PROIMMUNE
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Product Sheet
Pro5® Recombinant human MHC Pentamer
Unlabeled (Fxxx-0)

Pro5® Recombinant human MHC Pentamer:	Pro5® MHC class I Pentamers allow the enumeration of antigen-specific $CD8^+$ T lymphocytes in combination with the Pro5® Fluorotag secondary reagent. Multimeric MHC-peptide complexes bind to T cell receptors (TCRs) of a particular specificity (as determined by the MHC allele and peptide combination). $CD8^+$ T cells stained with Pro5® Pentamer can be analyzed by flow cytometric analysis and the frequency of antigen-specific T cells determined. Additional co-staining for intracellular cytokines (e.g. IFNγ / IL-2) and/or surface markers (e.g. CD69 / CD45RO) can yield functional data of the antigen-specific subset. **For Research Use Only. Not for use in therapeutic or diagnostic procedures.**
Test specification:	One test contains sufficient reagent to stain approximately 1×10^6 cells. Less reagent may be sufficient and it is recommended that the customer determine the optimum amount appropriate for each application.
Test volume:	2 µl / test
Concentration/ Formulation:	The Pro5® Pentamer concentration is approximately 0.5 mg/ml in 20 mM Tris, pH 8.0, containing 50 mM NaCl and 2 mM EDTA. The product is stabilized with 1% BSA and 0.01% sodium azide.
Storage Condition:	4°C for 3 months. -80°C for 12 months.
Shelf Life:	12 months if stored at -80°C.
Hazards:	This reagent is formulated in 0.01% sodium azide. Under acid conditions the toxic compound hydrazoic acid may be released. Solutions containing sodium azide should be flushed with running water while being discarded.
Conjugation Options:	It is recommended that unlabelled pentamers be used in 2-layer staining as described below.

Quality Control Assay Results

Appearance	Clear, colorless solution
Protein Characterization:	Passed
MHC Conformation Immunoassay:	Passed

Released by:

(Date as per product label above)

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Cellular Staining Protocol

Materials required Wash buffer (0.1% sodium azide, 0.1% BSA in PBS), Fix solution (1% fetal calf serum, 2.5% formaldehyde in PBS), Pro5® Fluorotag with your fluorescent label of choice, anti-CD8 antibody, anti-CD19 antibody

Staining procedure

1. **Centrifuge Pro5® Pentamer in a chilled microcentrifuge at 14,000×*g* for 5-10 minutes.** This will collect any protein aggregates present in the solution at the bottom of the vial. These aggregates may contribute to non-specific staining if included in test volume. Pentamer test volume should be taken from the supernatant fraction. Maintain reagents on ice, shielded from light, until required.
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 tube when using T cell clones or lines due to the high frequency of antigen-specific T cells).**
3. **Wash cells with 2ml wash buffer, spin down (500 × *g* for 5 minutes), discard supernatant and resuspend in residual liquid (~ 50µl).** Keep tubes chilled on ice for all subsequent steps, except where indicated.
4. **Add one test (2 µl) of unlabeled Pentamer to the cells and mix by pipetting.**
5. **Incubate at room temperature (22ºC) for 10 minutes.**
6. **Wash cells with 2ml wash buffer as for step 3, and resuspend in residual liquid (~ 50µl).**
7. **Add 8 µl Pro5® Fluorotag and optimal amounts of anti-CD8 and anti-CD19 antibodies (and any other secondary antibodies) and mix by pipetting.** 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 minutes, shielded from light.**
9. **Wash cells twice with 2ml wash buffer as for step 3. 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.

    Flow cytometric analysis: The Pentamer-positive cells are most conveniently viewed by gating first on live, CD19-negative lymphoid cells (use a histogram to visualize CD19 staining) and then analyzing on a two-color plot showing CD8 on the x-axis and Pentamer on the y-axis.

Protocol Optimization

The detection of antigen-specific T cell populations as rare as 0.02% of total $CD8^+$ T cells requires the design of suitably controlled experiments. If the number of Pentamer-positive events is expected to be low, it is important to acquire a suitably large number of events within the live lymphocyte gate in order to collect sufficient events of the population of interest. The binding affinity of the MHC molecule for the TCR varies depending on the allele/peptide combination. Thus, different complexes will have slightly different characteristics in the way they stain. The following guidelines will assist with protocol optimization for the best possible results:

For further tips on optimization refer to www.proimmune.com/ecommerce/page.php?page=protocol_optimization or request a Pro5® MHC Pentamer Handbook which contains useful protocols and advice on how to achieve the best possible staining for your samples (http://www.proimmune.com/ecommerce/html/form/handbook.html).

Live lymphocyte gate Ensure the forward-scatter and side-scatter gates are set correctly on the population of interest.

Find the optimum quantity of Pro5® Pentamer and/or Fluorotag to use Although a single test quantity of Pro5® Pentamer and Fluorotag should normally be sufficient to stain 1 - 2 × 10^6 cells, it is important to first titrate them both. Carry out a range of doubling dilutions from 1 test per 10^6 cells down to 1/16 test per 10^6 cells.

CD8 antibody Investigate the effect of titrating the anti-CD8 antibody. This will prevent any antibody-mediated blocking of the Pro5® Pentamer-binding site.

Reduction of background staining Pro5® Pentamers may bind non-specifically to B cells. It is therefore strongly recommended to include an anti-CD19 antibody when staining in order to gate on CD19-negative cells before plotting Pentamer versus CD8.

Temperature Although staining at room temperature (22°C) is recommended in the first instance, incubating at 4°C or 37°C may be beneficial to reduce background. The higher the incubation temperature, the shorter the incubation time required.

Positive control Pro5® Pentamers should be tested against a specific T cell line / clone. Use T cells that have not been recently stimulated as this causes TCR down-regulation. If a cell line is not available, use PBMCs from a known positive donor - the frequency of positive cells will be much lower and more cells will be required (at least 1 × 10^6) per stain.

Negative control To control for non-specific staining, stain T cells of a different peptide specificity or MHC restriction. For example, T cells from unexposed individuals may be used when detecting T cell responses to a specific antigen.

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