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Labeled Pentamer Staining Protocol for multiple specificities

Introduction

It is possible to stain a single cell sample for up to three different antigen-specificities using Pro5™ Recombinant MHC Pentamers. Any combination of R-PE-labeled, APC-labeled and biotin-labeled Pro5™ Pentamers can be used for this purpose.

Materials Required

- Cell sample, e.g. Blood sample (RBC-depleted), PBMCs or T cell line
- Up to 3 different Pro5™ Recombinant MHC Pentamers conjugated to R-PE, APC and biotin. *Ensure that the stock Pentamers are stored consistently at 4ºC in the dark, with the lid tightly closed.*
- Fluorescent-labeled anti-CD8 antibody
- Fluorescent-labeled streptavidin
- Wash buffer (0.1% sodium azide, 0.1% BSA in PBS)
- Fix solution (1% fetal calf serum, 2.5% formaldehyde in PBS)

Standard Staining Protocol

1. **Spin Pro5™ Pentamers in a chilled microcentrifuge at 14,000 × *g* for 3 minutes.** This will help to remove protein aggregates that may contribute to non-specific staining. Maintain reagents on ice, shielded from light, until required. Ensure that you do not aspirate any part of the pelleted aggregates when taking test volumes for staining.
2. **Allocate 1-2 ´ 10^6 lymphoid cells (PBMC or splenocytes) per staining condition.**

   This ensures that you have a sufficient number of cells to collect up to 500,000 events during flow cytometric analysis.
3. **Wash the cells* with wash buffer and resuspend in the residual volume (~ 50µl).** Keep tubes chilled on ice for all subsequent steps, except where otherwise indicated.
4. **Add one test (10 µl) of each labeled Pentamer to the cells and mix by pipetting.**
5. **Incubate at room temperature (22ºC) for 10 minutes, shielded from light.**
6. **Wash the cells with 2ml wash buffer per tube and resuspend in residual liquid (~ 50µl).**
7. **Add an optimally titrated amount of anti-CD8 antibody (and fluorescent-labeled streptavidin (optimally titrated) if biotin-labeled Pentamer was added in step 4) and mix by pipetting.**
8. **Incubate samples on ice for 20 minutes, shielded from light.**
9. **Wash cells twice with 2ml wash buffer per tube. 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 cells are now ready for flow cytometric analysis. The Pentamer-positive cells can be viewed by gating first on live lymphoid cells and then analyzing on two-color plots showing either CD8 on the x-axis and each individual Pentamer on the y-axis, or by plotting one Pentamer against another.

* To wash cells: Dispense 1 ml wash buffer per tube and spin 500 × *g* for 5 minutes in a chilled centrifuge at 4ºC. Check for presence of a cell pellet before discarding the supernatant.

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Protocol Optimization

The detection of rare antigen-specific T cells by Pro5™ Pentamer staining is a powerful and versatile tool for immunological analysis. However, the detection of events as rare as 0.02% of total $CD8^+$ T cells requires both the design of suitably controlled experiments as well as a well-maintained flow cytometer. If the number of Pro5™ 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. This means that different complexes will have slightly different characteristics in the way they stain. 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-H) and side-scatter (SSC-H) gates are set correctly on the cell population of interest. For further information and examples, please visit http://proimmune.com/ecommerce/page.php?page=Flow-Cytometry.

Find the optimum quantity of Pro5™ Pentamer to use Although a single test quantity of Pro5™ Pentamer should normally be sufficient to stain 1 - 2 $\times$ 10^6 cells, it is important that you first titrate the Pro5™ Pentamers. 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.

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

Fluorescent Streptavidin Investigate also the effect of titrating the fluorescent-labeled streptavidin.

Temperature The temperature at which you carry out the staining can affect the background considerably. Although staining at room temperature (22°C) is recommended in the first instance, incubating at 4°C or 37°C may be beneficial in optimizing the signal to noise ratio depending upon the MHC/peptide combination. The higher the incubation temperature, the shorter the incubation time required.

Positive control Pro5™ Pentamers should be tested against a specific T cell line (or clone). Be sure to use T cells that have not been recently stimulated as this has been shown to cause down-regulation of T cell receptors. If a T cell line is not available it is possible to use PBMCs from a known positive donor. In this situation 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 it is also useful to stain T cells of a different peptide specificity or MHC restriction. For example, T cells from unexposed individuals may be used in the case of detecting T cell responses to a specific antigen.

Use of previously frozen cells Pro5™ Pentamers can also be used to stain frozen PBMC samples, although the number of live cells will be reduced. Thaw the cells carefully and wash once or twice before proceeding with the staining protocol.

Ficoll purification of cells before use may reduce background staining (this has been reported to be particularly beneficial with mouse splenocytes).

Intracellular staining It is possible to co-stain samples with antibodies against intracellular markers. Cells should first be stained with the Pro5™ Pentamer and other antibodies specific for extracellular epitopes, followed by membrane permeabilization and intracellular staining. Please contact us if you would like a detailed protocol.

Fluorescence Activated Cell Sorting Live, Pro5™ Pentamer-positive cells can be sorted and collected for further manipulation. In this case it is advisable to remove the azide preservative in the Pro5™ Pentamer reagent beforehand, using centrifugal concentrators (e.g. Microcon 10kD from Millipore). Dilute the reagent to maximum volume with PBS and concentrate down to original volume. Repeat the procedure 2-3 times until the azide is sufficiently diluted. Make sure that you use an appropriate amount of Pro5™ Pentamer reagent and other antibodies, and sort the cells immediately after staining, without treating them with any fixative. If you are sorting a large number of cells then it is useful to chill the tube during the procedure to prevent clumping.

Choice of fluorescent label Pro5™ Pentamers can be labeled with R-Phycoerythrin (R-PE), APC or biotin. The anti-CD8 antibody and streptavidin are normally conjugated to FITC and PE-Cy5.

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