PROIMMUNE
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PRODUCT SHEET

ProM2® MHC Class II Monomer Negative Control:

- MTN02X-1X-

Negative ProM2® human MHC Class II Monomer:	In order to identify antigen-specific $CD4^+$ T lymphocytes, fluorochrome-labeled Class II tetramers are required. ProM2® human MHC Class II Monomer reagents can be made into Class II tetramers when combined with Streptavidin fluorochrome conjugates. Streptavidin has four biotin-binding sites, enabling biotinylated ProM2® human MHC Class II Monomer reagents to form Class II tetramers. $CD4^+$ T cells stained with Class II tetramers can be analyzed by flow cytometry and the frequency of antigen-specific T cells determined. Negative ProM2® human MHC Class II Monomer is loaded with CLIP (PVSKMRMATPLLMQA). When tetramerized, 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 size:	ProM2® Monomers are provided in 35 µg and 100 µg sizes.
Concentration/ Formulation:	Negative ProM2® human MHC Class II Monomer is supplied at 0.4 mg/ml in 20 mM Tris, pH 8, 50 mM NaCl, stabilized with 0.5% BSA and 0.025% sodium azide.
Storage Condition:	-80°C. **Avoid freeze-thaw cycles.**
Shelf Life:	Use liquid nitrogen to flash-freeze upon receipt of material, or tetramerize immediately. The monomer is stable for 12 months if stored as instructed above.
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: Colorless solution

Protein Characterization: Passed

Released by:
(Date as per product label above)

Class II Tetramer Production Protocol

Additional materials required: Streptavidin-R-PE or Streptavidin-APC for ProM2® Monomer, PBS containing 0.025% sodium azide.

1. Spin Streptavidin-R-PE or Streptavidin-APC in a chilled microcentrifuge at 14,000 ×*g* for 3 minutes. Maintain reagents on ice, shielded from light, until required. Do not aspirate any part of the pelleted aggregates when taking test volumes for conjugation.

2. *To conjugate 35 µg Negative ProM2® human MHC Class II Monomer with Streptavidin-R-PE:*

   Add 13 µl of 0.8 mg/ml Streptavidin-R-PE to 35 µg Negative ProM2® human MHC Class II Monomer, mix gently and incubate at 4˚C for 15 minutes. Repeat the addition of Streptavidin-R-PE four times with a 15 minute gap between each addition. Make up to a final volume of 400 µl with PBS/0.025% sodium azide.

   To conjugate 35 µg Negative ProM2® human MHC Class II Monomer with Streptavidin-APC:

   Add 23 µl of 0.09 mg/ml Streptavidin-APC to 35 µg Negative ProM2® human MHC Class II Monomer, mix gently and incubate at 4˚C for 15 minutes. Repeat the addition of Streptavidin-APC four times with a 15 minute gap between each addition. Make up to a final volume of 400 µl with PBS/0.025% sodium azide.

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3. *To conjugate 100 µg Negative ProM2® human MHC Class II Monomer with Streptavidin-R-PE:*

   Add 36 µl of 0.8 mg/ml Streptavidin-R-PE to 100 µg Negative ProM2® human MHC Class II Monomer, mix gently and incubate at 4˚C for 15 minutes. Repeat the addition of Streptavidin-R-PE four times with a 15 minute gap between each addition. Make up to a final volume of 1.15 ml with PBS/0.025% sodium azide.

   To conjugate 100 µg Negative ProM2® human MHC Class II Monomer with Streptavidin-APC:

   Add 65 µl of 0.09 mg/ml Streptavidin-APC to 100 µg Negative ProM2® human MHC Class II Monomer, mix gently and incubate at 4˚C for 15 minutes. Repeat the addition of Streptavidin-APC four times with a 15 minute gap between each addition. Make up to a final volume of 1.15 ml with PBS/0.025% sodium azide.

Store Class II tetramers at 4˚C, protected from light. **Do not freeze.**

We recommend that Class II tetramers are used at 5 µl / test.

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 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 5 µl of 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 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 tetramer versus CD4.

Protocol Optimization:

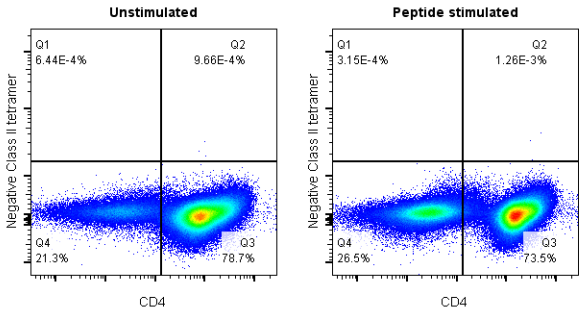
The following guidelines will help you optimize your protocol for the best possible results:

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 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.



1 × 10^6 cells were incubated with 1 test size R-PE-labeled CLIP-Class II tetramer at 37°C for 2 hours. Non-specific staining was eliminated from the plot by gating on $CD19^-$ cells before plotting CD4 vs Class II tetramer.

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