CAR Detection Reagents - Applications for flow cytometry, magnetic cell enrichment, and microscopic imaging

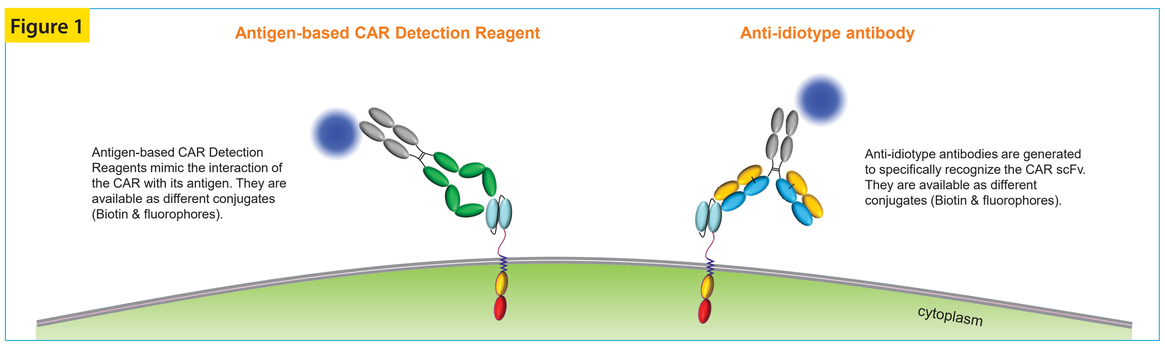
Stephan Wilmes, Desiré Missing, Anja Brauchle, Nadine Borgelt, Johanna Müllers, Monika Winkels, Larissa Möckel, Michaela Niemöller, Katrin Lange, Laura Meinersen, Dagmar Häußler-Kuballa, Franziska Antonia Bertram, Olga Goldbach, Dominik Poschen, Astrid Imann, Anne Bodenhausen, Silvia Rüberg, Anijutta Appelshoffer, Fabio El Yassouri, Christian Dose, Marsilius Mues, and Anne Richter I Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany

Scan the QR code to download the poster (PDF):

Introduction

Chimeric Antigen Receptor (CAR) cell therapies represent a key milestone in the treatment of hematological malignancies and show remarkable efficacy. Specific and reproducible detection of CAR expression is of utmost importance, not only for research and pre-clinical applications, but also for clinical manufacturing of cellular products and patient immunomonitoring. To fulfill these critical requirements, we developed CAR Detection Reagents (CAR DRs) for flow cytometry with outstanding quality and high specificity. In addition, our CD19 CAR Detection Reagent can also be used for the fast magnetic isolation of CAR cells from freshly drawn anticoagulated whole blood or PBMC, as well as for the precise detection and enumeration of CD19 CAR (FMC63) T cells by immunofluorescence microscopy.

Figure 1



We developed different CAR Detection Reagents that can be classified into two groups (Fig. 1). Antigen-based CAR Detection Reagents consist of the extracellular domain of the target protein that is fused to a Fc part and thereby mimic the target-epitope-binding. We also developed anti-idiotype antibodies that specifically target the scFv of the CAR. Both CAR detection strategies take advantage of a recombinantly engineered human IgG Fc part that prevents Fc receptor binding ("REAfinity" technology) and thereby reduces unspecific background staining. These CAR Detection Reagents are provided either as Biotin-conjugates or they are directly conjugated to a variety of fluorophores (FITC, VioBright515, PE, APC).

