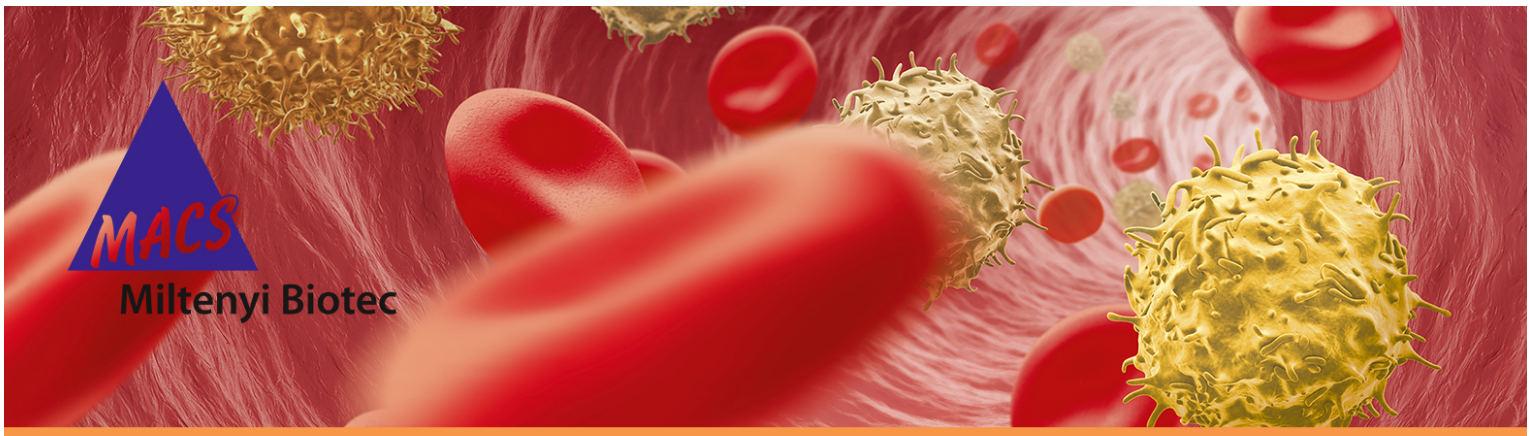

Human CD4$^+$CD25$^+$ regulatory T cell isolation, *in vitro* suppression assay and analysis

In vitro human regulatory T cell suppression assay

Introduction

Regulatory T (Treg) cells are a subpopulation of T cells. Basic as well as clinical research has focused on the study of Treg cells for many years due to their unique function, which is the suppression of immune responses. The suppression assay is one of the most common assays to test the functionality of Treg cells *in vitro*. For this purpose, Treg cells are co-cultured with responder T (Tresp) cells and a proliferative stimulus (either polyclonal or antigen-specific). During co-culture, Treg cells suppress the proliferation of Tresp cells. The selection of an appropriate proliferative stimulus is challenging and requires a fine-tuning as it must stimulate the Tresp cells in a way that Treg cells can suppress their proliferation.

To address this problem, we have developed the Treg Suppression Inspector, human, which provides for optimal polyclonal stimulation of Tresp cells. Tresp cells stimulated with the Treg Suppression Inspector remain susceptible to the suppression by Treg cells, while Treg cells remain hypoproliferative.

This application note describes every step from human Treg and Tresp cell isolation, their co-cultivation in an *in vitro* suppression assay using the Treg Suppression Inspector, to flow cytometry analysis.

Workflow

Day 0
- Treg and Tresp cell isolation
- Flow cytometry analysis
- Start *in vitro* suppression assay

Day 5
- Stop *in vitro* suppression assay: cell harvest
- Flow cytometry analysis of *in vitro* suppression assay

Materials

Treg cell isolation from PBMCs

Treg cell isolation kit

- $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, human (# 130-091-301)

Separation buffer

- 1x PBS (pH 7.2), BSA, EDTA
- Alternative: 6×1.5 L autoMACS® Running Buffer – MACS® Separation Buffer (# 130-091-221)

Magnetic separation

- LD Columns, 1 for 10^8 labeled cells (# 130-042-901)
- MS Columns, 2x per isolation (# 130-042-201)
- MidiMACS™ Separator (Separator for LD Column), (# 130-042-302)
- MiniMACS™ Separator (Separator for MS Column), (# 130-042-102)
- MACS® MultiStand (# 130-042-303)
- Optional: Pre-Separation Filters, 30 µm (# 130-041-407)

Staining of Treg cells for flow cytometry analysis

Miltenyi Biotec provides flow cytometry antibodies coupled to a variety of different fluorochromes. We recommend using the antibodies listed below.

