Tetramer enrichment for HBV-specific CD8+ T cells

Immunology Assays

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Introduction

The frequency of HBV-specific CD8+ T cells can be very low, depending on the specificity. Therefore, they cannot always be detected by conventional *ex vivo* tetramer staining using 10^6 PBMCs. Tetramer-based magnetic enrichment enables the detection of these rare virus-specific CD8+ T-cell population by concentration of tetramer-labeled cells. Additionally, if an *ex vivo* response by conventional tetramer staining can be detected, this enrichment approach can increase the frequency of tetramer-positive cells for the following analysis.

Materials and Reagents

- Tetramers of HLA-A*02-restricted HBV-derived epitopes ($core_{18}$:FLPSDFFPSV, pol_{455}: GLSRYVARL, env_{183}: FLLTRILTI) (The tetramers are not commercially available but gained from a cooperation with David Price (Cardiff University, UK.)
- Anti-fluorochrome MACS MicroBeads
    - Anti-PE, order no: 130-048-801
    - Anti-APC, order no: 130-090-855
- MACS LS columns (Miltenyi Biotec, order no.: 130-042-401)
- MACS manual separator (MidiMACS™ or QuadroMACS™ Separators)
- MACS buffer (500 ml PBS, 2.5 g BsA (0,5%), 2 ml EDTA 0,5 M (2 mM))
- RPMI media (RPMI supplemented with 1640 with 10% fetal bovine serum, 1% penicillin-streptomycin and 1.5% 1 M HEPES)
- Benzonase nuclease (Milipore, order no.: 70746-3)

Experimental Procedures

1. Prepare the cells you want to use for enrichment.

- Use freshly isolated peripheral blood mononuclear cells (PBMCs) or thaw frozen PBMCs in pre-warmed RPMI.
- Thawing can be performed with Bezonase nuclease (e.g. if cells are frozen for >10 years or cell clumping is expected for whatever reason).
- Use at least 10^7 PBMCs
- Depending on the frequency of the analyzed virus-specific CD8+ T cells, enrichment can be performed also with less cells.

2. Centrifuge cells (500xg, 10 minutes) and discard the supernatant.
3. Optional: Incubation of cells with Benzonase-containing RPMI for 10-30min (50Uml $^{-1}$ Benzonase) at 37 °C, 5% CO2 (after incubation wash cells once with RPMI (500xg, 10

minutes)).
4. Resuspend cell pellet in 100µl MACS buffer.
5. Centrifuge tetramers at full speed for 4 min at 4°C before use. Add optimized volume of tetramers (labelled with APC or PE), resuspend, incubate 30 min at room temperature in the dark.

- Avoid light exposure when working with fluorochrome-conjugated tetramers!
- Consider tetramer titration for the appropriate amount you have to add!

6. After incubation add 5ml MACS buffer.
7. Centrifuge the cells (500xg, 10 minutes), discard the supernatant.
8. Add 50µl anti-APC and/or anti-PE beads (Miltenyi Biotec) and fill up to a final volume of 250µl with MACS buffer (the optimal concentration of beads should be titrated).

- If you use more cells (e.g. $8x10^7$ PBMCs) or the frequency of your virus-specific CD8+ T cells is very high you probably have to adjust the volume of beads to catch all your tetramer+ cells (the original enrichment protocol used 100µl of beads and a final volume of 500µl).

9. Incubate 20 minutes at 4°C in the dark.
10. Add 5ml MACS buffer and centrifuge the cells (500xg, 10 minutes), discard the supernatant.
11. Resuspend cell pellet in 1ml MACS buffer.
12. Remove 5µl for counting the “pre”-fraction if you want to calculate your frequency.
    - Caution! Your total volume is about 1.2ml (because the cells also have a volume), consider this in your calculation of the total “pre” cell number.

13. Remove 5µl for staining of the “pre”-fraction and plate them in your staining plate.

- before removing the cells you can add 5µl into the “pre” wells of the staining plate to prevent evaporation of the low volume of 5µl.
- in general, staining of the pre-fraction is required for the calculation of the frequency of enriched virus-specific CD8+ T cells (number of tetramer+ cells/ number of CD8+ cells).

14. Perform a magnetic separation with according to the manufacturer’s instructions (use a LS column even if your initial cell count was very low – the enrichment does not really work well with MS columns).

- Place the column into the MACS magnet.
- Place a 15ml falcon tube under the column.
- Equilibrate LS column with 3ml of MACS buffer, let the whole buffer run through the column.
- Discard the falcon tube and place a new tube under the column.
- Add your cell suspension onto the column, let run through.
- Add 3ml of MACS buffer onto the column, let run through -> the cells collected in the falcon represent the “depleted” fraction.
- Place the column onto a fresh 15ml falcon tube (outside of the MACS magnet) and add 5ml MACS buffer.
- Elute the labeled cells with the plunger -> this is your “enriched” fraction.

All further steps are for staining the enriched population (for flow cytometric analysis).

15. Centrifuge eluted cells (500xg, 10 minutes).
16. During centrifugation remove cells from the depleted fraction for the single stains you may need for compensation (about 100µl each).
17. After centrifugation discard supernatant (but leave about 150µl).
18. resuspend cells in the leftover MACS buffer and transfer them to your staining plate (you can also rinse the falcon once again with another 100-150µl MACS buffer).

Handling & storage All reagents used for this protocol should be stored at 4°C. In the case of tetramers and antibodies: avoid light exposure. The benzonase is stored at -20 °C.

References

For more information visit homepage of manufacturer: https://www.miltenyibiotec.com