Results

1 Antigen-based CAR Detection Reagents for flow cytometry

Figure 2

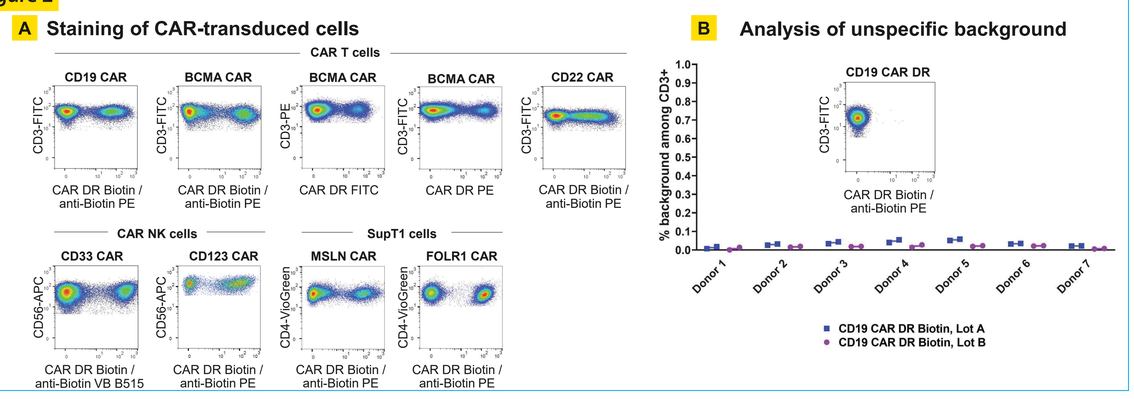


Figure 2a: Representative density plots of CAR T cells, CAR NK cells or CAR-transduced SupT1 cells stained with various antigen-based CAR Detection Reagents (DR), based on ectodomains of CD19, BCMA, CD22, CD33, CD123, MSLN, and FOLR1. CAR T and CAR NK samples were gated on CD45 (REA737) positive, 7-AAD negative and CD3 (REA613) positive cells. CAR SupT1 samples were gated on 7-AAD negative, CD4 (REA623) positive and CD8 (REA734) positive cells.

Figure 2b: Analysis of unspecific background of two different CD19 CAR DR Biotin lots detected on seven different whole blood donors. Secondary staining of biotinylated CAR Detection Reagents was done with anti-Biotin antibody (REA746) conjugated to PE or VioBright B515 (VB B515). Inset shows a representative density plot of one donor. Flow cytometric data was generated using MACSQuant Analyzer 10.

2 Anti-idiotype antibodies for flow cytometry

Figure 3

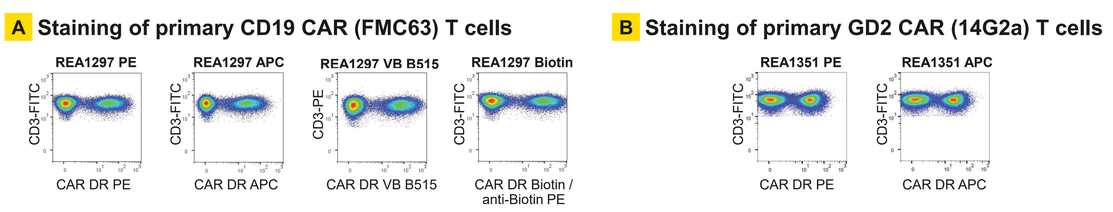


Figure 3a: Representative density plots of CD19 CAR (FMC63) T cells stained with the CD19 CAR FMC63 idiotype antibody REA1297 conjugated to PE, APC, VioBright B515, or Biotin. Samples were gated on CD45 (REA737) positive, 7-AAD negative, and CD3 (REA613) positive cells. Figure 3b: Representative density plots of GD2 CAR (14G2a) T cells stained with the GD2 CAR 14G2a idiotype antibody REA1351 conjugated to PE or APC. Samples were gated on CD45 (REA737) positive, 7-AAD negative, and CD3 (REA613) positive cells. Flow cytometric data was generated using MACSQuant Analyzer 10.

