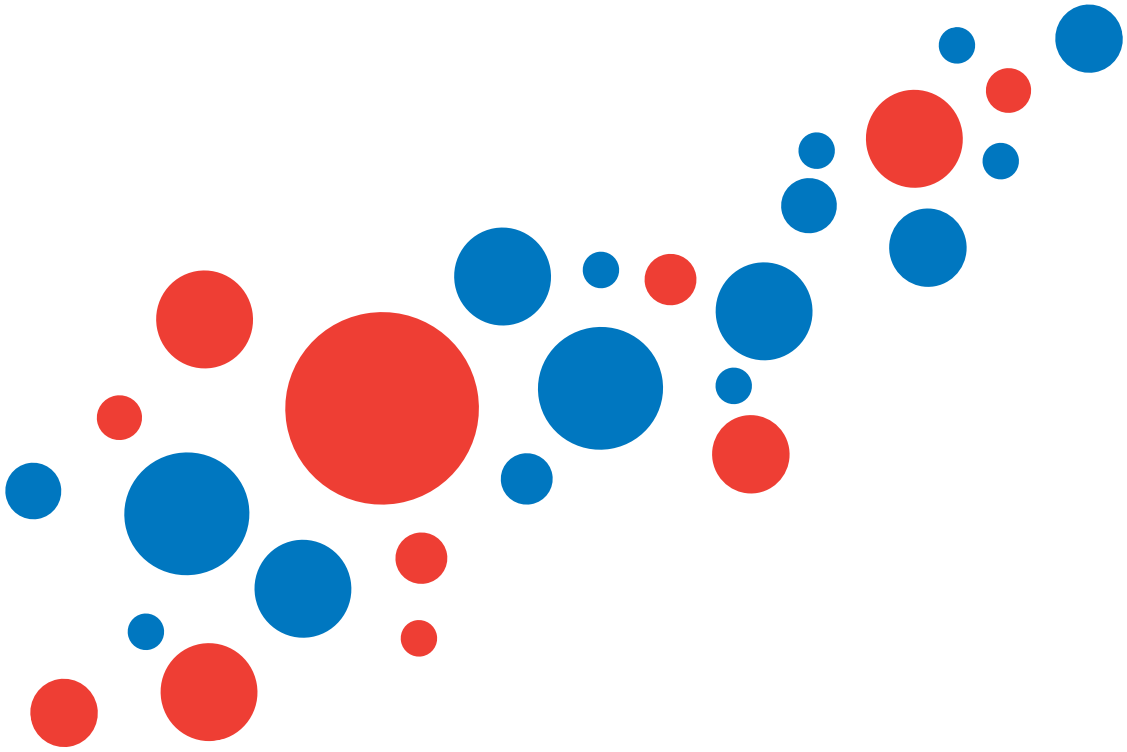
ImmunoSpot®
High content ELISPOT

Protocols and Guidelines for Working with Human PBMC in ELISPOT Assays



C.T.L.
The Immune Monitoring Company

PROTOCOL:
Isolation of PBMC from Fresh Whole Blood

Isolation of PBMC from Fresh Whole Blood

PREPARATIONS:

Draw venous blood: Use sodium heparin as an anti-coagulant. Mix thoroughly. Process within 24 hours, with best results obtained by prompt processing. If storing is needed before processing, store the blood at room temperature in the dark and on a rocker. Always keep cells at 20-37°C, do not refrigerate!

Cell Counting: Count cells with fluorescent dyes that distinguish between live and dead cells, such as CTL-LDC™ Reagent (CTL-LDC-100). Few samples can be counted by eye with a UV microscope. For high-throughput cell counting, CTL offers a dedicated software module — please inquire.

AFTER BLOOD DRAW:

1. Pool the heparinized blood of individual donor and dilute blood 1:1 with Ca^{2+}-free PBS (e.g., add 15ml PBS to 15ml blood). PBS should be at room temperature (20-37°C). Gently mix by inverting the tubes/flasks two times; do not mix by pipetting since shear forces will induce apoptosis in lymphocytes.
2. In a sterile, 50ml conical tube, add 15ml of Ficoll®. To avoid mixing the two phases, gently overlay the Ficoll® with 30ml of the diluted blood using a sterile serological pipette. Alternatively, the diluted blood can be gently poured onto the Ficoll® or it can be added first, and the Ficoll® gently underlaid with a serological pipette (videos at www.immunospot.com).
3. *Carefully balance the weight of the tubes.* Centrifuge the samples at 740g for 30 minutes at room temperature, or according to density gradient manufacturer's recommendation.
   Accelerate the centrifuge slowly so the gradient does not mix. If the centrifuge starts shaking, immediately stop and weight-balance the tubes. The brake should be OFF to ensure that the deceleration does not disrupt the density gradient.
4. After the centrifuge stops, immediately collect the mononuclear cells from the plasma/Ficoll® interphase and transfer into a sterile 50ml conical tube. Be sure to aspirate as little Ficoll® as possible while collecting the cells. Interphase cells from a maximum of two 50ml tubes can be combined into one tube. If the proportion of Ficoll® is too high (>5ml), a significant cell loss will occur. Fill the tube to the 45ml mark with 37°C CTL-Wash™ Medium. The use of Ca^{2+}-free PBS at this stage can result in reduced cell viability and functionality and/or cell clumping.
5. Centrifuge cells again, this time at 330g for ten minutes at room temperature. Acceleration should be high and the centrifuge brake ON. As soon as centrifugation is complete, cautiously decant the supernatant and discard it. Resuspend the cell pellet by tapping the tube until no clumps are visible. ***Do not vortex or pipette the cells because shear forces will damage the cells. Do not let cells sit in the pellet for a prolonged time (more than a minute): pelleted cells start dying if not resuspended immediately.*** Promptly add 37°C CTL-Wash™ Medium (or CTL-Test™ Medium), 5ml per 50ml tube.
6. Pool cells of the same donor and count the cells (see Cell Counting above). The cell yield should be one-two million PBMC for each ml of blood drawn. Fill tubes to the 45ml mark with warm CTL-Wash™ (or CTL-Test™) Medium, and spin at 330g for ten minutes with the break on.
7. *Decant supernatant and follow the appropriate option below:*
   - *Option One* — If plating cells in an assay, resuspend the cells in 37°C CTL-Test™ Medium in the desired concentration for direct pipetting into the assay.
   - *Option Two* — If cryopreserving PBMC, resuspend cells in warm CTL-Cryo™ C, adjusting the cell concentration to $20x10^6$/ml (twice the intended final concentration).
8. If the PBMC cannot be plated in the ELISPOT assay immediately, keep cells in a 37°C CO_2 incubator with the lids of the tubes loosened until plating. Optimal functionality and viability is seen when PBMC are isolated and plated or frozen as quickly as possible.

