**PBMCs isolation from CPT™ tube**

**Chemicals and Reagents:**

1. Fetal Bovine Serum (FBS) (Sigma, Cat. no. F2442)
2. Phosphate-Buffered Saline (PBS), pH 7.4 (Gibco, Cat. no. 10010049)
3. ACK Lysing Buffer (Thermo Fisher Scientific, Cat. no. A10492)
4. CryoStor CS10 (Stem Cell Tech, Cat. no. 07955)
5. UltraPure 0.5 M EDTA, pH 8.0 (Invitrogen, Cat. no. 15575020)
6. 0.4% Ready-made Trypan Blue (Gibco, Cat. no. 15250061)

**Consumables:**

1. Vacutainer Cell Preparation Tubes (CPT) with sodium heparin (BD, Cat. no. 362753)
2. Cryovial 1.8 mL Internal Thread PP \*VS\* (Nunc (Fisher Scientific), Cat. no. NNC368632-PK)
3. 3 mL Pasteur pipettes, sterile 1's (Alpha Laboratories, Cat. no. LW4112)
4. Counting slides for TC20 cell counter (150 slides) (Bio-Rad, Cat. no. 1450015)
5. CoolCell LX Freezing Container, 12 x 1-2ml cryo vials, Purple (Biocision, Cat. no. BCS-405)

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| Wash Buffer composition (1% FBS, 1 mM EDTA), store at 4°C. |  |
| PBS, pH 7.4 (Gibco, Cat. no. 10010049) | 500 mL |
| Fetal Bovine Serum (FBS) (Sigma, Cat. no. F2442) | 5 mL |
| UltraPure 0.5 M EDTA, pH 8.0 | 1 mL |

**Procedure:**

1. The BD Vacutainer® CPT™ Tube (Cat. no.362753) should be at room temperature (18-25ºC) and properly labeled for patient identification.
2. After blood collection, the CPT tube should be stored upright at room temperature (18-25ºC) until centrifugation. Blood samples should ideally be centrifuged within two hours of blood collection for best results.
3. Mix the blood sample immediately prior to centrifugation by gently inverting the tube 8 to 10 times.
4. Centrifuge the CPT tube at 1500 RCF (Relative Centrifugal Force) in a horizontal rotor (swing-out head) for 30 minutes at 20ºC (Speed change of accel/decel: Soft). WARNING: Excessive centrifuge speed (over 2000 RCF) may cause tube breakage and exposure to blood and possible injury.
5. After centrifugation, PBMCs will be in a whitish layer just under the plasma layer. Using a Pasteur pipette, aspirate approximately half of the plasma without disturbing the PBMC cell layer. Collect cell layer by pouring and transferring cell layer to a 50 mL size conical centrifuge tube with cap. Collection of cells immediately following centrifugation will yield best results.
6. Spin down the collected mixture at 300 RCF for 15 minutes at 20 ⁰C. (Speed change of accel/decel: Soft). Use Pasteur pipette to remove as much supernatant as possible without disturbing cell pellet.
7. Using a 5-mL serological pipette, gently resuspend the cell pellet with 3 mL of ACK lysing buffer and incubate for 3 minutes at room temperature.
8. First wash: Add wash buffer to bring volume to 50mL. Cap tube. Mix cells by inverting tube 5 times.
9. Centrifuge at 300 RCF (accel/decel: Soft) for 15 minutes at 20ºC. Aspirate as much supernatant as possible without disturbing cell pellet.
10. Second wash: Add wash buffer to bring volume to 20 mL. Cap tube. Mix cells by inverting tube 5 times.
11. Centrifuge at 300 RCF (accel/decel: Soft) for 15 minutes at 20⁰C. Aspirate as much supernatant as possible without disturbing cell pellet.
12. Re-suspend cell pellet in an appropriate volume of wash buffer to bring to a concentration of ~1×106 cells/mL for counting.
13. Cell counting: Mix 10 µL of cell suspension with 10 µL of trypan blue. Apply 10 µL of the mixture to a counting slide. Count the cells using an automated cell counter within 5 minutes (concentration tends to range from 5×104 to 1×107 cells/mL).
14. Centrifuge the remaining suspension at 300 RCF (accel/decel: Soft) for 10 minutes at 20ºC. Aspirate as much supernatant as possible without disturbing cell pellet.
15. Resuspend cell pellet in 1mL of cold CryoStor CS10 in cryotubes and aliquot into two cryotubes per sample (0.5 mL X 2).
16. Store the cryotubes into CoolCell LX Freezing Container in a -80⁰C freezer overnight before permanent storage in liquid nitrogen.