3 Comparison of antigen-based CD19 CAR DR and anti-CD19 (FMC63) idiotype antibody REA1297

Figure 4

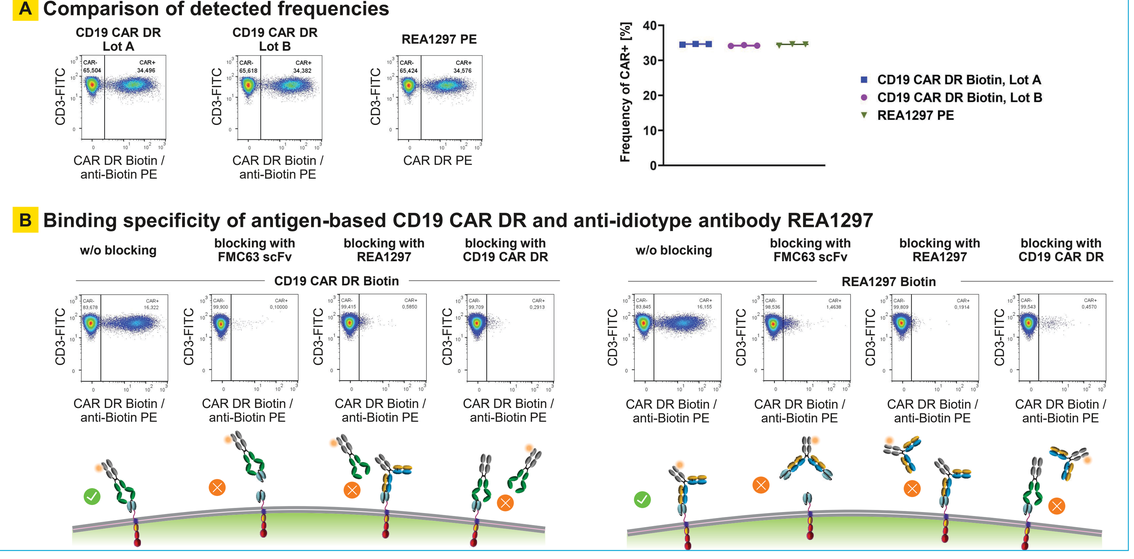
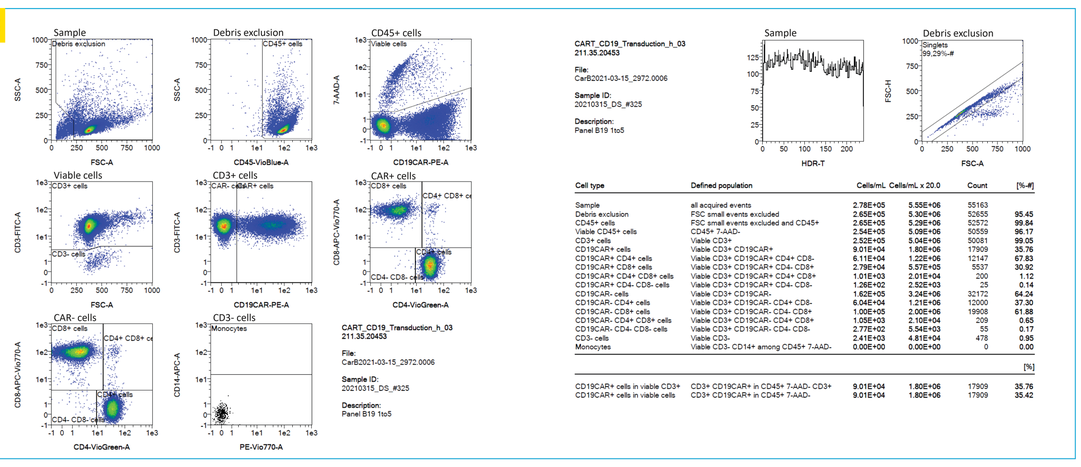


Figure 4a: Example density plots of CD19 CAR (FMC63) T cells stained with two CD19 CAR DR Biotin lots and one lot of anti-idiotype antibody REA1297 PE. CAR+ frequencies were reproducibly detected with all tested reagents. The test was performed in technical triplicates. Figure 4b: Example density plots of CD19 CAR (FMC63) T cells stained either with CD19 CAR DR Biotin or anti-idiotype antibody REA1297 Biotin. Staining specificity was tested by blocking with FMC63 scFv, pure CD19 Fc protein, or pure REA1297 antibody. Samples were gated on CD45 (REA737) positive, 7-AAD negative, and CD3 (REA613) positive cells. Secondary staining of biotinylated CAR Detection Reagents was done with anti-Biotin antibody (REA746). Flow cytometric data was generated using MACSQuant Analyzer 10.

