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Cryopreservation Protocol for PBMCs and T Cells Using PRIME-XV FreezIS DMSO-Free Cryopreservation Medium

The following protocol has been optimized for freezing PBMCs and T cells using PRIME-XV FreezIS DMSO-Free (Catalog # 91140) Cryopreservation Medium. The procedure was performed using buffy coat from six healthy donors as starting material, both with a manual method and an automated method.

MATERIALS: REAGENTS

- PRIME-XV T Cell CDM (FUJIFILM Irvine Scientific; Cat. # 91154)
- IL-2 (CellGenix; Cat. # 1420-050)
- CD3 (Miltenyi Biotec; Cat. # 130-093-387)
- CD-28 (BioLegend; Cat. # 302934)
- PRIME-XV FreezIS DMSO-Free (FUJIFILM Irvine Scientific; Cat. # 91140)
- PRIME-XV FreezIS Cryopreservation Medium (FUJIFILM Irvine Scientific; Cat. # 91139)
- FBS (Gibco, Cat. # 10101-145)
- DPBS 1x (Lonza; Cat. # 17-512F)
- WFI (Galenica Senese, Cat. # AA0013004)
- Cryoserv DMSO (Mylan/Lagitre, Cat. # 67457-178-50)
- Includes Human Albumin solution (Kedrion; Cat. # AIC 021111024)
- NaCl Solution 9 mg/mL (Galenica Senese; Cat. # AA0492027)
- Human Serum Albumin Antibody (R&D System; Cat. # IC1455P)
- CD3 (BD Biosciences Cat. # 51-9007098)
- CD4 (BD Biosciences Cat. # 561843)
- CD8 (BD Biosciences Cat. # 561947)
- CD14 (BD Biosciences Cat. # 561712)
- CD19 (Miltenyi Biotec; Cat. #130-110-351)
- CD56 (BD Biosciences Cat. # 561903)
- PD1 (BD Biosciences Cat. # 561272)
- SHEATH Fluid (Sony; Cat. # AE700713)
- FACS beads (Sony; Cat. # LE-B3001)

EXPERIMENTAL PROCEDURE: MANUAL METHOD

Isolation and freezing of PBMCs from buffy coat using Ficoll-Paque reagent

1. Buffy Coats were diluted 4x in DPBS.
2. 16 mL of Ficoll-Paque reagent was pipetted into a Leucosep tube. Tubes were centrifuged at 1000 xg for 30 sec.
3. Diluted cell suspension was transferred into prepared Leucosep tube and filled up with PBS to a total volume of 50 mL/tube.
4. Tubes were centrifuged at 1000 xg for 10 minutes at Room Temperature (RT) in a swinging-bucket rotor centrifuge without brake. Three layers formed above the barrier of Leucosep Tube: a plasma layer, the interphase containing PBMCs and a small layer of Ficoll-Paque. The plasma layer was discarded.
5. The interphase above the barrier, which contains PBMCs, was transferred to a new 50 mL conical tube.
6. The conical tube was filled with DPBS and centrifuged at 300 xg for 10 minutes at RT. Supernatant was carefully removed.
7. For removal of platelets, cellular pellet was resuspended in 50 mL of DPBS and centrifuged at 200 xg for 15 minutes at RT. Supernatant was carefully removed. Step 7 was repeated twice to ensure platelet removal.
8. Cell pellet was re-suspended in RPMI 1640. Cell counting was performed using trypan blue and Countess II automated cell counter.
9. An aliquot of freshly isolated PBMCs (1×10^5 cells/marker) was analyzed by FACS to assess $CD3^+$, $CD14^+$, $CD56^+$, and $CD19^+$ population percentages.
10. Cells were frozen at 2.5×10^6 cells/mL and 5×10^6 cells/mL in four different freezing media:
    - **Freezing medium solution 1:** 90% FBS + 10% DMSO
    - **Freezing medium solution 2:** PRIME-XV FreezIS Cryopreservation Medium
    - **Freezing medium solution 3:** 90% Human Serum Albumin + 10% DMSO
    - **Freezing medium solution 4:** PRIME-XV FreezIS DMSO-Free Cryopreservation Medium
11. Cells were transferred to a freezing container, Nalgene Mr. Frosty, and cells were incubated at -80°C for 48 hours.
12. After 48 hours, the vials were moved to a liquid nitrogen vapor tank.

