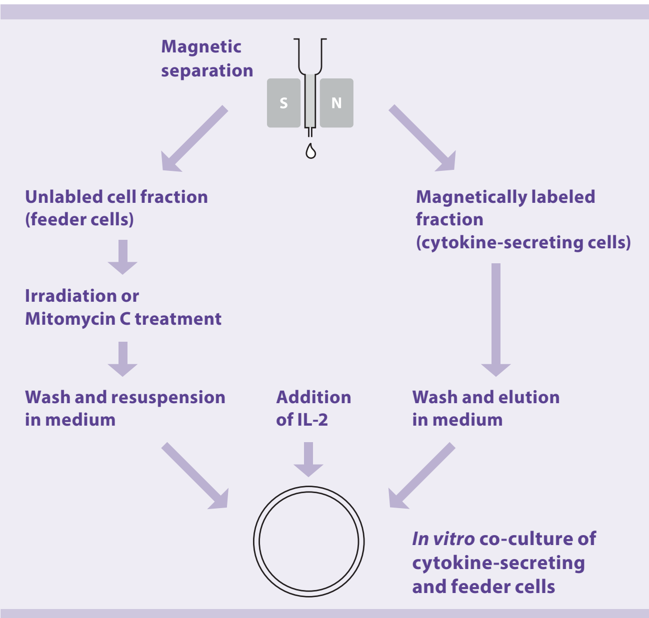
Expansion of antigen-specific cytokine-secreting T cells

Contents

1. Description

1.1 Principle of *in vitro* expansion of T cells isolated with the MACS® Cytokine Secretion Assay

This expansion system is designed for *in vitro* expansion of cytokine-secreting, antigen-specific T cells. The antigen-specific T cells in PBMCs or leukapheresis are restimulated with antigen. The responding cells are magnetically isolated, according to their secretion of cytokines, using the MACS® Cytokine Secretion Assay. The non-cytokine secreting cell fraction, i.e., the unlabeled flow through, is used as a source of antigen-presenting cells (referred to as "feeder cells" throughout the protocol) after irradiation or treatment with Mitomycin C. The cytokine-secreting cells and the feeder cells are co-cultured *in vitro* in the presence of IL-2.



1.2 Background information

This protocol is designed to expand antigen-specific T cells which have been isolated using the MACS® Cytokine Secretion Assay after restimulation with antigen. These T cells are therefore of a memory phenotype. The memory phenotype, particularly for $CD8^+$ cells, is heterogeneous. It contains cells, which divide rapidly in response to antigen-"central memory cells" and cells, which exhibit recall effector function, such as cytotoxicity-"effector memory cells". These subsets are not mutually exclusive, e.g., cells can divide rapidly but still lyse antigen-loaded targets. The mixture of memory cells isolated with the MACS Cytokine Secretion Assay is entirely dependent on the antigen the T cells responded to, e.g., immune responses against chronic virus infections are often characterized by a pre-dominance of effector memory T cells. As a general rule, central memory cells have a higher proliferation rate *in vitro*, compared to effector memory cells. **Therefore, the rate of proliferation of T cells expanded with this method will depend on the memory phenotype of the cells that have been isolated.** This protocol has been optimized to expand both central and effector memory T cells very efficiently. However, the individual results obtained may vary depending on the antigen and the Cytokine Secretion Assay used to isolate the cells. This protocol has been established for the expansion of CMV-specific, IFN-γ-secreting T cells. It may require optimization when working with other antigens or expanding cells which were isolated according to the secretion of cytokines other than IFN-γ.

1.3 Reagent and instrument requirements

- Cytokine Secretion Assay – Cell Enrichment and Detection Kit, e.g. IFN-γ Secretion Assay – Cell Enrichment and Detection Kit (# 130-054-201) or Large Scale IFN-γ Secretion Assay – Enrichment Kit (# 130-091-329)
- Buffer: Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2, 0.5% human serum albumin (HSA) or AB serum (do **not use BSA** or **FCS** because of non-specific stimulation!) and 2 mM EDTA (e.g. 4 mL of a 0.5 M EDTA stock solution per 1 liter of buffer). Keep buffer cold (2–8 °C). Degas buffer before use, as air bubbles could block the column.

  - 0.5 M EDTA stock solution: dissolve 56 g sodium hydroxide (NaOH) in 900 mL double-distilled water (ddH_2O). Add 146.2 g ethylene-diamine-tetraacetic acid (EDTA), adjust pH to 7.5 and fill up to 1000 mL with ddH_2O.
- Culture medium, e.g. RPMI 1640 containing 5–10% human serum, e. g., autologous or AB serum (do **not use BSA** or **FCS** because of non-specific stimulation!) or serum-free media may be used.

