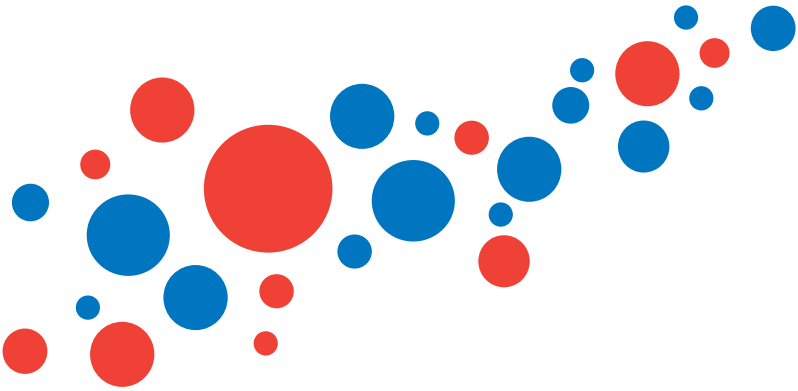
IMMUNOSPOT®

THE ELISPOT Source

Protocols and Guidelines for Working with Human PBMC in ELISPOT Assays



C.T.L.

Cellular Technology Limited

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Protocols

The following protocols provide all necessary procedures to successfully handle human PBMC for subsequent use in ELISPOT. A high recovery rate and vitality of the cells maximize the successful performance of the assay.

Protocol 1. Acquisition 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 20-37ºC, 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 (20-37ºC). Gently mix by inverting the tubes/flasks 2 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.
3. *Carefully balance the weight of the tubes.* Centrifuge the samples at 600g for 20 minutes at room temperature. Accelerate the centrifuge slowly so the gradients do 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. At this point, 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 room temperature 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 10 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 CTL-Wash™ Medium (or CTL-Test™ Medium) at room temperature, 5ml per 50ml tube.
6. Pool cells of the same donor and count the cells (see Cell Counting). The cell yield should be 1-2 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 10 minutes.
7. *Decant supernatant and follow the appropriate option below:*
   - ***Option 1*** — If plating cells in an assay, resuspend the cells in CTL-Test™ Medium in the desired concentration for direct pipetting into the assay.
   - ***Option 2*** — If cryopreserving PBMC, resuspend cells in warm CTL-Cryo™ C, adjusting the cell concentration to $20x10^6$/ml (twice the intended final concentration).
9. If the PBMC cannot be processed immediately, keep cells in a 37°C CO_2 incubator with the lids of the tubes loosened until plating or adding the freezing medium. The cells can be kept in the incubator for up to 24h without major loss or gain of function. (Keep cells on benchtop for as little time as possible; never store them on ice.)

Protocol 2. 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 MSDS, included.)
2. Warm the resulting CTL-Cryo™ AB Mix 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 will contain approximately $10x10^6$ cells (10-15 million). 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 1-2 million 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 ~2 minutes, add an equal volume of warm CTL-Cryo™ AB Mix to the CTL-Cryo™ C containing the PBMC. (Add CTL-Cryo™ AB Mix drop-by-drop while gently whirling the tube to ensure complete mixing of the two solutions.)
4. Aliquot the resulting CTL-Cryo™ ABC 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. The cells can sit in the complete CTL-Cryo™ ABC Media for 10-20 minutes without loss of viability or function.

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. After a minimum of 12 hours and no more than 48 hours, transfer the cryovials into vapor/liquid nitrogen tanks for storage.

Note: If Nalgene® cryofreezing containers (Mr. Frosty) are not available, the following "low technology" method works equally well: Place the cryovials in a Styrofoam rack, e.g., the racks in which15ml conical tubes are sold. Insert the Styrofoam container with the vials into a plastic bag leaving abundant air in the bag before taping it closed. Place in a -80°C freezer for a minimum of 12 hours, then transfer into vapor/liquid nitrogen tank.

Protocol 3. Thawing Cryopreserved PBMC

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 10 minutes. Dilute 1:20 by adding 19ml of RPMI-1640. 10ml of 1x diluted CTL Anti-Aggregate Wash™ Solution is needed for each PBMC vial.

