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Improved sensitivity in flow cytometry by in-line pre-enrichment of CAR T cells and antigen-specific T cells on the MACSQuant® Analyzer

Introduction

The detection of rare cells, such as circulating chimeric antigen receptor (CAR) T cells after infusion or antigen-specific T cells, presents a challenge for conventional immune monitoring assays due to their typically low frequencies. Standard flow cytometry delivers a lower limit of detection (LOD) of 1 cell in 10,000 (0.01%), which is often insufficient for tracking these rare populations.[1] More sensitive techniques like qPCR can detect frequencies as low as 0.01%, but they cannot distinguish between viable cells and debris or aggregates.[2]

To harness the phenotypic and kinetic insights of flow cytometry without sacrificing sensitivity, we evaluated the MACS® Cell Enrichment Unit, which integrates Miltenyi Biotec's MACS Technology for magnetic cell separation into the workflow of a MACSQuant Flow Cytometer. In this application note, we show that magnetic cell pre-enrichment enhances analytical sensitivity in the detection of both CAR T cells and antigen-specific T cells. This increased sensitivity improves the reliability of quantification and facilitates deeper characterization of these rare immune cell subsets. We present a robust method that combines detection reagents for different CAR entities and T cell receptors (TCR), illustrating its broad applicability for detecting engineered and naturally occurring rare T cell populations. Finally, we demonstrate the method's performance by determining the assay's limit of detection (LOD) and limit of quantitation (LOQ) according to ICH Q2 guidelines.[3]

Materials and methods

Sample preparation

CAR T cells (CD19 or BCMA) were spiked into 1×10^7 fresh peripheral blood mononuclear cells (PBMCs) and serially diluted to achieve 1, 0.1, 0.01, and 0.001% spike-in ratios. PBMCs from a CMV-seropositive donor were used for the MHC multimer experiments (fig. 1).

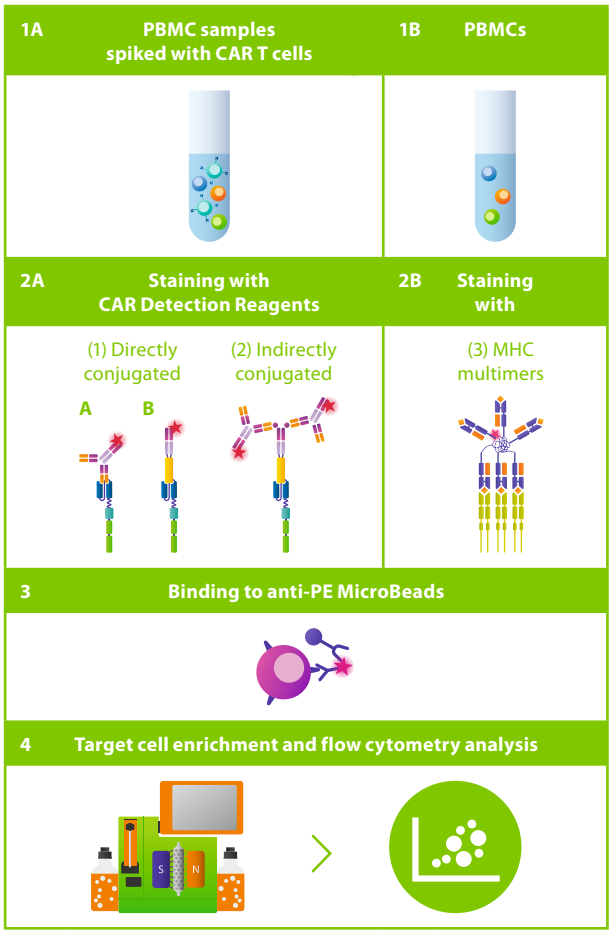

Figure 1: Experimental setup to evaluate the sensitivity of flow cytometric analyses after pre-enrichment with the MACS Cell Enrichment Unit. PBMCs spiked with CAR T cells (1A) were stained using three different CAR Detection Reagents (2A). PBMCs from a CMV-seropositive donor (1B) were labeled with MHC multimers (2B). After staining, the cells were incubated with Anti-PE MicroBeads (3), enriched, and analyzed via flow cytometry (4).

