Application protocol

Engineering of CAR T cells for research use

Isolation of donor T cells, *in vitro* activation, transduction, expansion, phenotyping, and functional analysis

CAR T cells have to be developed, optimized, and validated in a pre-clinical setting by small-scale benchtop experiments. This application protocol describes a complete workflow for the engineering of CAR T cells for research. It includes isolation of donor T cells, followed by activation, gene-transfer of the CAR construct, CAR T cell expansion, as well as the phenotyping and analysis of the final CAR T cell product.

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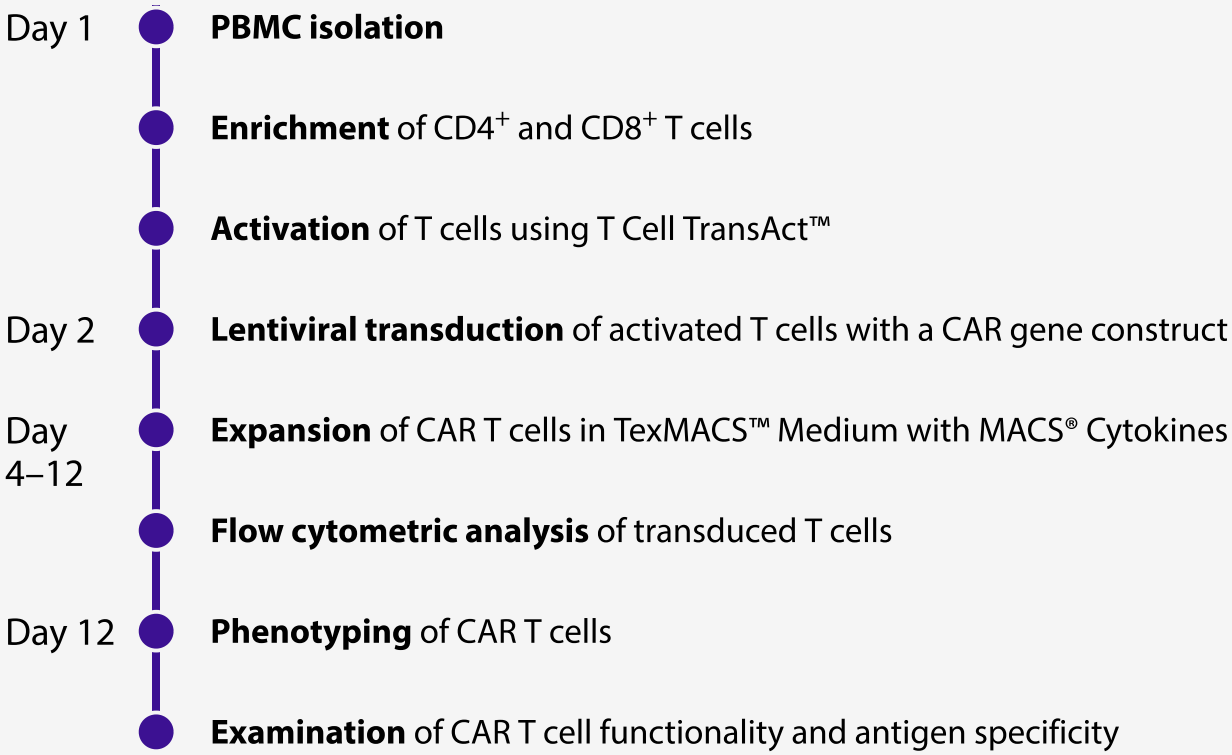
[https://www.miltenyibiotec.com/_Resources/Persistent/761c3be7be470fc995206924676e45625273581a/IM0022471.pdf]

Protocol

Timeline

Engineering of CAR T cells for research use



This protocol includes detailed instructions for preparing necessary reagents and materials and then carrying out each step.

1. Reagent and instrument preparation

TexMACS™ Medium supplemented with MACS® Cytokines, premium grade

Supplement TexMACS™ Medium with MACS® Cytokines, Human IL-7, premium grade (500 U/mL) and Human IL-15, premium grade (84 U/mL).
▲ **Note:** Make sure to **freshly** add Human IL-7, premium grade and Human IL-15, premium grade to the T cell medium for effective T cell expansion.

Standard wash and dilution buffer

Prepare buffer by diluting the MACS® BSA Stock Solution 1:20 with autoMACS® Rinsing Solution or PBS/EDTA.
▲ **Note:** Keep buffer cold (2–8 °C). Degas buffer before use, as air bubbles could block the column.

CFSE stock solution for cell labeling

Prepare the 10 mM CFSE stock solution in 1 mL of DMSO. Use CFSE at a final concentration of 1 µL by diluting the stock solution 1:10,000 in PBS.

Antibody panels

Freshly prepare the following staining cocktails for each sample.
▲ **Note:** Store staining cocktails in the dark in the refrigerator (2–8 °C) until use. Do not store for extended periods.

Table 1: Staining cocktail for immune cell composition antibody panel.