  ▲ **Note:** Serum-free media should not be used in the stimulation phase of the Cytokine Secretion Assay: there is a tendency towards higher background reactivity and more variability in the response to protein antigen.
- Human IL-2 IS, premium grade (# 130-097-744).

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- MACS Columns and MACS Separators:

Column	Max. number of labeled cells	Max. number of total cells	Separator
MS	10^7	2×10^8	MiniMACS, OctoMACS, VarioMACS, SuperMACS II
LS	10^8	2×10^9	MidiMACS, QuadroMACS, VarioMACS, SuperMACS II,
autoMACS	2×10^8	4×10^9	autoMACS Pro
CliniMACS Tubing Set		4×10^{10}	CliniMACS Plus Instrument

▲ **Note:** Column adapters are required to insert certain columns into the VarioMACS™ or SuperMACS™ II Separators. For details refer to the respective MACS Separator data sheet.

- Appropriate cell culture containers: culture dishes or gas permeable culture bags, e.g. MACS GMP Cell Expansion Bags (# 170-076-403)
- (Optional, not required when feeder cells are irradiated) Mitomycin C (Sigma). Mitomycin C is extremely toxic. Observe all manufacturer's handling instructions.

  - (Optional) Prepare a stock solution of 1 mg/mL Mitomycin C in culture medium. It can be kept at 4 °C for at least one month.
- (Optional) Irradiation device
- Pre-Separation Filters (30 µm) (# 130-041-407)

2. Protocol

▲ All steps in the following protocol have to be performed under sterile conditions.

2.1 Cell preparation

Prepare and restimulate cells with antigen and perform the Cytokine Secretion Assay referring to the respective data sheet (refer to 4. Protocol for Cytokine Secretion Assay, steps 4.1–4.4).

2.3 Magnetic separation using MS or LS Columns

▲ Choose an appropriate MACS Column and MACS Separator according to the number of total cells and the number of IFN-γ–secreting cells. For details refer to the table in section 1.4.

▲ Always wait until the column reservoir is empty before proceeding to the next step.

▲ When enriching antigen-specific T cells, **always perform two consecutive column runs** to achieve best results.

Magnetic separation with MS or LS Columns

1. Prepare **two columns** by rinsing with the appropriate amount of cold buffer. :

   MS: 500 µL LS: 3 mL

2. Discard effluent.
3. Place the first column into the magnetic field of a MACS Separator (use column adapter with VarioMACS or SuperMACS Separator).
4. (Optional) Pass the cells through Pre-Separation Filters (30 µm) to remove clumps.
5. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells.
6. Wash column with the appropriate amount of cold buffer. Collect unlabeled cells that pass through and combine with the flow-through from step 5 in a sterile tube. Keep it sterile for further processing as feeder cells.

   MS: 3×500 µL LS: 3×3 mL

   ▲ **Note:** Perform washing steps by adding buffer aliquots as soon as the column reservoir is empty.

7. Remove first column from the separator, place the second column into the separator and put the first column on top of the second one.
8. Pipette the appropriate amount of cold buffer onto the first column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the first column directly onto the second column.

   MS: 1 mL LS: 5 mL

9. Collect unlabeled cells that pass through and wash column with appropriate amount of cold buffer and medium. Perform the first washing step by adding buffer, perform two further washing steps by adding cold medium, successively once the column reservoir is empty.

   MS: 1×500 µL LS: 1×3 mL with **cold buffer.**
   MS: 2×500 µL LS: 2×3 mL with **cold medium.**

10. Remove the second column from separator and place column on a suitable sterile tube.
11. Pipette appropriate amount of **cold medium** onto the column. Immediately flush out the magnetically labeled T cells by firmly pushing the plunger into the column. Collect T cells in a sterile tube and keep it under sterile conditions.

    MS: 1 mL LS: 5 mL

12. Take an aliquot for cell counting and flow cytometric analysis and proceed to cell culture (refer to 2.3.2).

    ▲ **Note:** For flow cytometry cells should be washed with buffer to remove medium before analysis.

    ▲ **Note:** The remaining cells may be stored at room temperature until cultured further.

13. Take an aliquot for cell counting from the unlabeled fraction and proceed to preparation of feeder cells (refer to 2.3.1).

Magnetic separation with the autoMACS® Pro Separator

▲ Refer to the respective user manual for instructions on how to use the autoMACS® Pro Separator.

▲ Buffers used for operating the autoMACS Pro Separator should have a temperature of ≥10 °C.

1. Prepare and prime the instrument.
2. Pass cells through Pre-Separation Filters (30 µm) to remove cell clumps.
3. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube in row A of the tube rack and the fraction collection tubes in rows B and C.
3. For a standard separation choose following program:

   Positive selection: Posseld
   Collect magnetically labeled cells (positive fraction) in a sterile tube in row C of the tube rack. Collect feeder cells (negative fraction) in a sterile tube in row B of the tube rack.

