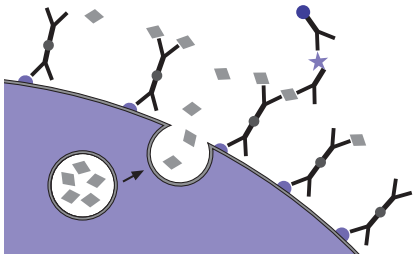
Miltenyi Biotec

Large Scale IFN-γ Secretion Assay – Enrichment Kit

human

For 10^9 cells, up to 100 separations Order no. 130-091-329



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Contents

1. Description

Components

7.5 mL **IFN-gamma Catchmatrix Reagent:** Anti-IFN-gamma monoclonal antibody (mouse IgG1) conjugated to cell surface specific monoclonal antibody (mouse IgG2a).

7.5 mL **IFN-gamma Enrichment Reagent:** Anti-IFN-gamma monoclonal antibody (mouse IgG1) conjugated to MACS® paramagnetic MicroBeads.

1 mL **IFN-γ Detection Antibody:** monoclonal antibody (mouse IgG1) conjugated to R-Phycoerythrin (**PE**).

Size	For 10^9 total cells, up to 100 separations.
Product format	IFN-γ Detection Antibody is supplied in buffer containing stabilizer and 0.05% sodium azide. IFN-gamma Catchmatrix Reagent and IFN-gamma Enrichment Reagent are supplied in buffer containing stabilizer.
Storage	Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label.

1.1 Principle of the MACS® Separation

Antigen-specific T cells are analyzed and enriched using the Large Scale IFN-γ Secretion Assay starting from leukapheresis harvests, PBMCs, or other leukocyte-containing single-cell preparations. The cells are restimulated for a short period of time with specific peptide, protein, or other antigen preparations. Subsequently, the cell suspension is incubated with the **IFN-gamma Catchmatrix Reagent** which binds to the cell surface of all leukocytes. The cells are then incubated for a short period of time at 37 °C to allow cytokine secretion. The secreted IFN-γ is bound to the **IFN-gamma Catchmatrix Reagent** on the antigen-specific secreting cells. These cells are subsequently labeled with the **IFN-gamma Enrichment Reagent,** for magnetic enrichment using either a manual MACS® Separator, the autoMACS™ Separator, the autoMACS™ Pro Separator or the CliniMACS® Plus Instrument. Therefore, the cell suspension is loaded onto a MACS Column which is placed in the magnetic field of the MACS Separator. The magnetically labeled cells are retained within the column. The unlabeled cells run through; this cell farction is thus depleted of IFN-γ–secreting cells. After removing the column from the magnetic field, the magnetically retained IFN-γ–secreting cells can be eluted as positively selected cell fraction. The quality of the separation can be analyzed using the Anti-IFN-γ-PE antibodies for sensitive detection by flow cytometry.

After the separation, the viable cells can be used for cell culture or analysis. For analysis, non-specific background can be minimized by dead cell exclusion. This provides highest sensitivity of analysis.

1.2 Background information

The Large Scale IFN-γ Secretion Assay is designed for the enrichment and analysis of viable IFN-γ-secreting leukocytes. After restimulation with specific antigen *in vitro,* secretion of IFN-γ is induced. IFN-γ is predominantly secreted by activated $CD4^+$ and $CD8^+$ memory and effector T cells and by NK cells upon activation.
Quantitative analysis of antigen-specific T cell populations can provide important information on the natural course of immune responses. Enrichment of the antigen-specific T cells by using MACS Technology increases the sensitivity of analysis, allowing detection of frequencies as low as one in a million cells.
The enrichment by using MACS Technology also allows further functional characterization of the antigen-specific cells and downstream experiments, as well as the expansion of antigen-specific cells, thus allowing research on potential future immunotherapeutical applications.

1.3 Applications

- Enrichment of IFN-γ-secreting, antigen-specific T cells for expansion and functional characterization.
- Isolation and expansion of antigen-specific T cells for research in immunotherapy.
- Enrichment and analysis of IFN-γ-secreting cells for determination of functional antigens in disease and for T cell receptor (TCR) epitope mapping.

