

## Contents

1. Description
  - 1.1 Principle of T Cell Activation/Expansion Kit
  - 1.2 Background information
  - 1.3 Applications
  - 1.4 Reagent and instrument requirements
2. Protocol
  - 2.1 Sample preparation
  - 2.2 Loading of Anti-Biotin MACSiBead™ Particles
  - 2.3 T cell activation protocol
  - 2.4 T cell expansion protocol
  - 2.5 Immunofluorescent staining
  - 2.6 Removal of Anti-Biotin MACSiBead™ Particles
3. Examples of T cell activation and T cell expansion using the T Cell Activation/Expansion Kit
4. Appendix: Flask and dish sizes for T cell expansion

## 1. Description

<b>Components</b>	2 mL Anti-Biotin MACSiBead™ Particles, cell culture grade, corresponding to $4 \times 10^8$ MACSiBead Particles; MACSiBead Particles conjugated to monoclonal anti-biotin antibodies, 0.4 mL CD2-Biotin, human (100 µg/mL), 0.4 mL CD3-Biotin, human (100 µg/mL), 0.4 mL CD28-Biotin, human (100 µg/mL).
<b>Product format</b>	All components are supplied in azide-free buffer containing stabilizer. Low endotoxin.
<b>Storage</b>	Store protected from light at 2–8 °C. Do not freeze. The expiration date is indicated on the vial label.

### 1.1 Principle of T Cell Activation/Expansion Kit

The T Cell Activation/Expansion Kit is designed to activate and, if required, expand human T cells. The kit consists of Anti-Biotin MACSiBead Particles and biotinylated antibodies against human CD2, CD3 and CD28. Anti-Biotin MACSiBead Particles loaded with biotinylated antibodies are used to mimic antigen-presenting cells and activate resting T cells from peripheral blood mononuclear cells (PBMCs) as well as purified T cells. T cell expansion is achieved by culturing and reactivation at day 14 of culture.

### 1.2 Background and product applications

The Anti-Biotin MACSiBead Particles are in a first step loaded with biotinylated antibodies. An optimal activation is achieved by using equal amounts of the provided biotinylated antibodies against CD2, CD3 and CD28.

▲ **Note:** Other combinations of biotinylated antibodies may be experimentally tested for their suitability, if required.

Loaded Anti-Biotin MACSiBead Particles are subsequently used

for the activation of T cells. An optimal activation of T cells is accomplished by using one loaded Anti-Biotin MACSiBead Particle per two cells (bead-to-cell ratio 1:2). The cells are cultured for up to 3 days and can be further expanded, if desired. Expansion is achieved by adding IL-2 and fresh medium every 3–4 days. Cells are restimulated at day 14 by adding additional loaded Anti-Biotin MACSiBead Particles at a bead-to-cell ratio of 1:2.

▲ The efficiency of the T cell activation depends on their differentiation status, which will often be heterogeneous. For special applications, it is therefore recommended to experimentally determine the best stimulation ratio.

▲ Over-activation of T cells carries a risk of activation-induced cell death.

T cells, activated by using Anti-Biotin MACSiBead Particles, can be used for any downstream processing such as cytokine analysis or immunoprecipitation. Also, activated T cells can be transfected with high efficiency.

Anti-Biotin MACSiBead Particles show no autofluorescence and normally do not need to be removed prior to flow cytometric analysis. However, if desired, removal of Anti-Biotin MACSiBead Particles is easily achieved by using the MACSiMAG™ Separator (see 2.6).

### 1.3 Applications

- Activation and expansion of resting T cells from PBMCs.
- Activation and expansion of T cells positively selected with MicroBeads, e.g. CD4 MicroBeads (# 130-045-101) or CD8 MicroBeads (# 130-045-201), or untouched T cells isolated using Pan T Cell Isolation Kit II (# 130-091-156), CD4<sup>+</sup> T Cell Isolation Kit II (# 130-091-155) or CD8<sup>+</sup> T Cell Isolation Kit II (# 130-091-154)
- Expansion of antigen-specific T cells.

