



Special protocol for Cytokine Secretion Assays

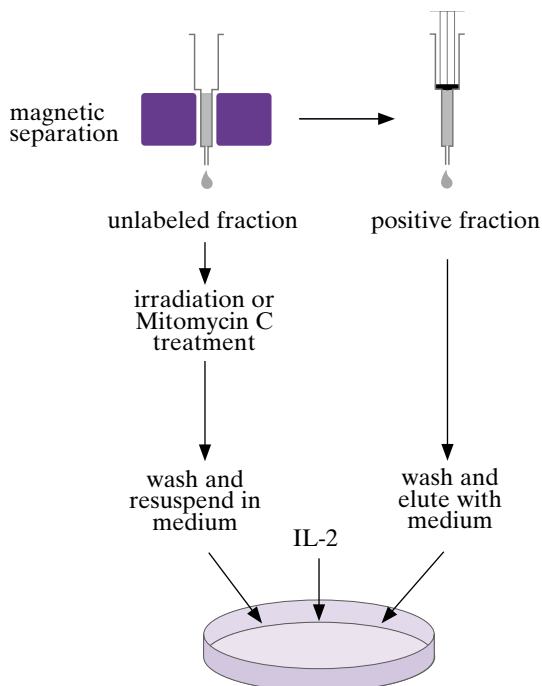
Index

1. Description
 - 1.1 Principle of *in vitro* expansion of T cells isolated with the MACS® Cytokine Secretion Assay
 - 1.2 Background
 - 1.3 Reagent and instrument requirements
2. Protocol
 - 2.1 Cell preparation
 - 2.2 Magnetic separation
 - 2.3 Expansion protocol
 - 2.3.1 Preparation of feeder cells
 - 2.3.2 Initiation of T cell expansion
 - 2.3.3 Maintenance of culture
3. Appendix: Flask and dish sizes for T cell expansion

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 PBMC or leukapheresis are restimulated with antigen. The responding cells are magnetically isolated, according to their secretion of cytokines, using the MACS® Cytokine



Expansion of antigen-specific cytokine-secreting T cells

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

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 predominance 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** (degassed): phosphate buffered saline pH 7.2, containing 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)
 - (0.5 M EDTA stock solution: dissolve 56 g sodium hydroxide (NaOH) in 900 mL dd H₂O. Add 146.2 g ethylene-diamine-tetraacetic acid (EDTA), adjust pH to 7.5, fill up to 1000 mL with dd H₂O).

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page 1/4

- **Culture medium**, e.g. RPMI 1640 (# 130-091-440) 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, e.g. X-VIVO15 (Biowhittaker), 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 Interleukin-2 (IL-2)**

- Biological activity of IL-2 may vary depending on manufacturer and batch. It needs to be determined individually (approximately 20 IU correlate with 10 ng).

- **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
LS	10 ⁸	2×10 ⁹	MidiMACS, QuadroMACS, VarioMACS, SuperMACS
autoMACS	2×10 ⁸	4×10 ⁹	autoMACS
CliniMACS tubing set		6×10 ¹⁰	CliniMACS ^{plus}

▲ **Note:** Column adapters are required to insert certain columns into VarioMACS or SuperMACS. For details, see MACS Separator data sheets.

- Appropriate cell culture containers: culture dishes, or gas permeable culture bags, e.g. T cell Expansion Bags (# 130-091-458, or # 130-091-459).
- (Optional, not required when feeder cells are irradiated) **Mitomycin C** (Sigma). **Warning: Mitomycin C is extremely toxic!** Observe all manufacturer's handling instructions.
- (Optional) Prepare a stock solution of 1mg/mL Mitomycin C in culture medium. It can be kept at 4 °C for at least one month.
- (Optional) Irradiation device.
- (Optional) Pre-Separation Filter (# 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 (see 4. Protocol for Cytokine Secretion Assay, steps 4.1– 4.4).

2.2 Magnetic separation



Magnetic separation using MS or LS Columns

▲ Choose an appropriate MACS Column and MACS Separator according to the number of total cells (see table in 1.3).

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

1. Prepare **two columns** per sample by rinsing with cold buffer:
MS: 500 µL LS Column: 3 mL.
and discard effluent.
2. Place the first column into the magnetic field of a MACS

Separator (use column adapter with VarioMACS™ Separator or SuperMACS™ Separator).

