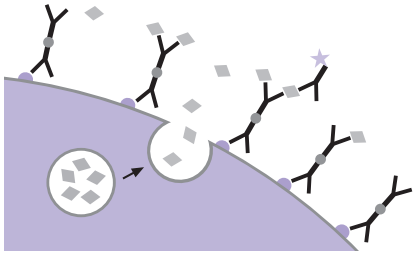
Miltenyi Biotec

IFN-α Secretion Assay – Detection Kit (PE)
human

For 100 tests with 10^6 cells

Order no. 130-094-161



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Contents

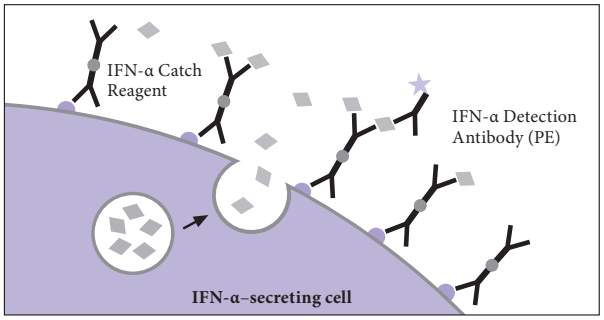
1. Description

Components	1 mL **IFN-α Catch Reagent, human**: anti-IFN-α monoclonal antibody (mouse IgG1) conjugated to cell surface-specific monoclonal antibody (mouse IgG2a). 1 mL **IFN-α Detection Antibody, human**: anti-IFN-α monoclonal antibody (mouse mIgG1) conjugated to PE (phycoerythrin).
Size	For 100 tests with 10^6 cells.
Product format	All components are supplied in buffer containing stabilizer and 0.05% sodium azide.
Storage	Store protected from light at 2–8 °C. Do not freeze. The expiration dates are indicated on the vial labels.

1.1 Principle of the IFN-α Secretion Assay

IFN-α-secreting cells can be analyzed using the IFN-α Secretion Assay starting from whole blood, PBMCs, or other leukocyte containing single-cell preparations.

The cells are stimulated for a certain period of time with a suitable stimulation reagent (e.g. viral compounds for the stimulation of monocytes or CpG motif containing DNA for plasmacytoid dendritic cells).



Subsequently, an IFN-α–specific **Catch Reagent** is attached to the cell surface of all leukocytes. The cells are then incubated for a short time at 37 °C to allow cytokine secretion. The secreted IFN-α binds to the IFN-α Catch Reagent on the positive, secreting cells. These cells are subsequently labeled with a second IFN-α–specific antibody, the **IFN-α Detection Antibody** conjugated to phycoerythrin (PE) for sensitive detection by flow cytometry.

Since viable cells are analyzed, non-specific background can be minimized by dead cell exclusion. This provides highest sensitivity of analysis.

1.2 Background information

Type I interferons (IFNs), including IFN-α, are a family of cytokines that have pleiotropic functions in the immune system.[1] Besides its potent antiviral activity, IFN-α exerts a variety of immunomodulatory effects, including positive and negative regulation of its own production, regulation of IL-12 and IFN-γ production, promotion of natural killer (NK) cell cytotoxicity, activation and maturation of dendritic cells, and the promotion of B cell development towards plasma cells.[2,3]

IFN-α is expressed predominantly by $CD123^{+}CD11c^{-}CD303$ (BDCA2)$^{+}$ plasmacytoid dendritic cells, also referred to as natural interferon producing cells. These cells representing approximately 0.4% of total PBMCs, produce up to 1000-fold more type I IFNs than any other cell type. However, biologically relevant levels of IFN-α are also produced by other cell types, e.g. monocytes and myeloid dendritic cells.[4]