CD4-VioBlue™
Clone: VIT4
30 tests # 130-098-163
100 tests # 130-094-153

Clone: M-T466
30 tests # 130-099-683
100 tests # 130-097-333

CD25-PE
Clone: 4E3
30 tests # 130-098-211
100 tests # 130-091-024

In vitro suppression assay

Suppression medium

- TexMACS™ Medium (500 mL) (# 130-097-196), human AB Serum, Penicillin/Streptamycin (PenStrep)

Suppression assay

- Treg Suppression Inspector, human (# 130-092-909), for stimulation of $5x10^7$ total cells
- 96-well flat-bottom plate
- CellTrace™ Violet Cell Proliferation Kit, for flow cytometry

Material preparation

Treg cell isolation from PBMCs

Separation buffer

- 1x PBS (pH 7.2)
  + 0.5% BSA
  + 2 mM EDTA

In vitro suppression assay

Suppression medium

- 500 mL TexMACS Medium
  + 5% human AB serum
  + 1% PenStrep

The suppression medium can be stored for up to seven days under sterile conditions at 4°C.

Methods

1. Generation of peripheral blood mononuclear cells (PBMCs)

When working with anticoagulated peripheral blood or buffy coat, PBMCs should be isolated by density gradient centrifugation, for example, using Ficoll-Paque™ according to the manufacturer's instructions. Collect a sample of PBMCs for subsequent flow cytometry analysis.

2. Isolation of $CD4^+CD25^+$ Treg cells using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, human

The isolation of Treg cells with the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, human, enables the simultaneous isolation of Treg and Tresp cells with a single kit from one blood sample. The Treg cells ($CD4^+CD25^+$) are the final positive fraction and the Tresp cells ($CD4^+CD25^-$) are the final negative fraction.

2.1 Magnetic labeling of non-$CD4^+$ cells

▲ Volumes for magnetic labeling given below are for up to 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).

1. Determine cell number.

2. Centrifuge cell suspension at 300×g for 10 minutes. Discard supernatant.

3. Resuspend cell pellet in 90 µL of separation buffer per 10^7 total cells.

4. Add 10 µL of $CD4^+$ T Cell Biotin-Antibody Cocktail II per 10^7 total cells.

5. Mix well and incubate for 5 minutes in the refrigerator (2–8 °C).

6. Add 20 µL of Anti-Biotin MicroBeads per 10^7 total cells.

7. Mix well and incubate for 10 minutes in the refrigerator (2–8 °C).

8. Adjust the volume to 500 µL with separation buffer.

▲ **Note:** Resuspend up to 10^8 cells in 500 µL of separation buffer. For higher cell numbers, scale up buffer volume accordingly.

2.2 Magnetic depletion of non-$CD4^+$ cells

▲ The first step of Treg cell isolation is a depletion of non-$CD4^+$ cells. Here, an LD Column is used, which has a capacity of 10^8 labeled cells and $5x10^8$ total cells. When using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, human, we do not recommend to process more than $1.3x10^8$ total cells on an LD Column. When exceeding this cell number, it is strongly recommended to split the sample and use additional LD Columns.

1. Place LD Column(s) in the magnetic field of a MidiMACS™ Separator.

2. Prepare column by rinsing with 2 mL of separation buffer. Always wait until the column reservoir is empty before proceeding to the next step.

3. Apply single cell suspension onto the column.

4. Collect unlabeled cells that pass through and wash column twice with 1 mL of separation buffer each. **Collect total effluent; this is the unlabeled pre-enriched $CD4^+$ cell fraction which is needed for further Treg and Tresp cell isolation.**

5. Determine cell number.

2.3. Magnetic labeling of $CD25^+$ cells

▲ Volumes for magnetic labeling given below are for up to 10^7 total cells. For higher cell numbers, scale up all volumes accordingly.