### 1.4 Reagent and instrument requirements

- Buffer: Phosphate-buffered saline (PBS) pH 7.2, supplemented with 0.5% human serum albumin (HSA) and 2 mM EDTA. Keep buffer cold (2–8 °C). Degas buffer before use, as air bubbles could block the column.

▲ **Note:** HSA can be replaced by other proteins such as bovine serum albumin, fetal calf serum or human AB serum. Buffers or media containing Ca<sup>2+</sup> or Mg<sup>2+</sup> are not recommended for use.

- Medium: RPMI 1640 (# 130-091-440) 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.
- (Optional) Cell Expansion Bags (6 ports: # 130-074-351; tube: # 130-074-352) or flat bottom cell culture plates with lids.
- Humidified incubator.

- MACSmix™ Tube Rotator (# 130-090-753) for loading of MACSiBead Particles.
- (Optional) MACSiMAG Separator for removal of Anti-Biotin MACSiBead Particles after T cell expansion prior to downstream experiments.
  - ▲ **Note:** Do not remove MACSiBead Particles by using MACS Columns and MiniMACS™, MidiMACS™, VarioMACS™, SuperMACS™, autoMACS™ or autoMACS Pro Separators.
- (Optional) Fluorochrome-conjugated antibodies for flow cytometric analysis, for example CD4-FITC (# 130-080-501), CD4-PE (# 130-091-231), CD4-APC (# 130-091-232), CD8-FITC (# 130-080-601), CD8-PE (# 130-091-084), CD8-APC (# 130-091-076) or CD25-PE (# 130-091-024). For more information about fluorochrome-conjugated antibodies see [www.miltenyibiotec.com](http://www.miltenyibiotec.com).
- (Optional) Propidium Iodide Solution (# 130-093-233) or 7-AAD for the flow cytometric exclusion of dead cells.
- (Optional) Recombinant Interleukin-2 (rIL-2), e.g. Human IL-2 (# 130-093-901) or Human IL-2 (v126) (# 130-093-906).

## 2. Protocol

▲ All steps in the protocol have to be performed under sterile conditions.

### 2.1 Sample preparation

When working with anticoagulated peripheral blood or buffy coat, peripheral blood mononuclear cells (PBMCs) should be isolated by density gradient centrifugation, for example, using Ficoll-Paque™.

▲ **Note:** To remove platelets after density gradient separation, resuspend cell pellet in buffer and centrifuge at 200×g for 10–15 minutes at 20 °C. Carefully aspirate supernatant. Repeat washing step.

When working with tissues or lysed blood, prepare a single-cell suspension using standard methods.

For details see the protocols section at [www.miltenyibiotec.com/protocols](http://www.miltenyibiotec.com/protocols).

▲ Dead cells may bind non-specifically to MACS MicroBeads. To remove dead cells, we recommend using density gradient centrifugation or the Dead Cell Removal Kit (# 130-090-101).

### 2.2 Loading of Anti-Biotin MACSiBead™ Particles

▲ Resuspend Anti-Biotin MACSiBead™ Particles thoroughly by vortexing **before use**, to obtain a homogenous suspension.

▲ Anti-Biotin MACSiBead Particles are supplied without preservative. Remove aliquots under aseptic conditions.

▲ It is recommended to load Anti-Biotin MACSiBead Particles in batches of  $1 \times 10^8$  Anti-Biotin MACSiBead Particles. Loaded Anti-Biotin MACSiBead Particles are stable for up to 4 months when stored at 2–8 °C.