1.3 Applications

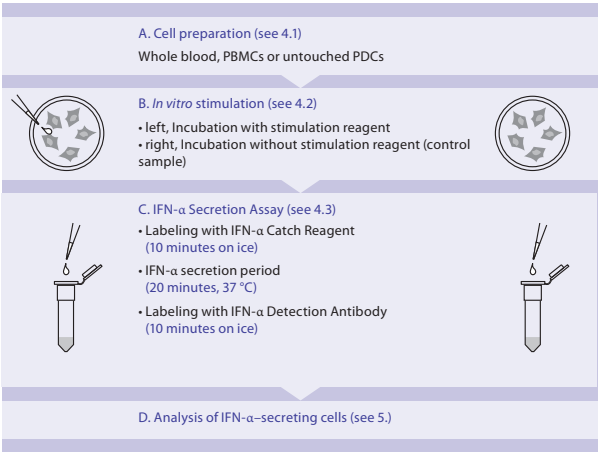
- Detection of viable IFN-α–secreting leukocytes.
- Detection of IFN-α–secreting cells for enumeration and phenotypic analysis.
- Monitoring and analysis of the stimulatory or regulatory functions of cytokine-secreting monocytes and dendritic cells.

1.4 Reagent and instrument requirements

- Buffer: Prepare a solution containing phosphate-buffered saline (PBS) pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS® BSA Stock Solution (# 130-091-376) 1:20 with autoMACS™ Rinsing Solution (# 130-091-222). Keep buffer cold (2–8 °C). Degas buffer before use, as air bubbles could block the column.
- Culture medium, e.g., RPMI 1640 (# 130-091-440), containing 5% human serum, such as autologous or AB serum, or 10% fetal bovine serum (FBS).
- Propidium Iodide Solution (# 130-093-233) or 7-AAD to exclude dead cells from analysis.
- (Optional) Dead Cell Removal Kit (# 130-090-101) for the depletion of dead cells.

- (Optional) Staining reagents such as CD303 (BDCA-2)-FITC (# 130-090-510), CD303 (BDCA-2)-APC (130-090-905), CD14-FITC (# 130-080-701), CD14-APC (# 130-091-243), CD123-FITC (# 130-090-897), or CD123-APC (# 130-090-901). For more information about other fluorochrome conjugates see www.miltenyibiotec.com.
- Refrigerated centrifuge (2–8 °C).
- Rotation device for tubes: MACSmix™ Tube Rotator (# 130-090-753).

2. Protocol overview



3. Experimental set-up

3.1 Controls

Negative control

For accurate detection of IFN-α–secreting cells, a negative control sample should always be included. This will provide information about IFN-α secretion unrelated to the *in vitro* stimulation but, for example, due to ongoing *in vivo* immune responses. The control sample should be treated in exactly the same way as the stimulated sample, with the exception of not adding the stimulus.

3.2 Counterstaining of cytokine-secreting cells

The IFN-α-secreting cells are stained with PE-conjugated anti-IFN-α detection antibodies. To identify cells of interest, counterstaining of cells with, for example, CD303 (BDCA-2)-FITC (# 130-090-510), CD303 (BDCA-2)-APC (# 130-090-905), CD14-FITC (# 130-080-701), CD14-APC (# 130-091-243), CD123-FITC (# 130-090-897), or CD123-APC (# 130-090-901) is recommended.

▲ The samples should be stained with propidium iodide (PI) or 7-AAD prior to acquisition, to exclude dead cells from analysis. This will reduce non-specific background staining and increase sensitivity.

4. Protocol for the IFN-α Secretion Assay

4.1 Cell preparation

To detect cytokine-secreting cells, best results are achieved by starting the assay with fresh PBMCs or isolated untouched PDCs.

▲ **Note:** PBMCs and isolated untouched PDCs may be stored over night. The cells should be resuspended and incubated in culture medium as described in 4.2 step 2. However, the stimulus should not be added to the culture until the next day.

▲ **Note:** Remove platelets after density gradient separation. Resuspend cell pellet, fill tube with buffer, and mix. Centrifuge at 200×g for 10–15 minutes at 20 °C. Carefully remove supernatant.

4.2 *In vitro* stimulation

▲ Always include a **negative control** in the experiment (see 3.1).

Protocol for *in vitro* stimulation

1. Wash cells by adding medium, centrifuge at 300×g for 10 minutes.
2. Resuspend cells in culture medium containing 10% fetal bovine serum, adjust to 10^7 cells/mL and 5×10^6 cells/cm^2.

