PBMC Isolation, cryopreservation and thawing

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INTRODUCTION

The separation of PBMC (peripheral blood mononuclear cells) is essential for all subsequent analyses in immune monitoring. Without an effective and careful isolation, freezing and thawing of the cells, immune monitoring will be not successful and will not provide reliable results, especially for antigen-specific T cells with lower frequency in the circulation.

For separation of mononuclear cells from whole blood or bone marrow the density gradient centrifugation is the most widely used robust method. Here we describe the separation of PBMC using Leucosep® tubes containing a porous polyethylene barrier. This very efficient, user friendly and reproducible method is especially suitable for multicenter studies with a central immune monitoring facility.

MATERIALS

REAGENTS:

- 100 ml anticoagulated whole blood
  (as an anticoagulant we use sodium heparin for functional assays; others use sodium citrate because it seems to work better for PCR analyses)
- Na-Heparin (25000 IE/5ml, Braun-Melsungen), 500 units per 50 ml syringe
- Ficoll-Paque™ Plus (Amersham/GE Healthcare #17-1440-03)
- HANK's balanced salt solution (HBSS, Cambrex # 10-547 F)
- 20% human serum albumin (HSA; Kabi low salt, Octapharma # PZN-0504841)
- RPMI-1640 (Invitrogen # 21875034)
- L-glutamine (Invitrogen # 25030-024)
- Sodium-pyruvate (Invitrogen #11360-039)
- Penicillin/Streptomycin (Invitrogen 15140-122)
- Isopropanol p. a. (for refill of "Mr. Frosty®" freezing boxes)

- Dimethylsulfoxid (DMSO, CryoSure-DMSO #WAK-DMSO-10, WAK-Chemie Medical GmbH)
- 70% ethanol for disinfection
- Benzonase Nuclease (purity >99%, Novagen Merck Biosciences # 71206-3)
- C.T.L. wash™ supplement (Cellular Technology Ltd. Europe #CTLW-010)
- C.T.L. Test™ medium (Cellular Technology Ltd. Europe #CTLT-010)
- Trypan blue solution (Sigma #T8154-20ML)

EQUIPMENT:

- "class II" biological safety hood
- hemacytometer "Neubauer improved" with fitting cover slips
- Leucosep® tubes, 50 ml (Greiner bio-one #227290)
- 250 ml grade cell culture flask (sterile, pyrogen free)
- 5, 10 and 25 ml graded serological pipettes
- calibrated variable pipettes (10-1000 µl)
- pipette tips (sterile, pyrogen free)
- pipetting aid for 5-25 ml graded serological pipettes (sterile, pyrogen free)
- 15 and 50 conical polypropylene tubes (graded, sterile, pyrogen free)
- 96 round-bottom well microtiter plates
- 1,8 ml cryopreservation tubes (Nalgene Nunc # 377267)
- tube racks for 15 and 50 ml tubes
- tube racks for crypreservation tubes
- freezing boxes "Mr. Frosty®" (Nalgene Nunc # 9400945)
- refrigerator, adjusted to +4-8°C
- freezer, adjusted to -20°C
- freezer, adjusted to -80°C
- water bath, adjusted to 37°C
- humidified CO_2-incubator
- light microscope (200x-400x magnification)
- tabletop centrifuge (refrigated) with swinging buckets and suitable inserts (with brake optionally to be switched off)

GENERAL REMARKS:

- *Strictly observe local safety regulations for handling uncharacterized blood samples.*
- Once drawn, storing the blood sample at room temperature should not exceed 4 hrs. Gently agitate the blood to store over 30 min.
- The samples and all reagents and equipments must be handled aseptically (wear disposable gloves and lab coats, and work under a "class II" biological safety hood).
- Avoid mechanical stress (resuspend and pipette gently, minimize pellet time and avoid air bubbles).

REAGENT SETUP:

- Fill eight **Leucosep® tubes** with 15 ml Ficoll and centrifuge the tubes for 1 min. at 800g (room temperature). The Ficoll is now under the barrier.

- **RPMI III;**
  RPMI 1640 should be supplemented with 1% sodium-pyruvate, 1% L-glutamine and 1% penicillin/streptomycin solution

- **Serum-free C.T.L.-Test™;**
  Storage at 4°C, protected from light; should be supplemented with 1% fresh glutamine, sterile filtered and pre-warmed prior to use

- **C.T.L.-Wash™ supplement medium;**
  Storage at 4°C, protected from light; should be diluted 1+9 with RPMI-1640 and supplemented with 1% fresh glutamine prior to use;
  optional: add Benzonase to the C.T.L.-Wash™ supplement medium (50 U/ml), sterile filter and use warm for washing PBMC.

- **Freezing solution I;**
  95% RPMI III, 5% HSA, freshly prepared

- **Freezing solution II;**
  80% HSA, 20% DMSO; while gently swirling a 50 ml tube with HSA, add dropwise DMSO and cool the solution at +4°C

- Fill **"Mr. Frosty®" freezing boxes** with 250 ml room temperature isopropanol as indicated. (Isopropanol has to be changed every fifth use or at least once per month. When isopropanol has to be changed, first discard any old isopropanol). Before use cool the boxes for 1 h at +4°C.