1. Pipette 100  $\mu$ L of CD2-Biotin, 100  $\mu$ L CD3-Biotin and 100  $\mu$ L CD28-Biotin into sealable 2 mL tube and mix well.
  - ▲ **Note:** This antibody combination, with a final antibody concentration of 10  $\mu$ g per antibody per 1 mL loaded Anti-Biotin MACSiBead Particles, is optimized for achieving maximal T cell activation.
2. **Resuspend** Anti-Biotin MACSiBead Particles **thoroughly** by vortexing.
3. Remove 500  $\mu$ L Anti-Biotin MACSiBead Particles ( $1 \times 10^8$  Anti-Biotin MACSiBead Particles) and add to antibody mix.

4. Add 200  $\mu$ L buffer to adjust to a total volume of 1 mL.
  - ▲ **Note:** Anti-Biotin MACSiBead Particles can be loaded in a flexible manner with biotinylated antibodies or ligands other than those supplied. If desired, add other biotinylated antibodies or ligands at appropriate concentrations and adjust with buffer to a total volume of 1 mL, accordingly.
5. Incubate for 2 hours at 2–8 °C under constant, gentle rotation by using the MACSmix Tube Rotator (# 130-090-753) at approximately 4 rpm (slowest permanent run program).
6. The **loaded Anti-Biotin MACSiBead Particles** ( $1 \times 10^8$  Anti-Biotin MACSiBead Particles/mL) are now ready to use. **Do not remove the loaded Anti-Biotin MACSiBead Particles from the antibody mix.** Store at 2–8 °C for up to 4 months.

### 2.3 T cell activation protocol

This T cell activation protocol is optimized for strong activation of PBMCs, using one loaded Anti-Biotin MACSiBead Particle per two PBMCs or T cells (bead-to-cell ratio 1:2). The same bead-to-cell ratio is recommended when activating purified T cells.

▲ **Note:** Other ratios than 1:2 of loaded Anti-Biotin MACSiBead Particles per cell may be required for other applications (see also 1.2 Background and product applications).

▲ Volumes for activation given below are for  $5 \times 10^6$  PBMCs. When working with higher cell numbers, scale up all reagent volumes and total volumes, accordingly (e.g. for  $1 \times 10^7$  total cells, use twice the volume of all indicated reagent volumes and total volumes).

▲ For the activation of purified T cells, cultivate T cells at  $2.5 \times 10^6$  cells per mL per  $\text{cm}^2$ .

1. Resuspend **loaded Anti-Biotin MACSiBead Particles** thoroughly and transfer 25  $\mu$ L ( $2.5 \times 10^6$  loaded Anti-Biotin MACSiBead Particles) per  $5 \times 10^6$  PBMCs to a suitable tube.
  - ▲ **Note:** If unloaded MACSiBead Particles shall be used for negative control experiments, replace loaded Anti-Biotin MACSiBead Particles by adding 12.5  $\mu$ L ( $2.5 \times 10^6$  beads) of unloaded Anti-Biotin MACSiBead Particles per  $5 \times 10^6$  PBMCs.
2. Add 100–200  $\mu$ L culture medium to the **loaded Anti-Biotin MACSiBead Particles** and centrifuge at 300×g for 5 minutes.
3. Aspirate supernatant and resuspend **loaded Anti-Biotin MACSiBead Particles** in 100  $\mu$ L fresh culture medium.
4. Resuspend PBMCs at a density of  $5 \times 10^6$  cells per 900  $\mu$ L of culture medium RPMI 1640 (# 130-091-440) supplemented with 10% AB serum, or X-VIVO 15™ (optional: supplemented with 5% AB serum).
  - ▲ **Note:** Defined media, e.g. X-VIVO-15, might be advantageous for T cell expansion.
5. Add the prepared Anti-Biotin MACSiBead Particles from step 3 to the 900  $\mu$ L of cell suspension and mix well.
6. Add the mixture to a suitable cell culture vessel at a density of  $5 \times 10^6$  cells per mL per  $\text{cm}^2$ .
7. Incubate at 37 °C, 5–10% CO<sub>2</sub> for up to 3 days.
  - ▲ **Note:** Inspect cultures daily, and add fresh medium if required.
8. For T cell expansion, proceed to step 2 of the T cell expansion protocol (see section 2.4). For immunofluorescent staining proceed to 2.5.

