Miltenyi Biotec

MSC Suppression Inspector
human

Order no. 130-096-207

Contents

1. Description

Components	2.5 mL MSC Suppression Inspector, human: 5×10^7 Anti-Biotin MACSiBead™ Particles pre-loaded with biotinylated CD2, CD3, and CD28 antibodies.
Product format	MSC Suppression Inspector is supplied in an azide-free suspension.
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 a suppression assay using the MSC Suppression Inspector

Mesenchymal stem cells (MSCs) are often functionally analyzed *in vitro* by a so-called suppression assay. For this purpose, MSCs are co-cultured with $CD4^+CD25^-$ or $CD4^+$ responder T cells (Tresp) at different ratios in the presence of a polyclonal stimulus, in this case the MSC Suppression Inspector. Tresp cells alone show a proliferative response. Co-culture of MSCs with Tresp cells results in reduced proliferation of Tresp cells. Cell proliferation is determined by ^{3}H-thymidine incorporation but can also be detected by carboxyfluorescein succinimidyl ester (CFSE) staining. The suppression assay is performed with a dilution series ranging from a ratio of 1 : 1 to 8 : 1 of Tresp cells : MSCs as outlined in tables 1 and 3. As additional control, Tresp and MSCs are cultured alone with and without the MSC Suppression Inspector. The dilution series is carried out in triplicate to achieve significant results. All volumes given in the protocol are calculated for one assay.

1.2 Background information

MSCs are fibroblast-like plastic-adherent cells that can be isolated from a variety of tissues, such as bone marrow or adipose tissue. During the last few years the attention of scientists was redirected away from the multipotentiality of MSCs towards their possibility for immunomodulation. It was observed that bone marrow derived MSCs suppress T cell proliferation.[1,2] This function of MSCs can be analyzed using the MSC Suppression Inspector which contains an optimal T cell stimulation reagent for a MSC suppression assay. The MSC Suppression Inspector consists of Anti-Biotin MACSiBead Particles that are pre-loaded with biotinylated CD2, CD3, and CD28 antibodies.

1.3 Applications

- Functional characterization of human MSCs by *in vitro* suppression assays.

1.4 Reagent and instrument requirements

- (Optional) $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, human (# 130-091-301), $CD4^+CD25^+CD127^{dim/-}$ Regulatory T Cell Isolation Kit II, human (# 130-094-775).
- Cell culture medium, for example, RPMI 1640 supplemented with 10% AB serum, X-VIVO 15™ (Cambrex), or X-VIVO 15™ supplemented with 5% AB serum.

  ▲▲ **Note:** 2-Mercaptoethanol (0.01 mM) can be added to preserve cell viability in case of rapid cell growth.
- 96-well culture plates (flat bottom).
- Humidified incubator.
- (Optional) StemMACS™ MSC Expansion Media (# 130-091-680) or StemMACS MSC Expansion Media Kit XF (# 130-104-182).
- (Optional) CytoMix – MSC, human (# 130-093-552).
- (Optional) CD271 MicroBead Kit (PE) (# 130-092-819) or CD271 MicroBead Kit (APC) (# 130-092-283).
- (Optional) Anti-MSCA-1 (W8B2) MicroBead Kit, human (# 130-093-583)

Additional requirements for tritium-based suppression assay:

- PBS buffer: Prepare a solution containing phosphate-buffered saline (PBS), pH 7.2.
- PBS/10% FBS buffer: Prepare a solution containing PBS, pH 7.2 and 10% fetal bovine serum (FBS). Keep buffer cold (2–8 °C).
- ^{3}H-thymidine.

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Additional requirements for CFSE-based suppression assay:

- CFSE (5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester: Prepare a stock solution of CFSE as indicated by the manufacturer in DMSO with a concentration of 1 mM. Aliquot stock solution and store at –20 °C.

2. Protocol

2.1 Sample preparation

▲ All steps in the protocol have to be performed under aseptic conditions. In this protocol one MACSiBead™ Particle per cell (bead-to-cell ratio 1:1) is used for stimulation.