- Enrichment of IFN-γ-secreting T cells for the generation of antigen-specific CD4 and CD8 positive T cell lines or clones.
- Pre-evaluation of protocols for the clinical scale enrichment and subsequent modifications of antigen-specific cells using the CliniMACS Cytokine Capture System (IFN-gamma).
- **Important note:**
  This product is for research use only and not for human use.

1.4 Reagent and instrument requirements

1.4.1 Requirements for research scale cell preparations

- 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, autoMACS Pro

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

- Refrigerated centrifuge

For 10^9 cells:

- Up to 1 liter of culture medium (RPMI 1640) (4 °C)
- 1.1 liter of culture medium, 10% AB serum (37 °C)
- 500 mL of culture medium, 2% AB serum (4 °C)
- 1 liter of buffer (PBS, 2 mM EDTA) containing 0.5% AB serum or HSA or autologous serum.
- Propidium iodide (final conc. 0.5 µg/mL) or 7-AAD (final conc. 1.0 µg/mL)

- (Optional for phenotyping) CD8-APC (# 130-091-076), CD8-FITC (# 130-080-601), CD4-APC (# 130-091-322), or CD4-FITC (# 130-080-501)
- (Optional) Pre-Separation Filters (# 130-041-407)
- (Optional) MACSmix™ Tube Rotator (# 130-090-753)
- (Optional) 250 mL or 500 mL conical tubes for processing of cell numbers higher than 10^8.

1.4.2 Requirements for clinical scale cell preparations using the CliniMACS® Plus Instrument

- CliniMACS® Plus Instrument (# 151-01)
- CliniMACS Tubing Set (# 161-01)
- Plasma extractor stand for bags (e.g. Terumo® Plasma Separation Stand)
- Sterile tubing welder (e.g. Terumo® TSCD)
- Transfer Bags 600 mL (# 190-01)
- 120 mL of AB serum or alternatively autologous serum as supplement for the media used
- Up to 1 liter of culture medium (4 °C) in bag(s)
- 1.1 liter of culture medium containing 10% AB serum (37 °C)
- 500 mL of culture medium containing 2% AB serum (4 °C)

- 3×1 liter of CliniMACS PBS/EDTA Buffer (# 700-25) or CliniMACS PBS/EDTA Buffer for research use (# 705-25)[1)] containing 0.5% of AB serum
- Human serum albumin (HSA)
- Orbital rotator
- Transfer Bag 150 mL (# 183-01)
- Transfer Set Coupler/Needle (# 185-01)
- Luer/Spike Interconnectors (# 187-01)
- Sampling Site Couplers (# 189-01)
- Cell Expansion Bag (tube) (# 200-074-301) or Cell Expansion Bag (tube) for research use (# 130-074-351)[1)]
- Slide clamps
- Pre-System Filter (# 181-01)
- Syringes (1 mL; 10 mL, 20 mL, 50 mL) and hypodermic 20 gauge needles
- 24 well culture dish (e.g. Corning® COSTAR® 24 well culture plate, Sigma Aldrich # CLS3526)
- MiniMACS™ Separation Unit (# 130-042-102)
- MS Column (# 130-042-201)
- MACSmix™ Tube Rotator (# 130-090-753)
- Pre-Separation Filters (# 130-041-407)

- Centrifuge suitable for bag processing (e.g. Sorvall®, Model RC3BP) and buckets for centrifugation with aerosol containment caps
- Table-top balance (e.g. Mettler Toledo, # 11274-998)
- Tubing heat sealer (Fenval, Hematron II, # 4R4335)
- Tubing stripper (Fenval, # 4R415)
- Propidium iodide (final conc. 0.5 µg/mL) or 7-AAD (final conc. 1.0 µg/mL)
- (Optional for phenotyping) CD8-APC (# 130-091-076), CD8-FITC (# 130-080-601), CD4-APC (# 130-091-322), or CD4-FITC (# 130-080-501)

For availability in your country please contact your local representative.

1) Available in the US only.

2. Protocol (research scale)

Starting from a maximum of 10^9 total cells, either from leukapheresis harvest or peripheral blood, antigen-specific T cells can be enriched using the Large Scale IFN-γ Secretion Assay Enrichment Kit.
In the following a **research scale** protocol for the enrichment of antigen-specific cells from **10^8 total cells** is given (for the processing of other cell numbers volumes must be adjusted accordingly). The protocol is optimized for cell samples containing <5% of IFN-γ–secreting cells.