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PROTOCOL:
Cryopreservation of PBMC from Fresh Whole Blood

Cryopreservation of PBMC

Cell permeability, reagent toxicity, and cooling rates must be considered for each cell type when freezing. The osmotic pressure caused by DMSO (more than DMSO's intrinsic toxicity) is one of the primary factors that needs to be controlled for successful freezing and thawing of PBMC. Maintaining the metabolic activity of the cells is important so they can compensate for the osmotic pressure and their membrane lipid fluidity. All reagents should be used at room temperature (preferably at 37ºC).

PREPARATION:

1. Mix CTL-Cryo™ A with CTL-Cryo™ B in an 80% to 20% (v/v) ratio (4:1), by slowly adding CTL-Cryo™ B into CTL-Cryo™ A. (CTL-Cryo™ B contains DMSO as a component, please refer to included SDS.) Filter CTL-Cryo™ A-B, through a 0.22µm filter.
2. If the addition of CTL-Cryo™ B to CTL Cryo™ A and the filteration doesn't have the resulting CTL-Cryo™ A-B mixture at 35-37ºC place CTL-Cryo™ A-B mixture and CTL-Cryo™ C in a 37ºC CO_2 incubator. (It is advised to start with this step while the Ficoll® gradient runs).
3. Each cryotube should contain approximately 10-15x10^6. Freezing more cells per tube may lead to cell loss. Label the appropriate number of cryotubes per sample based on the anticipated cell count (expect 1-2x10^6 PBMC per ml of blood drawn).

AFTER WASHING:

1. After Ficoll® purification and washing, resuspend PBMC in warm CTL-Cryo™ C, adjusting the cell concentration to 20x10^6/ml (or twice the intended final concentration).
2. Mix cells gently by tapping the tube without using a pipette, avoid foam formation!
3. Slowly, over a time period of ~two minutes, add an equal volume of warm CTL-Cryo™ A-B mix to the CTL-Cryo™ C containing the PBMC. (Add CTL-Cryo™ A-B mix drop-by-drop while gently whirling the tube to ensure complete mixing of the two solutions.)
4. Aliquot the resulting CTL-Cryo™ A-B-C suspension containing the PBMC into the pre-labeled cryovials. Pipette gently and slowly to minimize shear forces; do not attempt additional mixing with the pipette.
5. Place cryovials into a room temperature Nalgene® cryofreezing container (Mr. Frosty) filled with propanol and transfer into a -80°C freezer for a minimum of 12 hours. Do not open the freezer during this time period. Use a dedicated -80°C freezer in order to prevent shaking the samples or fluctuation of the freezer's temperature by opening the freezer.
6. Optimal functionality and viability is seen when cells are transferred to cryovials in a cryofreezing container and into a -80ºC freezer without delay.
7. After a minimum of 12 hours and no more than 48 hours, transfer the cryovials into vapor/liquid nitrogen tanks for storage.

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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PROTOCOL:
Thawing Cryopreserved PBMC

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.

PROTOCOL:
Plating PBMC into the ELISPOT Assay

PLATE_PBMC_080519

1. Keep the PBMC that have been adjusted to the desired concentration in CTL-Test™ Medium in a 37°C CO_2 incubator with the lids of the tubes loosened slightly until plating (Keep the tubes containing the cells on the bench for as little time as possible).
2. Plate antigens or test substances first. Before adding the cells, place the plates in a 37°C CO_2 incubator for at least ten minutes to equilibrate to the temperature and CO_2 level of the incubator.
3. Plate cells with wide orifice pipette tips. Gently resuspend the cells before plating the cells as they sediment in conical tubes and resevoirs quickly. Before plates are put into the incubator, gently tap the plate from all sides with a firm grip of the top and bottom of the plate to help evenly distribute the cells within the wells. Place plates into the incubator as soon as the cells are plated—do not stack plates.

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