Antigen/buffer	Clone	Fluorochrome	Volume (µL)
CD45	REA747	VioBlue	2
CD3	REA613	FITC	2
CD4	REA623	VioGreen	2
CD8	REA734	APC-Vio 770	2
CD16	REA423	PE	2
CD56	REA196	PE	2
CD14	REA599	APC	2
CD20	REA780	PE-Vio 770	2
7-AAD	-	-	2
Buffer	-	-	82

Table 2: Staining cocktail for transduction efficiency antibody panel.

Antigen/buffer	Clone	Fluorochrome	Volume (µL)
CD45	REA747	VioBlue	2
CD3	REA613	FITC	2
CD4	REA623	VioGreen	2
CD8	REA734	APC-Vio770	2
CD14	REA599	APC	2
CAR DR	-	PE	to be defined
7-AAD	-	-	2
Buffer	-	Fill up to 100 µL with buffer	

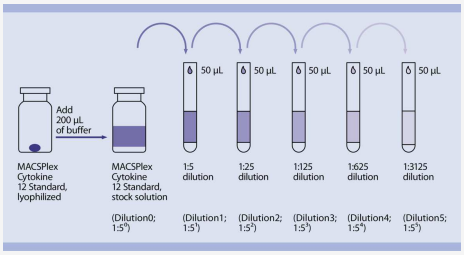
On day 12:
Preparation of the standard of MACSPlex Cytokine 12 Assay

Notes:

- Reconstitute and dilute standard with MACSPlex Buffer or use the same medium as it is used for the dilution of the sample.
- Only use freshly prepared standard solutions. Do not store or reuse reconstituted or diluted standards.
- Use polypropylene or polystyrene reagent tubes. Do not use glass vials. The generation of standard curves requires eight samples: seven samples of the standard, and one blank control. These samples will be measured as duplicates. For details refer to respective data sheet.

1. Thaw one vial containing the lyophilized MACSPlex Cytokine 12 Standard.
2. Open the vial and add 200 µL of MACSPlex Buffer or media to the pellet and mix gently.
   This is the stock solution (10,000 pg/mL for IL-4, IL-5, IL-17A, IL-23, and 50,000 pg/mL for IL-2, IL-10, IL-12p70, IFN-γ, TNF-α, and GM-CSF).
3. Label six reagent tubes and arrange them in the following order:
   a. 1:5 ($1:5^1$; 2,000 pg/mL or 10,000 pg/mL)
   b. 1:25 ($1:5^2$; 400 pg/mL or 2,000 pg/mL)
   c. 1:125 ($1:5^3$, 80 pg/mL or 400 pg/mL)
   d. 1:625 ($1:5^4$; 16 pg/mL or 80 pg/mL)
   e. 1:3125 ($1:5^5$; 3.2 pg/mL or 16 pg/mL)
   f. 1: 15,625 ($1:5^6$; 0.6 pg/mL or 3.2 pg/mL)
4. Pipette 200 µL of MACSPlex Buffer or TexMACS Medium into each tube.
5. Perform a 1:5 dilution by transferring 50 µL from the stock solution to the tube labeled 1:5 and mix thoroughly. Continue making 1:5 serial dilutions by transferring 50 µL from the tube labeled 1:5 to the tube labeled 1:25 and so on to the tube labeled 1:15,625. Mix each dilution before performing the next transfer.
6. Keep 200 µL MACSPlex Buffer or media as blank control (0 pg/mL).

Serial dilution of the MACSPlex Cytokine 12 Standard



2. PBMC isolation

The peripheral blood or buffy coat should not be older than 8 hours and supplemented with anticoagulants (e.g. heparin, EDTA, citrate, ACD-A, or citrate phosphate dextrose (CPD)). PBMCs should be isolated by density gradient centrifugation, e.g., using Ficoll-Paque™.
Resuspend cell pellet in an appropriate amount of PBS-EDTA and proceed to magnetic labeling.
▲ **Note:** PBMCs may be stored in the refrigerator overnight in PBS containing 0.5% BSA or autologous serum. Do not store cells longer than one day in the refrigerator. Wash at least once before proceeding magnetic labeling and resuspend cells in an appropriate buffer. For details see MACS® Cell Separation Reagents data sheets.

3. Enrichment of $CD4^+$ and $CD8^+$ T cells

Fully automated cell labeling and separation using the autoMACS® Pro Separator

Notes:

- For optimal performance it is important to obtain a single-cell suspension before magnetic labeling. Pass cells through Pre-Separation Filters to remove cell clumps which may clog the column. Wet filter with buffer before use.
- Refer to user manual for instructions on how to use the autoMACS® Pro Separator.
- All buffer temperatures should be ≥10 °C.
- Place tubes in the following Chill Rack positions: position A = sample, position B = negative fraction, position C = positive fraction.

