Guidelines for PBMC freezing.

This technical note describes a protocol for freezing and thawing PBMC and provides details on functional validations after freezing the procedure compared to fresh PBMC.

Abstract

Peripheral Blood Mononuclear Cells (PBMC) are often used as a relevant biological model to investigate the function of the immune system. The crosstalk of the different subsets of immune cells present in a PBMC population enables studying of more physiological environment compared to most common immortalized immune cell lines.

The use of cryopreserved PBMC has dramatically increased in recent years. Cryopreserved PBMC are particularly useful because they allow all experimental studies to be performed on a unique PBMC preparation, either from a single donor or a pool of different donors. Thus, it eliminates the main drawbacks of immunology studies which includes changes of immunological parameters over time and among donors.

This technical note describes a simple protocol for freezing and thawing PBMC, as well as functional validations after freezing procedure compared to fresh PBMC.

Materials and methods

PBMC were obtained from a buffy coat purification following the process described in the technical note: Guidelines from PBMC isolation to cytokine assay optimisation.

HTRF cytokines assays: Revvity Bioassays, Human IL6 # 62HIL06PEG, Human IL8 # 62HIL08PEG, Human IFNγ # 62HIFNGPEG.

Agonists: LPS (Sigma #L4391)

Antagonists: Dexamethasone (Sigma-Aldrich #D2915)

Flowchart

Freezing steps

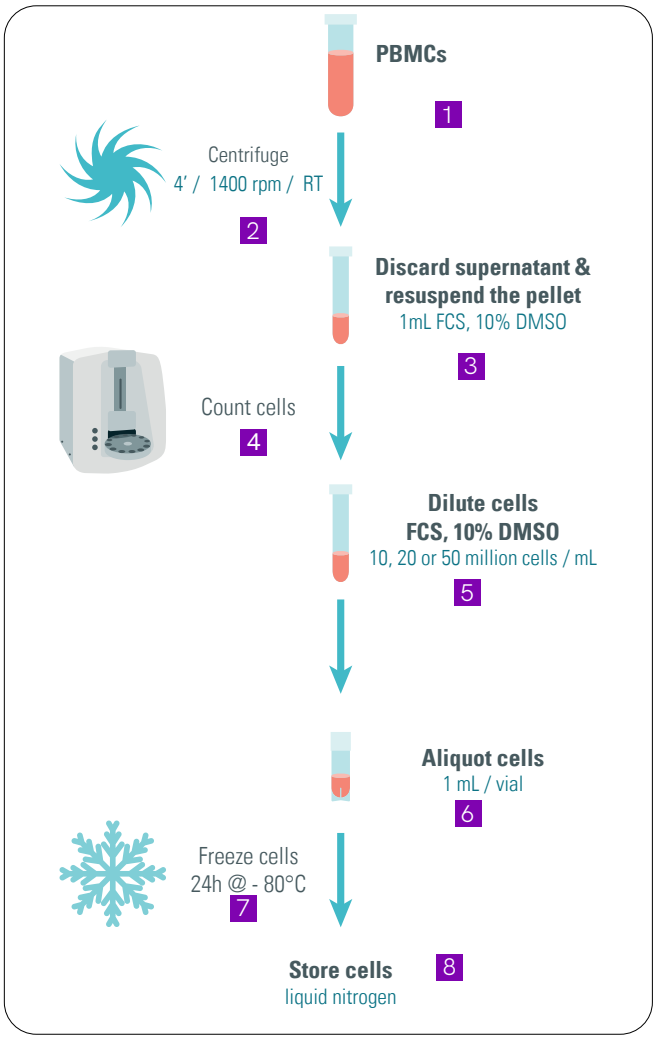

Figure 1: Flowchart for freezing steps

1. The PBMC stock can be purified from a buffy coat, following the procedure described in the Revvity technical note "Guidelines from PBMC isolation to cytokine assay optimization".
2. Centrifuge the PBMC stock 4 minutes at 1400 rpm, at RT.
3. Discard the supernatant and resuspend the pellet with 1mL FCS containing 10% DMSO, warmed up to RT.
4. Count the cells to determine the cell density in 1 mL.
5. Dilute the cells with FCS containing 10% DMSO, to obtain a solution at 10, 20 or 50 million cells per mL.