1. Centrifuge cell suspension at 300×g for 10 minutes. Discard supernatant.

2. Resuspend cell pellet in 90 µL of separation buffer per 10^7 total cells.

3. Add 10 µL of CD25 MicroBeads per 10^7 total cells.

4. Mix well and incubate for 15 minutes in the refrigerator (2–8 °C).

5. Wash cells by adding 1–2 mL of separation buffer and centrifuge at 300×g for 10 minutes. Discard supernatant.

6. Resuspend up to 10^8 cells in 500 µL of separation buffer.
▲ **Note:** For higher cell numbers, scale up buffer volume accordingly.

2.4. Magnetic separation of $CD25^+$ cells

The second step during Treg and Tresp cell isolation is a positive selection of $CD25^+$ cells. Here, two consecutive MS Columns are used, with a capacity of 10^7 labeled cells. To not exceed the capacity, it is recommended to determine the frequency of $CD25^+$ cells in your cell suspension by flow cytometry analysis beforehand.

1. Place an MS Column in the magnetic field of a MiniMACS™ Separator.

2. Prepare column by rinsing with 500 µL of separation buffer.

3. Apply cell suspension onto the column. **Collect flow-through containing unlabeled Tresp cells ($CD4^+CD25^-$).**

4. Wash column 3 times with 500 µL of separation buffer each. Collect unlabeled cells that pass through and combine with the effluent from step 3.

5. Remove column from the separator and place it on a suitable collection tube.

6. Pipette 1 mL of separation buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.

7. To increase purity of $CD4^+CD25^+$ cells, the eluted fraction can be enriched over a second MS Column (recommended). Repeat the magnetic separation procedure as described in steps 1 to 6 using a new MS Column. **The isolated Treg and Tresp cells are now ready-to-use for *in vitro* suppression assay.**

To assess the purity of the isolated Treg and Tresp cells, a flow cytometry analysis must be performed. Please refer to section 3 for a detailed protocol.

3. Surface immunofluorescent staining of Treg and Tresp cells for flow cytometry analysis

▲ To determine the purity of the isolated Treg and Tresp cells, perform the flow cytometry analysis for the final positive (Treg cells) and negative fraction (Tresp cells). It is also recommended to analyze the starting fraction (collect a sample after PBMC preparation).

▲ Volumes given below are **for up to 10^7** cells. When working with fewer 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 nucleated cells, use twice the volume of all indicated reagent volumes and total volumes).

3.1. Immunofluorescent staining

1. Determine cell number.

2. Centrifuge cell suspension at 300×g for 10 minutes. Discard supernatant.

3. Resuspend up to 10^7 nucleated cells per 70 µL of separation buffer.

4. Add 10 µL of CD4-VioBlue® and 20 µL of CD25-PE.

5. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).

6. Wash cells by adding 1–2 mL of separation buffer per 10^7 cells and centrifuge at 300×g for 10 minutes at 4 °C. Discard supernatant.

7. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry

3.2. Flow cytometry analysis

To assess the purity of the isolated Treg and Tresp cells, the cells were analyzed by flow cytometry.

1. Identify lymphocytes according to forward scatter (FSC) and sideward scatter (SSC, data not shown).

2. Exclude dead cells from the analysis by using a live/dead cell exclusion marker (e.g. PI) (data not shown).

3. Analyze the lymphocytes further for their expression of CD4 (x-axis) and CD25 (y-axis) to assess the frequency of $CD4^+CD25^+$ Treg cells and of $CD4^+CD25^-$ Tresp cells in the starting fraction (Fig. 1 A) as well as in the isolated fractions (Fig. 1 B).

