PROTOCOL:
Thawing Cryopreserved PBMC

The CTL cryopreserved PBMC from the ePBMC® library have been mailed to you under conditions that secure their full functionality during the shipment. Please store the cryopreserved PBMC vials in liquid nitrogen (vapors) immediately upon receipt, and keep them in liquid nitrogen (vapors) until the day they will be thawed and used. Avoid storing at anything other than LN2 temperatures and avoid transient warming events during storage. When stored under these conditions, the CTL cryopreserved PBMC will maintain full-functionality for several years. CTL has developed an optimized Serum-free Media platform for standardized work with cryopreserved PBMC. Typically, PBMC show higher antigen-specific T cell responses over lower background when tested using the CTL Serum-free Media platform. These PBMC can be processed and tested in serum-containing media as well, but careful testing of the serum batch is recommended in order to avoid serum-mediated mitogenic or suppressive effects. The following protocol provides instructions for the thawing of PBMC using the CTL Serum-free Media platform.

PREPARATION:

To reduce the risk of contaminating the cells during thawing, we recommend the use of a CTL Bead Bath™ (CTL-BB-001) instead of a water bath. Make sure that the temperature of the Bead Bath (or water bath) is at 37ºC.

PREPARE CTL ANTI-AGGREGATE WASH™ MEDIUM:

For each vial of PBMC to be thawed, thaw 1 vial of CTL Anti-Aggregate Wash™ Supplement 20x (1ml, CTL-AA-001) by placing in a 37ºC CTL Bead Bath™ (or water bath) for ten minutes. Dilute 1:20 by adding 19ml of RPMI-1640. 20ml total of 1x diluted CTL Anti-Aggregate Wash™ solution is needed for each PBMC vial.

For best results, prepare CTL Anti-Aggregate Wash™ Medium within one hour of use. Place the medium in a 37ºC CO_2 incubator with a loose cap for a minimum of 20 minutes. This allows the pH and the temperature to equilibrate.

PREPARE CTL-TEST™ MEDIUM:

CTL Test™ Medium is a ready-to-use formulation, except for the need to supplement it with 1 vol % fresh L-glutamine before use. (L-glutamine is unstable at 2-8ºC and needs to be frozen for long-term storage). Thaw L-glutamine and add 1 vol % (e.g., 5ml L-glutamine to 500ml CTL-Test™ Medium).

For best results, pre-warm the L-glutamine-supplemented CTL Test™ Medium before adding it to the PBMC by placing the medium in a 37ºC CO_2 incubator with a loose cap for a minimum of 20 minutes. This allows the pH and the temperature to equilibrate. After use, the medium should be stored at 2-8ºC.

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THAWING CRYOPRESERVED PBMC:

1. Raise the temperature in the cryovial that contains the PBMC rapidly to 37°C by placing it in a CTL Bead Bath™ to thaw (Use of a 37°C water bath is acceptable but it increases the chance of contamination).
2. Invert the cryovial twice to resuspend the cells.
3. Use 1ml pipette to aspirate all medium from the cryovial, transfer into a 50ml conical tube (make sure the tube is labeled with the sample ID). The contents of up to 4 cryovials from the same sample can be pooled.
4. To recover the residual cells from the cryovial, pipette 1ml warm (37°C) CTL Anti-Aggregate Wash™ Medium into each cryovial, and add to the rest of the cells.
5. Using a 10ml pipette, add warm (37°C) CTL Anti-Aggregate Wash™ Medium to the 50ml tube. The first 3ml should be added slowly, 1ml at a time every five seconds, while gently swirling the tube. Add the remaining 5ml of CTL Anti-Aggregate Wash™ Medium more quickly from the pipette. The PBMC are now suspended in ~10ml.
6. Centrifuge cell suspension at room temperature at 330g for 10 minutes with rapid acceleration and brake on high.
7. Decant the supernatant and carefully resuspend the cell pellet by tapping the tube (avoid pipetting or vortexing). Add 10ml (37°C) CTL Anti-Aggregate Wash™ Medium. Mix the cells by inverting the tube twice with cap tightly closed. Take a sample for cell counting (CTL Live-Dead cell counting dye and CTl Cell Counting suite is recommended).
8. Centrifuge cell suspension at room temperature at 330g for 10 minutes with rapid acceleration and brake on high.
9. Once centrifuge stops, decant the supernatant, and resuspend the pellet by tapping the tube. Add warm (37°C) CTL-Test™ Medium, adjust the cells to the concentration for plating into the assay (e.g., adjust to 3 million PBMC per ml if 300,000 PBMC are to be plated in 100µl/well).

The above protocol summarizes the ideal thawing conditions as established in "Optimal Thawing of Cryopreserved Peripheral Blood Mononuclear Cells for Use in High-Throughput Human Immune Monitoring Studies," *Cells,* 2012. 1:313-324. Ramachandran, et al.

CTL does not recommend "overnight resting" of ePBMC®, but to test the cells right after thawing "Resting of Cryopreserved PBMC Does Not Generally Benefit the Performance of Antigen-Specific T Cell ELISPOT Assays," *Cells,* 2012. 1:409-427. S. Kuerten, et al.