For best results, prepare CTL Anti-Aggregate Wash™ Medium within 1 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 reset.

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 4ºC 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 reset. The medium should be protected from light while working on the bench top (by wrapping it in aluminum foil). After use, the media should be stored at 4ºC.

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™ for 8 minutes. (Using a 37°C water bath is adequate, but it increases the chance of contamination.)
2. Flip the cryovial twice 180° 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 at this point.

4. To recover the residual cells from the cryovial, pipette 1ml warm (37°C) CTL Anti-Aggregate Wash™ Medium into each cryovial, aspirate it, 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 5 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 180° with cap tightly closed. Take a sample for cell counting.
7. Centrifuge cell suspension at room temperature at 330g for 10 minutes with rapid acceleration and brake on high.
8. Once centrifuge stops, decant the supernatant, and resuspend the pellet by tapping the tube. Add warm (37°C) CTL-Test™ Medium adjusting the cells to the concentration as planned 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 4. Plating PBMC into the ELISPOT Assay

1. Keep the PBMC that has 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. The cells can be stored this way in the incubator for up to 24h without major loss or gain of function. (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 10 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 every minute while plating as cells sediment fast. Place plates into the incubator as soon as the cells are plated—do not stack plates on the bench top. 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.

Scientific Background and Utility of Serum-free Media in ELISPOT

Serum-free cell culture media allow users to standardize their cell culture conditions by avoiding the use of undefined and highly-variable serum products derived from humans or animals, e.g. human AB serum or fetal calf serum (FCS).

The high variability in the biological properties of different serum batches makes it necessary to pre-screen many batches in order to obtain a batch that is well-suited for a given application. Even a brief exposure of PBMC to a mitogenic serum batch, for example during washing or freezing of these cells, will result in a high background in cytokine assays. In addition, toxic or inhibitory serum batches will jeopardize the assay results. The unique performance of each serum batch necessitates the purchase of large lots to assure consistency of assay conditions as long as possible. This adds to the inconvenience and cost associated with the use of serum. Switching to or using a different batch of serum introduces a significant unpredictable variable to the test conditions each time, making test results difficult to compare. Furthermore, infectious risks associated with serum are leading to increasingly tighter restrictions for international shipments and exchange of any material that has been exposed to serum.

For all these reasons, there is considerable pressure from regulatory agencies and from the scientific community to avoid the use of serum and move to consistent, defined substitutes. However, because serum is naturally rich in a multitude of growth factors and other essentials for cell growth and functionality, it has been challenging to develop a serum substitute for primary cells in general, and for PBMC in particular. Most serum-free media contain enough additives to permit the growth of robust tumor cells, and hybridomas, but freshly-isolated human PBMC require more stringent conditions to maintain their viability and functionality in serum-free media.

CTL has been working for years with US governmental, industrial, and academic partners to standardize ELISPOT and other direct ex vivo cytokine assays performed on freshly-isolated or cryopreserved PBMC. These efforts have resulted in the CTL Serum-free Media Platform, which consists of three product lines that have been carefully designed to cover the four critical steps of working with human PBMC: washing, testing, freezing, and thawing. All four previously required the use of serum.

CTL-Test™ Medium (Cat# CTLT-005, CTLT-010)

CTL-Test™ Medium has been formulated to permit direct ex vivo testing of PBMC in T cell cytokine assays (ELISPOT, ELISA, CBA, CPA), it exhibits an equal or better performance than pre-screened, non-mitogenic serum. CTL-Test™ Medium contains injection-grade water supplemented with amino acids, vitamins, inorganic salts, and human serum albumin in a proprietary composition to provide a nutritionally complete and balanced environment for PBMC.

CTL-Test™ Medium is a ready-to-use formulation, except for the need to supplement with L-glutamine before use. (L-glutamine is unstable at 4ºC and needs to be frozen for long-term storage.) On the day of use, supplement CTL-Test™ Medium with freshly-thawed L-glutamine at a concentration of 1%. The supplemented medium can be stored at 4ºC protected from light for up to 1 month, however, L-glutamine must be replenished every 10 days. For best test results, pre-warm media shortly prior to use by placing it in a 37ºC, CO_2 incubator in a tube with a loose cap for 20 minutes. This allows the pH to reset itself. The media should be protected from light while working on bench top (e.g. by wrapping in aluminum foil). CTL-Test™ Medium is buffered. Slight fluctuations in pH might occur resulting in fluctuation of color. Such changes do not affect the medium's performance.

