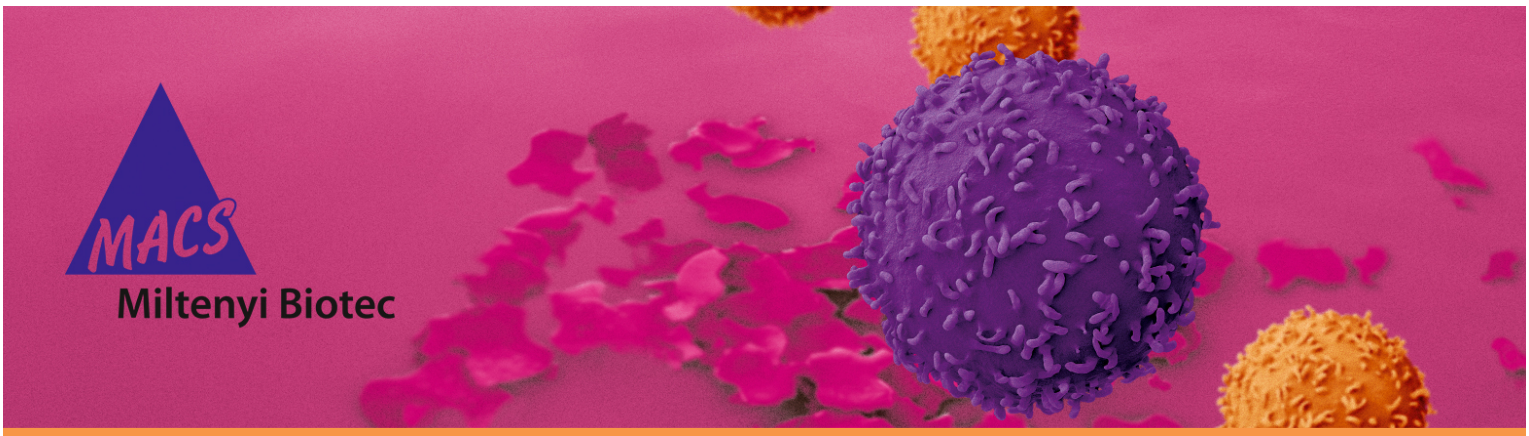


Miltenyi Biotec

## Direct *ex vivo* characterization of human antigen-specific $CD154^+CD4^+$ T cells

# Rapid antigen-reactive T cell enrichment (Rapid ARTE)

## Introduction

Antigen (ag)-specific T cells play a central role in mediating specific immune responses as well as in the formation of immunological memory. Information about their frequency, phenotype, and functional capacity is essential to estimate the specific immune status of an individual, to understand the mechanisms of protective immunity or immunopathology and to predict immune protection or diagnose immune-related diseases. Due to the very low frequency of ag-specific T cells in peripheral blood the reliable detection, enumeration and phenotypical characterization require the processing of high cell numbers and highly specific analysis methods. CD154 is transiently up-regulated on activated $CD4^+$ T cells and plays an important role as a costimulatory molecule in T cell ag-presenting cell interactions through ligation of CD40. Due to its transient expression within hours after activation, CD154 can be used as a marker for activated ag-specific $CD4^+$ T cells. Adding a CD40-blocking antibody during cell stimulation prevents down-regulation of CD154 expression. CD154 is induced by the interaction with CD40 expressed on ag-presenting cells.

Combining magnetic cell enrichment and multiparameter flow cytometry analysis of $CD154^+CD4^+$ T cells allow direct *ex vivo* detection and characterization of rare ag-specific T cells.

## Workflow

| | |
|---|---|
| 6 h | **PBMC stimulation** |
| 40 min | **Magnetic and immune-fluorescent surface labeling** |
| 60 min | **Magnetic enrichment and intracellular staining** |
| | **Flow cytometry analysis** |

# Materials

**PBMC cultivation**

- RPMI 1640 medium
- 100× L-Glutamine stock solution (200 mM)
- Human AB Serum
- 24-well plate (e.g. Gas-permeable Culture Plate, # 150-000-362)

▲ Note: With the Gas-permeable Culture Plate (# 150-000-362), up to $2.5\times10^7$ PBMCs/well/mL can be stimulated as opposed to $1\times10^7$ PBMCs/well/mL in standard 24-well plates.