4. Take aliquots for cell counting and flow cytometric analysis from the labeled and the unlabeled fraction.

5. Wash the magnetically labeled cells by adding an appropriate amount of medium and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely. Proceed to cell culture (refer to 2.3.2).
   ▲ **Note:** The remaining cells may be stored at room temperature until cultured further.

6. Proceed to preparation of feeder cells from the unlabeled fraction (refer to 2.3).

Magnetic separation using the CliniMACS® Plus Instrument

▲ For sample preparation refer to the protocol in the data sheet of the Large Scale IFN-γ Secretion Assay – Enrichment Kit.

▲ For set-up of the CliniMACS® Plus Instrument, selection of the separation program, and installation of the CliniMACS Tubing Set follow the detailed instructions given in the CliniMACS Plus User Manual.

1. Connect sample bag and CliniMACS Buffer bag to CliniMACS Tubing Set.

2. Separate cells using program version ENRICHMENT 3.1 or higher. The magnetically labeled cell fraction, i.e., the antigen-specific T cells will appear in the target T cell bag and the feeder cells in the non-target T cell bag.

3. Take aliquots for cell counting and flow cytometric analysis from the labeled and unlabeled fraction.

4. Wash the magnetically labeled cell fraction by adding an appropriate amount of medium and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely. Proceed to cell culture (refer to 2.3.2).
   ▲ **Note:** The remaining cells may be stored at room temperature until cultured further.

5. Proceed to preparation of feeder cells from the unlabeled fraction (refer to 2.3).

2.3 Expansion protocol

The protocol is based on standard T cell expansion procedures where specific T cells are cultured with autologous feeder cells, antigen, and IL-2. Using the cells collected from the unlabeled fraction, provides a convenient source of antigen pre-loaded feeder cells. As the target T cells are already highly activated and enriched they have high co-stimulation requirements to grow properly and require more feeder cells than in standard expansion procedure, where the initial frequency of antigen-specifi c T cells is low. Therefore, in the first phase of expansion, 100 feeder cells are required per antigen-specific T cell in the isolated positive fraction. After two weeks of expansion the T cells require to be restimulated with feeder cells (which still contain the antigen), at a ratio of 10 feeder cells per T cell. This cycle can be repeated every 2 weeks.

As there will be only a limited amount of cells from the unlabeled fraction, long term culture or quickly growing cells will require a new source of feeder cells. For this purpose, fresh PBMCs from the same donor may be used. The cells should be used in exactly the same way as for the unlabeled fraction, with the addition of antigen at the same concentration as used to originally stimulate the T cells. It is also possible that restimulation of T cells with frozen cells from the unlabeled fraction can be enhanced by addition of fresh antigen.

2.3.1 Preparation of feeder cells

In order to have feeder cells for later restimulation of the T cells, cells of the unlabeled fraction (or PBMCs from the same donor) can be frozen and later resuscitated.
To prevent the feeder cells growing out during culture, these cells need to be immobilised using irradiation or Mitomycin C treatment.

▲ **Note:** Cells should not be frozen after irradiation or Mitomycin C treatment.

Irradiation
For example, irradiate cells with 35–50 Gy (3500–5000 rads) using a caesium 137 source. Individual irradiators may vary in performance, and the optimum degree of irradiation has to be determined.

Mitomycin C
▲ Mitomycin C is extremely toxic. Observe all manufacturer's safety warnings.

1. Determine cell number of the unlabeled fraction.

2. Wash cells of the unlabeled fraction once in culture medium, centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.

3. Prepare a fresh working solution of 25 μg/mL Mitomycin C in culture medium.

4. Add 500 μL of Mytomycin C working solution per 10^7 cells.

5. Incubate cells for 30 minutes at 37 °C.

6. Wash the cells four times in culture medium. Each time centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.

7. Determine cell number and adjust cells to a final density of 10^7 cells/mL.
   ▲ **Note:** It is normal to see a reduction in cell numbers by up to 50% after Mitomycin C treatment. This is due to the death of many B cells, and some general cell loss during the washing steps.

2.3.2 Initiation of T cell expansion

1. Determine total number of antigen-specific T cells in the positive fraction.
   ▲ **Note:** Cell count of antigen-specific T cells needs to be determined, not total cell count, as monocytes or dead cells may be co-enriched with the antigen-specific T cells. Flow cytometric analysis should be used to get an accurate count.

2. Adjust the antigen-specific T cell fraction to 10^5 cells/mL in medium.

3. Combine the antigen-specific T cells with an equal volume of prepared feeder cells (at 10^7 cells/mL). Mix well and plate cells in an appropriate cell culture dish at 5×10^6 cells/cm², e.g. 2 mL of cell suspension in one well of a 24 well plate (see 3. Appendix: Flask and dish sizes for T cell expansion).

