# Highly sensitive and antigen-specific detection of CAR expression cells using novel CD19, BCMA, CD20 and TROP2 soluble fusion proteins by flow cytometry

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## Abstract

**Introduction:** As CAR-T therapies advance through development, analytical methods must provide meaningful characterization of product identity and function. While FDA guidance emphasizes direct CAR detection for identity testing, anti-idiotype reagents are not always available, and surrogate methods remain common during early development. Recombinant bioactive target proteins offer attractive antigen-specific CAR-T detection, which enables direct flow cytometric detection of CAR expression while simultaneously confirming target-binding specificity and affinity in a single, biologically relevant measurement.

**Methods:** To further advance the anti-specific CAR-T evaluation, we have developed novel cell surface fusion proteins including Fc-free dimeric CD19 and TROP2, tetrameric BCMA, and scaffold CD20 extracellular loop 2 (ECL2) proteins for sensitive, and specific CAR detection by flow cytometry.

**Results:** The results demonstrated that the CD19-dimer could potently bind to the FDA approved therapeutic antibody clones including 1) Lisocabtagene maraleucel CAR-T therapeutics, FMC63; 2) Loncastuximab antibody conjugate drug (ADC); 3) Blinatumomab bispecific therapeutic antibody, clone HD37; and 4) Tafasitamab therapeutic monoclonal antibody, clone 4G7. It had excellent sensitivity and specificity for CD19-CAR mRNA transfected human CD8 and CD4 T cells with EC50 <2ug/ml and no binding to un-transfected T cells. The CD19-specific CAR T detection is more relevant to tumor-cell killing although the linker-detected CAR expression levels are very similar. Validation of CAR detection using the CD19-dimer method showed a strong positive correlation with a surrogate marker. Similar results are achieved using BCMA, TROP2 or CD20 fusion proteins for specific antibody binding and CAR detections on transfected HEK293 cells.

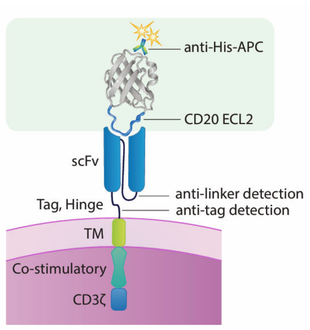
**Conclusions:** The novel CD19, BCMA, CD20, and TROP2 soluble proteins preserve the desired conformations and epitopes binding to specific antibodies and CAR expressing cells. Antigen-specific CAR binding is more relevant to CAR T function. These proteins can be readily incorporated into multicolor flow cytometry panels, enabling detailed phenotypic characterization of antigen-specific CAR-positive subpopulations through CAR design and development.

## 1. Conigen Novel Soluble Dimer Protein For Measuring CAR-T

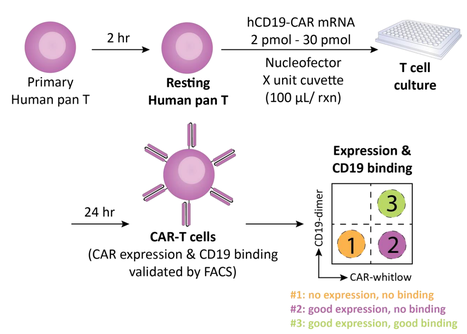
CAR-T cell therapy is a revolutionary new pillar in cancer treatment. There is an urgent need for CAR-T cell monitoring by CAR-T cell researchers, manufacturers, and clinician.

**Challenge:**
CAR-T-cells are often detected through tags added to the CAR construct. However, the tag detection methods often lack sensitivity and can not address CAR receptors recognition of its target.

**Solution**:
We developed a direct CAR T detection method using novel Fc-free target protein reagents taking advantage of their high avidity to detect both high and low affinity CAR-T cells.

