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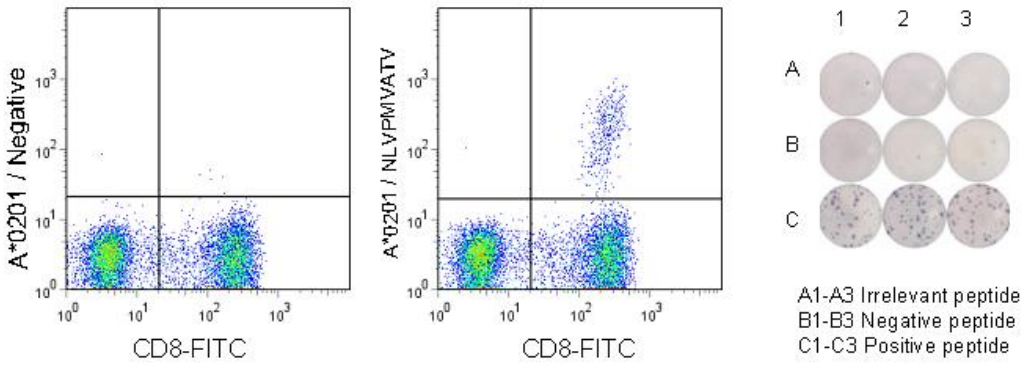
Product Sheet
Fluorescent-labeled Pro5® Recombinant HLA-A*0201 Negative Control Pentamer (FN01)

Pro5® Recombinant HLA-A*0201 Negative Control Pentamer:	Pro5® Class I MHC Pentamers allow the enumeration of antigen-specific $CD8^+$ T lymphocytes. HLA-A*0201 negative control Pro5® Pentamers can be used to assess non-specific binding in flow cytometric analysis. The product consists of multimeric HLA-peptide complexes, assembled with an irrelevant peptide antigen that is known to have no T cell response. The HLA-A*0201 Negative Pentamer is recommended for use with samples where a low frequency T cell response is expected, e.g. some cancer or autoimmune epitopes. The use of a negative control reagent in conjunction with experimental Pentamer epitopes will allow low frequency positive populations to be accurately quantified. **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:	10 µl / test for biotin- or fluorescent-labeled Pentamers
Concentration / Formulation:	The Pro5® Pentamer concentration is approximately 0.05 mg/ml in PBS stabilized with 1% BSA and 0.01% sodium azide.
Storage Condition:	4°C. Protect from light. **Do not freeze.**
Shelf Life:	6 months if stored as instructed above.
Fluorochromes and other labels:	R-phycoerythrin (R-PE): excites at 480, 565 nm; emits at 578 nm (FL-2) Allophycocyanin (APC): excites at 650 nm; emits at 660 nm (FL-4) Biotin-labeled for use in conjunction with Fluorescent-labeled streptavidin
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.
Example staining:	Figures of PBMC stained with anti-CD8 (LT8) on *x*-axis and Pro5® MHC Pentamer on *y*-axis. Each plot shows approx. 10,000 live lymphoid events. Left plot: HLA-A*0201 Negative Control Pentamer; center plot: positive Pentamer; right image: ELISPOT plate showing responses to an irrelevant peptide (A), the negative control Pentamer peptide (B) and a positive control peptide (C). 

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Cellular Staining Protocol for Labeled Pro5® Pentamers

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-CD8 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 staining condition 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 (10 µl) of labeled Pentamer to the cells and mix by pipetting.**
5. **Incubate at room temperature (22ºC) for 10 minutes, shielded from light.**
6. **Wash cells with 2ml wash buffer as for step 3 and resuspend in residual liquid (~ 50µl).**
7. **Add an optimal amount of anti-CD8 antibody (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.** 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 lymphoid cells and then analyzing on a two-color plot showing CD8 on the x-axis and Pentamer on the y-axis.

Protocol Optimization

For further tips on protocol 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).

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