**PBMC stimulation**

- CD40 pure – functional grade, human (# 130-094-133)
- Reagents for ag-specific T cell stimulation (e.g. PepTivator® BKV VP1 – research grade, human, # 130-097-272 and PepTivator BKV LT – research grade, human, # 130-096-504)
- brefeldin A
- FcR Blocking Reagent, human (# 130-059-901)

**Buffer (standard wash and dilution buffer)**

- autoMACS® Rinsing Solution (# 130-091- 222)
- Bovine serum albumin (BSA Stock Solution, # 130-091-376)

**Magnetic and immunofluorescent surface labeling**

- CD154 MicroBead Kit, human (# 130-092-658)
- Inside Stain Kit (# 130-090-477)
- Orbital shaker
- CD3-VioBlue®, human (# 130-094-363)
- CD4-APC, human (# 130-092-374)
- CD8-PerCP, human (# 130-094-972)
- CD14-PerCP, human (# 130-094-969)
- CD20-PerCP, human (# 130-094-976)

**Magnetic enrichment and intracellular staining**

- MS Columns (# 130-042-201)
- MACS® Separator for MS Columns (e.g. MiniMACS™ Separator # 130-042-102)
- MACS MultiStand (# 130-042-303)
- CD154-FITC (# 130-096-233)
- Anti-cytokine antibodies for intracellular staining (e.g. Anti-IFN-γ-PE, # 130-091-653 or Anti-IL-17A-PE, # 130-094-521)

# Material preparation

**PBMC cultivation medium**

Prepare a solution of 500 mL RPMI 1640 and 5 mL 100× L-Glutamine stock solution (2 mM final concentration). Add 25 mL human AB Serum (5% final concentration).

**Buffer (standard wash and dilution buffer)**

Prepare a solution of PBS, pH 7.2, 2 mM EDTA and 0.5% BSA by diluting MACS BSA Stock Solution 1:20 with autoMACS Rinsing Solution.

**Reconstitution of PepTivator® Peptide Pool**

For reconstitution of the lyophilized peptide pool take the vial from –20 °C and warm-up to room temperature.

▲ Note: Do not open the vial by removing the rubber plug.

2. To dissolve the 6 nmol packing unit of the PepTivator® Peptide Pool fill a sterile syringe (0.5 mL) with 200 µL of sterile water. To dissolve the 60 nmol packing unit of the PepTivator Peptide Pool fill a sterile syringe (5 mL) with 2 mL of sterile water.

3. Slowly inject the water with a sterile needle through the center of the rubber plug into the vial containing the lyophilized peptide pool.

4. Vortex the solution to completely dissolve the lyophilized peptide pool. The concentration of the stock solution of PepTivator® Peptides is 30 nmol (approximately 50 µg) of each peptide per mL.

5. Remove the rubber plug and aspirate the stock solution with a pipette.

6. To avoid repeated freeze-thaw cycles prepare working aliquots from the stock solution.

7. Store the working aliquots at –80 °C.

**Staining Mix**

**Freshly** prepare 200 µL of staining mix for each well:

- 12.5 µL CD3-VioBlue®
- 25 µL CD4-APC
- 25 µL CD8-PerCP
- 25 µL CD14-PerCP
- 50 µL CD20-PerCP
- 25 µL CD154-Biotin (from CD154 MicroBead Kit)
- 37.5 µL FcR Blocking Reagent

# Methods

## 1. *In vitro* stimulation for induction of CD154 expression

1. Sterile preparation of PBMCs from fresh buffy coats or whole blood 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.

2. Resuspend cells at a density of $10^7$ cells/mL in PBMC cultivation medium.

3. For each non-stimulated control and antigen-stimulated sample transfer $10^7$ cells to one well of a 24-well plate.

4. Add 10 µL of CD40 pure – functional grade (1 µg/mL; 1:100) to each well.

5. Add antigen according to manufacturer's recommendation to the antigen-stimulated wells, but not to control wells. When using PepTivators: Add 20 µL of reconstituted stock solution per well.

6. Incubate cells for 4 hours at 37 °C, 5% $CO_2$.

7. Add brefeldin A to a final concentration of 2 µg/mL.

8. Incubate cells for another 2 hours at 37 °C, 5% $CO_2$.

## 2. Magnetic and immunofluorescent surface labeling

1. Centrifuge plate for 5 minutes at 300×g at room temperature.

2. Very carefully aspirate 800 µL of cell culture supernatant. Avoid resuspension of the cells.

3. Add 200 µL of antibody staining mix to each well:

- 12.5 µL CD3-VioBlue®
- 25 µL CD4-APC,
- 25 µL CD8-PerCP
- 25 µL CD14-PerCP
- 50 µL CD20-PerCP
- 25 µL CD154-Biotin (from CD154 MicroBead Kit)
- 37.5 µL FcR Blocking Reagent

4. Mix well for 2 minutes using an orbital shaker.

5. Incubate for 3 minutes in the dark at room temperature.

6. Add 20 µL of Anti-Biotin MicroBeads UltraPure (from CD154 MicroBead Kit) to each well. Mix well for 2 minutes using an orbital shaker.