CTL-Test™ Medium is available in 100ml or 500ml bottles. The 100ml bottles are convenient for average-sized experiments, avoiding the need for sterile filtration, saving the cost of filters.

Each batch of CTL-Test™ Medium is thoroughly tested for an equally high performance in ELISPOT assays as the previous batches, in addition to standard quality assurance procedures.

CTL-Wash™ Supplement 10x (Cat# CTLW-010)

CTL-Wash™ is a formulation specially made to keep PBMC in a nutrient-rich environment while maintaining their full viability and functionality during washing. The two to three washing steps required after the Ficoll® isolation or during thawing of PBMC and the time required for their counting typically adds up to 30-40 minutes. During this time, the PBMC quality will deteriorate if kept in a nutrition-deficient environment. If uncharacterized serum is added, mitogenic or toxic effects can affect the cells.

CTL-Wash™ Supplement is a 10x serum-free formulation which has to be added to RPMI-1640 medium. It contains injection-grade water supplemented with amino acids, vitamins, inorganic salts, and human serum albumin in a proprietary composition to provide a balanced environment for maximizing PBMC survival.

CTL-Wash™ should be stored at 4ºC and protected from light. For best results, it is diluted 1+9 in warm (20-37ºC) RPMI-1640 and used warm for washing. Diluted CTL-Wash™ can be safely stored at 4ºC. CTL-Wash™ Supplement 10x is sold in convenient sterile 100ml units ready for dilution.

CTL-Cryo™ ABC Kit (Cat# CTLC-ABC)

The CTL-Cryo™ ABC Kit is a serum-free freezing media kit formulated to cryopreserve freshly-isolated PBMC. When adhering to the CTL protocols (see the following), CTL-Cryo™ ABC ensures a higher than 90% viability (typically >95%) of PBMC after thawing, and a recovery rate of greater than 70% (up to 95%).

The CTL-Cryo™ ABC has been designed to ensure unimpaired PBMC function in T cell cytokine recall assays, such as ELISPOT, ELISA, CBA and CPA. It contains injection-grade water supplemented with amino acids, vitamins, inorganic salts, and human serum albumin in a proprietary composition. CTL-Cryo™ requires the addition of 20% high-grade DMSO at the time of use, which is included separately with the kit.

CTL Anti-Aggregate Wash™ Supplement 20x (Cat#, CTL-AA-001, CTL-AA-005)

Cells that die during freezing-thawing release DNA strands that can cause cell clumping and aggregation. Therefore, the use of DNAse-containing wash solutions is recommended while thawing PBMC. Moreover, cells show increased metabolic activity after thawing as they compensate for the stress reaction — this needs to be accounted for by using a nutrient-rich medium. CTL Anti-Aggregate Wash™ Supplement has been specifically formulated for optimal PBMC recovery and functionality after thawing. It contains injection-grade water supplemented with amino acids, vitamins, inorganic salts, and human serum albumin in a proprietary composition that comes in a ready-to-use formulation, providing a balanced environment for PBMC. For each vial of PBMC to be thawed, 1 vial of CTL Anti-Aggregate Wash™ Supplement 20x is needed.

Equipment and Reagents

EQUIPMENT:	VENDOR:	CATALOG NUMBER:
Conical Tubes, 50ml, sterile	Fisher	14-432-22
Serological Pipette, 2ml	Fisher	13-678-11C
Serological Pipette, 5ml	Fisher	13-678-11D
Serological Pipette, 10ml	Fisher	13-678-11E
Pipette Aids, Drummond	Fisher	13-681-19
Centrifuge, Allegra 6R	Beckman Rotor Gh3.8A	ALR6
Variety Micropipette (20µl)	Eppendorf	21-371-6
Pipette Tips (200µl)	VWR	53508-783
Gloves	Fisher	19-048-575A
Hemocytometer	Reichert Bright-Line	02-671-5
Hemotology Mixer	Barnstead/Thermolyne	2-814-2
Vials, cryogenic, 1.8ml, sterile	Nalgene	12-565171N
Nalgene Cryo Freezing Container, filled with 2-Propanol	Nalgene	15350-50
-80°C Freezer	Forma scientific Inc.	55703-430
Liquid nitrogen storage tank	Thermo Forma	8030
Biological Safety Cabinet	Forma Scientific	Model 1286
Plate Washer	CTL	405LSR