## 2.4 T cell expansion protocol

As T cells are activated and expand, the addition of further growth factors and stimuli, such as more loaded Anti-Biotin MACSiBead Particles is required. The following procedure is a guideline for the stimulation and expansion of T cells.

1. Perform steps 1–7 as described under section 2.3 (T cell activation protocol).
2. At day 3 gently pipette cell suspension up and down to break up clumps.
3. Split cell suspension into two equal parts and add culture medium supplemented with 20 units rIL-2 per mL. Incubate at 37 °C, 5–10% CO<sub>2</sub>.  
▲ **Note:** If rIL-2 interferes with downstream experiments, it may be omitted. However, omission will lower the cell viability.
4. At day 7 gently pipette cell suspension up and down to break up clumps. Count T cells and dilute to 1.2–2.5×10<sup>6</sup> T cells per mL by adding fresh medium supplemented with 20 units rIL-2 per mL.
5. At day 11, split cell suspension into two equal parts and add fresh medium supplemented with 20 units rIL-2 per mL.
6. At day 14, resuspend T cells at 2.5×10<sup>6</sup> cells per mL in fresh medium supplemented with 20 units rIL-2 per mL.
7. For longer expansion, cells are now restimulated by adding one **loaded Anti-Biotin MACSiBead Particle** per two cells: Count cells and add 12.5 µL **loaded Anti-Biotin MACSiBead Particles** per 2.5×10<sup>6</sup> T cells.
8. Further cultivate cells and repeat steps 2 and 3 every 3–4 days.  
▲ **Note:** Daily inspect culture. Depending on the expansion rate, it might be necessary to split culture more frequently than every 3–4 days.
9. Proceed to downstream application, e.g. analysis of cells.  
▲ **Note:** Removal of Anti-Biotin MACSiBead Particles is not required for immunofluorescent staining. For assays where T cells are required to return to a fully resting state before further stimulus, Anti-Biotin MACSiBead Particles should be removed at least 24 hours before restimulation (see section 2.6).

## 2.5 Immunofluorescent staining

▲ Volumes for fluorescent labeling given below are for 10<sup>6</sup> total cells. When working with fewer than 10<sup>6</sup> cells and up to 10<sup>7</sup> cells, use the same volumes as indicated.

▲ MACSiBead Particles show no autofluorescence and do not need to be removed prior to flow cytometric analysis.

▲ After short term stimulus for up to 24 h, scatter properties of cells may be altered due to strong interaction between cells and MACSiBead Particles.

▲ Upon stimulation, expression of CD3 might be transiently down-regulated. Thus, the staining of CD3 on the cell surface of activated cells might be affected.

1. Resuspend cells to break up cell clumps.  
▲ **Note:** In short-term stimulus of cells (up to 24 hours), the Anti-Biotin MACSiBead Particles may be bound strongly to cells. Care should be taken to thoroughly resuspend cells before analysis.
2. Wash cells by adding 1–2 mL of buffer per 10<sup>6</sup> cells and centrifuge at 300×g for 10 minutes. Pipette off supernatant completely.
3. Add 10 µL of each staining antibody, e.g., CD4-FITC (# 130-080-501) and CD25-PE (# 130-091-024) to 10<sup>6</sup> cells resuspended with buffer to a total volume of 110 µL.

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

▲ **Note:** Working on ice requires increased incubation time. Higher temperatures and/or longer incubation times lead to non-specific cell labeling.

5. Wash cells by adding 1–2 mL of buffer per 10<sup>6</sup> cells and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
6. Resuspend cell pellet in a suitable amount of buffer for analysis by flow cytometry or fluorescence microscopy.