1. Determine cell number.
2. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend cell pellet in 120 µL of buffer per 2×10^7 total cells.
   ▲ **Note:** (Optional) To compare purity before and after enrichment, remove a small aliquot (e.g. 50–100 µL) from the fraction and keep it on ice.
4. Place sample (position A) and collection tubes (position B and C) into the Chill Rack.
   ▲ **Note:** Refrigerate Chill Rack overnight to ensure samples are kept at 4 °C.
5. Mix CD4 and CD8 MicoBeads (1:1).
   ▲ **Note:** Mix at least 45 µL CD4 MicroBeads and 45 µL CD8 MicroBeads.
   ▲ **Note:** Shelf life of the CD4 and CD8 MicroBead mixture corresponds to the shelf life of the single CD4 or CD8 MicroBeads and can be stored at 2–8 °C until expiration date.
6. Switch on the instrument for automatic initialization.
7. Go to the **Reagent** menu and press the **Read Reagent** button. Scan the 2D barcode below. Alternatively, select **Enter Reagent** in the **Reagent** menu. Enter '130-999-995' and select **Custom_MicroBead_Mix**.
   ▲ **Note:** Software update 2.4.7.0 is required.



8. Place the reagent into the appropriate position on the reagent rack.

9. Go to the **Separation** menu and select the position for each sample. Select **Cust_MB** from the **Labeling** submenu (the correct labeling, separation, and wash protocols will be selected automatically).

10. Enter sample volume (min. 120 µL) into the **Volume** submenu. Press **Enter**.

11. Select **Run**.

12. The positive fraction containing $CD4^+$ and $CD8^+$ T cells is collected in row C.

13. Remove positive fraction and proceed with section "(Optional) Analysis of purity before and after T cell enrichment".

4. (Optional) Analysis of purity before and after T cell enrichment

Note:

- Additional antibodies can be included in the analysis according to the respective needs. For additional antibodies refer to www.miltenyibiotec.com/antibodies.

1. Remove a small aliquot from the fraction representing the enriched $CD4^+$ and $CD8^+$ T cells (e.g. 50–100 µL).
   ▲ **Note:** For comparative analysis of $CD4^+$ and $CD8^+$ T cells before and after enrichment use also the aliquot from step 3.3.
2. Determine cell numbers.
3. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
4. For each sample resuspend up to 10^6 nucleated cells in 100 µL immune cell composition staining cocktail.
   ▲ **Note:** Refer to section “Reagent and instrument preparation”, table 1 “Staining cocktail for immune cell composition antibody panel”.
5. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).
   ▲ **Note:** Higher temperatures and/or longer incubation times may lead to non-specific cell labeling.

Working on ice requires increased incubation times.

6. Wash cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
7. Resuspend cell pellet in a suitable amount of buffer (e.g. 500 µL) for analysis by flow cytometry.
   ▲ **Note:** Cells must be acquired within 1 hour after staining.

5. (Optional) Examination of T cell proliferation and expansion rates

Note:

- CFSE is added to enriched $CD4^+$ and $CD8^+$ T cell suspension to assess cell proliferation at any given point in time and for every condition tested. Use an additional separate tube for each condition (e.g. transduced, non-transduced, stimulated, non-stimulated, different CAR constructs,…) because antibodies for flow analysis are conjugated to fluorochromes that exhibit the same spectrum as CFSE (λ_{ex} 492 nm; λ_{em} 517 nm).
  Alternatively, every other cell proliferation dye can be used that does not interfere with the fluorochromes of the antibody panel.
  ▲ **Note:** Cell proliferation is usually analyzed on days 3, 6, 8, 10, and 12. However, please feel free to choose time points that are appropriate for the experimental needs.
  ▲ **Note:** To assess expansion rates, please also determine cell numbers, e.g., on days 3, 6, 8, and 10 using a Neubauer Chamber or the counting function of the MACSQuant® Analyzer 10.

1. Determine cell numbers.
2. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
3. Resuspend cells to a final concentration of 2×10^7 cells/mL in PBS.
4. Add the CFSE stock solution to a final concentration of 1 µM to the cell suspension (e.g. add 1 µL of 10 mM CFSE stock solution to 10 mL cell suspension).
5. Mix well and incubate for 10 minutes at 37 °C.
6. Add one volume of human AB serum, mix gently, and incubate for 5 minutes at room temperature (e.g. add 10 mL human AB serum to 10 mL cell suspension)
7. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
8. Resuspend cells to a final concentration of 2×10^7 cells/mL in TexMACS™ Medium without supplements.
9. Repeat steps 7 and 8 to wash cells twice. Cells are now ready for activation and expansion.

6. Activation of T cells using T Cell TransAct™

Note:

- For optimal T cell stimulation EDTA must be removed. Ensure removal of EDTA (e.g. over 200-fold reduction).