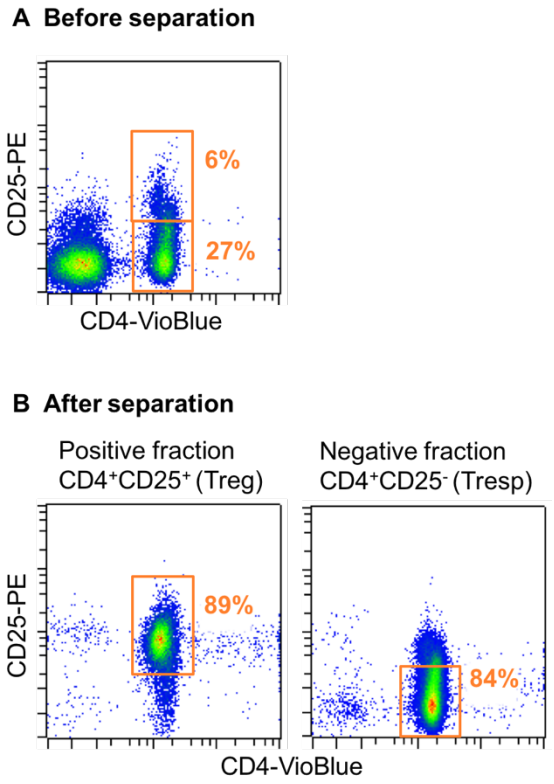

Figure 1: Purity of isolated Treg and Tresp cells. $CD4^+CD25^+$ Treg cells and $CD4^+CD25^-$ Tresp cells were isolated from human PBMCs using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, human. The cells were fluorescently labeled with CD4-VioBlue® and CD25-PE before (A) and after (B) separation.

4. *In vitro* Suppression Assay

4.1 General information

▲ In this protocol one MACSiBead™ Particle per cell (bead-to-cell ratio 1:1) is used for stimulation.

For *in vitro* suppression, Treg cells, Tresp cells and the Treg Suppression Inspector (amount of MACSiBead Particles) are co-cultured in different ratios as depicted in table 1 in a 96-well flat-bottom plate. To improve the final flow cytometry resolution, the cell numbers can be scaled up accordingly (i.e. $2x10^5$ Tresp per well, upscaling of Treg cells and MACSiBead Particles accordingly).

Ratio Tresp: Treg	Tresp	Treg	Treg Suppression Inspector
1:0	$5x10^4$	–	$5x10^4$
1:1	$5x10^4$	$5x10^4$	$10x10^4$
2:1	$5x10^4$	$2.5x10^4$	$7.5x10^4$
4:1	$5x10^4$	$1.3x10^4$	$6.3x10^4$
8:1	$5x10^4$	$0.6x10^4$	$5.6x10^4$
0:1	–	$5x10^4$	$5x10^4$
1:0	$5x10^4$	–	–
0:1	–	$5x10^4$	–
Total	$3x10^5$	$2x10^5$	$4x10^5$
Total triplicates	$9x10^5$	$6x10^5$	$12x10^5$

Table 1: Number of Treg cells, Tresp cells and Treg Suppression Inspector (MACSiBead Particles) per well of a 96-well flat-bottom plate.

4.1.1. Fluorescent labeling of Tresp cells

▲ To monitor the proliferation of Tresp cells during the *in vitro* suppression assay, the cells have to be stained with a fluorescent dye, which allows tracking of cell division, e.g. by using the CellTrace™ Violet Cell Proliferation Kit. For more information please refer to the manufacturer´s instruction. It is also possible to use the CSFE-method or the 3^H-thymidine incorporation method with this *in vitro* suppression assay protocol. Data shown in this application note have been acquired with the CellTrace Violet Cell Proliferation Kit.

4.1.2. Cell preparation

1. Determine the total number of Treg cells and Tresp cells. For one assay, as outlined in table 1, 9×10^5 Tresp cells and 6×10^5 Tregs are needed (if suppression assay is performed with higher cell numbers, scale up the number of cells accordingly).

2. Transfer required cell numbers of cell suspension to suitable tubes.

3. Add 5–10 volumes suppression 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 and the Treg cells (6×10^5) in 1200 µL of suppression medium.
▲ **Note:** The concentration in both cell suspensions is now 5×10^5 cells/mL.