## 2.6 Removal of Anti-Biotin MACSiBead™ Particles

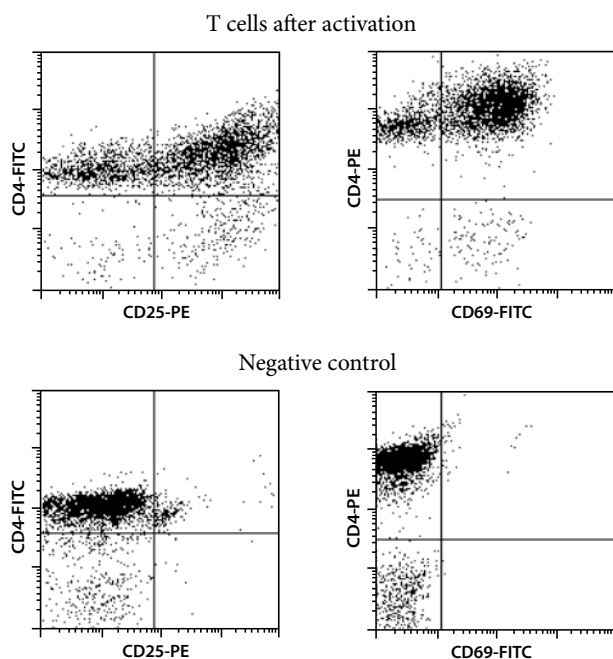
▲ Removal of MACSiBead™ Particles may be required before magnetic separation of cells with MACS MicroBeads or before restimulation with different agents or antigens.

1. Harvest cells and transfer to a 5 mL, 15 mL, or 50 mL tube and wash once with buffer.
2. Resuspend cells in buffer at a density of up to 2×10<sup>7</sup> cells per 1 mL and vortex thoroughly.
3. Place the tube in the magnetic field of the MACSiMAG Separator.  
▲ **Note:** Use tube rack to insert 5 mL tube into the magnetic field of the separator. For details see MACSiMAG Separator data sheet.
4. Allow the MACSiBead Particles to adhere to the wall of the tube:  
5 mL tubes: 2 minutes  
15 mL or 50 mL tubes: 4 minutes
5. Retaining the tube in the magnet, carefully remove the supernatant containing the MACSiBead-depleted cells and place in a new tube.
6. Remove the tube from the separator and add buffer to the same volume as before.
7. Vortex sample, replace tube in the MACSiMAG Separator and repeat steps 4–5.
8. Collected cells can now be further processed as required.

### 3. Examples of T cell activation and T cell expansion using the T Cell Activation/Expansion Kit

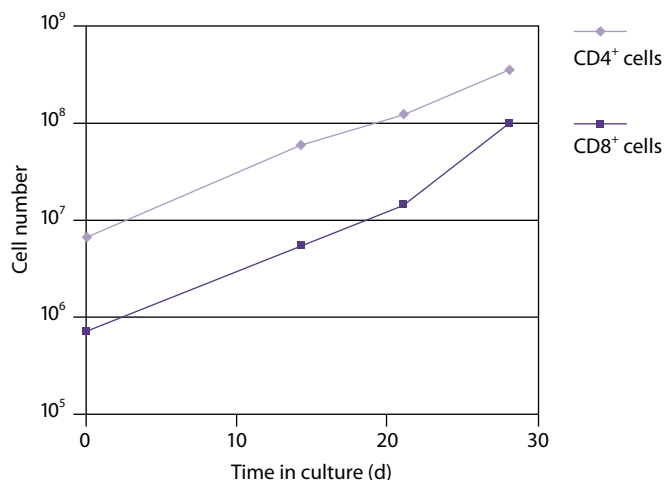
#### A) Example of a T cell activation

Anti-Biotin MACSiBead Particles were loaded with CD2, CD3, and CD28 antibodies. T cells were isolated using the Pan T Cell Isolation Kit II. Purified T cells were activated using 1 loaded Anti-Biotin MACSiBead Particle per 2 T cells, for 72 hours. The negative control experiment was performed without adding MACSiBead Particles. Cells were fluorescently stained using CD4-FITC and CD25-PE or CD4-PE and CD69-FITC. Dead cells and debris were excluded from the analysis based on scatter signals and PI fluorescence.



