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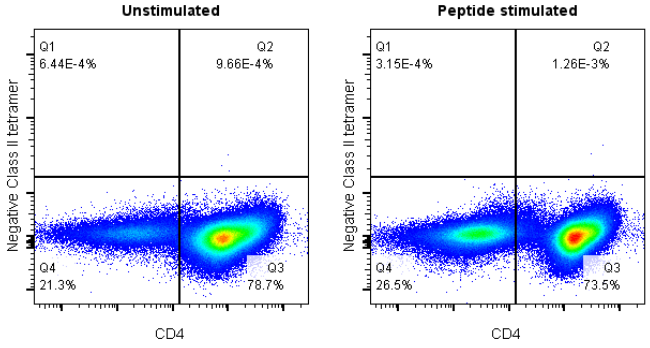
PRODUCT SHEET
ProT2® MHC Tetramer
Negative Control:
- TTN02X-2X-
- TTN02X-4X-

ProT2® MHC Class II Tetramer:	In order to identify antigen-specific $CD4^+$ T lymphocytes, fluorochrome-labeled Class II tetramers are required. Fluorescent-labeled ProT2® MHC Class II Tetramers are used to identify antigen-specific $CD4^+$ T lymphocytes. Multimeric MHC-peptide complexes bind to T cell receptors (TCRs) of a particular specificity (as determined by the MHC allele and peptide combination). $CD4^+$ T cells stained with ProT2® MHC Class II Tetramers can be analyzed by flow cytometry and the frequency of antigen-specific T cells determined. 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. The ProT2® Negative Control MHC Class II Tetramer is loaded with CLIP (PVSKMRMATPLLMQA). These Class II tetramers will not bind to $CD4^+$ T cells, and so the use of this negative control reagent in conjunction with a peptide-loaded Class II tetramer will allow low frequency positive populations to be accurately quantified. **For Research Use Only. Not for use in therapeutic or diagnostic procedures**.
Test Volume:	5 µl / test.
Test Specification:	One test contains sufficient reagent to stain approximately 1-2 × 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 ProT2® MHC Class II Tetramer concentration is approximately 0.2 mg/ml in PBS, stabilized with 0.5% 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; emits at 578 nm. Allophycocyanin (APC): excites at 650 nm; emits at 660 nm.
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.



The figure on the left shows a cell sample that has not been stimulated with peptide; the figure on the right shows the same cells that have been stimulated with peptide. No population of $CD4^+$ CLIP tetramer-positive cells is visible in the upper right quadrant. Non-specific staining was eliminated from the plot by gating on $CD19^-$ cells before plotting CD4 vs. ProT2® Negative Control MHC Class II Tetramer.

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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-CD4 antibody, anti-CD19 antibody (optional[†]).

1. Centrifuge ProT2® MHC Class II Tetramer in a chilled microcentrifuge at 14,000 ×*g* for 5 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 tests for staining.
2. Allocate 1-2 × 10^6 lymphoid cells (PBMC) per staining condition.
3. Wash the cells with wash buffer and resuspend them in the residual volume (~50 µl).
4. Add one test (5 µl) of fluorescent-labeled ProT2® MHCa Class II Tetramer to the cells and mix by pipetting.
5. Incubate at 37°C for 2 hours in the dark.
6. Wash the cells in wash buffer.
7. Add an optimally titrated amount of anti-CD4 antibody per staining condition. At this stage, it is also recommended to add anti-CD19 antibody in order to gate out B cells when performing analysis.
8. Incubate on ice for 20-30 minutes in the dark.
9. Wash the cells twice in wash buffer and store them in fix solution in the dark until 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 CD4 on the *x*-axis and Class II tetramer 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 $CD19^-$ cells before plotting CD4 versus Class II tetramer.

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 ProT2® MHC Class II tetramer Although a single test quantity of Class II tetramer should normally be sufficient to stain 1-2 × 10^6 cells, it is important that you first titrate the Class II 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.

CD4 antibody Investigate the effect of titrating the anti-CD4 antibody.

Temperature The temperature at which cells are stained can affect signal considerably. Varying time and temperature of incubation is necessary to determine optimal signal to noise ratio depending upon the MHC/peptide combination and T cell receptor. We recommend incubation at 37°C for 2 hours in the first instance.

Amplification of cell samples for Class II tetramer analysis:

$CD4^+$ T-cell expansion of Class II tetramer positive cells may be used to aid detection of low frequency populations, particularly when analyzing PBMCs. It must be noted that some low affinity antigens may not give detectable staining.

A suggested protocol for expansion of Class II tetramer positive cells is as follows:

1. Plate PBMCs in the presence of 5 µM of the peptide of interest at a density of 5 x 10^6 cells/ml. Incubate cells at 37°C and add 10 U/ml IL-2 from day 5, every 2-3 days.
2. Stain with ProT2® MHC Class II Tetramer after 12-15 days. Alternatively, 2-3 rounds of restimulation may be required for additional amplification. Allow at least 6 days following the final stimulation before staining with Class II tetramer to allow re-expression of the T cell receptor on the cell surface.

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