3. Add stimulus or control reagent, for example, CpG-ODN 2216 (5 µg/mL) for 5 hours at 37 °C, 5–7% CO_2.

   For comparison of different experiments, the stimulation time should always be the same.
4. Collect cells carefully by using a cell scraper or by pipetting up and down when working with smaller volumes. Rinse the dish with cold buffer. Check microscopically for any remaining cells. If necessary, rinse the dish again.

4.3 Cytokine Secretion Assay

General considerations

▲ The assay is optimized for cell samples from PBMCs containing <0.2% of total IFN-a-secreting cells and for negatively selected PDCs containing more than 2% and less than 20% of total IFN-α–secreting cells. For details see table on page 15. If higher percentages of IFN-α–secreting cells are expected, it is necessary to dilute the cells further during the cytokine secretion period. A larger test tube will then be needed (see table below). The dilution prevents non-specific staining of cells not secreting IFN-α during this period.

▲ For each test with 10^6 total cells, prepare:

50 mL of **cold buffer** (on ice)

1 mL (or 10 mL; see table below) of **warm medium** (37 °C).

▲ Work fast, keep the cells cold, use pre-cooled solutions which will prevent capping of antibodies on the cell surface and a non-specific cell labeling (exception: warm medium during secretion period).

▲ Volumes shown below are for 10^6 total cells. When working with fewer than 10^6 cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly (e.g. for 2×10^6 total cells, use twice the volume of all indicated reagent volumes and total volumes).

▲ Do not remove supernatant by decanting. This will lead to cell loss and incorrect incubation volumes. Aspirate supernatant.

▲ Dead cells may bind non-specifically to MACS MicroBeads or antibodies. Therefore, when working with cell preparations containing large amounts of dead cells, they should be removed before starting the IFN-α Secretion Assay, e.g., by density gradient centrifugation or by using the Dead Cell Removal Kit (# 130-090-101).

Labeling cells with IFN-α Catch Reagent

1. Use 10^6 total cells in a 15 mL closable tube per sample.

   ▲ **Note:** For larger cell numbers, scale up all volumes accordingly. For fewer than 10^6 cells, use same volumes.

2. Wash cells by adding 10 mL of **cold buffer**, centrifuge at 300 × g for 10 minutes at **2–8 °C**, aspirate supernatant completely.

   ▲ **Note:** Do not remove supernatant by decanting. This will lead to cell loss and incorrect incubation volumes.

3. Resuspend cell pellet in 90 µL of **cold buffer** per 10^6 total cells.

4. Add 10 µL of **IFN-α Catch Reagent** per 10^6 total cells, mix well and incubate for 10 minutes **on ice**.

IFN-α secretion period

1. Add **warm** (37 °C) medium to dilute the cells according to the following table:

Expected number of IFN-α–secreting PBMCs	Dilution	Amount of medium to add per 10^6 total cells
< 0.2 %	10^6 cells/mL	1 mL
≥ 0.2 %	10^5 cells/mL	10 mL

Expected number of IFN-α–secreting PDCs	Dilution	Amount of medium to add per 10^6 total cells
< 2 %	10^6 cells/mL	1 mL
≥ 2 %	10^5 cells/mL	10 mL

   ▲ **Note:** For frequencies of cytokine-secreting cells > 20% the cells need to be further diluted, e.g., by a factor of 5.

2. Incubate cells in closed tube for 20 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.

   ▲ **Note:** During this step, it is crucial to prevent contact of cells to avoid cross contamination with cytokines.

Labeling cells with IFN-α Detection Antibody

1. Put the tube **on ice**.

2. Wash the cells by filling up the tube with **cold buffer**, and centrifuge at 300 × g for 10 minutes at **2–8 °C**. Aspirate supernatant completely.

3. Resuspend cell pellet in 90 µL of **cold buffer** per 10^6 total cells.

4. Add 10 µL of **IFN-α Detection Antibody (PE)** per 10^6 total cells.

5. (Optional) Add additional staining reagents, e.g., 10 µL of CD303 (BDCA-2)-FITC (# 130-090-510), or 10 µL of CD303 (BDCA-2)-APC (# 130-090-905), or 10 µL of CD14-FITC (# 130-080-701), or 10 µL of CD14-APC (# 130-091-243), or 10 µL of CD123-FITC (#130-090-897), or 10 µL of CD123-APC (#130-090-901).