Ratio Tresp cells : MSCs	Tresp cells	MSCs	MSC Suppression Inspector (amount of MACSiBead Particles)
For tritium-based suppression assay			
1:0	5×10^4	–	5×10^4
0:1	–	5×10^4	5×10^4
1:1	5×10^4	5×10^4	10×10^4
2:1	5×10^4	2.5×10^4	7.5×10^4
4:1	5×10^4	1.3×10^4	6.3×10^4
8:1	5×10^4	0.6×10^4	5.6×10^4
Control 1:0	5×10^4	–	–
Control 0:1	–	5×10^4	–
Total cells/ MACSiBeads	3×10^5	2×10^5	4×10^5
Total cells/ MACSiBeads for 1 assay (triplicates)	9×10^5	6×10^5	12×10^5
For CFSE-based suppression assay			
1:0	1×10^5	–	1×10^5
0:1	–	1×10^5	1×10^5
1:1	1×10^5	1×10^5	2×10^5
Control 1:0	1×10^5	–	–
Control 0:1	–	1×10^5	–
Total cells/ MACSiBeads	3×10^5	3×10^5	4×10^5
Total cells/ MACSiBeads for 1 assay (duplicates)	6×10^5	6×10^5	8×10^5

Table 1: Number of responder T cells (Tresp), mesenchymal stem cells (MSCs), and MSC Suppression Inspector (MACSiBead Particles) per well.

2.2 Preparation of cells

Determine the concentration and the total number of responder T cells (Tresp)

▲ Start with $CD4^+CD25^-$ or $CD4^+$ responder T cells isolated under aseptic conditions, e.g., with the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, human (# 130-091-301). For details concerning Treg isolation refer to the respective data sheet.

Human PBMCs	
Depletion of non-$CD4^+$ cells	1. Indirect magnetic labeling of non-$CD4^+$ cells with $CD4^+$ T Cell Biotin-Antibody Cocktail and Anti-Biotin MicroBeads. 2. Magnetic separation using an LD Column or an autoMACS Column (program "Depl05").
Pre-enriched $CD4^+$ cells (flow-through fraction)	
Depletion of $CD4^+CD25^+$ regulatory T cells (Tregs)	1. Direct magnetic labeling of $CD25^+$ T cells with CD25 MicroBeads. 2. Magnetic separation using two MS Columns or an autoMACS Column (program "Posseld2").
$CD4^+CD25^-$ Tresp (flow-through fraction, first column) $CD4^+CD25^+$ Tregs (eluted cells, second column)	

Table 2: Isolation of Tresp with the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, human.

2.2.1 For tritium-based suppression assay

Preparation of mesenchymal stem cells (MSCs) and Tresp

▲ Use MSCs after isolation from human tissue, e.g., bone marrow or adipose tissue.

▲ When using frozen MSCs it is suggested to use the cells after 2–3 days of cultivation.

1. Determine the concentration and the total number of MSCs and Tresp cells. For one assay, as outlined in table 1, 9×10^5 Tresp cells and 6×10^5 MSCs are needed.
2. Transfer required volumes of cell suspension to suitable tubes.
3. Add 5 mL of culture medium to the cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
4. Resuspend the Tresp cells (9×10^5) in 1800 µL of medium and the MSCs (6×10^5) in 1200 µL. The concentration of the cell suspensions is now 5×10^5 cells/mL.
5. Pipette the appropriate volumes of MSCs and Tresp cell suspension in a 96-well culture plate. Refer to table 3 for the respective volumes.

2.2.2 For CFSE-based suppression assay

CFSE-staining of Tresp

1. Resuspend Tresp in PBS/10% FBS buffer at a density of 2×10^7 cells per 1 mL and vortex thoroughly.
2. Prepare a CFSE working solution by diluting CFSE stock solution 1:100 with PBS buffer to a final concentration of 10 µM.
3. Add CFSE working solution to the same volume of cell suspension (final: 1×10^7 cells/mL, PBS with 5% FBS, 5 µM CFSE). Mix well.

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4. Incubate the cells for 5 minutes at room temperature in the dark.
5. Wash the cells twice with 1 mL of PBS/10% FBS buffer. Centrifuge at 300×g for 10 minutes.
6. Resuspend cells in medium with a concentration of 1×10^6 cells per mL.
7. Pipette the appropriate volume of Tresp cell suspension in a 96-well culture plate. Refer to table 4 for the respective volumes.

Preparation of mesenchymal stem cells (MSCs)

▲ Use MSCs after isolation from human tissue, e.g., bone marrow or adipose tissue.

▲ When using frozen MSCs it is suggested to use the cells after 2–3 days of cultivation.

1. Determine the concentration and the total number of MSCs. For one assay, as outlined in table 1, 6×10^5 MSCs are needed.
2. Transfer required volumes of cell suspension to suitable tubes.
3. Add 5 mL of culture medium to the cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
4. Resuspend the MSCs (6×10^5) in 600 µL. The concentration of the cell suspensions is now 1×10^6 cells/mL.
5. Pipette the appropriate volumes of MSCs in a 96-well culture plate. Refer to table 4 for the respective volumes.