REAGENTS	VENDOR:	CATALOG NUMBER:
CTL-Wash™ Supplement 10x	Cellular Technology Ltd.	CTLW-010
CTL-Test™ Medium	Cellular Technology Ltd.	CTLT-010 or CTLT-005
CTL-Cryo™ ABC Kit*	Cellular Technology Ltd.	CTLC-ABC
CTL Anti-Aggregate Wash™ 20x	Cellular Technology Ltd.	CTL-AA-001 or CTL-AA-005
CTL-LDC™ Reagent	Cellular Technology Ltd.	CTL-LDC-100
CTL-LDAC™ Reagent	Cellular Technology Ltd.	CTL-LDAC-100
Ficoll®, Isoprep	Robbins Scientific	1070-01-0
L-glutamine	Gibco	25030-081

*Classified as irritant. See included MSDS.

Technical Tips

- It is important to quick-spin vials before use to ensure content volumes.
- To maximize the use of each plate, an adhesive plate-sealing sheet has been included that can be adhered to the top of the plate to cover and protect unused wells that are intended to be used in subsequent assays. Use your thumbs to firmly adhere the sheet to the plate and use a razor blade to cut the sheet to expose only the necessary wells.
- We highly recommend the use of CTL Serum-free Media for freezing, washing, and testing PBMC. Even brief exposure to a mitogenic serum can cause high background while other sera can have suppressive effects. CTL also recommends using the CTL-LDC™ Kit for accurate live/dead cell counts.
- Deviations from specified temperatures, timing requirements, number of washing steps, and specified reagent preparation volumes may alter the performance of the assay.
- Plates can be washed manually or by a suitable automated plate washer with adjusted pin length and flow rate so membranes and spots are not damaged (CTL recommends BioTek ELx405).
- To avoid damage to the PVDF membrane in the wells, do not touch the membrane with pipette tips or with the plate washer. The PVDF membrane is permeable and protected by an underdrain. Avoid direct contact between the well bottom and wet surfaces, including paper towels or any other materials that can absorb liquid.
- While processing plates, the PVDF membrane at the bottom of the wells must remain wet.
- When underdrain and gloves are wet, the underdrain may be slippery and difficult to remove. Wipe gloves and underdrain with paper towel before removing.
- After completion of the experiment, do not dry the ELISPOT assay plates at temperatures exceeding 37°C as this may cause the membrane to crack.
- Spots may not be readily visible while the membrane is still wet. Scan and count plates only after membranes have completely dried.
- Higher background appearing in the control wells can be potentially overcome by the following steps:
  - When working with pre-cultured cells, wash the cells thoroughly in CTL-Wash™ prior to the experiment in order to avoid carryover of cytokines and other substances; use CTL-Test™ for testing PBMC.
  - SmartCount™ module of the ImmunoSpot® counting software automatically recognizes spots over high background or uneven background correcting background deviations. The Autogating™ module will help discern between T cell-derived and background spots. The CTL technical support team will gladly assist you with using the ImmunoSpot® software for the analysis of complicated test results.
- Data analysis: The CTL ImmunoSpot® Analyzers along with the ImmunoSpot® software have advanced features that permit automated, objective recognition of spots, gating and counting. An ELISPOT data management tool, SpotMap®, is also available for facilitation of high-throughput ELISPOT work.

The CTL team will gladly assist you with data analysis and troubleshooting, as well as customizing ELISPOT assays to suit your needs. Please contact us via ctl-europe@immunospot.eu.

Visit our website for several helpful videos on working with ELISPOT assays and PBMC: www.immunospot.com.

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