#### B) Example of a T cell expansion

Anti-Biotin MACSiBead Particles were loaded with CD2, CD3, and CD28 antibodies. T cells were isolated using the Pan T Cell Isolation Kit II and expanded using 1 loaded Anti-Biotin MACSiBead Particle per 2 T cells. T cells were cultured (X-VIVO 15 supplemented with 5% AB serum and 20 units rIL-2/mL) at an initial density of  $2.5 \times 10^6$  T cells per mL per  $\text{cm}^2$ . At day 14, additional loaded Anti-Biotin MACSiBead Particles were added at a bead-to-cell ratio of 1:2. Cells were further expanded for 2 weeks.



### 4. Appendix: Flask and dish sizes for T cell expansion

For T cell expansion the cells should be resuspended in culture medium at  $2.5 \times 10^6$  cells/mL. The cells should be plated at a density of  $2.5 \times 10^6$  cells/ $\text{cm}^2$ . Both the dilution and the cell density are important to assure optimal stimulation and cell growth.

The following table lists culture plate, dish and flask sizes suitable for different cell numbers. It also indicates the appropriate amount of medium to add.

Total cell number	Medium volume to add	Culture plate size	Well diameter
$0.75 \times 10^6$	0.3 mL	96 well	0.64 cm
$2.5 \times 10^6$	1 mL	48 well	1.13 cm
$5 \times 10^6$	2 mL	24 well	1.6 cm
$10 \times 10^6$	4 mL	12 well	2.26 cm
$25 \times 10^6$	10 mL	6 well	3.5 cm

Total cell number	Medium volume to add	Culture dish size	Dish diameter
$2.25 \times 10^7$	9 mL	small	3.5 cm
$5 \times 10^7$	20 mL	medium	6 cm
$12.5 \times 10^7$	50 mL	large	10 cm
$25 \times 10^7$	100 mL	extra large	15 cm

Total cell number	Medium volume to add	Culture flask size	Growth area
$6 \times 10^7$	24 mL	50 mL	25 $\text{cm}^2$
$20 \times 10^7$	80 mL	250 mL	75 $\text{cm}^2$
$40 \times 10^7$	160 mL	720 mL	162 $\text{cm}^2$
$60 \times 10^7$	240 mL	900 mL	225 $\text{cm}^2$

Total cell number	Medium volume to add	Culture bag size	Growth area
$6.25 \times 10^7$	25 mL	25 mL	27 $\text{cm}^2$
$12.5 \times 10^7$	50 mL	50 mL	58 $\text{cm}^2$
$25 \times 10^7$	100 mL	100 mL	112 $\text{cm}^2$

#### Warranty

The products sold hereunder are warranted only to be free from defects in workmanship and material at the time of delivery to the customer. Miltenyi Biotec GmbH makes no warranty or representation, either expressed or implied, with respect to the fitness of a product for a particular purpose. There are no warranties, expressed or implied, which extend beyond the technical specifications of the products. Miltenyi Biotec GmbH's liability is limited to either replacement of the products or refund of the purchase price. Miltenyi Biotec GmbH is not liable for any property damage, personal injury or economic loss caused by the product.

MACS is a registered trademark and autoMACS, MACSiBead, MACSiMAG, MACSmix, MidiMACS, MiniMACS, OctoMACS, QuadroMACS, SuperMACS, and VarioMACS are trademarks of Miltenyi Biotec GmbH.

Ficoll-Paque is a trademark of GE Healthcare companies.

Copyright © 2008 Miltenyi Biotec GmbH. All rights reserved.