1. Wash cells twice with TexMACS™ Medium (without supplements) by filling the tube to the maximum volume and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
2. Resuspend T cells in TexMACS Medium supplemented with Human IL-7, premium grade (500 U/mL) and Human IL-15, premium grade (84 U/mL) to a final density of 1×10^6 cells per mL per cm^2. Add the cell suspension into a suitable cell culture vessel (e.g. 2×10^6 cells in 2 mL/well of a 24-well plate). For details refer to table 4.
3. Add T Cell TransAct™ to a final dilution of 1:100 and carefully resuspend.
   ▲ **Note:** The T Cell TransAct is soluble and applied using an optimized titer and not using bead-to-cell ratio.
4. Incubate for in total 3 days at 37 °C, 5–10% CO_2.
   ▲ **Note:** Inspect culture daily and add fresh medium if required.
   ▲ **Note:** T Cell TransAct needs to be washed out after 3 days (refer to step 8.1).

Table 3: Optimal surface density when working with purified T cells.

Culture plate	Growth area	Max. working volume	Total T cell number	T Cell TransAct to add per well
96 well	0.31 cm^2	0.2 mL	0.3×10^6	2 µL
48 well	1 cm^2	1 mL	1×10^6	10 µL
24 well	2 cm^2	2 mL	2×10^6	20 µL
12 well	4 cm^2	4 mL	4×10^6	40 µL
6 well	10 cm^2	5 mL	5×10^6	50 µL

7. Lentiviral transduction of activated T cells with a CAR gene construct

Notes:

- When transducing T cells with a lentiviral vector the multiplicity of infection (MOI) should be calculated.
- In general, conditions for the viral transduction must be defined for each virus independently. The given step-by-step instruction is an example and must be adapted to the given conditions.
- Include mock-transduced T cells as negative control.

1. On day two (24 hours after application of T Cell TransAct™ to enriched $CD4^+$ and $CD8^+$ T cells), thaw lentiviral vector on ice following manufacturer's recommendations and mix gently by pipetting up and down.
2. Calculate the volume of lentiviral vector for transduction with a known T cell number and MOI. Please, refer to example below:

- Number of T cells to be transduced: 10^6 cells
- Viral titer: 2×10^8 TU/mL
- MOI: 2

Example:
(Number of cells/viral titer) × MOI = Volume of viral vector
(1×10^6 cells/2×10^8 TU/mL) × 2 = 10 µL

3. Add the required amount of lentiviral vector (e.g. CD19 CAR construct) to the T cell culture.
4. Mix gently and incubate T cells for 48 hours (until day 4) at 37 °C, 5–10% CO_2.

8. Expansion of CAR T cells in TexMACS™ Medium supplemented with MACS® Cytokines

Notes:

- Inspect culture daily. Depending on the expansion rate, it might be necessary to split culture more frequently than every 2–3 days.
- A 9-day expansion period should be adequate to obtain sufficient numbers of expanded lentiviral-transduced cells.
- Upon stimulation with T Cell TransAct, expression of CD3 will be transiently down-regulated. Thus, the antibody staining of CD3 on the cell surface of activated cells might be affected.

1. On day 4, remove as much supernatant (containing T cell TransAct™ and the lentiviral vector) as possible from T cell culture, taking care not to disturb the cells.
2. Adjust T cell culture medium to the previous volume by adding pre-warmed TexMACS™ Medium supplemented with Human IL-7, premium grade and Human IL-15, premium grade.
3. When media is exhausted, split cell suspension into two equal parts and add TexMACS Medium supplemented with Human IL-7, premium grade and Human IL-15, premium grade. Incubate at 37 °C, 5–10% CO_2.
4. Repeat step 3 when media is exhausted (e.g. every 2–3 days), or when cells reach 80% confluency.
5. Incubate for 9 days (until day 12) at 37 °C, 5–10% CO_2.
   ▲ **Note:** The ideal cell density for T cell expansion until next splitting is $1–2\times10^6$ T cells per mL.

9. Flow cytometric analysis of transduced T cells

1. On day 9 of expansion remove a small aliquot from the fraction representing the transduced CD19 CAR-expressing $CD4^+$ and $CD8^+$ T cells (e.g. 50–100 µL).
   ▲ **Note:** Transduction efficiency can also be determined before and within the CAR T cell expansion period.
2. Determine the cell number.
3. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.

4. For each sample resuspend up to 10^6 nucleated cells in 100 µL transduction efficiency staining cocktail.
   ▲ **Note**: Refer to section "Reagent and instrument preparation", table 2 "Staining cocktail for transduction efficiency antibody panel".
5. Mix well and incubate for 10 minutes in the dark in the refrigerator (2–8 °C).
   ▲ **Note:** Higher temperatures and/or longer incubation times may lead to non-specific cell labeling. Working on ice requires increased incubation times.
6. Wash cells by adding 1–2 mL of buffer and centrifuge at 300×g for 10 minutes. Aspirate supernatant completely.
7. Resuspend cell pellet in a suitable amount of buffer (e.g. 500 µL) for flow cytometry analysis.
   ▲ **Note:** Cells must be acquired within 1 hour after staining.