⚠ Caution: From 30% to 50% cells are lost during the freezing step. To obtain 10 M cells post thawing prepare a 20 M cell stock.

6. Aliquot the cells under 1mL per vial in a 2mL cryotube.
7. Freeze the cells for 24 hours at -80°C in a freezing box.
8. Transfer the vials in liquid nitrogen.

Thawing steps

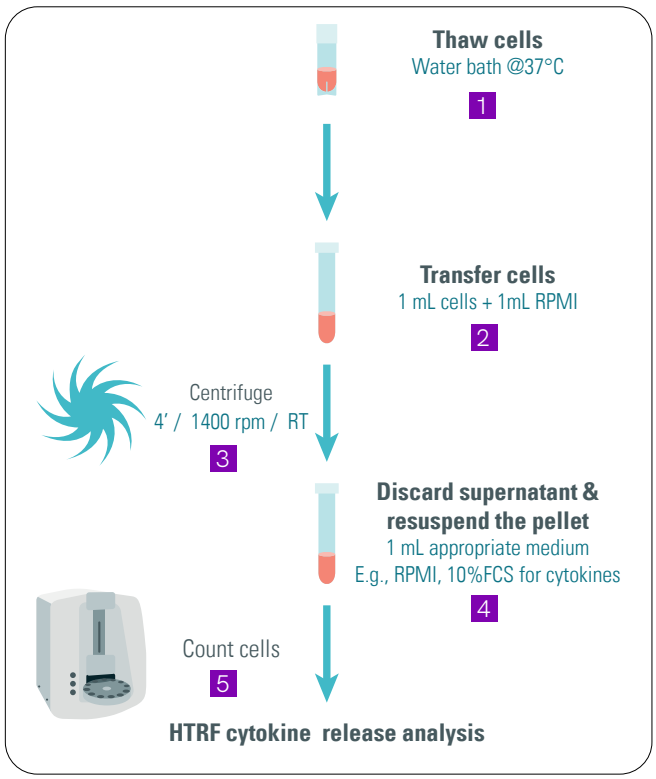

Figure 2: Flowchart for thawing steps

1. Thaw the cell vial in a water bath (37°C).
2. Transfer the cells into a 15 mL falcon vial containing 1 mL of cells and 1 mL of RPMI.
3. Centrifuge the cells 4 minutes at 1400 rpm (RT).
4. Discard the supernatant and resuspend the pellet with 1mL of the appropriate medium for your assay, in an appropriate cell density. For example, to run a phospho immunoassay on the PBMC lysate, resuspend the pellet with RPMI free FCS (more information on phospho assays run on frozen PBMC are described in the Revvity application note "TCR phospho signaling investigation with HTRF"). To run a cytokine immunoassay on the PBMC supernatant, resuspend the pellet with RPMI supplemented with 10% FCS.
5. Count the cells to determine the cell density in 1 mL.

⚠ Caution: From 30% to 50% cells are lost during the freezing step. The remaining cells present a viability > 95%.

