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Pro5® Recombinant MHC
Pentamer Staining Protocol
for whole blood

Materials Required

Pro5® Recombinant MHC Pentamer in PBS/azide conjugated to your fluorescent label of choice. **Ensure that the stock Pentamer is stored consistently at 4°C in the dark, with the lid tightly closed.**

Ammonium chloride lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$ pH 7.4, 0.4% EDTA);
alternatively, you can use commercial preparations such as Becton Dickinson PharmLyse™ (#555899)
Wash buffer (0.1% sodium azide, 0.1% BSA in PBS)
Fix solution (1% fetal calf serum, 2.5% formaldehyde in PBS)

Whole blood collection

1. **Collect 100µl whole blood per staining condition in tubes with suitable anti-coagulant (**e.g. EDTA :- lavender-capped vacutainer).

Option 1: Red cell lysis <u>before</u> staining

2. **Add approximately 2ml of ammonium chloride lysis buffer per 100µl whole blood.** Incubate for up to 2 minutes, until opaque solution clears, and vortex well.
3. **Centrifuge cells. If red cells remain, repeat lysis step.**
4. **Wash the cells twice in wash buffer** and resuspend them in the residual volume of wash buffer (approximately 50µl).
5. **Add one test of fluorescently-labeled Pro5® Pentamer (equivalent to 10µl test reagent) and an optimally titrated amount of anti-CD8 antibody (e.g. FITC-labeled) per staining condition.**
   This may best be carried out in 2 steps - see 'Protocol Optimization' section.
6. **Incubate at room temperature for 10-15 minutes in the dark.**
7. **Wash the cells twice in wash buffer and store in fix solution in the dark.**

Option 2: Red cell lysis <u>after</u> staining

2. **Add one test of fluorescently-labeled Pro5® Pentamer (equivalent to 10µl test reagent) and an optimally titrated amount of anti-CD8 antibody (e.g. FITC-labeled) per staining condition.**
   This may best be carried out in 2 steps - see 'Protocol Optimization' section.
3. **Incubate at room temperature for 10-15 minutes in the dark.**
4. **Add approximately 2ml of ammonium chloride lysis buffer per 100µl whole blood. Incubate for ~2 minutes and vortex well.**
   Red cells are lysed when the opaque solution clears.
5. **Centrifuge, then wash the cells twice in wash buffer and store in fix solution in the dark.**
6. **The cells are now ready for 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.

Tel: +44 (0)870 0427279 · Fax: + 44 (0)870 7120588 · Email: enquiries@proimmune.com · Web: www.proimmune.com
ProImmune Limited · The Magdalen Centre · The Oxford Science Park · Robert Robinson Avenue · Oxford OX4 4GA · United Kingdom
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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 0.01% 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 your 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 your 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 Pentamer is equivalent to 10µl of reagent it is important that you first titrate the Pentamer. Carry out a range of doubling dilutions from 1 test per 1 x 10^6 cells down to 1/16 test per 1 x 10^6 cells.

CD8 antibody Investigate the effect of titrating your anti-CD8 antibody. Also, try incubating your cells first with the MHC-peptide complex for approximately 10 minutes before adding the anti-CD8 antibody. This will prevent any antibody-mediated blocking of the Pentamer-binding site.

Temperature The temperature at which you carry out the staining can affect the background considerably. Although staining at 22°C (room temp.) is recommended in the first instance, incubating at 22ºC or 37°C may be beneficial in optimizing the signal to noise ratio depending upon the MHC/peptide combination (Whelan et al., 1999). The higher the incubation temperature, the shorter the incubation time required. Try comparing staining at 4°C, 22°C and 37°C.

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 x 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 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). You may also find that increasing the concentration of azide in the wash buffer helps to reduce any background staining.

Intracellular staining It is possible to co-stain samples with antibodies against intracellular markers. Cells should first be stained with the 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, Pentamer-positive cells can be sorted and collected for further manipulation. In this case it is advisable to remove the azide preservative in the 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 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-PE or APC. R-Phycoerythrin (PE) is most commonly used as it stains very brightly. The anti-CD8 antibody is normally conjugated to FITC or PE-Cy5.

Reference
Whelan, J.A., *et al.* 1999. Specificity of CTL interactions with peptide-MHC class I tetrameric complexes is temperature dependent. *J Immunol*. 163:4342-4348.

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