2.3 Preparation of MSC Suppression Inspector

2.3.1 For tritium-based suppression assay

1. Resuspend MSC Suppression Inspector thoroughly and transfer 60 µL to a suitable tube.

   ▲ **Note:** Concentration of MSC Suppression Inspector is **2×10^7** MACSiBead Particles per mL.

2. Add 0.6 mL of culture medium and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.
3. Resuspend MSC Suppression Inspector in 120 µL of culture medium. The reagent is now ready to use.

   ▲ **Note:** Concentration of prepared MSC Suppression Inspector is **1×10^7** MACSiBead Particles per mL.

2.3.2 For CFSE-based suppression assay

1. Resuspend MSC Suppression Inspector thoroughly and transfer 60 µL to a suitable tube.

   ▲ **Note:** Concentration of MSC Suppression Inspector is **2×10^7** MACSiBead Particles per mL.

2. Add 0.6 mL of culture medium and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.
3. Resuspend MSC Suppression Inspector in 60 µL of culture medium. The reagent is now ready to use.

   ▲ **Note:** Concentration of prepared MSC Suppression Inspector is **2×10^7** MACSiBead Particles per mL.

Ratio Tresp cells : MSCs	Tresp cells (5×10^5 cells/mL)	MSCs (5×10^5 cells/mL)	MSC Suppression Inspector (1×10^7 MACSiBead particles/mL)	Culture medium
For tritium-based suppression assay				
1 : 0	100 µL	–	5 µL	105 µL
0 : 1	–	100 µL	5 µL	105 µL
1 : 1	100 µL	100 µL	10 µL	–
2 : 1	100 µL	50 µL	7.5 µL	53 µL
4 : 1	100 µL	25 µL	6.5 µL	79 µL
8 : 1	100 µL	12.5 µL	6.0 µL	92 µL
Control 1 : 0	100 µL	–	–	110 µL
Control 0 : 1	–	100 µL	–	110 µL
Total volume	600 µL	387.5 µL	40 µL	654 µL
Total volume for 1 assay (triplicates)	1800 µL	1200 µL	120 µL	approx. 2 mL

Table 3: Pipetting scheme for one assay with a total volume of 210 µL per well using cell suspensions that contain 5×10^5 cells/mL.

Ratio Tresp cells : MSCs	Tresp cells (1×10^6 cells/mL)	MSCs (1×10^6 cells/mL)	MSC Suppression Inspector (2×10^7 MACSiBead particles/mL)	Culture medium
For CFSE-based suppression assay				
1 : 0	100 µL	–	5 µL	105 µL
0 : 1	–	100 µL	5 µL	105 µL
1 : 1	100 µL	100 µL	10 µL	–
Control 1 : 0	100 µL	–	–	110 µL
Control 0 : 1	–	100 µL	–	110 µL
Total volume	300 µL	300 µL	20 µL	430 µL
Total volume for 1 assay (duplicates)	600 µL	600 µL	40 µL	860 µL

Table 4: Pipetting scheme for one assay.

2.4 Stimulation and suppression

2.4.1 For tritium-based suppression assay

1. Resuspend the prepared MSC Suppression Inspector thoroughly and add the required amount to the wells (bead-to-cell ratio 1:1). For a detailed pipetting scheme refer to table 3.

   ▲ **Note:** The bead-to-cell ratio refers to the total cell number per well.

2. Fill up wells to a total volume of 210 µL with culture medium (refer to table 3).
3. Incubate at 37 °C and 5–7% CO_2 for 4–5 days.
4. Add 1 µCi ^{3}H-thymidine to each well and incubate at 37 °C and 5–7% CO_2 for 16 hours.
5. Measure ^{3}H-thymidine incorporation, e.g., by using a liquid scintillation counter.

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2.4.2 For CFSE-based suppression assay

1. Resuspend the prepared MSC Suppression Inspector thoroughly and add the required amount to the wells (bead-to-cell ratio 1:1). For a detailed pipetting scheme refer to table 4.