5. Pipette the appropriate volumes of Treg and Tresp cell suspension in a 96-well flat-bottom plate. Refer to table 2 for the respective volumes.

4.1.3. Preparation of Treg Suppression Inspector, human

▲ MACSiBead™ Particles are bigger in size than MicroBeads and sediment rapidly. It is therefore mandatory to bring the MACSiBead Particles in suspension by vortexing prior to use.

1. Resuspend Treg Suppression Inspector thoroughly by vortexing and transfer 60 µL to a suitable tube.
▲ **Note:** Concentration of Treg Suppression Inspector is 2×10^7 MACSiBead Particles per mL.

2. Add 300–600 µL of suppression medium and centrifuge at 300×g for 5 minutes. Aspirate supernatant completely.

3. Resuspend Treg Suppression Inspector in 120 µL of suppression medium. The reagent is now ready to use.
▲ **Note:** Concentration of prepared Treg Suppression Inspector is now 1×10^7 MACSiBead Particles per mL.

4. Pipette the appropriate volumes of Treg Suppression Inspector (amount of MACSiBead Particles) to the Treg/Tresp co-cultured cells into the 96-well flat-bottom plate. Refer to table 2 for the respective volumes.

5. Fill up wells to a total volume of 210 µL with suppression medium (see table 2).

Ratio Tresp: Treg	**Tresp** ($5x10^5$ cells/mL)	**Treg** ($5x10^5$ cells/mL)	**Treg Suppression Inspector** ($1x10^7$ MACSiBead Particles/mL)	**Culture medium**
1:0	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 µL	92 µL
0:1	–	100 µL	5 µL	105 µL
1:0	100 µL	–	–	110 µL
0:1	–	100 µL	–	110 µL
Total volume	600 µL	387.5 µL	40 µL	654 µL
Total volume triplicates	1800 µL	1200 µL	120 µL	~ 2 mL

Table 2: Pipetting scheme for one assay with a total volume of 210 µL of cell suspension per well with a concentration of $5x10^5$ cells/mL.

4.1.4. Incubation

Incubate the suppression assay at 37 °C and 5–7% CO_2 for 5 days.

1. If a proliferation dye (e.g. CellTrace™ or CFSE) was used, refer to section 5 for detailed description of final flow cytometry analysis.

2. If ^{3}H-thymidine was used: after 4 days add the appropriate volume of ^{3}H-thymidine to each well and incubate at 37 °C and 5–7% CO_2 for 16 hours. Measure ^{3}H-thymidine incorporation, e.g., by using a liquid scintillation counter.

5. Flow cytometry analysis

5.1. Immunofluorescent staining

1. Harvest the cells by transferring the cell suspension of each well into a separate 5 mL tube.

2. Wash cells by adding 1–2 mL of separation buffer for up to 10^7 cells and centrifuge at 300×g for 10 minutes at 4 °C. Discard supernatant.

3. Resuspend cells in 90 µL of separation buffer.
4. Add 10 µL of CD4-VioBlue® and 20 µL of CD25-PE.

5. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).

6. Wash cells by adding 1–2 mL of separation buffer per 10^7 cells and centrifuge at 300×g for 10 minutes at 4 °C. Discard supernatant.

7. Resuspend cell pellet in a suitable amount of separation buffer for analysis by flow cytometry.

5.2. Flow cytometry analysis

1. Identify lymphocytes according to FSC and SSC (data not shown).

2. Exclude dead cells from the analysis by using a live/dead cell exclusion marker (e.g. PI) (data not shown).

3. Gate CD4 cells according to CD4 (y-axis) and FSC (x-axis) (data not shown).

4. $CD4^+CD25^+$ Treg cells can be distinguished from the $CD4^+CD25^-$ Tresp cells according to CD25 (y-axis) and CellTrace (x-axis) (fig. 2 A). Gate the CellTrace-positive cells for further cell proliferation analysis (fig. 2 A, red gate).

5. Apply the gating strategy to all samples. Analyze the frequency of proliferating cells by using a histogram plot (fluorescence intensity of the CellTrace on the x-axis) (fig. 2B, right) and determining of CellTrace dilution (red bar).