HTRF cytokine release analysis

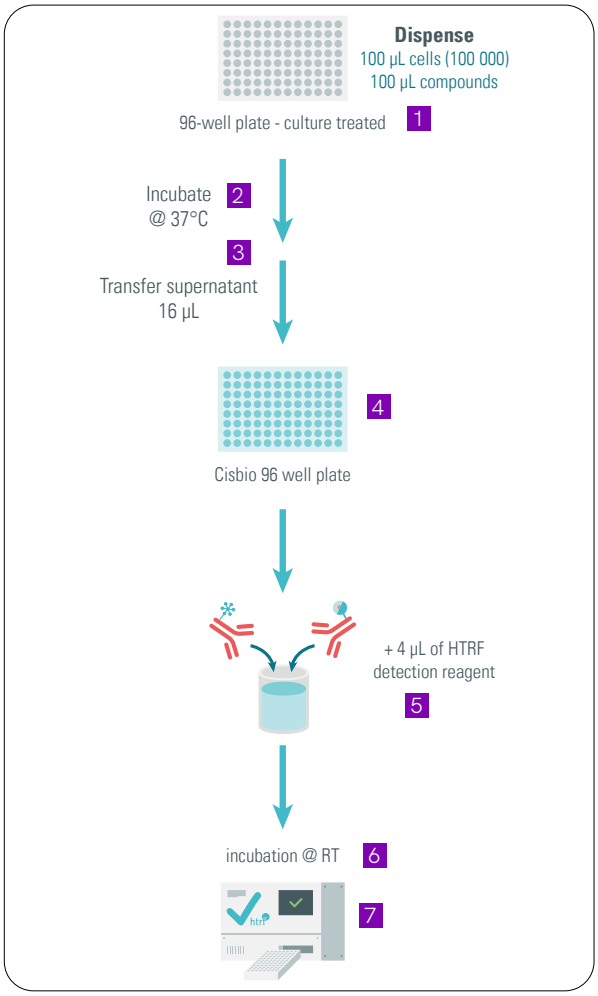

Figure 3: Flowchart for HTRF cytokine release analysis

1. To analyze PBMC cytokine secretion, plate the PBMC under 100 µL in a 96 well plate, culture treated, In the cytokine assay described here, PBMC are plated at 100 000 cells per well.
2. Add the compound of interest in 100 µL. E.g. Add 100 µL of 0.2 µg/mL LPS or 100µL of a mixture of 0.2 µg/mL LPS and dexamethasone ranging from 10 µM to 10 pM.
3. Incubate the cells for the appropriate time at 37°C. In the example, cells are incubated overnight.
4. Transfer 16 µL of PBMC supernatant into a detection plate. E.g. Revvity 96 well low volume white micro-plate # 66PL96001, or a 384 well small volume white micropltate.
5. Dispense 4 µL of pre-mixed HTRF detection reagents.
6. Incubate according to the kit protocol.
7. Record the HTRF signal on an HTRF-compatible reader.

Results

Comparison of the cytokine release by fresh and frozen cells

The figure below shows the concentration of IL6, TNFα and IL8 cytokines secreted either by fresh or frozen PBMC after LPS stimulation. Whereas the basal cytokine secretion is higher in frozen PBMC compared to fresh cells, the cytokine levels remain unchanged after LPS stimulation.

These results are consistent with previous studies which reported a preactivated state of PBMC after freezing (R. Mallone, S.I. Mannering, B.M. Brooks-Worell, I. Durinovic-Bello, C.M. Cilio, F.S. Wong, N.C. Schloot, Clinical and experimental immunology, 2010, 163:33-49).

