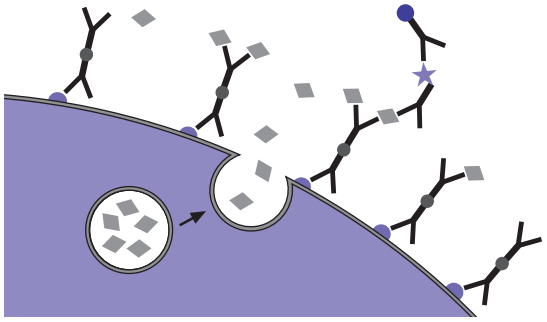
Miltenyi Biotec

IFN-α Secretion Assay – Cell Enrichment and Detection Kit (PE)

human

For 50 tests with 10^7 cells

Order no. 130-092-605



Unless otherwise specifically indicated,
Miltenyi Biotec products and services are for research use only and not for diagnostic or therapeutic use.

Miltenyi Biotec

Miltenyi Biotec B.V. & Co. KG
Friedrich-Ebert-Straße 68, 51429 Bergisch Gladbach, Germany
Phone +49 2204 8306-0, Fax +49 2204 85197
macsde@miltenyi.com, **www.miltenyibiotec.com**

140-001-824.02

Contents

1. Description
   1.1 Principle of the IFN-α Secretion Assay
   1.2 Background information
   1.3 Applications
   1.4 Reagent and instrument requirements
2. Protocol overview
3. Experimental set-up
   3.1 Controls
   3.2 Counterstaining of cytokine-secreting cells
   3.3 Detection without prior enrichment
4. Protocol for the IFN-α Secretion Assay
   4.1 Cell preparation
   4.2 *In vitro* stimulation
   4.3 Cytokine Secretion Assay
   4.4 Magnetic labeling
   4.5 Magnetic separation
5. Detection and analysis of IFN-α–secreting cells
6. References

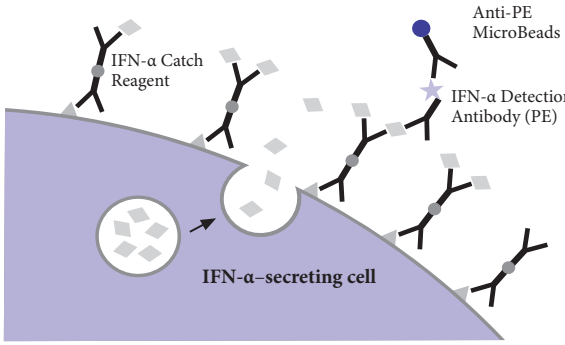
1. Description

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). 1 mL **Anti-PE MicroBeads**: colloidal superparamagnetic MicroBeads conjugated to monoclonal mouse anti-PE antibody (mouse IgG1).
Size	For 50 tests with 10^7 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 and isolated 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.

The IFN-α–secreting cells can now be magnetically labeled with **Anti-PE MicroBeads** and enriched over a MACS® Column which is placed in the magnetic field of a MACS Separator. The magnetically labeled cells are retained in the MACS Column while the unlabeled cells run through. After the column has been removed from the magnetic field,

the magnetically retained cells can be eluted as positively selected cell fraction, enriched for cytokine-secreting cells. The cells can now be used for cell culture or analysis. 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]

Enrichment of the IFN-α–secreting leukocytes using the MACS Technology increases the sensitivity of analysis and also enables further functional characterization, culturing of the cells, as well as downstream applications.

1.3 Applications

- Detection and enrichment of viable IFN-α–secreting leukocytes.
- Detection and enrichment of IFN-α–secreting cells for enumeration and phenotypic analysis as well as for culturing and functional characterization.
- 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.