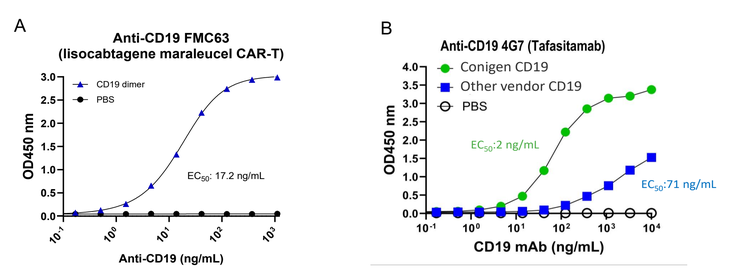



A. Schematic illustration of CAR on cell surface



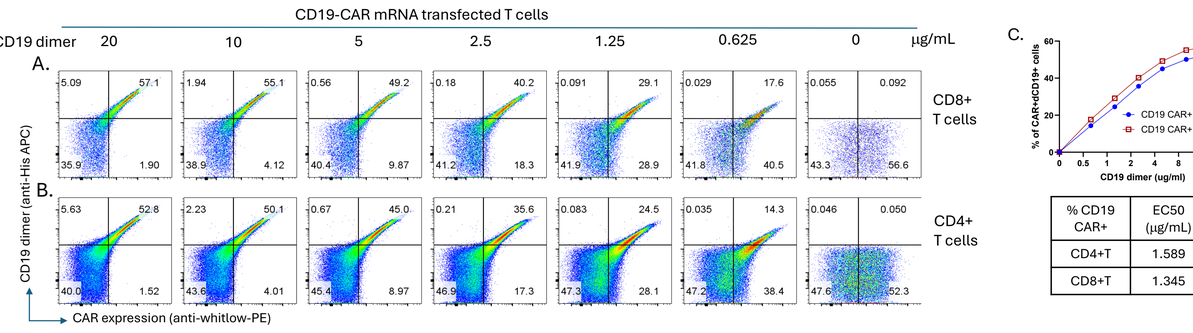

B. The workflow to measure CD19 CAR expression on mRNA transfected human primary T cells.

## 2. CD19 Dimer Antigenicity




CD19 dimer preserves the natural epitopes. It can be potently and specifically recognized by FDA approved CD19 therapeutic antibody measured by ELISA. A. Lisocabtagene maraleucel CAR-T therapeutics, clone FMC63, and other therapeutic antibodies; B. Cyno CD19 dimer is significantly more potent binding to Tafasitamab therapeutic monoclonal antibody clone 4G7 than other vendor's Cyno CD19 monomer.

## 3. CD19 Dimer Specific Detection of CD19-CAR mRNA–Transfected Human T Cells







| % CD19 CAR+ | EC50 (µg/mL) |
|---|---|
| CD4+T | 1.589 |
| CD8+T | 1.345 |

Titration of CD19 dimer protein for antigen-specific CD19 CAR-T detection by flow cytometry. The representative CAR detection of flow cytometry signals on CD8+ T cells (A) and CD4+ T cells (B) are shown. The CAR expression detected by anti-linker (whitlow)-PE antibody is shown on the x-axis. The CD19-dimer specific CAR detection is shown on the y-axis. C. CD19 dimer dose response curves of % linker-positive dimer-bound (CAR+Dimer+) cells within total CD8+ or CD4+ T cells.

CD19 antigen-specific CAR T detection is more relevant to tumor-cell killing

CD19 CAR T test methods

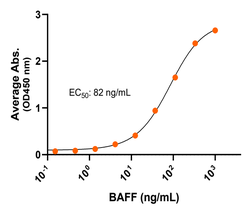
| Method | Description |
|---|---|
| Method 1 | Anti-Flag-Tag binding |
| **Method 2** | **CD19 antigen binding** |
| Method 3 | CD8+ T cell mediated leukemia tumor cell killing |

CD19 CAR binding and tumor cell killing correlation

| Correlation | Pearson r | P value | Significance |
|---|---|---|---|
| Method 1 vs Method 3 | 0.446 | 0.146 | No |
| **Method 2 vs Method 3** | **0.685** | **0.014** | **Yes** |

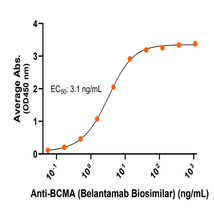
## 4. BCMA Ligand, mAb and CAR Detection