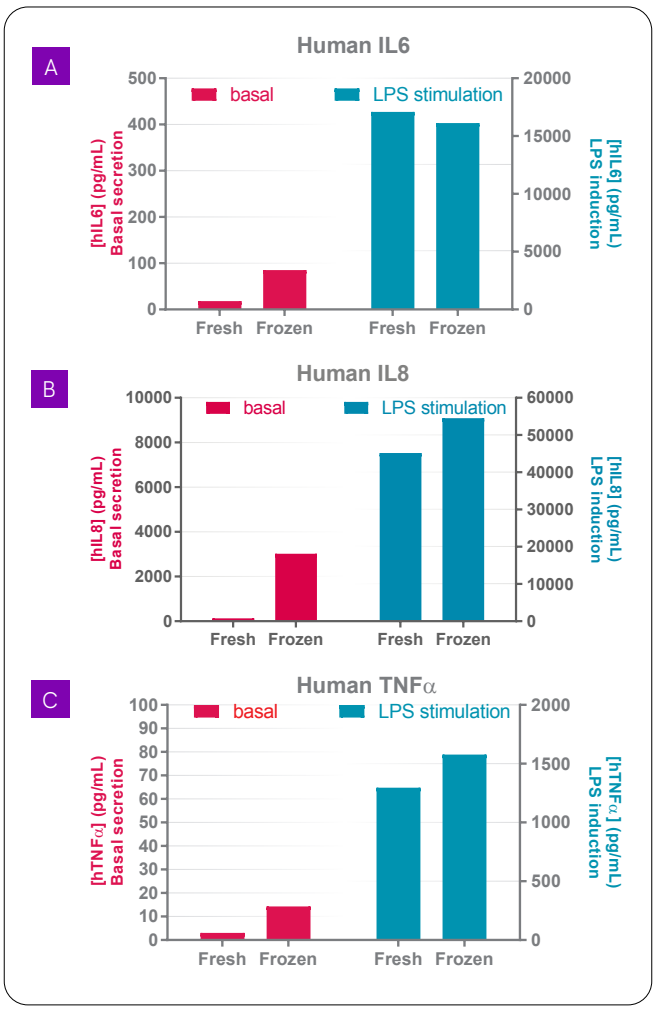

Figure 4: Comparison of cytokine release. Concentration of IL6 (A), IL8 (B), and TNFα (C) secreted by either fresh or frozen PBMC after LPS stimulation. Basal cytokine secretion is higher in frozen PBMC compared to fresh cells, however, the cytokine levels remain unchanged after LPS stimulation.

Assessment of inter-assay reproducibility

Three independent experiments were run on cryopreserved PBMC. 100,000 PBMC per well were stimulated with 0.2 µg/mL LPS in the presence of increasing doses of dexamethasone overnight (37°C). Then IL6, TNFα and IL8 secretion were quantified by HTRF.

As illustrated in the figures below, the dexamethasone treatment leads to inhibition of IL6, TNFα and IL8 release. The estimated IC50 determined for each 3 independent experiments are ranged on a semi-log scale. The data prove that the good reproducibility of the PBMC freezing enables day to day experimental robustness.