4. Add 20 IU of IL-2 per mL of medium.

5. Incubate at 37 °C, 5–7.5% CO_2.

The final concentrations are:
- 100 feeder cells per antigen-specific T cell
- 5×10^6 cells per mL
- 5×10^6 cells per cm²
- 20 IU IL-2/mL

▲ **Note:** 20 IU IL-2 is a low dose, some cells may need 20–100 IU IL-2.

2.3.3 Maintenance of culture

The following are guidelines as to how cells should grow; the rates of growth will depend on the phenotype of the cells generated. After one or two days it is normal that large clumps of cells appear in the culture. Cells should be observed daily to ensure that medium is not depleted of nutrition. A basic schedule is to feed the T cells every 3–4 days with fresh medium supplemented with 20 IU IL-2 at day 3, 7, and 11 (start is day 0). By day 14, cells need to be restimulated with feeder cells and antigen, followed by a repeated cycle of feeding with medium and IL-2.

In detail

▲ If at any point the indicator of the medium turns yellow, remove about 70% of the medium with a pipette without resuspending the cells. Replace volume with medium supplemented with 20 IU/mL of IL-2, and transfer half of the culture to a new identical culture dish.

Day 0:

Start (see 2.3.2)

Day 3:

- Fast-growing cells
  - Gently resuspend the cells using a pipette and transfer half of the culture to a new identical culture dish. Add an equivalent volume of medium supplemented with 20 IU/mL of IL-2 to each well.
- Slow-growing cells
  - Gently resuspend the cells using a pipette and add 20 IU/mL IL-2 (no fresh medium).

Day 7:

- Count cells and determine culture viability.

▲ **Note:** At this point the culture is a mixture of growing antigen-specific T cells and dying feeder cells, so total viabilities of 50% are common.

- Adjust the cell concentration to 5×10^5 viable T cells per mL with medium supplemented with 20 IU/mL of IL-2.

Day 11:

- Repeat procedure from Day 3.

Day 14:

- Harvest T cells, and determine cell count and viability.
- If desired, or if there are large numbers of dead cells in the culture, the cells may be purified by density gradient centrifugation.
- Wash cells by adding medium and centrifuge at 300×g for 10 minutes. Aspirate supernatant.
- Remove supernatant and resuspend cells in 1 mL of medium per 10^6 cells.
- Prepare feeder cells as before, and resuspend the cells in 1 mL of medium per 10^7 cells (see 2.3.1).
- Combine equal volumes of the feeder cells and T cells in a suitable culture vessel and incubate as before.
- Add 20 IU of IL-2 per mL of medium.
- This 2-week cycle may be repeated to continue proliferation of T cells.

▲ Long term (> 4 weeks) culture of T cells selects the cells that grow best in culture – the longer the cells are cultured, the less they will reflect the original effector phenotype of the isolated cells (antigen-specificity is unaltered).

▲ Some T cells may require to be restimulated with feeder cells on a weekly basis after the initial 14-day period.

3. Appendix: Flask and dish sizes for T cell expansion

For T cell expansion the cells should be resuspended in culture medium, containing 5% of human serum and 20 IU of IL-2 at 5×10^6 cells/mL. The cells should be plated at a density of 5×10^6 cells/cm². Both the dilution and the cell density are important to assure optimal stimulation and cell growth.

The following table lists culture plate, dish and flask sizes suitable for different cell numbers. It also indicates the appropriate amount of medium to add.

Total cell number	Medium volume to add	Culture plate	Well diameter
0.15×10^6	0.3 mL	96 well	0.64 cm
0.50×10^6	1 mL	48 well	1.13 cm
1×10^7	2 mL	24 well	1.60 cm
2×10^7	4 mL	12 well	2.26 cm
5×10^7	10 mL	6 well	3.50 cm
Total cell number	**Medium volume to add**	**Culture dish**	**Dish diameter**
4.5×10^7	9 mL	small	3.5 cm
10×10^7	20 mL	medium	6 cm
25×10^7	50 mL	large	10 cm
50×10^7	100 mL	extra large	15 cm
Total cell number	**Medium volume to add**	**Culture flask**	**Growth area**
12×10^7	24 mL	50 mL	25 cm²
40×10^7	80 mL	250 mL	75 cm²
80×10^7	160 mL	720 mL	162 cm²
120×10^7	240 mL	900 mL	225 cm²
Total cell number	**Medium volume to add**	**Culture bag**	**Growth area**
12.5×10^7	25 mL	25 mL	27 cm²
25×10^7	50 mL	50 mL	58 cm²
50×10^7	100 mL	100 mL	112 cm²

Refer to **www.miltenyibiotec.com** for all data sheets and protocols.