Separation

Carefully transfer the blood (100 ml) into the 250 ml cell culture flask and dilute with 100 ml HBSS. Rinse the original blood tubes with HBSS. Mix carefully by inverting the closed flask but avoid foaming.

Aliquot the diluted blood very slowly onto the barrier of the eight Leucosep® tubes. (Rinse the flask with 10 ml HBSS).

Switch-off the brake and centrifuge the separation tubes at room temperature for 15 min. at 800 g.

Carefully remove the tubes from the centrifuge while not disturbing the layering.

Identify the PBMC containing interphase (opaque) above the barrier between Ficoll (colorless) and plasma (yellow). Part of the Ficoll and the pellet containing erythrocytes and most of the granulocytes are located under the barrier.

Remove the plasma using a pipette until approx. 5 ml above the PBMC interphase.

Pour or pipette the upper content of the tubes containing the PBMC, residual plasma and Ficoll into eight 50 ml conical polypropylene tubes and fill the tubes with HBSS up to the 30 ml graduation.

Switch-on the brake and centrifuge at room temperature for 10 min. at 320 g.

Carefully discard the supernatant and resuspend the PBMC by forcefully tapping until no larger clumps can be seen.

Join all cells in one tube by pipetting 15 ml HBSS from tube to tube. Rinse tubes by pipetting again 15 ml HBSS.

Count the cells with the Neubauer hemacytometer and determine the cell viability with trypan blue by mixing a small volume of the PBMC with trypan blue solution 1:1 in a microtiter plate. Load the hemacytometer with the cell mixture and wait for at least 30 sec. before counting.

While counting centrifuge the cells again for 10 min. at 320 g, discard the supernatant and resuspend the cells.

Cryopreservation

DMSO reduces the amount of ice present during the freezing procedure and lowers solute concentration, thus reducing ionic stress. However, DMSO can cause osmotic damage, especially during its addition or removal at warm temperatures, because it is hypertonic. Therefore, careful but uninterrupted working is important during freezing and thawing.

Calculate 500 µl freezing solution I and 500 µl freezing solution II per cryotube with $5x10^6$ PBMC.

Add the calculated volume freezing solution I to the PBMC.

Dropwise add the same volume freezing solution II at a rate of approx. 2 drops per sec., while swaying the tube to gently mix the cell suspension.

Aliquot 1 ml cell suspension to each cryotube, firmly close the lid and put the tubes into the freezing boxes.

Place the boxes immediately into a -80°C freezer for 24 hrs, then into the liquid nitrogen tank. *Follow the safety rules for handling with liquid nitrogen!*

Thawing

In general, cells should be thawed quickly but diluted slowly to remove DMSO. We incorporated a Benzonase treatment step in the thawing procedure to avoid cell clump formation as a result of dying cells.

Warm C.T.L.-Wash™ supplement medium to 22°-37°C in a 37°C water bath before beginning thawing procedure.

Remove the cryotube from the liquid nitrogen, place the tube on ice and immediately transfer into a 37°C water bath.
Hold the tube in the surface of the water bath while gently swayling. *Do not leave the cryotube unattended during the thawing process and do not thaw more than two tubes at the same time.*

With a small bit of ice remaining in the tube, transfer the tube into the biosafety hood.
Dry off the outside of the cryotube and wipe with 70% ethanol for disinfection before opening to prevent contamination.

Add 500 µl warm C.T.L.-Wash™ supplement medium (with Benzonase) dropwise into the cryovial containing the cell suspension.

Transfer the diluted cell suspension dropwise to a 15 ml conical polypropylene tube containing 5 ml of C.T.L.-Wash™ supplement medium (with Benzonase), rinse the cryotube with 1 ml medium to recover all cells, and fill the 15 ml tube up to 10 ml. Gradual dilution of DMSO avoids the osmotic stress.

Centrifuge the cells at room temperature for 4 min. at 472 g with the brake switched-on.

Discard the supernatant, and gently tap the tube with a finger to break up the pellet.

Resuspend the cells and wash again first with 10 ml C.T.L.-Wash™ supplement medium and then with serum-free C.T.L.-Test™ medium.
Check for cell clumps and remove them carefully with a pipette.

Determine cell number and viability as described above.

Resuspend the cells in the appropriate volume for the immune monitoring assay.

Resting

Resuspend cells to a concentration of up to $10x10^6$ cells in a 50 ml conical polypropylene tube with 5 ml of warm serum-free C.T.L.-Test™ medium and incubate at 37°C in a humidified CO_2-incubator with lid slightly loosened for gas exchange.

 2 – 18 hrs

After the resting phase, take again a sample for determination of the cell count and viability.

Resuspend the cells in the appropriate volume for the immune monitoring assay.