10. Phenotyping, assessing functionality and antigen specificity of generated CAR T cells

10.1 Analysis of cell proliferation and expansion rates

1. For monitoring **T cell proliferation rates** samples of 25–50 µL are taken at appropriate points in time (e.g. day 3, 6, 8, 10, and 12) and diluted 1:2 with buffer. CFSE dilution is measured on the MACSQuant® Analyzer 10. At least 20,000 events are recorded.
2. For determination of the **T cell expansion rates** samples of 25–50 µL are taken at appropriate points in time (e.g. days 3, 6, 8, and 10) and the count of viable cells is measured on the MACSQuant Analyzer 10. For dead cell exclusion propidium iodide is added prior to flow cytometric acquisition. Expansion rate is calculated afterwards.
   ▲ **Note:** Points in time can be adapted.

10.2 CAR T cell phenotyping

For CAR T cell phenotyping, e.g. to assess activation, differentiation, proliferation, and exhaustion of CAR T cells, please feel free to directly contact our experts at mquant@miltenyibiotec.de. All CAR T cell phenotyping panels are compatible with MACSQuant® Express Modes.

10.3 Quantification of CAR T cell-mediated cytotoxicity (tumor cell killing rate)

Notes:

- Antigen-expressing target cells must be chosen upon the respective CAR construct utilized for the viral transduction (e.g. co-culture of GFP^+CD19^+ JeKo-1 B cell lymphoma target cell line with CD19 CAR T cells).
- If not working with GFP^+ target cells: target cell labeling can be performed using CellTrace™ Violet Cell Proliferation Kit. For detailed information refer to end of this section "(Optional) Target cell labeling for killing assay".

1. On day 12 set CD19 CAR T cells cytokine-free: Wash the cells twice with TexMACS™ Medium by filling the tube to the maximum volume and centrifuge at 300×g for 10 minutes. Completely aspirate supernatant and resuspend in plain TexMACS Medium without any additional Human IL-7, premium grade and Human IL-15, premium grade.
2. Seed target cells (e.g. JeKo-1 GFP^+ CD19 surface protein-expressing target cells) and control cells (e.g. JeKo-1 GFP^+ CD19 knockout cells), respectively, in a 96-well plate.
3. After 24 hours of cytokine-free cultivation determine CAR T cell number.
4. Centrifuge cell suspension at 300×g for 10 minutes. Aspirate supernatant completely.
5. Resuspend cells to a final concentration of 2×10^7 cells/mL in PBS.
6. Add CAR T cells (effector cells (E)) to each well plate containing target cells (T) or control cells at different
   E:T ratios (e.g. 10:1, 5:1, 1:1, 0.2:1).
7. Co-culture CAR T cells with respective target cells for 24 hours.
8. Remove 96-well plate from the incubator and incubate for 20 minutes at 2–8 °C.
   ▲ **Note:** (Optional) Supernatant of the E:T 1:1 condition can be used to determine secreted cytokines using MACSplex Assays (please refer to section 10.4).
9. (Optional) Take out 100 µL of the supernatant of each well of the 96-well plate for the cytokine analysis (section 10.4) and fill up with 100 µL of fresh TexMACS Medium without supplements.
10. The killing rate is determined by analyzing the GFP fluorescence intensity of the target cells using the MACSQuant® Analyzer. Make sure that for measurement sufficient running buffer and washing solution are in the containers of the MACSQuant Analyzer. Refill if the levels are low. Additionally, the waste container must be empty.
11. Make sure that propidium iodide is automatically added by the MACSQuant Analyzer using the autolabel function.
12. Define the sample volume (200 µL) and the uptake volume (100 µL). Choose the Acquisition Mode “High” and activate mixing.
13. Start the acquisition of all samples. For results refer to figure 8 in section “Results”.

(Optional) Target cell labeling for killing assay

Note:

- If not working with GFP^+ target cells: target cell labeling can be performed using CellTrace™ Violet Cell Proliferation Kit.

1. Dilute CellTrace Violet dye stock solution to 1 µM using sterile 1× standard buffer.
2. Determine the cell number.
3. Calculate the volume to adjust the cells to a final concentration of 5×10^6 cells/mL.
4. Centrifuge cells at 300×g for 10 minutes.
5. Discard supernatant and resuspend the cell pellet in the calculated volume of CellTrace Violet dye staining solution, mix well, and incubate for 5 minutes at 37 °C, 5% CO_2.
6. After incubation add 5 mL of FCS and 5 mL of TexMACS Medium (without supplements).
7. Centrifuge the cells at 300×g for 10 minutes.
8. Discard supernatant and resuspend the cell pellet in 5 mL TexMACS™ Medium.
9. Determine the cell number.

10. Calculate the volume to adjust the cells to a concentration of 1×10^5 cells/mL.
11. Centrifuge the cells at 300×g for 10 minutes.
12. Discard supernatant and resuspend the cell pellet in the calculated volume of TexMACS Medium.