Staining CAR T cells and antigen-specific T cells
The performance of the MACS Cell Enrichment Unit was evaluated using distinct detection reagents (fig. 1, 2A and 2B): (1) an either idiotype antibody-based (A; CD19) or antigen-based (B; BCMA), PE-conjugated CAR Detection Reagent; (2) an antigen-based, biotin-conjugated CD19 CAR Detection Reagent with secondary fluorescent anti-biotin-PE labeling; and (3) a peptide-based approach using our innovative MHC multimers (MHC MACSimer A*0201 CMV pp65 (NLVPMVAT)-PE). The results from the CAR T cell detection were used to evaluate the analytical sensitivity with and without pre-enrichment.

After labeling the cells according to the protocol for each detection method, the samples were washed twice and then stained with a fluorescent antibody cocktail, as detailed in the product table. This cocktail included 7-AAD Staining Solution to discriminate dead cells.

Binding to magnetic beads
After staining, the cells were washed and then incubated with Anti-PE MicroBeads, followed by resuspension in autoMACS® Running Buffer.

CAR T cell and antigen-specific T cell enrichment
The magnetic column integrated into the MACSQuant Analyzer was used in the "EnrichS" Mode, found under the Pickup and Measure section of the Experiment tab. More information about pre-enrichment functions and corresponding instrument settings is detailed in the MACSQuantify™ Software User Manual.

The results presented in the following section were acquired on a MACSQuant Analyzer 10. Similar results were obtained with the MACSQuant Analyzer 16 (data not shown here).

Results

In-line pre-enrichment of CAR T cells improved the analytical sensitivity of the MACSQuant Analyzer
Samples with spiked concentrations of CD19 or BCMA CAR T cells ranging from 0.001% to 1% were analyzed with and without automated pre-enrichment using the MACSQuant column. A direct comparison of the results demonstrated that in-line pre-enrichment substantially increased the proportion of CAR T cells even at the lowest concentration tested. Figure 2 shows results for the CD19 CAR T cells (similar results obtained for BCMA CAR T cells are not shown here).

In addition, the limit of detection (LOD) and limit of quantitation (LOQ) were determined to assess gains in analytical sensitivity attributable to in-line pre-enrichment. The MACS Cell Enrichment Unit built into the MACSQuant Analyzer enabled automated pre-enrichment of rare cells, which enhanced assay sensitivity and provided reliable results down to 0.001% CAR T cells (fig. 3).