BCMA and ligand binding



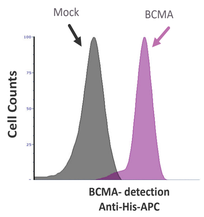

A. BCMA-tetramer preserves the natural conformation bound to its ligand BAFF

BCMA specific antibody binding




B. BCMA-tetramer specifically and potently binds therapeutic antibody Belantamab.

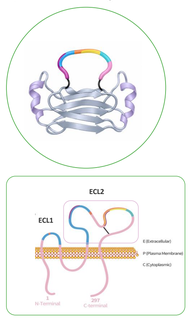
BCMA specific CAR detection



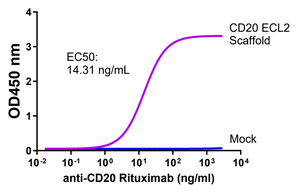

C. BCMA-tetramer specifically detected anti-BCMA CAR on transfected HEK293 cells.

## 5. CD20-ECL2 mAb Binding and CAR Detection

CD20-ECL2 scaffold protein design

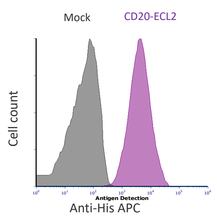



CD20-ECL2 specific antibody binding



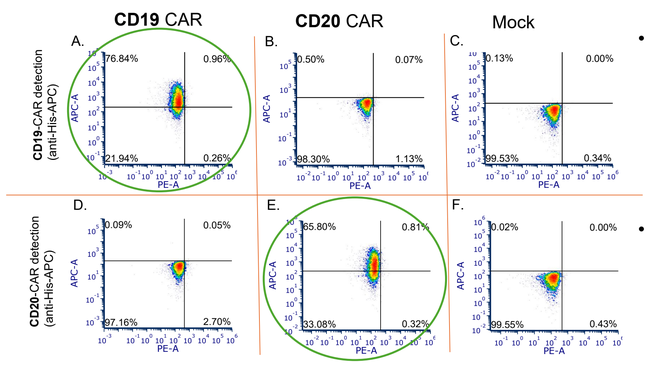

A. CD20-ECL2 scaffold protein preserves the epitopes potently binding to specific antibody Rituximab

CD20-ECL2 specific CAR detection




B. CD20-ECL2 scaffold protein specifically detected Rituximab CAR on transfected HEK293 cells

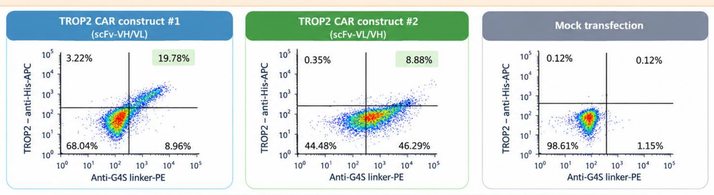
## 6. CD19 and CD20 CAR Detection Specificity




- CD19 or CD20 protein only binds to the respective CD19 CAR (A) or CD20 (B) CAR construct transfected HEK293 cells, not to the non-relevant CAR or Mock transfected cells.
- Antigen CAR recognition is very specific only to its relevant CAR construct that cannot differ by universal detection.

## 7. TROP2 Detecting Different CAR Designs

- Use TROP2 dimer His-AviTag protein for antigen-specific CAR recognition (detected by anti-His-APC).
- Use anti-G4S linker to measure total CAR expression.




| CAR Construct | Orientation | % Linker+ (Total CAR expression) | % TROP2+ (Antigen binding) | Double positive (Linker+ / TROP2+) |
|---|---|---|---|---|
| 1 | VH–VL | 29.7 | 23.0 | 19.8 |
| 2 | VL–VH | 55.2 | 9.2 | 8.9 |

TROP2 antigen-specific detection reveals differences in CAR binding competence of differently designed CAR Constructs that anti-linker staining alone cannot.

## Conclusions

- Novel Fc-free novel cell surface antigen-based fusion proteins are designed to mimic the natural structure and epitopes for specific antibody and ligand binding, and sensitive CAR recognition.
- CD19 Fc-free homodimer shows strong binding to FDA approved therapeutic antibodies by ELISA; and specifically measures the CD19 CAR-T cells using flow cytometry.
- CD19-specific CAR detection is more relevant to CD8+ CAR T cell function measured as tumor-cell killing
- CD20-ECL2 scaffold protein can specifically and potently bind therapeutic antibody Rituximab and detect anti-CD20 CAR expression on transfected HEK293 cells.
- BCMA-tetramer protein can specifically and potently bind its natural ligand BAFF, therapeutic antibody Belantamab and measure anti-BCMA CAR expression on transfected HEK293 cells.
- Native-like TROP2 dimer antigen-specific detection reveals differences in differently designed CAR binding competence that linker staining alone cannot resolve.
- Antigen-based CAR detections are specific and can reveals the differences in CAR binding competence of differently designed CAR Constructs that anti-linker staining alone cannot.
- These novel fusion proteins provide a reliable, robust, and widely applicable method for detection of antigen-specific CAR recognition.