4 Automated acquisition and analysis of transduction efficiency and viability of CAR T cells

Figure 5

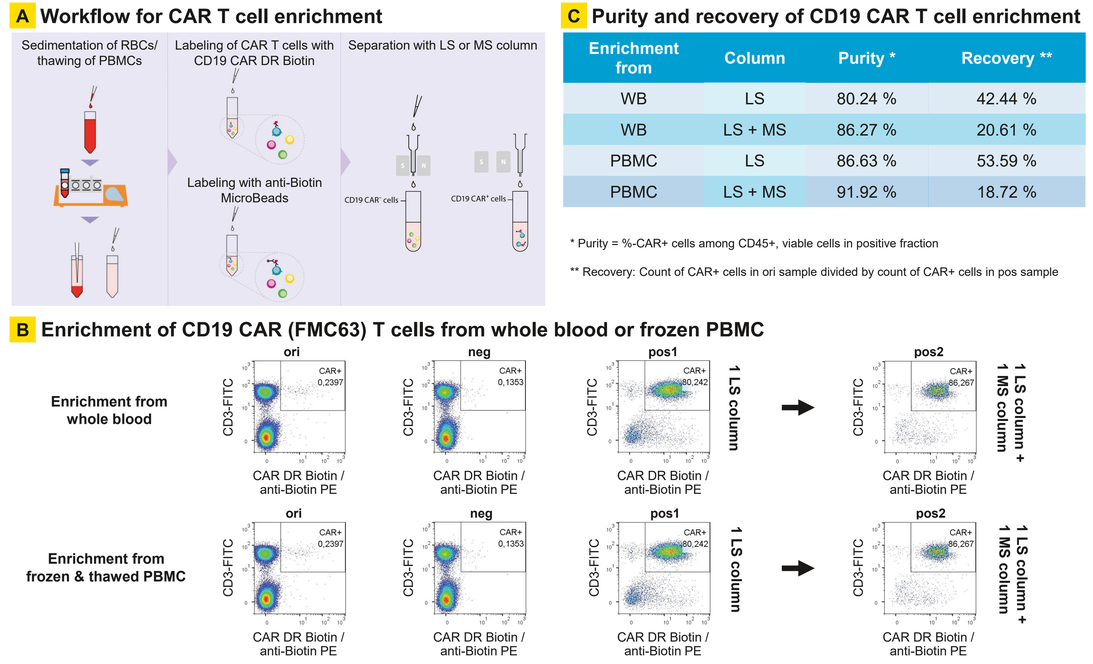


Cell type	Defined population	Cells/mL	Cells/mL x 20.0	Count	[%-#]
Sample	all acquired events	2.78E+05	5.55E+06	55163	
Debris exclusion	FSC small events excluded	2.65E+05	5.30E+06	52655	95.45
CD45+ cells	FSC small events excluded and CD45+	2.65E+05	5.29E+06	52572	99.84
Viable CD45+ cells	CD45+ 7-AAD-	2.54E+05	5.09E+06	50559	96.17
CD3+ cells	Viable CD3+	2.52E+05	5.04E+06	50081	99.05
CD19CAR+ cells	Viable CD3+ CD19CAR+	9.01E+04	1.80E+06	17909	35.76
CD19CAR+ CD4+ cells	Viable CD3+ CD19CAR+ CD4+ CD8-	6.11E+04	1.22E+06	12147	67.83
CD19CAR+ CD8+ cells	Viable CD3+ CD19CAR+ CD4- CD8+	2.79E+04	5.57E+05	5537	30.92
CD19CAR+ CD4+ CD8+ cells	Viable CD3+ CD19CAR+ CD4+ CD8+	1.01E+03	2.01E+04	200	1.12
CD19CAR+ CD4- CD8- cells	Viable CD3+ CD19CAR+ CD4- CD8-	1.26E+02	2.52E+03	25	0.14
CD19CAR- cells	Viable CD3+ CD19CAR-	1.62E+05	3.24E+06	32172	64.24
CD19CAR- CD4+ cells	Viable CD3+ CD19CAR- CD4+ CD8-	6.04E+04	1.21E+06	12000	37.30
CD19CAR- CD8+ cells	Viable CD3+ CD19CAR- CD4- CD8+	1.00E+05	2.00E+06	19908	61.88
CD19CAR- CD4+ CD8+ cells	Viable CD3+ CD19CAR- CD4+ CD8+	1.05E+03	2.10E+04	209	0.65
CD19CAR- CD4- CD8- cells	Viable CD3+ CD19CAR- CD4- CD8-	2.77E+02	5.54E+03	55	0.17
CD3- cells	Viable CD3-	2.41E+03	4.81E+04	478	0.95
Monocytes	Viable CD3- CD14+ among CD45+ 7-AAD-	0.00E+00	0.00E+00	0	0.00
					[%]
CD19CAR+ cells in viable CD3+	CD3+ CD19CAR+ in CD45+ 7-AAD- CD3+	9.01E+04	1.80E+06	17909	35.76