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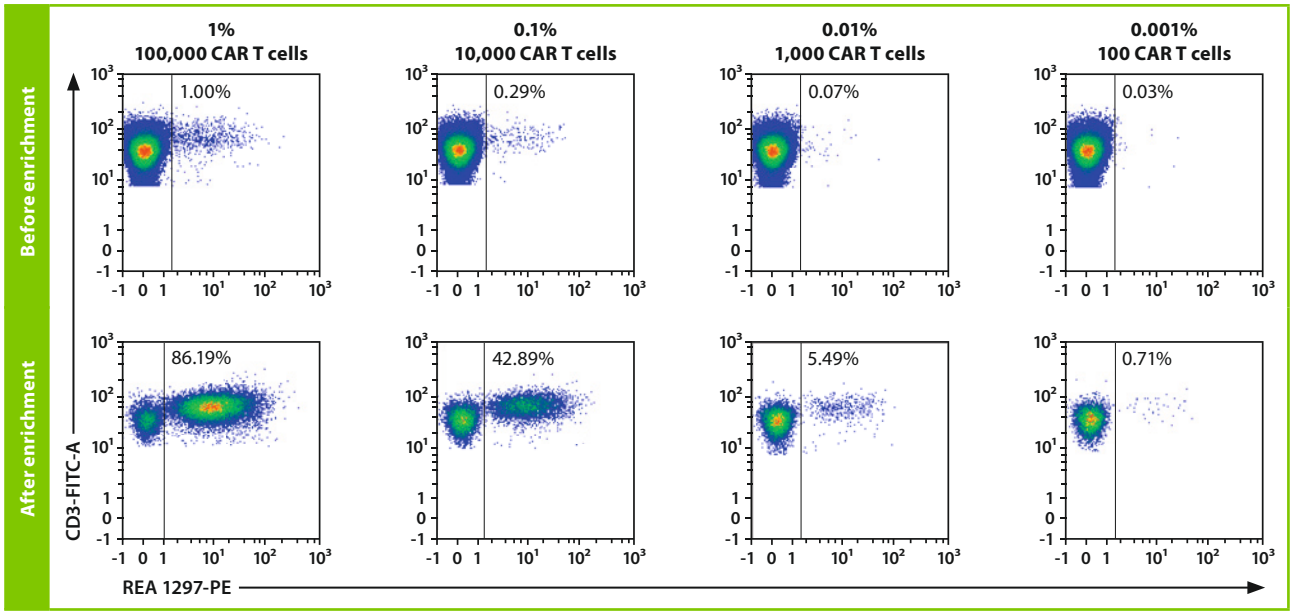
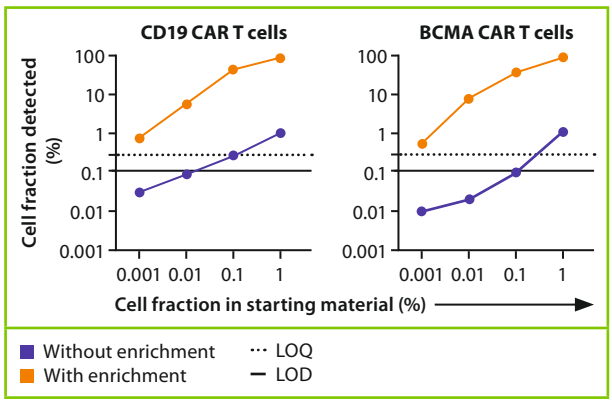


Figure 2: The MACS Cell Enrichment Unit of the MACSQuant Analyzer effectively increased the relative fraction of target cells, thereby improving the sensitivity of subsequent analyses. CD19 CAR T cells were spiked into PBMCs at four different concentrations, stained with the CD19 CAR FMC63 Idiotype Antibody, PE (REA1297), and magnetically enriched on the built-in MACSQuant Column. The CD19 CAR T cells were effectively enriched at all tested concentrations.



	CD19 CAR T cells	BCMA CAR T cells
LOQ	0.281	0.302
LOD	0.11	0.10

Figure 3: Improved analytical sensitivity of CAR T cell detection with in-line pre-enrichment on the MACSQuant Analyzer. Pre-enrichment markedly increased assay sensitivity across all tested dilutions for both CD19 CAR T cells (left) and BCMA CAR T cells (right). CD19 CAR T cells were detected using the CD19 CAR FMC63 Idiotype Antibody, PE (REA1297) and BCMA CAR T cells were detected using the BCMA CAR Detection Reagent, human, PE.

Enrichment performance was comparable between direct and indirect labeling approaches

To assess the impact of detection reagent type on the performance of the MACS Cell Enrichment Unit, the enrichment performance of CD19 CAR T cells stained with the idiotype antibody (direct labeling) was compared to the efficiency using an antigen-based biotin-conjugated CAR Detection Reagent with secondary fluorescent labeling with the Biotin Antibody, PE, REAfinity® (REA746). Figure 4 shows the results from samples with a 1% spike-in concentration. The enrichment efficiency was comparable between both CAR Detection Reagents, with an approximately 80-fold increase. These results suggest that magnetic enrichment can be performed with several detection strategies, and the assay can be tailored to various CAR T cell products.