2.1 Sample preparation

Cells can be processed in standard laboratory cell-culture vessels.

When working with anticoagulated peripheral blood or buffy coat, peripheral blood mononuclear cells (PBMCs) should be isolated by density gradient centrifugation, for example, using Ficoll-Paque™.

Starting from PBMCs a maximum of 10^8 total cells should be transferred into a 50 mL conical tube.

1. Add cold culture medium up to 50 mL.
2. Centrifuge at 200×g for 10 minutes at 4 °C to remove platelets.
3. Carefully remove supernatant, add cold culture medium and repeat washing step.
4. After second wash, resuspend cells in 10 mL of culture medium containing 10% AB serum.

2.2 *In vitro* restimulation

▲ For *in vitro* restimulation the cells must be cultured in appropriate culture vessels.

▲ Optimal cell density for all samples is 5×10^6 cells/cm² and 10^7 cells/mL.

▲ Take an aliquot (e.g. 10^7 cells) of the cells to serve as control sample (unstimulated).

Control sample (unstimulated):

Culture the aliquot under optimal conditions in 1 mL of culture medium in one well of a 24 well-plate.

Target sample:

1. Transfer cells into a cell culture dish with 6 cm diameter.
2. Add antigen to the culture vessel at appropriate concentration using a pipette.

If proteins or cell lysates are used as specific antigen, incubation time is between 4 and 16 hours. If cells are to be stimulated with peptide, incubation time is 3–6 hours. In this case cells can either be stimulated directly or after overnight cultivation. In the latter case stimulation with antigen can then be carried out the following morning.

Stimulation conditions:

for peptides:	3–6 hours	at 37 °C,	5–7.5% CO_2
for proteins / cell lysates:	4–16 hours	at 37 °C,	5–7.5% CO_2

▲ **Note:** A sample stimulated with CytoStim (# 130-092-172 or # 130-092-173) can be used as positive control. For details refer to the respective data sheet.

2.3 Labeling cells with the IFN-gamma Catchmatrix Reagent

▲ All centrifugation steps must be carried out in a cold centrifuge (4 °C)!

Control sample (unstimulated):

1. Transfer the cells to a 15 mL closable tube and fill up with cold culture medium supplemented with 2% AB serum.
2. Centrifuge cells at 300×g for 10 minutes and carefully remove supernatant.
3. Resuspend cell pellet in 100 µL of cold medium, add 75 µL of the IFN-gamma Catchmatrix Reagent, and incubate the suspension for 5 minutes on ice.

Stimulated sample:

1. Transfer cells to 50 mL conical tube and fill up with cold culture medium containing 2% AB serum.
2. Centrifuge cells at 300×g for 10 minutes and carefully remove supernatant.
3. Resuspend cell pellet in 1 mL of cold medium, add 750 µL of the IFN-gamma Catchmatrix Reagent, and incubate for 5 minutes on ice.

2.4 Secretion phase

▲ **For the secretion phase the vessel of choice must be of sufficient size to allow the addition of at least 1 volume of cold buffer at the end of the secretion phase. Cell density must not exceed 10^6 cells/mL**.

▲ If larger cell numbers are to be processed, it may be necessary to divide the sample and distribute it among several vessels.

Control sample:

1. Add warm culture medium (37 °C) containing 10% AB serum to dilute the sample to a minimum of 10^6 cells/mL, i.e., a total volume of 10 mL must be used for the 10^7 cells.
2. Incubate cells in closed tubes for 45 minutes at 37 °C under slow continuous rotation using the MACSmix Tube Rotator (# 130-090-753) or turn tubes every 5 minutes to resuspend settled cells.

3. Add 5 mL of cold buffer (4 °C), put the tube on ice for 5 minutes, and centrifuge cells at 300×g for 10 minutes at 4 °C.
4. Completely remove supernatant, resuspend cells, and wash once in 15 mL of cold buffer(4 °C).
5. Centrifuge cells at 300×g for 10 minutes at 4 °C.