10.4 MACSPlex Assay for determining cytokine concentrations in co-culture supernatant

MACSPlex Assays are designed for determining concentrations of soluble analytes in a single sample. The analysis is based on MACSPlex Capture Beads, which display defined fluorescence properties and can be identified using standard flow cytometry techniques (figure 4).

The MACSPlex Cytokine 12 Kit, human is used to detect GM-CSF, IFN-α, IFN-γ, IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, IL-17A, and TNF-α.

Detection of MACSPlex Cytokine 12 Capture Bead populations

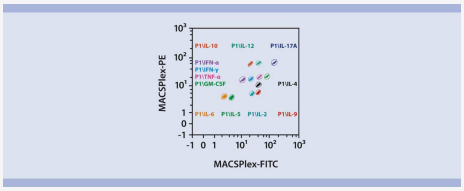


Figure 4: Detection of MACSPlex Cytokine 12 Capture Bead populations in a MACSPlex-FITC (B1) versus MACSPlex-PE (B2) dot plot.

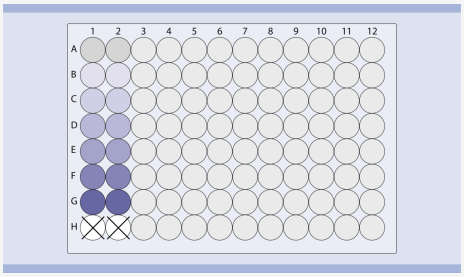
Notes:

- Run the assay at room temperature. Work fast and keep samples protected from light, for example, cover plate or tubes with aluminum foil, especially during incubation steps.
- Unknown samples should be run in replicates, for example, in duplicates or triplicates and in different dilutions to make sure the fluorescence values are within the dynamic range of the standard curve.
- Additional cytokines can be included in the analysis according to the respective needs. For additional cytokines refer to www.miltenyibiotec.com/cytokines.

Setup of the assay in a 96-well plate

Design the assay using two columns of the MACSPlex Filter Plate for the standards. Add each of the seven standard samples in duplicates next to each other. Standards should be run in order from the lowest concentration (blank control: 0 pg/mL) to the highest concentration (stock solution: 10,000 pg/mL or 50,000 pg/mL). Start with the unknown sample in the next column of the plate. For more details, refer to the respective data sheet, section ”Protocol for the assay using the MACSPlex Filter Plate”.

Setup of the assay using a 96-well plate



Well position	Sample	Dilution
A1/A2	Blank control	
B1/B2	MACSPlex Cytokine 12 Standard (3.2 pg/mL)	1:3125 (Dilution5; $1:5^5$)
C1/C2	MACSPlex Cytokine 12 Standard (16 pg/mL)	1:625 (Dilution4; $1:5^4$)
D1/D2	MACSPlex Cytokine 12 Standard (80 pg/mL)	1:125 (Dilution3; $1:5^3$)
E1/E2	MACSPlex Cytokine 12 Standard (400 pg/mL)	1:25 (Dilution2; $1:5^2$)
F1/F2	MACSPlex Cytokine 12 Standard (2 ng/mL)	1:5 (Dilution1; $1:5^1$)
G1/G2	MACSPlex Cytokine 12 Standard (10 ng/mL)	Stock solution (Dilution0; $1:5^0$)
H1/H2	Leave empty	
A3–H12	Add unknown samples	

1. Pre-wet required wells of the MACSPlex Filter Plate with 200 µL of MACSPlex Buffer per well and aspirate off using a vacuum manifold designed to accommodate the filter plate (max. -300 mbar) until the wells are drained.
2. Place the filter plate briefly on a tissue to remove any residual liquid.
3. Add 50 µL of MACSPlex Buffer or media as a blank control, 50 µL of each dilution, and the stock solution of the MACSPlex Cytokine 12 Standard to the corresponding wells of the filter plate.
4. Add 50 µL of each unknown sample per well.
5. Resuspend MACSPlex Cytokine 12 Capture Beads by vortexing for at least 30 seconds and transfer 20 µL of MACSPlex Capture Beads to each well.
6. Incubate filter plate for 2 hours protected from light on an orbital shaker (450 rpm).
7. Apply the filter plate to the vacuum manifold and aspirate until wells are drained. Place the filter plate briefly on a tissue to remove any residual liquid.
8. Add 200 µL MACSPlex Buffer to each well and apply the filter plate to the vacuum manifold and aspirate off until wells are drained. Place the filter plate briefly on a tissue to remove residual liquid.
9. Repeat step 8.
10. Add 80 µL of MACSPlex Buffer to each well.

11. Add 20 µL of MACSPlex Cytokine 12 Detection Reagent to each well.
12. Incubate filter plate for 1 hour protected from light on an orbital shaker (450 rpm).
13. Repeat wash steps 7 and 8.
14. Add 200 µL of MACSPlex Buffer to each well.
15. For sample acquisition using MACSQuant® Instruments and the Express Mode place the filter plate onto the Chill 96 Rack. To prevent liquid transfer from the wells, ensure that residual drops under the plate are completely removed by placing the plate briefly on a tissue.
    ▲ **Note:** Perform the flow cytometric acquisition on the same day, as prolonged storage of samples can result in increased background and reduced sensitivity.
    ▲ **Note:** Keep samples protected from light by using the protection lid during the flow cytometric acquisition with the MACSQuant Instrument.