Antigen-specific T cells are efficiently enriched with MHC MACSimers and the MACS Cell Enrichment Unit

In a final assessment of the versatility of the MACS Cell Enrichment Unit, MHC MACSimers were used to bind and detect antigen-specific T cells with and without enrichment. Figure 5 displays the results. Antigen-specific T cells were substantially enriched (approximately 700-fold). Thus, capturing the multimers and target T cells on the built-in MACSQuant Column using anti-PE MicroBeads strongly improved the sensitivity of our detection assay on the MACSQuant Analyzer Platform.

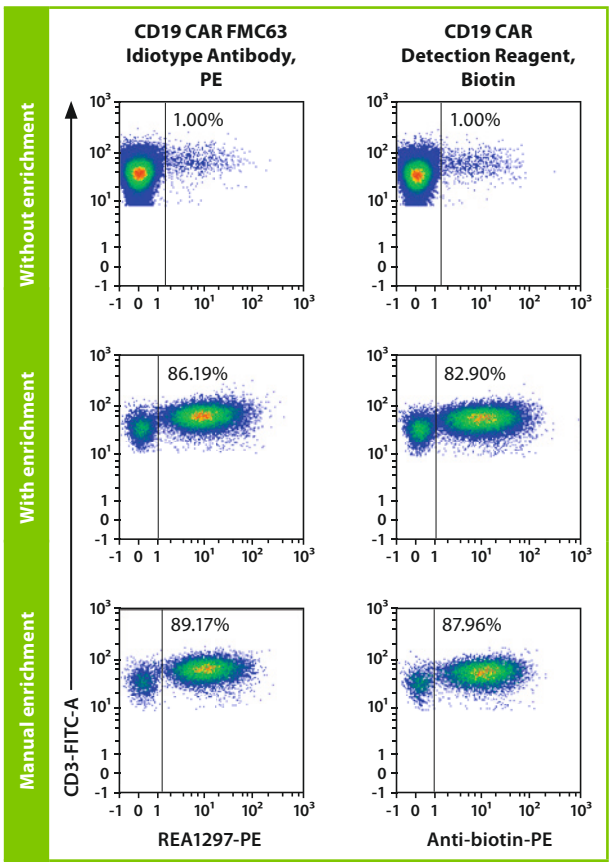


Figure 4: Comparable enrichment of target cells was achieved using direct and indirect labeling strategies. Spiked CD19 CAR T cells (1% among CD3+ cells in samples) were equally enriched with the MACS Cell Enrichment Unit, whether using an idiotype antibody-based (left column) or an antigen-based, biotinylated CAR Detection Reagent (right column). Moreover, the automated workflow on the MACSQuant Analyzer achieved enrichment efficiencies comparable to those of the conventional manual workflow using the MiniMACS™ Separator and Starting Kit (bottom row).

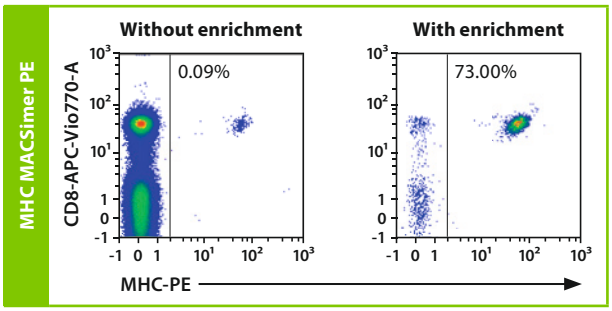


Figure 5: Antigen-specific T cells were effectively enriched using MHC MACSimers for detection. PBMCs were stained with peptide-loaded MHC MACSimer A*0201 CMV pp65. Using the MACSQuant Column and anti-PE MicroBeads, the stained antigen-specific T cells were efficiently enriched for subsequent characterization.