Target sample:

1. Add 4 mL of warm culture medium containing 10% AB serum and distribute suspension among four 50 mL conical tubes.
2. Add 24 mL of warm culture medium containing 10% AB serum to each of the tubes.
3. Incubate cells in closed tubes for 45 minutes at 37 °C under slow continuous rotation using the MACSmix Tube Rotator (# 130-090-753) or turn tubes every 5 minutes to resuspend settled cells.
4. After exactly 45 minutes fill the tubes with cold buffer (4 °C).
5. Put the tubes on ice for 10 minutes.
6. Centrifuge cells at 300×g for 10 minutes at 4 °C.
7. Resuspend the pellets each in 10 mL of cold buffer and recombine the cells by collecting the suspensions in one 50 mL conical tube.
8. Centrifuge cells at 300×g for 10 minutes at 4 °C.

2.5 Labeling with the IFN-gamma Enrichment Reagent

Control sample (unstimulated):

1. Carefully remove supernatant.
2. Resuspend the cell pellet in 100 µL of cold buffer, add 75 µL of the IFN-gamma Enrichment Reagent, and incubate for 15 minutes on ice.
3. Fill the vessel with cold buffer and centrifuge at 300×g for 10 minutes at 4 °C.
4. Carefully remove supernatant and resuspend cells in 1 mL of cold buffer (4 °C).

Target sample:

1. Carefully remove supernatant.
2. Resuspend cell pellet in 1 mL of cold buffer, add 750 µL of the IFN-gamma Enrichment Reagent to the cell pellet, and incubate for 15 minutes on ice.
3. Fill the vessel with cold buffer and centrifuge at 300×g for 10 minutes at 4 °C.
4. Carefully remove supernatant and resuspend cells in 1 mL of cold buffer (4 °C).

2.6 Magnetic separation using MS or LS Columns

▲ **Remove small aliquots of the cell suspensions before and after enrichment and store in the cold until analysis.**

▲ Choose an appropriate MACS Column and MACS Separator according to the number of total cells. For details see table in section 1.3.1.

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

Control sample:

Follow the instructions given below for the target sample.

Target sample:

1. Place one column per sample in the magnetic field of a suitable MACS Separator. For details see the respective MACS Column data sheet.
2. Prepare columns by rinsing with the appropriate amount of cold buffer:
   MS: 500 µL LS: 3 mL
3. (Optional) Pass cells through 30 µm nylon mesh (Pre-Separation Filters, # 130-041-407) to remove cells clumps which may clog the column. Wet filter with buffer before use.
4. Apply cell suspension onto the columns.

5. Collect unlabeled cells that pass through and wash with the appropriate amount of cold buffer. Collect total effluent; this is the unlabeled cell fraction. Perform washing steps by adding buffer three times. Only add new buffer when the column reservoirs are empty.
   MS: 3×500 µL LS: 3×3 mL
6. Remove columns from the separators and place them on suitable collection tubes.

   ▲ **Note:** To perform a second column run, you may elute the cells directly from the first onto the second, equilibrated column instead of a collection tube.
7. Pipette the appropriate amount of buffer onto each column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
   MS: 1 mL LS: 5 mL
8. To increase the purity of the antigen-specific T cells, the eluted fractions can be enriched over a second column. Repeat the magnetic separation procedure as described in steps 1 to 7 by using new columns.

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2.7 Magnetic separation using the autoMACS™ Separator or the autoMACS™ Pro Separator

▲ **Remove small aliquots of the cell suspensions before and after enrichment and store in the cold until analysis.**

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

▲ Program choice depends on the isolation strategy, the strength of magnetic labeling, and the frequency of magnetically labeled cells. For details refer to the section describing the cell separation programs in the respective user manual. Program recommendations below refer to separation of human heterogenous blood cell preparations.

2.7.1 Magnetic separation with the autoMACS™ Separator

1. Prepare and prime the instrument.
2. (Optional) Pass cells through 30 µm nylon mesh (Pre-Separation Filters, # 130-041-407) to remove clumps.
3. Apply tube containing the sample and provide tubes for collecting the labeled and unlabeled cell fractions. Place sample tube at the uptake port and the fraction collection tubes at port neg1 and port pos2.

4. For a standard separation choose the following separation program:

   Positive selection: “Posseld”.
   Collect positive fraction from outlet port pos2.
5. Proceed to analysis, cell culture, or other subsequent experiment.

