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PRODUCT SHEET

CD1d Tetramer R-PE Labeled Empty:

- D000-2X
- E000-2X

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. More recently, the lysosomal sphingolipid isoglobotrihexosylceramide (iGb3) has been identified as a CD1d ligand. This endogenous sphingolipid is thought to be responsible for NKT cell development.

Recombinant CD1d Tetramer for Ligand Loading:	ProImmune's fluorescent-labeled empty CD1d tetramers can be loaded with a ligand of choice and used to identify Natural Killer (NK) T cells. Tetrameric CD1d-lipid complexes bind to NKT cell receptors (TCRs) of a particular specificity (as determined by the lipid ligand used), and thus enable antigen-specific CD1d-restricted NKT cells to be identified and analyzed by flow cytometry. Additional co-staining for intracellular cytokines (e.g. IFNγ / IL-2) or surface markers (e.g. CD69 / CD45RO) can provide additional functional data on the antigen-specific sub-set. **NB. Empty CD1d Tetramers should <u>NOT</u> be used as a negative control reagent for ligand-loaded tetramers. The empty tetramer used alone will give a high background stain, which will impair further analysis. ProImmune can provide a specific negative control CD1d tetramer for this purpose (Catalog code D002 or E002).** **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.

<u>References</u>

1. Matsuda *et al.* 2000. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J Exp Med.* 192:741-753
2. Dougan *et al.* 2005. Microsomal triglyceride transfer protein lipidation and control of CD1d on antigen-presenting cells. *J Exp Med.* 202:529-539.
3. Zhou *et al.* 2004. Lysosomal glycosphingolipid recognition by NKT cells. *Science* 306:1786-1789.

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Ligand Loading Protocol

1. Prepare no more than 500 µl of a 0.2 mg/ml solution of lipid in PBS + 0.5% Tween-20. Incubate in a sonicating water bath (100W20kHz) at 80°C for 2 minutes.
2. After 2 minutes, check the solubility of the lipid and repeat this step if not completely solubilized. Only attempt to load fully solubilized lipid to the tetramer. Incomplete solubilisation will result in poor tetramer loading.
3. Add a 12 molar lipid excess to the CD1d Tetramer sample.
4. Incubate overnight at room temperature in the dark.

The optimal loading protocol will depend on the properties of the ligand to be loaded, such as its hydrophobicity. The protocol given here is therefore only supplied as a guide and researchers are advised to investigate optimal conditions for lipid loading.

Additional variations to the suggested conditions may be necessary and may include changing the lipid excess used, lipid concentration, solubilisation technique and CD1d loading temperatures and incubation times. Lipid binding proteins such as microsomal triglyceride transfer protein (MTP) and Saposin B have both been implicated in facilitating lipid loading.

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

For further tips on protocol optimization refer to http://www.proimmune.com/support-protocol-optimization or download the Pro5® MHC Pentamer Handbook which contains useful protocols and advice on how to achieve the best possible staining for your samples (https://www.proimmune.com/pro5-pentamer-handbook).

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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