CD19CAR+ cells in viable cells	CD3+ CD19CAR+ in CD45+ 7-AAD-	9.01E+04	1.80E+06	17909	35.42

Figure 5: Representative density plots of CD19 CAR (FMC63) T cells stained with the CD19 CAR Detection Reagent Biotin and anti-Biotin Antibody (REA746), as well as CD45 (REA747), CD3 (REA613), 7-AAD, CD8 (REA734), CD4 (REA623), and CD14 (REA599). Sample acquisition was done with the MACSQuant Analyzer 10. Analysis was done using the Express Mode "CART_ Transduction_h_03", allowing fully automated gating and calculations.

5 Fast magnetic isolation of CAR T cells

Figure 6



Enrichment from	Column	Purity *	Recovery **
WB	LS	80.24 %	42.44 %
WB	LS + MS	86.27 %	20.61 %
PBMC	LS	86.63 %	53.59 %
PBMC	LS + MS	91.92 %	18.72 %

* Purity = %-CAR+ cells among CD45+, viable cells in positive fraction

** Recovery: Count of CAR+ cells in ori sample divided by count of CAR+ cells in pos sample

Figure 6a: Workflow of CAR T cell isolation from red blood cell (RBC)-sedimented whole blood (WB) or frozen and thawed PBMC. Figure 6b: Representative density plots of CD19 (FMC63) CAR T cells stained with CD19 CAR DR Biotin before enrichment (original (ori) fraction) and after enrichment (negative (neg) fraction and positive (pos) fraction). Samples were gated on CD45 (REA737) positive, 7-AAD negative cells. Secondary staining of the biotinylated CAR DR was done with anti-Biotin antibody (REA746). Enrichment was done with CD19 CAR DR Biotin and anti-Biotin MicroBeads as illustrated in Figure 6a. Figure 6c: Rare CAR T cells were enriched to high purity.

