PROTOCOL:
Isolation of PBMC from Fresh Whole Blood

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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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