Flow cytometer setup

The kit includes MACSPlex Setup Beads for flow cytometer setup. MACSPlex Setup Beads are not required when using the MACSQuant Analyzer or MACSQuant Analyzer 10 but for all other flow instruments that can detect FITC, PE, and APC.
▲ **Note:** The kits are not suitable for use with the MACSQuant VYB.

Setup of the MACSQuant® Instrument

Calibrate the MACSQuant® Instrument using MACSQuant Calibration Beads (# 130-093-607). For details, refer to the data sheet of the MACSQuant Calibration Beads. After successfully completing the calibration, the MACSQuant Instrument is ready for measurement. No further steps are required as all necessary setup steps are performed automatically during calibration.

Setup of other flow cytometers

The analysis of MACSPlex Cytokine 12 Kit, human requires a flow cytometer with a blue (e.g. 488 nm) and a red (e.g. 635 nm) laser, which can detect FITC, PE, and APC. For setting up these cytometers use MACSPlex Setup Beads (included in the kit). For instructions on the setup procedures of other flow cytometers, please refer to the application note "General instructions for MACSPlex Cytokine Kits" available on the product page at www.miltenyibiotec.com/130-101-740 in the "Library" section.

Flow cytometric acquisition and data analysis using the MACSQuant® Express Mode

To perform the acquisition and data analysis of the MACSPlex Cytokine 12 Kit, human with the MACSQuant® Instrument it is recommended to use the Express Modes **MACSPlex_Standard** and **MACSPlex_Sample** to achieve automated measurement and data analysis. For details refer to the special protocol “Data acquisition and analysis of MACSPlex Cytokine Kits using the MACSQuant® Analyzer Express Modes” available at www.miltenyibiotec.com/130-099-169 in the "Resources" section. The minimum version number of the Express Mode package needed to run the assay on the MACSQuant Instrument can be found there as well. To check the version number of the Express Mode package available on the MACSQuant Instrument please select Help> Info> expressModes within the MACSQuantify™ Software. The version number of the Express Mode package is increasing with each Express Mode updates. Make sure the MACSQuant Instrument contains an Express Mode package with at least the same or higher version number than the special protocol is marked with.
For results refer to figure 9 in section “Results”.

11. Results

11.1 Analysis of purity after T cell enrichment

Analysis of purity after T cell enrichment

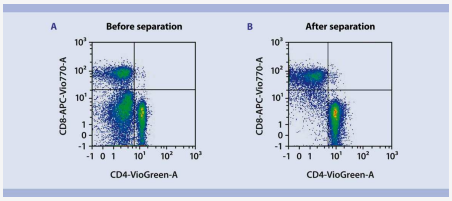


Figure 6: Flow cytometry analysis before and after magnetic cell separation. In order to generate functional CAR T cells, human $CD4^+$ and $CD8^+$ T cells were isolated from PBMCs by positive selection using a mixture of CD4 and CD8 MicroBeads (1:1). Isolated $CD4^+$ and $CD8^+$ T cells exhibited a purity of >98%.

11.2 Flow cytometric analysis of transduction efficiency

Flow cytometric analysis of transduction efficiency

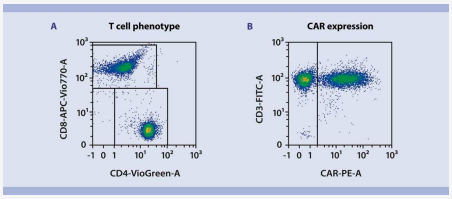


Figure 7: Flow cytometry analysis of transduced T cells after expansion on day 12. After transduction and expansion, cells were analyzed for $CD4^+$, $CD8^+$, and expression of CD19 CAR. (A) Of all viable leucocytes >99% were $CD4^+$ or $CD8^+$ (based on CD45 expression, 7-AAD fluorescence, and scatter signal). (B) More than 56% of all $CD3^+$ cells expressed the CD19 CAR construct.

