion PBMCs .

5.1.1 Ex: PBMCs from a 50 mL whole blood collection into 15 mL PBS.

5.1.2 Ex: PBMCs from a 65 mL buffy coat into 50 mL PBS, perform counting on a non-diluted sample and a 2-fold diluted sample.

5.2 Determine cell concentration and viability with the Cellometer 2000 (linearity: 2 to $12x10^6$ cells/mL):

5.2.1 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.

5.2.2 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, low RBC" program). Record the live cell concentration and the percentage of live cells.

5.3 QOC (**whole blood**) should show a fresh PBMC yield above $8x10^5$ PBMC/mL whole blood and viability above 95%. Long processing time and poor technique may adversely affect the viability.

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°C.
2. Put the NALGENE® Mr. Frosty or StrataCooler freezing boxes at 2 to 8°C overnight.
3. Prepare the 20% DMSO/FBS mixture and allow cooling at 2 to 8°C. 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 at room temperature but do not stop during the procedure:

5.1 Spin cells 350 x*g* (1300 rpm on Heraeus Megafuge 1.0R) for 8 min with the centrifuge brakes on.

5.2 Pipet off the supernatant.

5.3 Resuspend PBMCs in cold FBS at $4x10^7$ cells/mL: first add 0.5 to 1 mL FBS, mix/detach the cells by gentle pipetting and then add the remaining volume of FBS.

5.4 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).

5.5 Mix gently 3 times by tube inversion or gentle pipeting.

5.6 Aliquot 1 mL cell suspension to each cryotube, firmly close the lid and put the tubes into the NALGENE® Mr. Frosty.

5.7 If freezing boxes are not available, the following "low technology" method works equally well:

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

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

5.7.3 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.