2.7.2 Magnetic separation with the autoMACS™ Pro Separator

1. Prepare and prime the instrument.
2. (Optional) Pass cells through 30 µm nylon mesh (Pre-Separation Filters, # 130-041-407) to remove 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 row B and C.
4. For a standard separation choose the following separation program:

   Positive selection: “Posseld”.
   Collect positive fraction in row C of the tube rack.
5. Proceed to analysis, cell culture, or other subsequent experiment.

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3. Protocol (clinical scale)

3.1 Sample preparation

▲ A maximum of 10^9 cells should be taken and transferred into a Transfer Bag 600 mL.

▲ If centrifugation steps are carried out with cell suspensions in bags, centrifugation without brake is recommended to avoid disturbance of the cell pellet for best recovery of cells. For the washing steps the bags should be connected to the medium bags or to empty bags (Transfer Bag 600 mL) for removal of supernatants respectively. This is best achieved using a sterile tubing welder.

1. Add cold culture medium up to 500 mL.
2. Centrifuge at 200×g for 10 minutes without brake.
3. Remove supernatant by coupling the sample bag to a new, empty 600 mL bag using a sterile tubing welder and placing the bag in a plasma extractor stand.
4. After removing the supernatant add cold culture medium and repeat washing step.
5. Resuspend cells to a final volume of 100 mL in culture medium containing 10% AB serum. This can easily be controlled by weighing the bag during the filling process.

3.2 *In vitro* restimulation

▲ For *in vitro* restimulation the cells must be cultured in appropriate culture vessels. For clinical scale applications we recommend the use of gas-permeable bags.

▲ Optimal cell density for all samples is 0.5–1×10^7 cells/cm^2 and 10^7 cells/mL.

▲ Take a small aliquot (e.g. 10^7 cells) of the cells to serve as control sample (unstimulated).

Control sample (unstimulated):

Culture the aliquot under optimal conditions in 1 mL of culture medium in one well of a 24 well-plate.

Target sample:

1. Transfer cells in 100 mL of culture medium into gas-permeable bag.
2. Add antigen to the sample bag at appropriate concentration by injection using a syringe.

If proteins or cell lysates are used as specific antigen, incubation time is between 4 and 16 hours. If cells are to be stimulated with peptide, incubation time is 3–6 hours. In this case cells can either be stimulated directly or after overnight cultivation. In the latter case stimulation with antigen can then be carried out the following morning.

Stimulation conditions:

for peptides:	3–6 hours	at 37 °C,	5–7.5% CO_2
for proteins / cell lysates:	4–16 hours	at 37 °C,	5–7.5% CO_2

▲ **Note:** A sample stimulated with CytoStim (# 130-092-172 or # 130-092-173) can be used as positive control. For details refer to the respective data sheet.

3.3 Labeling cells with the IFN-gamma Catchmatrix Reagent

▲ All centrifugation steps must be carried out in a cold centrifuge (4 °C)!

Control sample (unstimulated):

1. Transfer the cells to a 15 mL closable tube.
2. Add cold culture medium containing 2% AB serum up to 15 mL.
3. Centrifuge cells at 300×g for 10 minutes (4 °C) and carefully remove supernatant.
4. Resuspend the cells in 75 µL of the IFN-gamma Catchmatrix-Reagent and incubate the suspension for 5 minutes on ice.

Target sample:

1. Transfer the cells to a Transfer Bag 600 mL.
2. Fill the bag up to 500 mL with cold culture medium containing 2% AB serum.
3. Centrifuge cells at 300×g for 10 minutes without brake and carefully remove supernatant as described in section 3.1.
4. Adjust volume to 10 mL.
5. Add 7.5 mL of the IFN-gamma Catchmatrix Reagent vial to the pellet by injecting it into the bag using a sterile syringe.
6. Resuspend the cells and incubate them for 5 minutes on ice.

3.4 Secretion phase

▲ For the secretion phase the vessel of choice must be of sufficient size to allow the addition of at least 1 volume of cold buffer at the end of the secretion phase. Cell density must not exceed 10^6 cells/mL.

Therefore, 10^9 cells must be incubated for secretion in 1000 mL of warm culture medium.