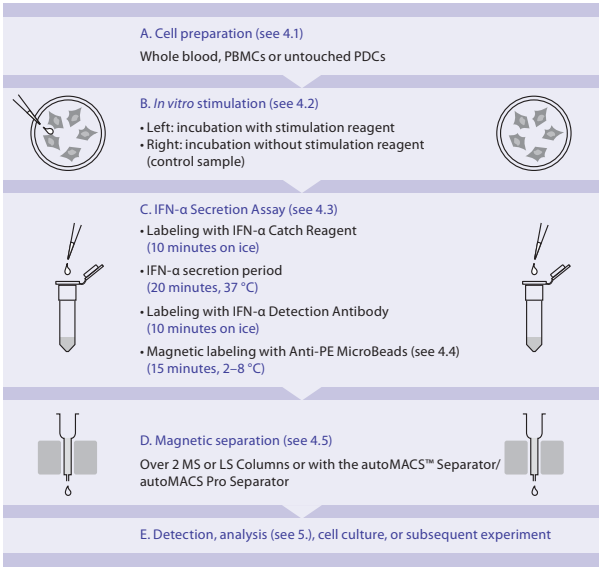
- (Optional) Staining reagents such as CD303 (BDCA-2)-FITC, CD303 (BDCA-2)-APC, CD14-FITC, CD14-APC, CD123-FITC, or CD123-APC. For more information about other fluorochrome conjugates see www.miltenyibiotec.com.
- MACS Columns and MACS Separators:

Column	Max. number of labeled cells	Max. number of total cells	Separator
MS	10^7	2×10^8	MiniMACS, OctoMACS, SuperMACS
LS	10^8	2×10^9	MidiMACS, QuadroMACS, SuperMACS
autoMACS	2×10^8	4×10^9	autoMACS or autoMACS Pro

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

- Refrigerated centrifuge (2–8 °C).
- Rotation device for tubes: MACSmix™ Tube Rotator (# 130-090-753).
- (Optional) Pre-Separation Filters (# 130-041-407) to remove cell clumps.

2. Protocol overview



140-001-824.02

140-001-824.02

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 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 important.

▲ Do **not use** tandem conjugates of phycoerythrin, such as Cy-Chrome™, PE-Cy5 etc., as they may also be recognized by the Anti-PE MicroBeads.

▲ 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.

3.3 Detection without prior enrichment

(Optional) If the sample contains more than 0.01–0.1% of IFN-α–secreting cells, you may be able to analyze IFN-α–secreting cells without prior enrichment (see also: IFN-α Secretion Assay – Detection Kit (PE), # 130-094-161).

140-001-824.02

140-001-824.02

4. Protocol for the IFN-α Secretion Assay

4.1 Cell preparation

To detect and isolate 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/cm².
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 10^7 total cells, prepare:

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

10 mL (or 100 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 10^7 total cells. When working with fewer than 10^7 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 2×10^7 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 10^7 total cells in a 15 mL closable tube per sample.

   ▲ **Note:** For larger cell numbers, scale up all volumes accordingly. For fewer than 10^7 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 80 µL of **cold buffer** per 10^7 total cells.

4. Add 20 µL of **IFN-α Catch Reagent** per 10^7 total cells, mix well and incubate for 10 minutes **on ice**.



IFN-α secretion period

1. Transfer cells in a 50 mL closable tube and add **warm** (37 °C) medium to dilute the cells according to the following table:

Expected number of IFN-α–secreting PDCs	Dilution	Amount of medium to add per 10^7 total cells
< 2 %	10^6 cells/mL	10 mL
≥ 2 %	10^5 cells/mL	100 mL

Expected number of IFN-α–secreting PBMCs	Dilution	Amount of medium to add per 10^7 total cells
< 0.2 %	10^6 cells/mL	10 mL
≥ 0.2 %	10^5 cells/mL	100 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 80 µL of **cold buffer** per 10^7 total cells.
4. Add 20 µL of **IFN-α Detection Antibody (PE)** per 10^7 total cells and transfer cells in a 15 mL closable tube per sample.
5. (Optional) Add additional staining reagents, e.g., 10 µL of CD303 (BDCA-2)-FITC, or 10 µL of CD303 (BDCA-2)-APC, or 10 µL of CD14-FITC, or 10 µL of CD14-APC, or 10 µL of CD123-FITC, or 10 µL of CD123-APC.
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.

140-001-824.02

4.4 Magnetic labeling



Magnetic labeling with Anti-PE MicroBeads

1. Resuspend cell pellet in 80 µL of cold buffer per 10^7 total cells.
2. Add 20 µL of **Anti-PE MicroBeads** per 10^7 total cells, mix well and refrigerate for 15 minutes on ice.
3. Wash cells by adding 10 mL of **cold buffer**, centrifuge at 300 × g for 10 minutes at **2–8 °C**. Aspirate supernatant.
4. Resuspend cell pellet in 500 µL of **cold buffer**. For higher cell numbers than 5×10^7 use a dilution of 10^8 cells/mL.
5. (Optional) Take an aliquot of the fraction before enrichment for flow cytometric analysis and cell count.
6. Proceed to magnetic separation (see 4.5).