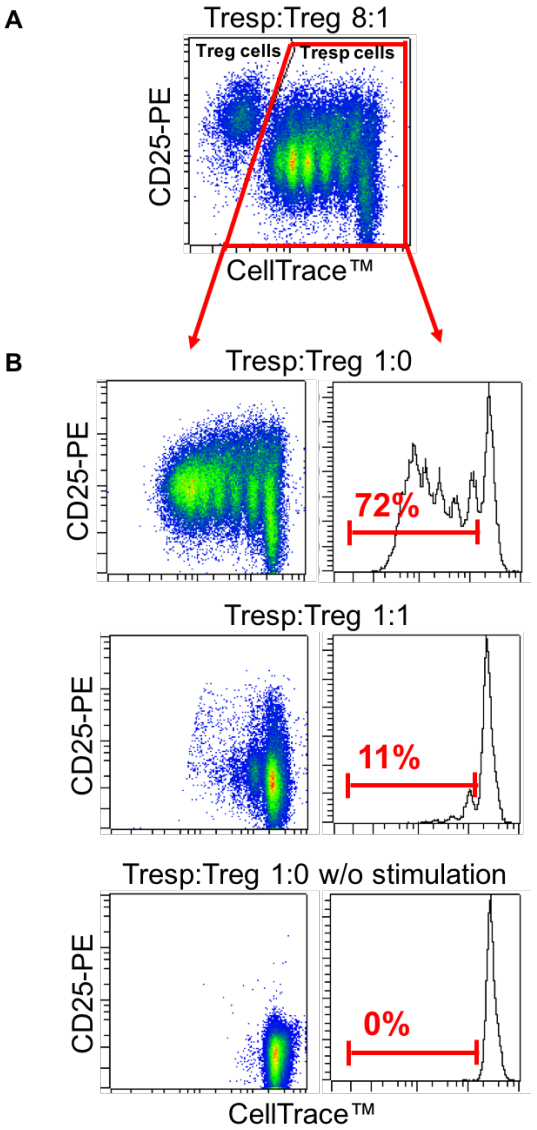

Figure 2: Analysis of an *in vitro* suppression assay. $CD4^+CD25^+$ Treg cells, $CD4^+CD25^-$ Tresp cells and Treg Suppression Inspector MACSiBead™ Particles were co-cultured in different ratios. After 5 days, cells were immunofluorescently stained with CD4-VioBlue and CD25-PE. (A) Gating strategy to distinguish Treg cells and Tresp cells to allow further analysis of Tresp cell proliferation (ratio Tresp:Treg 8:1). (B) Representative flow cytometry plots showing CellTrace MFI dilution of different culture conditions (upper row: Tresp:Treg 1:0, middle: Tresp:Treg 1:1, lower row: Tresp:Treg 1:0 cultured without Treg Suppression Inspector).

5.3 Final flow cytometry evaluation

The data obtained by flow cytometry analysis can be summarized in a graphic as depicted in figure 3.

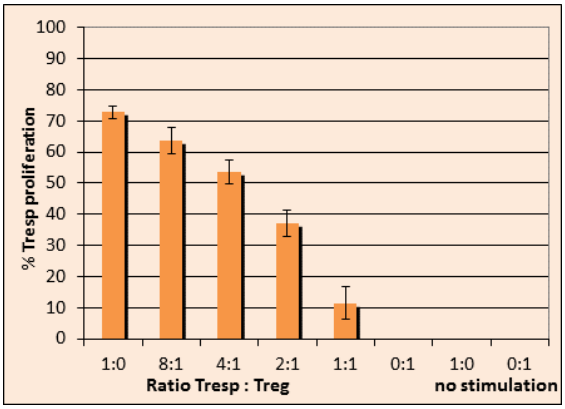

Figure 3: Summarized data of an *in vitro* suppression assay. $CD4^+CD25^+$ Treg cells and $CD4^+CD25^-$ Tresp cells were isolated from human PBMCs by using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit, human and co-cultured with the Treg Suppression Inspector in an *in vitro* suppression assay for 5 days. The suppressive capacity of Treg cells was determined by analyzinging the proliferation of Tresp cells under different co-culture conditions.

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