Control sample (unstimulated):

1. Add warm culture medium (37 °C) containing 10% AB serum to dilute the sample to a maximum of 10^6 cells/mL, i.e. a total volume of 10 mL must be used for the 10^7 cells.
2. Incubate cells in closed tubes for 45 minutes at 37 °C under slow continuous rotation using the MACSmix Tube Rotator (# 130-090-753) or turn tube every 5 minutes to resuspend settled cells.
3. After 45 minutes, fill up tube with cold CliniMACS PBS/EDTA Buffer (4 °C), put tube on ice for 5 minutes, and centrifuge cells at 300×g without brake for 10 minutes at 4 °C.
4. Carefully remove supernatant, resuspend cells, and wash once in cold buffer (4 °C).
5. Centrifuge cells at 300×g without brake for 10 minutes at 4 °C.

Target sample:

1. Prepare 3 new bags with 125 mL of warm culture medium (37 °C) containing 10% AB serum.
2. Add 500 mL of warm culture medium (37 °C) containing 10% AB serum to the cell sample in the bag.
3. Distribute 125 mL of the resulting cell suspension in each of the three 'new' bags resulting in a total of 250 mL of cell suspension.
4. Add 125 mL of warm culture medium to the 'original' cell bag.
5. Incubate cells at 37 °C for 45 minutes with continuous slow rotation (max. 50 rpm).
6. After 45 minutes, add at least one volume, i.e. 250 mL each, cold CliniMACS PBS/EDTA Buffer (4 °C) containing 0.5% AB serum. Allow the cells to cool by placing the vessels on ice for 10 minutes.
7. Centrifuge cells at 300×g without brake for 10 minutes at 4 °C and completely remove supernatant.
8. Resuspend cells and wash them in cold CliniMACS PBS/EDTA Buffer (4 °C).

▲ Note: **At this point divided samples should be recombined:**

9. Attach one of the four Transfer Bags to the bag containing the CliniMACS PBS/EDTA Buffer using the sterile tubing welder.
10. Fill the bag with 200 mL of buffer.
11. After detaching the buffer bag from the transfer bag containing the cell suspension attach one of the other transfer bags containing the cells and recombine both suspensions.
12. Repeat this procedure until the cell suspensions from all four transfer bags are recombined.
13. Centrifuge cells at 300×g without brake for 10 minutes at 4 °C.

3.5 Labeling with IFN-gamma Enrichment Reagent

Control sample (unstimulated):

1. Carefully remove supernatant.
2. Resuspend cells in 100 µL of cold buffer and add 75 µL of the IFN-gamma Enrichment Reagent to the cell pellet.
3. Resuspend the cells and incubate for 15 minutes on ice.
4. Fill the vessel with cold buffer and centrifuge at 300×g for 10 minutes at 4 °C.
5. Carefully remove supernatant and resuspend cells in 1 mL of cold buffer (4 °C).

Target sample:

1. Carefully remove supernatant and adjust volume to 10 mL.
2. Add 7.5 mL of the IFN-gamma Enrichment Reagent to the resulting cell suspension.
3. Resuspend the cells and incubate for 15 minutes on ice.
4. Fill bag with cold buffer (4 °C) and centrifuge at 300×g without brake for 10 minutes at 4 °C.
5. Carefully remove supernatant as described in section 3.1 and resuspend cells in 100 mL of cold buffer (4 °C).

3.6 Magnetic separation using the CliniMACS® Plus Instrument

▲ **Remove small aliquots of the cell suspensions before and after enrichment and store in the cold until analysis.**

Control sample (unstimulated):

Follow the instructions given above for the research scale enrichment.

Target sample:

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 Instrument user manual.

1. Connect sample bag and bag containing the CliniMACS PBS/EDTA Buffer containing 0.5% AB-Serum to CliniMACS Tubing Set.
2. Separate cells using separation program ENRICHMENT 3.2.

4. Analysis

4.1 Evaluation of the separation performance

The purity of the cell fractions can be evaluated by flow cytometry using the PE-conjugated anti-IFN-γ antibodies provided.

