Protocol for the isolation and expansion of antigen-specific cells from a mixed population

Materials and Equipment

- Cell sample, e.g. Blood sample (RBC-depleted), PBMCs or murine splenocytes
- Pro5® Recombinant MHC Pentamer conjugated to biotin.
- *Either:*
    - Streptavidin magnetic beads to detect the biotin label of the Pentamer, suitable for use with magnetic columns (e.g. Streptavidin Microbeads, Miltenyi Biotec #130-048-102)
    - Magnetic stand e.g. MACS Multistand, Miltenyi Biotec #130-042-303
    - Separation unit e.g. MiniMACS separation unit, Miltenyi Biotec #130-042-102
    - Magnetic columns e.g. MS Columns, Miltenyi Biotec #130-042-201

  Or:
    - Streptavidin magnetic beads to detect the biotin label of the Pentamer, suitable for use with an external magnet (e.g. Dynabeads® M-280 Streptavidin, Invitrogen #112-05D; Lodestars™ 2.7 Streptavidin, Polymer Laboratories #6727-1001)
    - Magnetic tube holder
- 96-well cell culture plates, U-bottom
- Complete IMDM (IMDM medium containing 5% fetal calf serum, 10% AB0 serum (for human cells) or 5% murine serum (for mouse cells) and 50 IU/ml IL-2 + penicillin/streptomycin)
- Wash buffer (0.1% sodium azide, 0.1% BSA in PBS) - remove air prior to use
- Benchtop centrifuge with swing-out rotor and appropriate carriers
- Hemacytometer and microscope for cell counting
- Humidified CO_2 incubator, heated to 37°C

Standard Procedures *(may be scaled up or down according to requirements and may need adjustment depending on the particular system used)*

Column-based isolation of antigen-specific cells using beads from Miltenyi Biotec

1. **Stain 5-10 × 10^6 cells with 5 tests biotin-labeled Pentamer in 200 µl wash buffer for 15 minutes at room temperature.**
2. **Wash samples with 8 ml wash buffer, spin 400 × *g* for 5-10 minutes in a chilled centrifuge at 4°C, and discard the supernatant.**
3. **Resuspend cells in 90 µl wash buffer and add 10 µl streptavidin magnetic beads per tube** (or as directed by the manufacturer).
4. **Incubate for 20 minutes in the refrigerator.**
5. **Wash cell-bead complexes with 8 ml wash buffer, spin 400 × *g* for 5-10 minutes in a chilled centrifuge at 4°C and resuspend in 500 µl wash buffer (de-gassed).**
6. **Meanwhile, wash a column suitable for positive-selection with 500 µl wash buffer (de-gassed) and place on magnetic stand.**
7. **Load cell-bead complexes onto the column.** Antigen-specific T cells labeled with Pentamer-bead complexes will be retained on the column (positive fraction).
8. **Collect the negative fraction that elutes from the column, including 3 washes of 500 µl each.**
9. **Remove column from magnet and flush out the positive fraction by adding 1 ml wash buffer onto the column and applying a plunger (provided with the column).**

10. **To obtain a purer antigen-specific cell population, the positive cell fraction may be passed over a second column.**

Tube-based isolation of antigen-specific cells using beads from Invitrogen or Polymer Laboratories

1. **Stain 5-10 × 10^6 cells with 5 tests biotin-labeled Pentamer in 200 μl wash buffer for 15 minutes at room temperature.**
2. **Meanwhile, calculate the volume of streptavidin beads required for incubation with the cells. Wash this volume of beads 3 times with 3 ml wash buffer, using a magnetic tube holder, then resuspend in the original volume.** 5 beads per cell is recommended in the first instance.
3. **Wash cells with 8 ml wash buffer, spin 400 × *g* for 5-10 minutes in a chilled centrifuge at 4ºC and discard the supernatant.**
4. **Resuspend cells in 200 μl wash buffer.**
5. **Add the pre-washed streptavidin beads.**
6. **Incubate for 20 minutes at room temperature, mixing every 5 minutes.**
7. **Bring the volume in the tube up to 2 ml with wash buffer then place in a magnetic tube holder.**
8. **Leave for 3-5 minutes.** If desired, supernatant can be retained for flow cytometric analysis to confirm removal of antigen-specific cells, otherwise discard supernatant.
9. **Wash the fraction containing cell-bead complexes (attracted to wall of tube) 3 times with 500 μl wash buffer and discard supernatant.**

Cell culture / expansion

1. **Spin the isolated cells at 400 × *g* for 5-10 minutes in a chilled centrifuge and discard the supernatant.**
2. **Resuspend cells in 100 μl complete IMDM and transfer to one well of a U-bottom 96-well tissue culture plate.** Expansion is best if 1 × 10^5 irradiated autologous feeder cells (negative fraction from bead isolation) are also added to each well.
3. **Place the culture plate at 37°C in a humidified CO_2 incubator for desired incubation period.**
4. **After 8-10 days of expansion the cells should be counted and/or analyzed by flow cytometry to determine the extent of expansion.** For flow cytometric analysis, cells should be re-stained with Pentamer for 10 minutes at room temperature, then washed and incubated with anti-CD8 antibody and Fluorescent-labeled Streptavidin for 30 minutes at 4°C.
5. **If further expansion is desired, re-stimulation with fresh irradiated autologous feeder cells should be carried out at day 10 - 12 of culture.**
6. **With careful culture technique and good re-stimulation every 10 - 12 days, the cells may be cultured for 1 - 2 months.**

n.b. Cellular stimulation generally results in down-regulation of the T cell receptor. For this reason, Pentamer staining should be carried out on cells that have been rested (i.e. no re-stimulation) for 7 - 10 days. This will ensure a good level of staining.

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PR20 Version 1.0 02/2007

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