Miltenyi Biotec

Treg Suppression Inspector
human

Order no. 130-092-909

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1. Description

This product is for research use only.

Components	2.5 mL Treg Suppression Inspector: 5×10^7 Anti-Biotin MACSiBead™ Particles pre-loaded with biotinylated CD2, CD3, and CD28 antibodies.
Product format	Treg 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 Treg Suppression Inspector

$CD4^+CD25^+$ regulatory T cells (Tregs) are often functionally analyzed *in vitro* by a so-called suppression assay. For this purpose, Tregs 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 Treg Suppression Inspector. Tregs alone show a hypoproliferative response (anergy). Tresp cells alone show a proliferative response. Coculture of Tregs with Tresp cells results in reduced proliferation of Tresp cells. Cell proliferation can be determined by ^{3}H-thymidine incorporation or by dilution of a cell tracker reagent (e.g. CFSE).
The suppression assay is performed with a dilution series ranging from a ratio of 1:1 to 8:1 of Tresp cells:Treg cells as outlined in tables 1 and 2. As additional control, Tresp and Treg cells are cultured alone with and without the Treg 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

The Treg Suppression Inspector has been developed for the functional characterization of human Tregs by *in vitro* suppression assays.
Tregs are a subset of T cells that have the ability to suppress harmful immunological reactions to self or foreign antigens. This function of Tregs can be analyzed using the Treg Suppression Inspector as an optimized T cell stimulation reagent in a Treg suppression assay. The Treg Suppression Inspector consists of Anti-Biotin MACSiBead Particles that are pre-loaded with biotinylated CD2, CD3, and CD28 antibodies.

1.3 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, human (# 130-093-337).
- Cell culture medium TexMACS™ Medium (# 130-097-196) supplemented with 5% human 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.
- ^{3}H-thymidine.
- Humidified incubator.

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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.

Table 1: Number of responder T cells (Tresp), regulatory T cells (Treg) and Treg Suppression Inspector (MACSiBead Particles) per well.

Ratio Tresp cells: Treg cells	Tresp cells	Treg cells	Treg Suppression Inspector (amount of MACSiBead Particles)
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

2.1.1 Preparation of cells

▲ Start with Tregs and $CD4^+CD25^-$ or $CD4^+$ responder T cells isolated under aseptic conditions, e.g., with the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, human (# 130-091-301) or the $CD4^+CD25^+CD127^{dim/-}$ Regulatory T Cell Isolation Kit, human (# 130-093-337). For details concerning Treg isolation refer to the respective data sheet.

1. Determine the concentration and the total number of Tregs and Tresp cells. For one assay, as outlined in table 1, 9×10^5 Tresp cells and 6×10^5 Tregs are needed.
2. Transfer required volumes of cell suspension to suitable tubes.
3. Add 5–10 volumes 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 Tregs (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 Treg and Tresp cell suspension in a 96-well culture plate. Refer to table 2 for the respective volumes.

2.1.2 Preparation of Treg Suppression Inspector

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

   ▲ **Note:** Concentration of Treg Suppression Inspector is $\mathbf{2\times10^7}$ MACSiBead Particles per mL.

2. Add 0.3–0.6 mL of culture medium and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.
3. Resuspend Treg Suppression Inspector in 120 µL of culture medium. The reagent is now ready to use.

   ▲ **Note:** Concentration of prepared Treg Suppression Inspector is $\mathbf{1\times10^7}$ MACSiBead Particles per mL.

Table 2: 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: Treg cells	Tresp cells (5×10^5 cells/ mL)	Treg cells (5×10^5 cells/ mL)	Treg Suppression Inspector (1×10^7 MACSiBead particles/ mL)	Culture medium
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	appr. 2 mL

2.2 Stimulation and suppression assay

1. Resuspend the prepared Treg Suppression Inspector thoroughly and add required amount to the wells (bead-to-cell ratio 1:1). For a detailed pipetting scheme see table 2.

   ▲ **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 (see table 2).
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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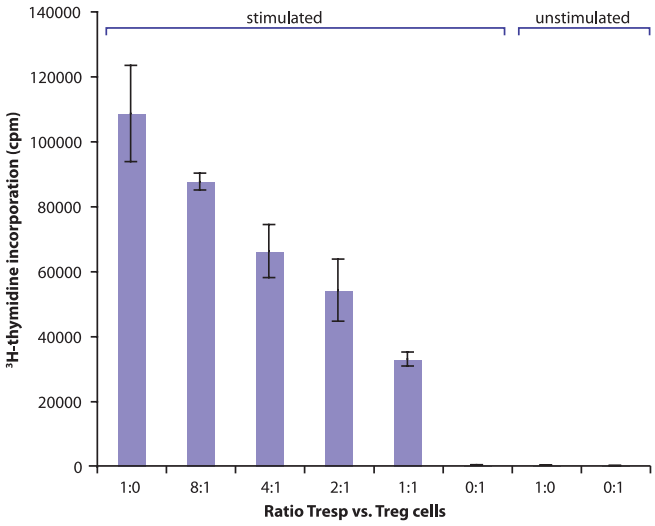
3. Example of a separation using Treg Suppression Inspector

$CD4^+CD25^+$ regulatory T cells were isolated with the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit and cocultured with $CD4^+CD25^-$ responder T cells at different ratios. For T cell stimulation, the Treg Suppression Inspector was added to the culture. As controls, $CD4^+CD25^+$ Treg cells 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.



Ratio Tresp : Treg	Counts per minute (cpm)			Mean cpm
1 : 0	97224	125318	102969	108504
8 : 1	84945	87442	90112	87500
4 : 1	63578	75205	59507	66097
2 : 1	60638	58488	43192	54106
1 : 1	34163	34081	30424	32889
0 : 1	316	291	253	287
1 : 0	288	232	223	248
0 : 1	172	177	141	163

4. Reference

1. Thornton, A. M. and Shevach, E. M. (1998) $CD4^+CD25^+$ immunoregulatory T cells suppress polyclonal T cell activation *in vitro* by inhibiting interleukin 2 production. J. Exp. Med.: 188: 287–296.

Refer to **www.miltenyibiotec.com** for all data sheets and protocols. Miltenyi Biotec provides technical support worldwide. Visit www.miltenyibiotec.com/local to find your nearest Miltenyi Biotec contact.

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