4.5 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 1.4).

▲ When enriching IFN-α–secreting cells, **always perform two consecutive column runs** to achieve best results.

1. Place column in the magnetic field of a suitable MACS Separator. For details see the respective MACS Column data sheet.
2. Prepare column by rinsing with the appropriate amount of cold buffer: MS: 500 µL LS: 3 mL
3. Apply cell suspension onto the column.
4. Collect unlabeled cells that pass through and wash column with the appropriate amount of 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 reservoir is empty.
   MS: 3×500 µL LS: 3×3 mL
5. Remove column from the separator and place it on a suitable collection tube.

   ▲ **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.

140-001-824.02

6. Pipette the appropriate amount of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.
   MS: 1 mL LS: 5 mL
7. To increase the purity of IFN-α-secreting cells, the eluted fraction should be enriched over a second MS or LS Column. Repeat the magnetic separation procedure as described in steps 1 to 6 by using a new column.

   ▲ **Note**: For subsequent cell culture, the cells can also be eluted with medium. If part of the cells are analyzed by flow cytometry, the medium should not contain phenol red.
8. Proceed to analysis (see section 5.), cell culture, or other subsequent experiment.



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

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

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

▲ 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.

Magnetic separation with the autoMACS™ Separator

1. Prepare and prime the instrument.
2. 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.
3. For a standard separation choose the following program:

   Positive selection: "Posseld2"

   Collect positive fraction from outlet port pos2.
4. Proceed to analysis (5.), cell culture, or other subsequent experiment.

Magnetic separation with the autoMACS™ Pro Separator

1. Prepare and prime the instrument.
2. 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 the following program:

   Positive selection: "Posseld2"

   Collect positive fraction in row C of the tube rack.
4. Proceed to analysis (5.), cell culture, or other subsequent experiment.

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.

- Acquire 2×10^5 viable cells from the fraction before enrichment (see 4.4 step 5.).
- For **enumeration** of low frequent IFN-α–secreting cells, acquire all of the positive fraction. For **preparative purposes**, acquire an aliquot of the positive fraction to determine the performance of the cell enrichment.

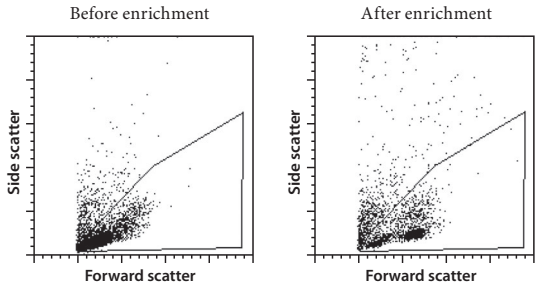
To illustrate the analysis, we describe the detection of IFN-α–secreting cells using the IFN-α Secretion Assay. The detailed description, including how to set gates, should serve as a model for the analysis of your own sample.

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.

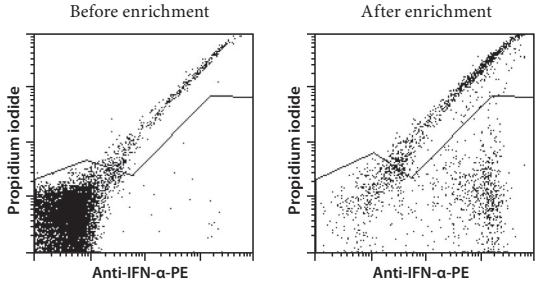
4. **Dead cells** were stained with propidium iodide (PI), which was added just prior to flow cytometric analysis to a final concentration of 0.5 µg/mL.
5. 200,000 viable cells of the fractions before enrichment and the complete enriched fractions were acquired by flow cytometry, from the stimulated 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 or MicroBeads. 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