Thawing, activation, and expansion of PBMCs

1. After four months, a vial of PBMCs for each of the aforementioned conditions was thawed. Cells were resuspended using double volume of PRIME-XV T Cell CDM (Cat. # 91154) expansion medium and softly centrifuged at 300 xg for 10 minutes. Acceleration and brake were set at the minimum value depending on the centrifuge model available. After centrifugation, cells were resuspended in 1 mL PRIME-XV T Cell CDM expansion medium supplemented with 300 IU/mL IL-2, 50 ng/mL of anti-CD3 (clone OKT-3), and 125 ng/mL of anti-CD28 (clone CD28.2).
2. Cell number, viability was assessed using trypan blue and Countess II automated cell counter.
3. An aliquot (1×10^5 cells/marker) of cells was analyzed by fluorescence-activated single cell sorting (FACS) to determine $CD3^+$, $CD14^+$, $CD19^+$, and $CD56^+$ population percentages.
4. Cells were seeded in a G-Rex 24-well plate using 8 mL/well of PRIME-XV T Cell CDM expansion medium supplemented with 300 IU/mL IL-2, 50 ng/mL of anti-CD3 (clone OKT-3), and 125 ng/mL of anti-CD28 (clone CD28.2). Seeding density was 1×10^6 cells/well.
5. After 72 hours, 6 mL of the medium was removed. Cell counting and viability assessment was performed using trypan blue and Countess II automated cell counter. Medium was replenished with 6 mL of fresh PRIME-XV T Cell CDM expansion medium supplemented with 300 IU/mL IL-2.
6. Step 5 was repeated on day 7 and day 10.
7. On day 13, the culture was stopped. 6 mL of spent medium were removed and the cells were resuspended in the remaining 2 mL. Cell counting and viability assessment was carried out using trypan blue and Countess II automated cell counter. Cells (1×10^5 cells/marker) were analyzed by FACS to evaluate $CD3^+$, $CD4^+$, $CD8^+$, and $PD1^+$ population percentages.
8. The remaining cells were frozen at 2.5×10^6 cells/mL in the four different freezing media (see section I., step 10).

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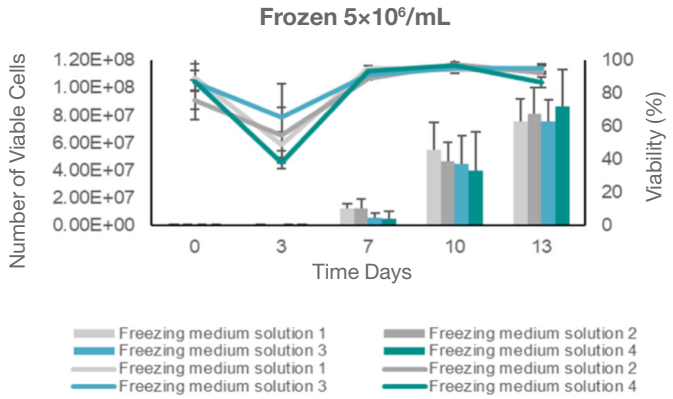
(CONTINUATION)

9. Cells were transferred to a freezing container, Nalgene Mr. Frosty, and cells were incubated at -80°C for 48 hours.
10. After 48 hours, cells were moved to a liquid nitrogen vapor tank.
11. After 1 month, 1 vial for each condition was thawed to perform cell counting and viability assessment, as well as FACS analyses on surface markers expression.

EXPERIMENTAL PROCEDURE: AUTOMATED METHOD

1. Buffy coats were diluted with 4 volumes of PBS.
2. Diluted buffy coats were washed to remove the platelet using Platelet Free protocol and settings recommended by instrument supplier with the kit CT 60.1.
3. The output bag obtained from PlateletFree protocol contained 220 mL of buffy coats washed to reduce the number of platelets. This bag was loaded as input for the separation protocol using Ficoll-Paque reagent (NeatCell protocol). PBMC isolation was performed using CT 90.1 kit with the setting recommended by instrument supplier.
4. A final bag containing 50 mL of PBMCs was resuspended in RPMI 1640 was automatically obtained. Cell number and viability were assessed using trypan blue and Countess II automated cell counter.
5. An aliquot of cell suspension corresponding to 2.4×10^7 cells for each donor was transferred to a 15 mL centrifuge tube and centrifuged at 300 xg for 5 minutes. The cell pellet was resuspended in PRIME-XV T Cell CDM expansion medium supplemented with 300 IU/mL IL-2, 50 ng/mL of anti-CD3 (clone OKT-3), and 125 ng/mL of anti-CD28 (clone CD28.2). The remaining cells were frozen in different DMSO-containing media and not used for this set of experiments.
6. Cells were seeded in a G-Rex 24-well plate. One G-Rex plate was prepared for each donor using 8 mL/well of PRIME-XV T Cell CDM expansion medium supplemented with 300 IU/mL IL-2, 50 ng/mL of anti-CD3 (clone OKT-3) and 125 ng/mL of anti-CD28 (clone CD28.2). Seeding density was set at 1×10^6 cells/well.
7. After 72 hours, 6 mL of medium was removed to perform cell counting and viability assessment using trypan blue and Countess II automated cell counter. Medium was replaced using 6 mL of fresh PRIME-XV T Cell CDM expansion medium supplemented with 300 IU/mL IL-2.
8. On day 7, cells were analyzed by FACS to assess $CD3^+$, $CD4^+$, and $CD8^+$ population percentages.
9. Expanded T cells were frozen at 5×10^6 cells/mL in four different freezing media:
   - **Freezing medium solution 1:** 90% FBS + 10% DMSO
   - **Freezing medium solution 2:** PRIME-XV FreezIS Cryopreservation Medium
   - **Freezing medium solution 3:** 90% Human Serum Albumin + 10% DMSO
   - **Freezing medium solution 4:** PRIME-XV FreezIS DMSO-Free Cryopreservation Medium
10. Cells were transferred to a freezing container, Nalgene Mr. Frosty, and incubated at -80°C for 48 hours.
11. After 48 hours, the cells were moved to a liquid nitrogen vapor tank.
12. After four months, a vial of expanded T cells for each of the aforementioned conditions was thawed and analyzed by FACS to test the percentage of cells positive for CD3, CD4, and CD8. Simultaneously, cell number and viability after thawing was evaluated using trypan blue and Countess II automated cell counter.



Protocol developed and owned by Anemocyte Srl.
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