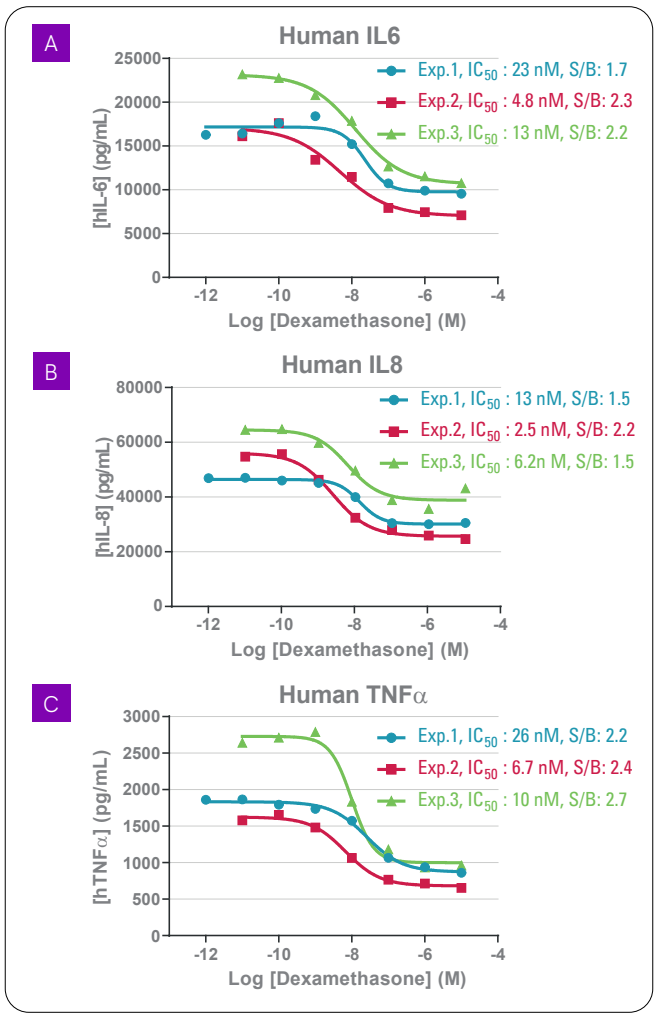

Figure 5: Inter-Assay reproducibility. Dexamethasone treatment leads to inhibitionof IL6 (A), IL8 (B), and TNFα (C) release, demonstrating that PBMC freezingenables day-to-day experimental robustness.

Assessment of inter-vial reproducibility

IInter-vial reproducibility is an important factor when using frozen cells and was assessed using 3 vials of PBMC frozen according to our protocol. Briefly, 100,000 PBMC per well were stimulated with 0.2 µg/mL LPS and treated with increasing concentrations of dexamethasone overnight (37°C). The quantification of IL6, TNFα and IL8 secretion was done using HTRF cytokine assays.

As expected, the release of IL6, TNFα and IL8 cytokines is inhibited by the dexamethasone treatment and to the same extent for the 3 different PBMC vials. These results demonstrate the high inter-vial reproducibility of PBMC frozen according to our protocol, as well as the reliability of HTRF-based cytokine quantification.

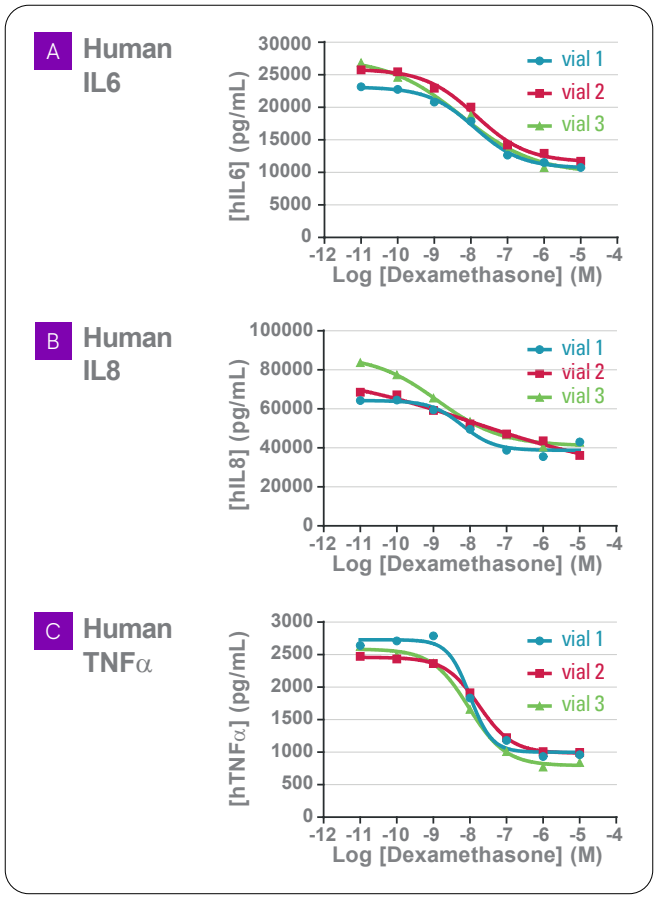

Figure 6: Inter-Vial Reproducibility. The release of IL6 (A), IL8 (B), and TNFα (C) are inhibited by dexamethasone treatment and to the same extent for the 3 different PBMC vials. This demonstrates high-inter-vial reproducibility of PBMC frozen according to our protocol.

Conclusion

This note is the perfect complement to the PBMC isolation technique we described earlier, in order to obtain highly reproducible functional responses from frozen PBMC. Good cell quality hand in hand with HTRF assay quality is the method of choice to maximize day to day experimental robustness and to deliver accurate results over time.

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