7. Incubate for 13 minutes in the dark at room temperature.

8. Add 300 µL of Inside Fix (from Inside Stain Kit) to each well. Mix well for 2 minutes using an orbital shaker.

9. Incubate for 13 minutes in the dark at room temperature.

10. Add 1 mL of buffer to each well and mix well.

11. (Optional) Take an aliquot of 200 µL cell suspension for staining of the original fraction.

## 3. Magnetic enrichment and intracellular staining (on column)

1. Place MS Column in the magnetic field of a suitable MACS® Separator.

2. Prepare MS Column by rinsing with 500 µL of buffer.

3. Apply cell suspension onto the column.

4. Wash cells by rinsing the column with 500 µL of buffer, followed by 2×500 µL of Inside Perm (from Inside Stain Kit).

5. Prepare a solution of 10 µL of CD154-FITC and 90 µL of Inside Perm (from Inside Stain Kit).

6. (Optional) Add additional staining antibodies to the solution, e.g., for the staining of cell surface antigens internalized upon cell activation or antigens which accumulate in the cell.
▲ **Note:** Do not exceed the total volume of 150 µL.

7. Apply the solution onto the column and incubate for 10 minutes at room temperature.
▲ **Note:** The MACS Column has a flow-stop mechanism that will retain the solution in the column.

8. Wash cells by rinsing the column with 2×500 µL of Inside Perm (from Inside Stain Kit) followed by 500 µL of buffer.

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

10. Pipette 500 µL of buffer onto the column. Immediately flush out the magnetically labeled cells by firmly pushing the plunger into the column.

11. Cells are now ready for analysis. Store cells at 2–8 °C in the dark until analysis. Mix well before flow cytometric acquisition.
▲ **Note:** Samples may be stored at 2–8 °C in the dark for up to 24 hours.
▲ **Note:** Do not use propidium iodide (PI) or 7-AAD staining in general.

## 4. Intracellular staining of original samples

1. Wash cells by adding 1 mL of buffer. Centrifuge cells for 5 minutes at 300×g at room temperature.

2. Aspirate supernatant.

3. Resuspend cells in 500 µL of Inside Perm (from Inside Stain Kit) and vortex well. Centrifuge cells for 5 minutes at 300×g.

4. Aspirate supernatant.

5. Prepare a solution of 10 µL of CD154-FITC and 90 µL of Inside Perm (from Inside Stain Kit).

6. (Optional) Add additional staining antibodies to the solution, e.g., for the staining of cell surface antigens internalized upon cell activation or antigens which accumulate in the cell.
▲ **Note:** Do not exceed the total volume of 150 µL.

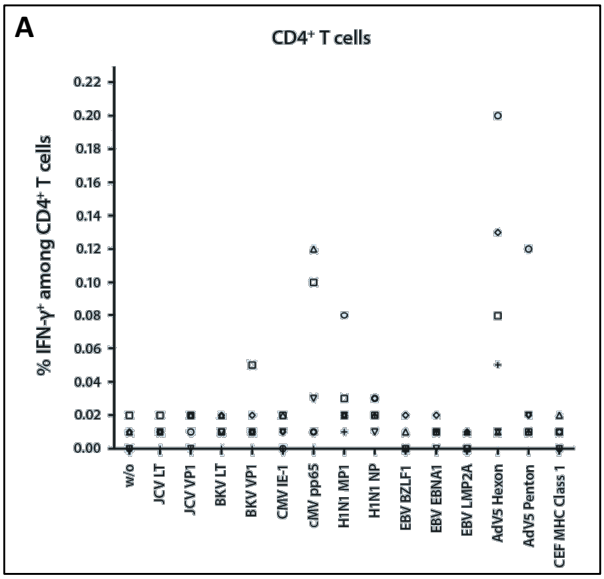
7. Add staining solution to the cells and incubate for 10 minutes at room temperature.

8. Wash cells by adding 1 mL of Inside Perm (from Inside Stain Kit). Centrifuge cells for 5 minutes at 300×g.

9. Aspirate supernatant.

10. Resuspend cells in 250 µL of buffer.

11. Samples are ready for measurement now.
▲ **Note:** Samples may be stored at 2–8 °C in the dark for up to 24 hours.



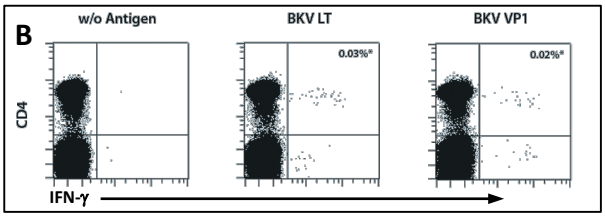




**Figure 1: Detection of virus-specific T cells without enrichment via Rapid ARTE. (A) and (B)** PBMCs from six randomly selected healthy donors were stimulated with various peptide pools for immunodominant antigens of BKV, JCV, CMV, influenza, EBV, and AdV, and for positive control with a mixture of CMV/EBV/influenza MHC class I–restricted peptides. After 6 hours, IFN-γ production within the $CD4^+$ T cell compartment was analyzed by intracellular staining using the Rapid Cytokine Inspector. CMV-, EBV-, and AdV-specific IFN-$\gamma^+$ T cells were clearly detectable in several donor samples. In contrast, IFN-$\gamma^+$-, BKV-, and JCV-specific T cells were detectable only at very low frequencies, between 0.01 and 0.03%, in five out of six donor samples.