3. (Optional) Pass the cells through Pre-Separation Filter (# 130-041-407) to remove clumps.
4. Apply cell suspension onto the column.
5. Collect unlabeled cells which pass through and wash with appropriate amount of cold buffer. Perform washing steps by adding buffer successively once the column reservoir is empty.
MS: 3x500 µL LS: 3x3 mL.
Collect total effluent, the unlabeled cell fraction, in a sterile tube and keep it sterile for further processing as feeder cells.
6. Remove the first column from separator, place the second column into the separator, and put the first column on top of the second one.
7. Pipette an appropriate amount of cold buffer onto the first column. Immediately flush out the fraction with the magnetically labeled cells by firmly applying the plunger, supplied with the column directly onto the second column.
MS: 1 mL LS: 5 mL.
8. 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: 1x500 µL LS: 1x3 mL with **cold buffer**.
MS: 2x500 µL LS: 2x3 mL with **cold medium**.
9. Remove the second column from separator, place the column on a suitable sterile tube.
10. Pipette appropriate amount of **cold medium** onto the column. Immediately flush out the fraction with the magnetically labeled cells by firmly applying the plunger, supplied with the column.
MS: 1 mL LS: 5 mL.
Collect T cells in a sterile tube and keep it under sterile conditions.
11. Take an aliquot for cell counting and flow cytometric analysis and proceed to cell culture.
▲ **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.
12. Take an aliquot for cell counting from the unlabeled fraction and proceed to preparation of feeder cells (see 2.3.1).



Magnetic separation using the autoMACS™ Separator

▲ Refer to the "autoMACS™ User Manual" for instructions on how to use the autoMACS™ Separator.

1. Prepare and prime autoMACS Separator.
2. (Optional) Pass cells through Pre-Separation Filter (# 130-041-407) to remove clumps.
3. Place tube containing magnetically labeled cells in autoMACS Separator. Choose separation program "Posseld". Collect the separated fractions from outlet ports, "pos2" the magnetically labeled fraction, and "neg" the feeder cell fraction, into sterile tubes.

4. Take aliquots for cell counting and flow cytometric analysis from the labeled and the unlabeled fraction.
5. Wash the magnetically labeled cell fraction by adding medium and centrifuge at 300xg. Remove supernatant completely, and resuspend in medium.
▲ **Note:** The remaining cells may be stored at room temperature until cultured further.
6. Proceed to preparation of feeder cells from the unlabeled fraction (see 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 Enrichment program 3.1. 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 the unlabeled fraction.
4. Wash the magnetically labeled cell fraction by adding medium and centrifuge at 300xg. Remove supernatant completely, and resuspend in medium.
▲ **Note:** The remaining cells may be stored at room temperature until cultured further.
5. Proceed to preparation of feeder cells from the unlabeled fraction (see 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-specific 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 PBMC 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 PBMC 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 35–50cGy (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 the cell count of the unlabeled fraction.
2. Wash cells of the unlabeled fraction once in culture medium, centrifuge at 300xg for 10 minutes. Remove supernatant.
3. Prepare a fresh working solution of 25 $\mu\text{g}/\text{mL}$ Mitomycin C in culture medium.
4. Add 500 μL of Mitomycin C working solution per 10^7 cells. Incubate cells for 30 minutes at 37 °C.
5. Wash the cells **four** times in culture medium. Each time centrifuge at 300xg for 10 minutes and **remove supernatant completely**.
6. Check cell count again, 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% CO₂.

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 300xg. 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 size	well diameter
0.15×10^7	0.3 mL	96 well	0.64 cm
0.5×10^7	1 mL	48 well	1.13 cm
1×10^7	2 mL	24 well	1.6 cm
2×10^7	4 mL	12 well	2.26 cm
5×10^7	10 mL	6 well	3.5 cm
total cell number	medium volume to add	culture dish size	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 size	growth area
12×10^7	24 mL	50 mL	25 cm ²
40×10^7	80 mL	250 mL	75 cm ²
80×10^7	160 mL	720 mL	162 cm ²
120×10^7	240 mL	900 mL	225 cm ²
total cell number	medium volume to add	culture bag size	growth area
12.5×10^7	25 mL	25 mL	27 cm ²
25×10^7	50 mL	50 mL	58 cm ²
50×10^7	100 mL	100 mL	112 cm ²