PROIMMUNE
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PRODUCT SHEET

CD1d Tetramer R-PE Labeled Negative Control:

- D002-2X
- E002-2X

CD1d molecules are highly-conserved non-classical major histocompatibility complex (MHC) molecules that are characterized as non-polymorphic and possessing narrow, deep, hydrophobic ligand binding pockets. These binding pockets are capable of presenting glycolipids and phospholipids to Natural Killer T (NKT) cells. NKT cells represent a unique lymphocyte population that co-express NK cell markers and a semi-invariant T cell receptor (TCR), and are implicated in the regulation of immune responses associated with a broad range of diseases.

The best-characterized CD1d ligand is α-galactosyl ceramide (α-GalCer), originally derived from marine sponge extract. Presentation of α-GalCer by CD1d molecules results in NKT cell recognition and rapid production of large amounts of IFN-γ and IL-4, bestowing α-GalCer with therapeutic efficacy.

Negative Control Recombinant CD1d Tetramer:	ProImmune's fluorescent-labeled CD1d negative control tetramers are mock-loaded with carrier only (no ligand loaded) and will not bind to NKT cells. Use of this negative control reagent in conjunction with a ligand-loaded CD1d Tetramer (e.g. α-GalCer) will allow low frequency positive populations to be accurately quantified. Please note that the negative control CD1d Tetramer cannot be reloaded with ligand by the end-user. ProImmune can provide a specific empty CD1d tetramer for this purpose (Catalog code D000 or E000). **For Research Use Only. Not for use in therapeutic or diagnostic procedures**.
Test Volume:	0.5 µl / test.
Test Specification:	One test contains sufficient reagent to stain approximately 1×10^6 cells. We recommend that the user titrate the reagent to determine the optimum amount to use in their specific application.
Concentration/ Formulation:	The CD1d Tetramer concentration is approximately 1µM. The Tetramer is supplied in PBS stabilized with 1% BSA and 0.025% sodium azide.
Storage Condition:	4°C. Protect from light. **Do not freeze.**
Shelf Life:	6 months if stored as instructed above.
Fluorochrome:	R-phycoerythrin (R-PE) excites at 480, 565 nm and emits at 578 nm (FL-2).
Hazards:	This reagent is formulated in 0.025% sodium azide. Under acid conditions the toxic compound hydrazoic acid may be released. Compounds containing sodium azide should be flushed with running water while being discarded.

Quality Control Assay Results

Appearance:	Clear, pale pink solution
Protein Characterization:	Passed
MHC Conformation Immunoassay:	Passed

Released by:
(Date as per product label above)

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Cellular Staining Protocol

Additional materials required: Wash Buffer (0.1% sodium azide, 0.1% BSA in PBS), Fix Solution (1% fetal calf serum, 2.5% formaldehyde in PBS), anti-CD3 antibody, anti-CD19 antibody.

1. Allocate 1-2 × 10^6 lymphoid cells (PBMCs or splenocytes) per staining condition. Allocate only 2-5 × 10^5 cells per staining condition when using NKT cell clones or lines, due to the higher frequency of antigen-specific NKT cells.
2. Wash the cells with 2 ml Wash Buffer and resuspend them in the residual volume (~50 µl). Keep tubes chilled on ice for all subsequent steps
3. Add one test (0.5 µl) of fluorescently-labeled CD1d tetramer to the cells and mix well.
4. Incubate samples on ice for 30 minutes, shielded from light.
5. Wash the cells with 2 ml Wash Buffer and resuspend them in the residual volume.
6. Add anti-CD3 and anti-CD19 antibodies to the cells and mix well. (Use of an anti-CD19 antibody enables non-specific staining of B cells to be excluded from the cytometry analysis.)
7. Incubate samples on ice for 20-30 minutes, shielded from light.
8. Wash the cells twice with 2 ml wash buffer and resuspend thoroughly before adding 200 µl Fix Solution. Store them in Fix Solution in the dark until analysis.

Tetramer-positive cells are most conveniently viewed by gating first on live, CD19-negative lymphoid cells and then analyzing on a two-color plot showing CD3 on the *x*-axis and tetramer on the *y*-axis.

Protocol Optimization

The following guidelines will help you optimize your protocol for the best possible results:

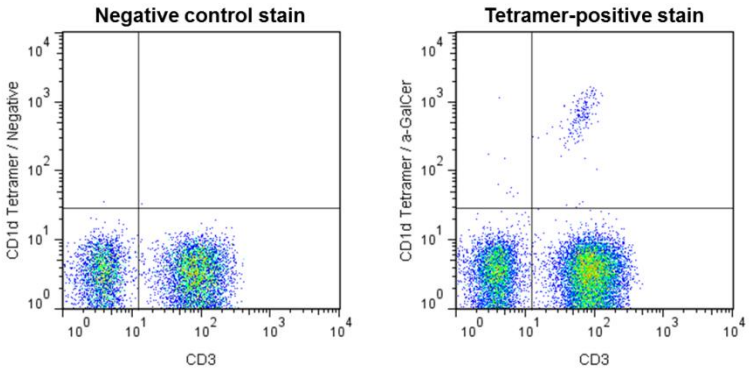
Setting the live lymphocyte gate It is important to ensure that the forward-scatter (FSC) and side-scatter (SSC) gates are set correctly on the cell population of interest. This is to ensure that dead cells, cell aggregates and cell debris are excluded from the fluorescence data.

Titrating the CD1d Tetramer 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, in order to determine the optimum amount of Pentamer reagent to use in your specific application.

Anti-CD3 antibody Investigate the effect of selecting different antibody clones or titrating the anti-CD3 antibody.

Positive control Pro5® MHC Pentamers 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, use PBMCs from a known positive donor - the frequency of positive cells will be much lower and therefore sufficient events must be collected to ensure a clear result.

Negative Control To control for non-specific staining it is also useful to stain T cells with the CD1d Negative Control Tetramer (Code D002 or E002), which are mock-loaded with carrier only (no ligand loaded) and will not bind to NKT cells. The use of this negative control reagent in conjunction with a ligand-loaded CD1d Tetramer (e.g. α-GalCer) will allow low frequency positive populations to be accurately quantified.



The figure on the left shows a cell sample stained with Negative Control Tetramer (Code D002 or E002). The figure on the right shows the same cells stained with the α-GalCer loaded CD1d Tetramer. A population of $CD3^+$ Tetramer-positive NKT cells is clearly visible in the upper right quadrant. Non-specific staining was eliminated from the plot by gating on CD19- cells before plotting CD3 vs. CD1d Tetramer.

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