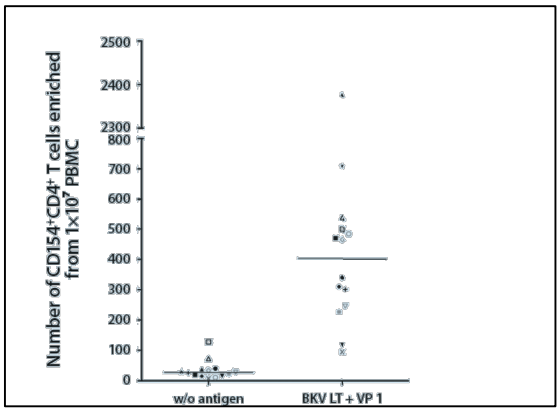



**Figure 2: Rapid ARTE successfully enriches ag-specific T cells.** In blood samples BKV-specific T cells are present only at very low frequencies, in the range of the detection limit of flow cytometry analysis. Therefore, we used the Rapid ARTE protocol for enumeration of BKV-specific T cells. Up to $2.5\times10^7$ PBMCs from 14 healthy donors were left untreated or stimulated with BKV peptide pools covering the complete sequence of the large T antigen (LT) and virion protein VP1. Both proteins are immunodominant targets for T cell immunity. After six hours, cells were treated as described in this protocol, and the absolute numbers of enriched $CD154^+CD4^+$ T cells were determined. In blood samples of each donor, BKV-reactive $CD4^+$ T cells were found. Absolute numbers ranged between 87 and 2340 per $1\times10^7$ PBMCs.

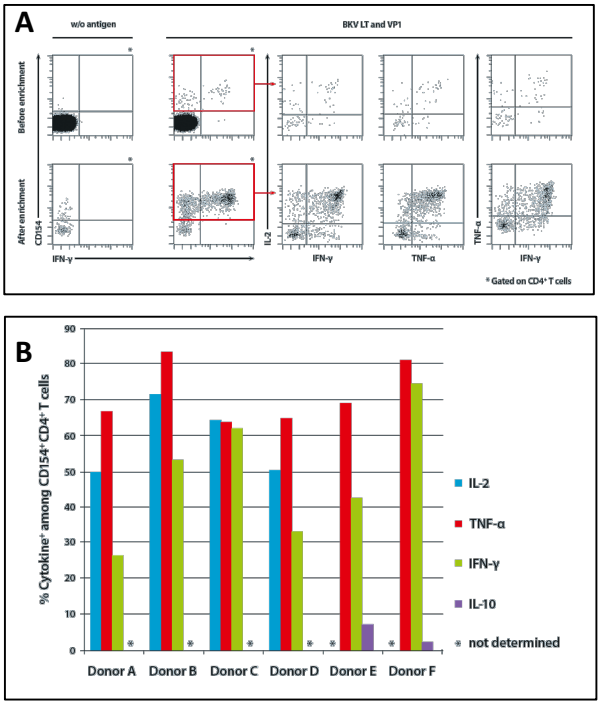



**Figure 3: The Rapid ARTE approach allows to process larger cell numbers compared to detection by flow cytometry alone. (A)** PBMCs ($2.5\times10^7$) from six healthy donors were processed as described in this protocol. Our new approach enables the reliable characterization of functional subsets of the entire BKV-reactive $CD154^+CD4^+$ T cell pool, i.e., TNF-α–, IFN-γ–, IL-10–, and IL-2–producing T cells. **(B)** Subsequently, the cytokine profile of the BKV-reactive $CD4^+$ T cells was analyzed. TNF-α and IL-2 were expressed by the majority of BKV-specific T cells in all samples analyzed for these cytokines. In contrast, the frequency of IFN-$\gamma^+$ cells among BKV-reactive $CD4^+$ T cells varied between 27 and 74% in the different donor samples. Furthermore, IL-10–producing T cells could be identified at very low frequencies, which could not have been detected by flow cytometry without pre-enrichment.

## References

**1.** Bacher, P. *et al.* (2013)
J. Immunol. 190: 3967–3976.

**2.** Bacher, P. *et al.* (2013)
Cytometry A 83: 692–701.