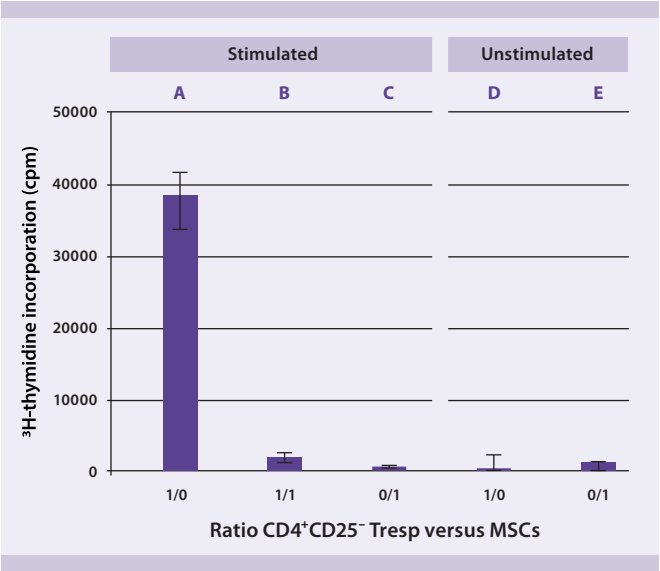
   ▲ **Note:** The bead-to-cell ratio refers to the total cell number per well.

2. Fill up wells to a total volume of 210 µL with culture medium (refer to table 4).
3. Incubate at 37 °C and 5–7% CO_2 for 5 days.
4. Analyse CFSE intensity by flow cytometric analysis.

3. Examples of a tritium- and CFSE-based suppression assay using the MSC Suppression Inspector

Tritium-based suppression assay

MSCs were isolated from human bone marrow and culture expanded with NH Expansion Medium (# 130-091-680). After two passages MSCs were co-cultured with $CD4^+CD25^-$ responder T cells at different ratios. For T cell stimulation, the MSC Suppression Inspector was added to the culture. As controls, MSCs and $CD4^+CD25^-$ responder T cells alone were cultured without any stimulus. Proliferation of T cells was determined by ^{3}H-thymidine incorporation. ^{3}H-thymidine was added for 16 hours after 5 days of culture.

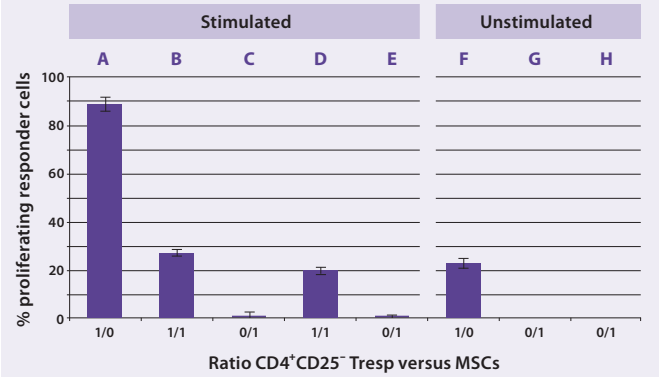


Tresp show high proliferation after stimulation with the MSC Suppression Inspector (A). When adding MSCs Tresp proliferation is suppressed dramatically (B). Unstimulated Tresp show no proliferation (D). MSCs alone show little proliferation with or without stimulation (C and E).

CFSE-based suppression assay

MSCs were isolated from human bone marrow either by plastic adherence (PA-MSCs) or by CD271 isolation (CD271-MSCs) using CD271 MicroBead Kit (APC) (# 130-092-283). Both PA-MSCs and CD271-MSCs were culture expanded with NH Expansion Medium (# 130-091-680).

After two passages MSCs were co-cultured with CFSE-labeled $CD4^+CD25^-$ responder T cells. For T cell stimulation, the MSC Suppression Inspector was added to the cultures. As control, MSCs and $CD4^+CD25^-$ Tresp were cultured without the MSC Suppression Inspector. Cells were harvested after 5 days and the percentage of proliferating Tresp was measured as CFSE dye dilution analyzed by flow cytometry using the MACSQuant® Analyzer.



Almost 90% of all Tresp proliferate after stimulation with the MSC Suppression Inspector (A). When adding PA-MSCs Tresp proliferation is suppressed to a level of about 28% (B). The addition of $CD271^+$ MSCs suppresses the Tresp proliferation to a level of 20% (D). MSCs alone show no proliferation with or without stimulation (C, E, G, and H). Unstimulated Tresp show a proliferation rate of 30% (F).

4. References

1. Di Nicola, M. *et al.* (2002) Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 99: 3838–3843.
2. Bartholomew, A. *et al.* (2002) Mesenchymal stem cells suppress lymphocyte proliferation *in vitro* and prolong skin graft survival *in vivo*. Exp. Hematol. 30: 42–48.

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