6 CD19 CAR (FMC63) T cell detection in tissue samples by fluorescence microscopy

Figure 7

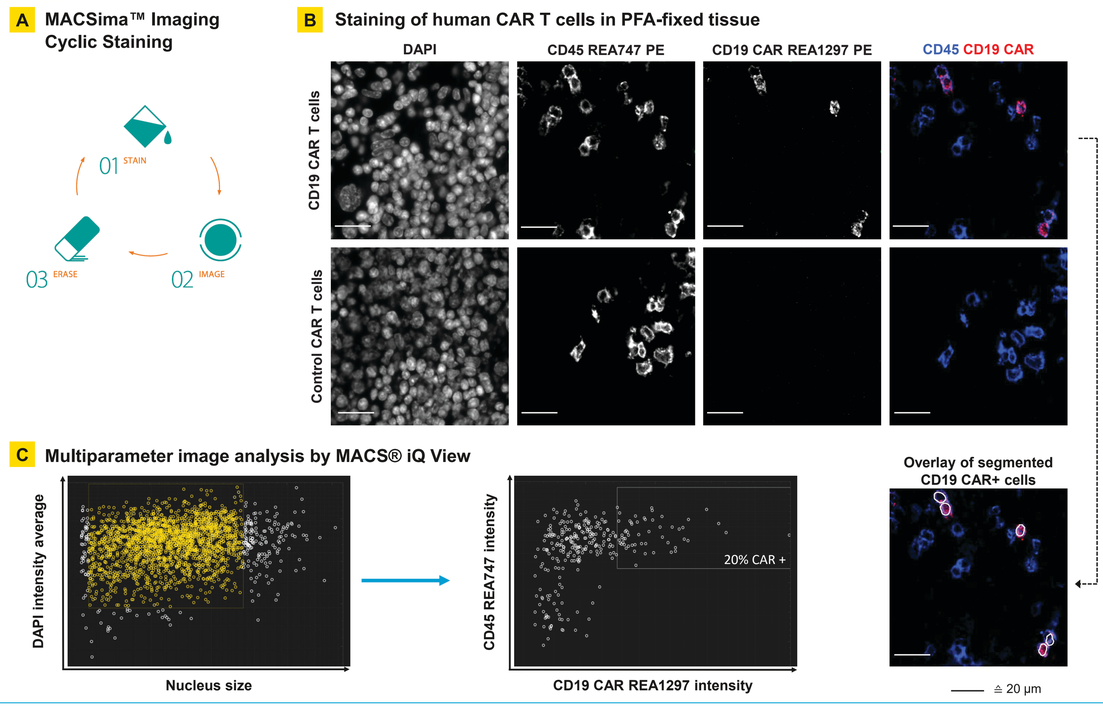


Figure 7a: Schematic representation of MACSima Image Cyclic Staining (MICS). Repetitive imaging cycles allow phenotyping of tissue sections with a broad selection of markers. Figure 7b: CD19 CAR (FMC63) T cell detection in mouse splenic tissue (fresh frozen, PFA fixed) harboring human tumors with CD19 or control CAR T cell treatment. The tissue section was stained by DAPI (nuclei). Human T cells were detected by CD45 REA747 PE and CAR T cells by anti-idiotype antibody REA1297 PE. CD19 CAR staining co-localizes with CD45 staining, indicating a specific CAR detection. Figure 7c: Multiparameter image analysis performed with MACS iQ View. Spacial segmentation allows easy and convenient gating as well as quantitative data analysis.

Conclusion

We developed reagents for flow cytometric analysis of CAR-transduced cells, fulfilling highest standards regarding brightness, specificity, lot-to-lot consistency, flexibility of panel design, and minimized unspecific background staining. These are fundamental prerequisites for quality control of CAR T cell products, immunomonitoring purposes, and research applications. Express Modes allow reproducible and user-independent gating of flow cytometric data. Additionally, our CAR DRs can be used for CAR T cell enrichment, relevant for technologies such as single cell RNA sequencing. Finally, we demonstrate that our CAR DRs can be applied for immunofluorescence microscopy. This enables detection of tissue infiltration by CAR T cells and detailed phenotyping. CAR Detection Reagents represent a versatile toolbox for various applications and we are aiming to further expand our CAR DR portfolio, for more specificities and considering fluorochrome-conjugated antigen-based CAR DRs.

Unless otherwise specifically indicated, Miltenyi Biotec products and services are for research use only and not for therapeutic or diagnostic use. For further regulatory notes, refer to miltenyibiotec.com/regulatory-notes.
MACSima,MACSQuant, the Miltenyi Biotec logo, REAfinity, and VioGreen are registered trademarks or trademarks of Miltenyi Biotec and/or its affiliates in various countries worldwide. All other trademarks mentioned in this document are the property of their respective owners and are used for identification purposes only.
Copyright © 2023 Miltenyi Biotec and/or its affiliates. All rights reserved.