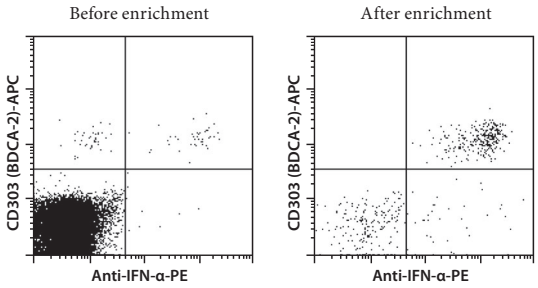


B. Dead cell exclusion

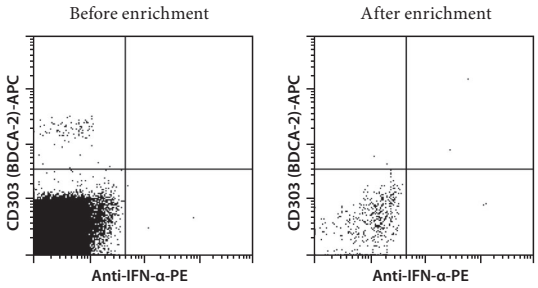


C. Counterstaining of IFN-α–secreting cells using CD303 (BDCA-2)-APC

Sample stimulated with CpG ODN 2216



Unstimulated control sample



140-001-824.02

6. References

1. Biron, C. A. (2001)). Interferons alpha and beta as immune regulators--a new look. Immunity 14: 661–664
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.

For further information visit our website **www.miltenyibiotec.com**.

140-001-824.02

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.

140-001-824.02

Legal notices

Limited product warranty

Miltenyi Biotec B.V. & Co. KG and/or its affiliate(s) warrant this product to be free from material defects in workmanship and materials and to conform substantially with Miltenyi Biotec's published specifications for the product at the time of order, under normal use and conditions in accordance with its applicable documentation, for a period beginning on the date of delivery of the product by Miltenyi Biotec or its authorized distributor and ending on the expiration date of the product's applicable shelf life stated on the product label, packaging or documentation (as applicable) or, in the absence thereof, ONE (1) YEAR from date of delivery ("Product Warranty"). Miltenyi Biotec's Product Warranty is provided subject to the warranty terms as set forth in Miltenyi Biotec's General Terms and Conditions for the Sale of Products and Services available on Miltenyi Biotec's website at www.miltenyibiotec.com, as in effect at the time of order ("Product Warranty"). Additional terms may apply. BY USE OF THIS PRODUCT, THE CUSTOMER AGREES TO BE BOUND BY THESE TERMS.

THE CUSTOMER IS SOLELY RESPONSIBLE FOR DETERMINING IF A PRODUCT IS SUITABLE FOR CUSTOMER'S PARTICULAR PURPOSE AND APPLICATION METHODS.

Technical information

The technical information, data, protocols, and other statements provided by Miltenyi Biotec in this document are based on information, tests, or experience which Miltenyi Biotec believes to be reliable, but the accuracy or completeness of such information is not guaranteed. Such technical information and data are intended for persons with knowledge and technical skills sufficient to assess and apply their own informed judgment to the information. Miltenyi Biotec shall not be liable for any technical or editorial errors or omissions contained herein.

All information and specifications are subject to change without prior notice. Please contact Miltenyi Biotec Technical Support or visit www.miltenyibiotec.com for the most up-to-date information on Miltenyi Biotec products.

140-001-824.02

Licenses

This product and/or its use may be covered by one or more pending or issued patents and/or may have certain limitations. Certain uses may be excluded by separate terms and conditions. Please contact your local Miltenyi Biotec representative or visit Miltenyi Biotec's website at www.miltenyibiotec.com for more information.

The purchase of this product conveys to the customer the non-transferable right to use the purchased amount of the product in research conducted by the customer (whether the customer is an academic or for-profit entity). This product may not be further sold. Additional terms and conditions (including the terms of a Limited Use Label License) may apply.

CUSTOMER'S USE OF THIS PRODUCT MAY REQUIRE ADDITIONAL LICENSES DEPENDING ON THE SPECIFIC APPLICATION. THE CUSTOMER IS SOLELY RESPONSIBLE FOR DETERMINING FOR ITSELF WHETHER IT HAS ALL APPROPRIATE LICENSES IN PLACE. Miltenyi Biotec provides no warranty that customer's use of this product does not and will not infringe intellectual property rights owned by a third party. BY USE OF THIS PRODUCT, THE CUSTOMER AGREES TO BE BOUND BY THESE TERMS.

Trademarks

autoMACS, MACS, MACSmix, MidiMACS, the Miltenyi Biotec logo, MiniMACS, OctoMACS, QuadroMACS, SuperMACS, and VarioMACS are registered trademarks or trademarks of Miltenyi Biotec and/or its affiliates in various countries worldwide. All other trademarks mentioned in this publication are the property of their respective owners and are used for identification purposes only.

Copyright © 2021 Miltenyi Biotec and/or its affiliates. All rights reserved.

140-001-824.02

140-001-824.02