1. Centrifuge aliquots of original and positive fraction of both stimulated and control sample in appropriate tube for flow cytometric analysis at 300×g for 10 minutes at 4 °C.
2. Remove supernatant and resuspend in 90 µL of cold buffer.
3. Add 10 µL of the IFN-γ Detection Antibody.
4. For further phenotyping analysis, antibodies specific for additional surface markers, for example, CD4-FITC (# 130-080-501) and CD8-APC (# 130-091-076) can be added.
5. Incubate for 10 minutes at 4 °C. Add 1 mL of cold buffer and wash cells by centrifugation at 300×g for 10 minutes at 4 °C.
6. Discard supernatant and resuspend cells in 500 µL of cold CliniMACS PBS/EDTA Buffer for research use. Add propidium iodide at a final concentration of 0.5 µg/mL just prior to flow cytometric analysis.

4.2 Example of a separation using the Large Scale IFN-γ Secretion Assay Enrichment Kit and the CliniMACS® Plus Instrument: Enrichment of CMV-specific T cells

Specimen: human PBMCs
Specific antigen: CMV pp65 – Recombinant Protein (# 130-091-823)
Stimulation time: 4 hours
Separation program: ENRICHMENT 3.2

Gating strategy:

For the analysis of cytokine-secreting cells it is crucial to exclude debris, monocytes, and dead cells. Therefore, 200,000 viable cells from the fractions before enrichment and the enriched fractions of the target sample should be acquired and all cells of the aliquots of the control sample.

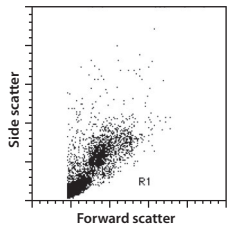
A lymphocyte gate based on forward and side scatter properties of the cells should be defined and activated prior to further gating.

Dead cells must then be excluded according to propidium iodide staining in a FL-2 versus FL-3 dot plot.

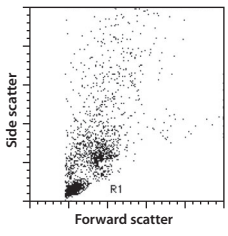
Analysis of secreted IFN-γ (-PE) versus CD4 (-FITC) or CD8 (-APC) staining by viable lymphocytes can thus be displayed as shown below.

Lymphocyte gate in the forward versus side scatter dot plot

Before enrichment

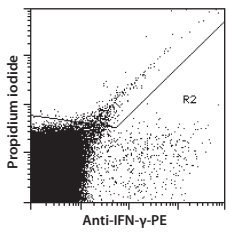


After enrichment

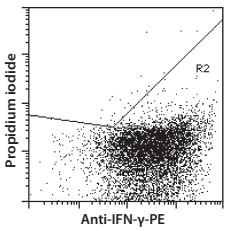


Dead cell exclusion in FL-2 versus FL-3

Before enrichment

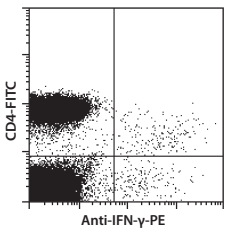


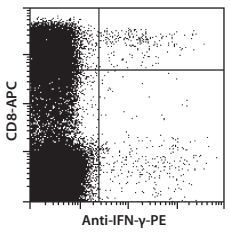
After enrichment



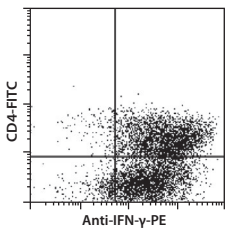
Antigen-specific CD4⁺ and CD8⁺ T cells stained for secreted IFN-γ

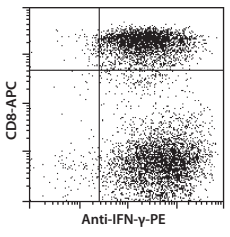
Before enrichment





After enrichment





Special protocols for flow cytometric analysis of antigen-specific T cells are available. For further information please contact our technical support team .

5. References

1. Bissinger, A. L. *et al.* (2002) Isolation and expansion of human cytomegalovirus-specific cytotoxic T lymphocytes using interferon-gamma secretion assay. Exp. Hematol. 30: 1178–1184.
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5. Rauser, G. *et al.* (2004) Rapid generation of combined CMV-specific $CD4^+$ and $CD8^+$ T-cell lines for adoptive transfer into recipients of allogeneic stem cell transplants. Blood 103: 3565–3572.

Refer to **www.miltenyibiotec.com** for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com for local Miltenyi Biotec Technical Support contact information.

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