6. Mix well and incubate for 10 minutes **on ice**.

7. Wash cells by adding 10 mL of **cold buffer**, centrifuge at 300 × g for 10 minutes at **2–8 °C.** Aspirate supernatant.

5. Detection and analysis of IFN-α-secreting cells

▲ Add propidium iodide (PI) or 7-AAD to a final concentration of 0.5 µg/mL **just prior** to acquisition to exclude dead cells from flow cytometric analysis. Incubating with PI for longer periods will affect the viability of the cells.
Do not fix the cells when using PI or 7-AAD.

▲ For optimized sensitivity, an appropriate number of viable cells has to be acquired from the stimulated sample as well as from the control sample.

To illustrate the analysis, we describe the detection of IFN-α–secreting cells using the IFN-α Secretion Assay. The detailed description, including how to set gates, should serve as a model for the analysis of your own sample.

1. 10^7 human PBMCs have been stimulated for 5 hours with CpG-ODN 2216 (5 µg/mL) or have been left untreated.

2. The IFN-α Secretion Assay was performed on the stimulated and the unstimulated sample.

3. **Counterstaining of cells** was performed using CD303 (BDCA-2)-APC.

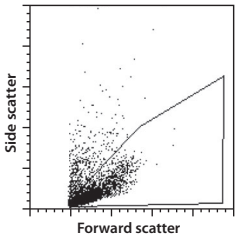
4. **Dead cells** were stained with propidium iodide (PI), which was added just prior to flow cytometric analysis to a final concentration of 0.5 µg/mL.

5. 200,000 viable cells were acquired by flow cytometry from the stimulated and the unstimulated samples.

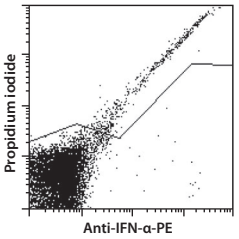
6. A **leukocyte gate** based on forward and side scatter (FSC/SSC) properties was activated prior to further gating to exclude debris (see A.).
7. Dead cells were excluded according to PI-staining in a fluorescence 2 (PE) versus fluorescence 3 plot (PI) (see B.).

   The dead cell exclusion is crucial for the analysis of rare cells, as dead cells may bind non-specifically to antibodies. This could lead to false positive events.
8. Analysis of secreted IFN-α (PE) versus CD303 (BDCA-2)-APC staining by viable leukocytes is displayed (see C.).

A. Leukocyte gate in the forward versus side scatter plot

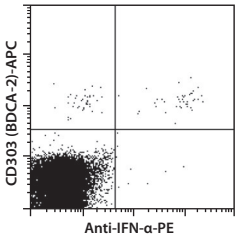


B. Dead cell exclusion

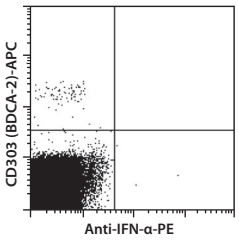


C. Counterstaining of IFN-α–secreting cells using CD303 (BDCA-2)-APC

Sample stimulated with CpG ODN 2216



Unstimulated control sample



6. References

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2. Montoya, M. *et al.* (2002) Type I interferons produced by dendritic cells promote their phenotypic and functional activation. Blood 99: 3263–3271.
3. Santini, S. M. *et al.* (2002) The natural alliance between type I interferon and dendritic cells and its role in linking innate and adaptive immunity. J. Interferon Cytokine Res. 22: 1071–1080.
4. Theofilopoulos, A. N. *et al.* (2005) Type I interferons (alpha/beta) in immunity and autoimmunity. Annu. Rev. Immunol. 23. 307–336.

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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.

Warnings

Reagents contain sodium azide. Under acidic conditions sodium azide yields hydrazoic acid, which is extremely toxic. Azide compounds should be diluted with running water before discarding. These precautions are recommended to avoid deposits in plumbing where explosive conditions may develop.

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