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Fluorescent-labeled CD1d Tetramer Staining Protocol

Materials Required

- Cell sample, e.g. Blood sample (RBC-depleted), PBMCs or NKT cell line
- CD1d tetramer conjugated to the fluorescent label of choice.

If using empty tetramers the ligand of choice should be loaded into the tetramer prior to staining

Ensure that the tetramer is stored consistently at 4ºC in the dark, with the lid tightly closed.

- Fluorescent-labeled anti-CD3 antibody
- Fluorescent-labeled anti-CD19 antibody†
- Wash buffer (0.1% sodium azide, 0.1% BSA in PBS)
- Fix solution (1% fetal calf serum, 2.5% formaldehyde in PBS)

Standard Staining Protocol

1. **Spin CD1d tetramer in a chilled microcentrifuge at 14,000 × *g* for 3 minutes.** This will remove protein aggregates that contribute to non-specific staining. Maintain reagents on ice, shielded from light, until required. Do not aspirate any part of the pelleted aggregates when taking test volumes for staining.
2. **Allocate 1-2 × 10^6 lymphoid cells (PBMC or splenocytes) per staining condition.**
   This ensures there is a sufficient number of cells to collect up to 500,000 events during flow cytometry.

   (Allocate only 2-5 × 10^5 cells per staining condition when using NKT cell clones or lines due to the high frequency of antigen-specific NKT cells).
3. **Wash cells* with wash buffer and resuspend in the residual volume (~ 50µl).** Keep tubes on ice for all subsequent steps, except where otherwise indicated.
4. **Add one test (0.5µl) of fluorescently-labeled tetramer to the cells and mix by pipetting.**
5. **Incubate at 4°C for 30 minutes, shielded from light.**
6. **Wash cells with 2ml wash buffer per tube and resuspend in residual liquid (~ 50µl).**
7. **Add an optimal amount of anti-CD3 antibody and mix by pipetting. At this stage, it is also recommended to add anti-CD19 antibody in order to gate out B cells when performing analysis.** If staining control samples with other primary antibodies, at this stage add an optimal amount to the cells in their respective tubes.
8. **Incubate samples on ice for 20 - 30 minutes, shielded from light.**
9. **Wash cells twice with 2ml wash buffer per tube. Mix each tube.**
10. **Add 200µl fix solution. Vortex tubes.** It is important to vortex well when adding fixative so that cells do not clump. Store tubes in the dark in the refrigerator until ready for data acquisition. The morphology of the cell changes after fixing, so it is advisable to leave the samples for 3 hours before proceeding with data acquisition. Samples can be stored for up to 2 days.

    The cells are now ready for flow cytometric analysis. The tetramer-positive cells are most conveniently viewed by gating first on live lymphoid cells and then analyzing on a two-color plot showing CD3 on the x-axis and Pentamer on the y-axis.

† Tetramers can bind non-specifically to B cells. It is therefore strongly recommended to include anti-CD19 antibody when staining in order to gate on CD19-negative cells before plotting tetramer versus CD3.

* To wash cells: Dispense 1 ml wash buffer per tube and spin 500 × *g* for 5 minutes in a chilled centrifuge at 4ºC. Check for presence of a cell pellet before discarding the supernatant.

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Protocol Optimization

The binding affinity of the CD1d molecule for the TCR varies depending on the lipid used. This means that different complexes will have slightly different characteristics in the way they stain. The following guidelines will help you optimize your protocol for the best possible results:

Setting the live lymphocyte gate It is important to ensure that the forward-scatter (FSC) and side-scatter (SSC) gates are set correctly on the cell population of interest. This is to ensure that dead cells, cell aggregates and cell debris are excluded from the fluorescence data.

Titrating the CD1d tetramer Although a single test quantity of CD1d tetramer should normally be sufficient to stain 1 - 2 × 10^6 cells, it is important that you first titrate the CD1d tetramer. Carry out a range of doubling dilutions from 1 test per 1 × 10^6 cells down to 1/16 test per 1 × 10^6 cells.

CD3 antibody Investigate the effect of titrating the anti-CD3 antibody.

Temperature The temperature at which you carry out the staining can affect the background considerably. Although staining at 4°C is recommended in the first instance, incubating at room temperature or 37°C may be beneficial in optimizing the signal to noise ratio depending upon the CD1d/lipid combination. The higher the incubation temperature, the shorter the incubation time required. Try comparing staining at 4°C, 22°C and 37°C.

Positive control CD1d tetramers should be tested against a specific NKT cell line (or clone). Be sure to use NKT cells that have not been recently stimulated as this has been shown to cause down-regulation of NKT cell receptors. If an NKT cell line is not available, it is possible to use PBMCs from a known positive donor. In this case the frequency of positive cells will be much lower and more cells will be required (>10^6) per stain.

Negative control To control for non-specific staining it is also useful to stain NKT cells with empty tetramer.

For further tips on protocol optimization please refer to
www.proimmune.com/ecommerce/page.php?page=protocol_optimization

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