Conclusions

This study demonstrates that pre-enrichment of target cells on the MACSQuant Analyzer Platform, equipped with the integrated MACS Cell Enrichment Unit, substantially enhances detection sensitivity. As a result, quantitative and qualitative analyses of rare cell populations by flow cytometry are more reliable. Furthermore, the automated and seamlessly integrated cell enrichment and analysis workflow eliminates manual steps, thereby reducing risk and enhancing reproducibility.

Using various CAR detection reagents, we reliably detected CAR T cells at spike-in concentrations as low as 0.001% following enrichment on the MACSQuant Analyzer. The integrated MACS Cell Enrichment Unit demonstrably improved the assay limit of detection (LOD) and limit of quantification (LOQ). Comparable enrichment was achieved for antigen-specific T cells using MHC MACSimers.

These findings position the MACSQuant Analyzer as a robust and efficient platform for the sensitive detection and precise characterization of low-frequency cell populations. Moreover, the achieved sensitivity underscores the promising potential of flow cytometry assays for long-term monitoring of infused CAR T cells. The enhanced detection capability we demonstrated paves the way for more precise, real-time assessment of CAR T cell populations, which could significantly improve treatment decisions and outcomes.

References

1. Mura, M. *et al.* (2020) Optimized flow cytometric protocol for the detection of functional subsets of low frequency antigen-specific CD4$^+$ and CD8$^+$ T cells. MethodsX 7: 101005.
2. Reichman, A. *et al.* (2022) Comparison of FACS and PCR for detection of BCMA-CAR-T cells. Int. J. Mol. Sci. 14: 903
3. ICH Q2(R2). Validation of analytical procedures – Scientific guideline. https://www.ema.europa.eu/en/ich-q2r2-validation-analytical-procedures-scientific-guideline (accessed July 2025)

Product	Order no.
MACSQuant Analyzer 10*	130-096-343
MACSQuant Analyzer 16*	130-109-803
MACSQuant Column	130-094-458
MiniMACS Separator and Starting Kit	130-090-312
7-AAD Staining Solution	130-111-568
autoMACS Running Buffer	130-091-221
CD19 CAR FMC63 Idiotype Antibody, PE, REAfinity (REA1297)	130-127-342
BCMA CAR Detection Reagent, human, PE	130-133-888
CD19 CAR Detection Reagent, human, Biotin	130-129-550
MHC MACSimer A*0201 CMV pp65 (NLVPMVATV), human, PE	130-134-651
MHC MACSimer A*0201 Control, human, PE	130-134-668
Anti-PE MicroBeads	130-048-801
Staining cocktail used with idiotype antibody and antigen-based, biotin-conjugated CAR Detection Reagents	
CD4 Antibody, anti-human, VioGreen™, REAfinity (REA623)	130-113-230
CD8 Antibody, anti-human, APC-Vio® 770, REAfinity (REA734)	130-110-681
CD14 Antibody, anti-human, PerCP-Vio 700, REAfinity (REA599)	130-110-523
CD15 Antibody, anti-human, PerCP-Vio 700 (VIMC6)	130-113-487
CD45RO Antibody, anti-human, VioBlue®, REAfinity (REA611)	130-119-620
Biotin Antibody, PE, REAfinity (REA746)	130-110-951
Staining cocktail used with MHC MACSimer	
CD3 Antibody, anti-human, FITC, REAfinity (REA613)	130-113-138
CD3 Antibody, anti-human, APC, REAfinity (REA613)	130-113-135
CD8 Antibody, anti-human, APC-Vio 770, REAfinity (REA734)	130-110-681
CD14 Antibody, anti-human, VioGreen, REAfinity (REA599)	130-110-525
CD20 Antibody, anti-human, VioGreen (LT20)	130-113-379
CD197 (CCR7) Antibody, anti-human, FITC, REAfinity (REA546)	130-120-468

*The MACSQuant Column is not integrated into the MACSQuant Analyzer X.

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