11.3 Quantification of CAR T cell-mediated cytotoxicity (tumor cell killing rate)

Quantification of CAR T cell-mediated cytotoxicity (tumor cell killing rate)

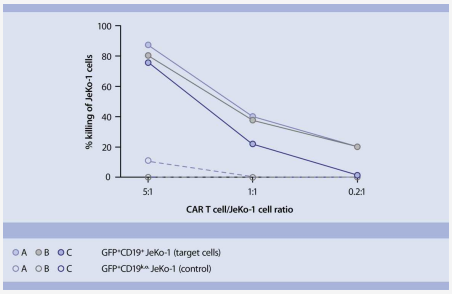


Figure 8: Killing of GFP$^+$CD19$^+$ JeKo-1 target cells by CD19 CAR T cells in a ratio-dependent manner. CD4$^+$ and CD8$^+$ T cells were isolated from PBMCs of three independent donors (A–C) and CD19 CAR T cells were manufactured as described. On day 12, CD19 CAR T cells were functionally tested in a killing assay. Transduced T cells were co-cultured with either the GFP$^+$CD19$^+$ JeKo-1 mantle cell lymphoma target cell line or a CD19 knock out (k.o.) variant control at indicated ratios. The killing rate was determined by analyzing the GFP fluorescence intensity of the target cells using the MACSQuant® Analyzer. Flow cytometry analysis showed about 80% killing of target cells at a 5-fold excess of CD19 CAR T cells. When changing the ratio by decreasing the number of CAR T cells, max. 18% of the target cells were killed. In contrast, the CD19$^{k.o.}$ JeKo-1 variant control cells were not killed by the CD19 CAR T cells, highlighting the specificity of the CAR T cells.

11.4 Analysis of GM-CSF, IFN-γ, IL-2, and TNF-α cytokine secretion in co-culture supernatant using the MACSPlex Cytokine Kits

Analysis of GM-CSF, IFN-γ, IL-2, and TNF-α cytokine secretion in co-culture supernatant using the MACSPlex Cytokine Kits

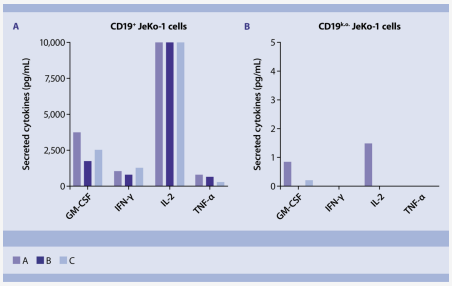


Figure 9: Cytokine analysis (GM-CSF, IFN-γ, IL-2, and TNF-α) of co-culture supernatant using MACSPlex Cytokine Kits. CD19 CAR T cells from three independent donors (A–C) were cultivated for 20 hours with the respective target cells (CD19$^+$ JeKo-1 and CD19$^{k.o.}$ JeKo-1). Cell culture supernatants were harvested and centrifuged to remove particulates. Undiluted samples were analyzed using MACSPlex Cytokine 12 Kit, human. Two standard curves were carried out and the concentrations of 12 analytes were determined using MACSQuant Express Mode. The four most relevant cytokines (GM-CSF, IFN-γ, IL-2, and TNF-α) were displayed. (A) Upon recognition of CD19 receptor on the surface of the target cell the CD19 CAR T cells respond by the secretion of pro-inflammatory cytokines, whereas (B) co-culture with CD19$^{k.o.}$ target cells induce negligible amounts of cytokine secretion, confirming the functionality and antigen-specificity of the CAR T cells.

Materials

The following is a listing of reagents, instruments, and consumables needed for each step of this protocol. These products are for research use only.

Reagent and instrument requirements

Isolation of PBMCs using Ficoll-Paque™

- PBS-EDTA buffer: Phosphate-buffered saline (PBS), pH 7.2, with 2 mM EDTA
  ▲ **Note:** EDTA can be replaced by other supplements such as anticoagulant citrate dextrose formula-A (ACD-A) or citrate phosphate dextrose (CPD).
- Ficoll-Paque™ (ρ = 1.077 g/mL)
- 50 mL conical centrifuge tubes
- Pre-Separation Filters (30 µm) (# 130-041-407)

T cell cultivation

- TexMACS™ Medium (# 130-097-196)
- Human IL-7, premium grade (# 130-095-362)
- Human IL-15, premium grade (# 130-095-764)
- 24-well plate (e.g. Gas-permeable Culture Plate, # 150-000-362)
  ▲ **Note:** Addition of penicillin/streptomycin to the T cell media is optional (e.g. 100× penicillin/streptomycin stock solution to a final concentration of 1×).

Standard wash and dilution buffer

- autoMACS® Rinsing Solution (# 130-091-222)
  ▲ **Note:** For manual cell separation autoMACS Rinsing Solution can be replaced by PBS-EDTA buffer.
- MACS® BSA Stock Solution (# 130-091-376)

Magnetic cell separation

- CD4 MicroBeads, human (# 130-045-101)
- CD8 MicroBeads, human (# 130-045-201)
- autoMACS Pro Separator (# 130-092-545)
- autoMACS Columns (# 130-021-101)
- Pre-Separation Filters (30 µm) (# 130-041-407)
  ▲ **Note:** For manual separation a MidiMACS™ Separator, MACS MultiStand, and MACS LS Columns can

be used in place of the autoMACS Pro Separator.

CFSE labeling for cell proliferation analysis

- 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE; molecular weight: 557.46 kDa, e.g. from Sigma-Aldrich; # 21888-25MG-F)
- Dimethylsulfoxide (DMSO; e.g. CryoMACS® DMSO 10 (EP, USP); # 170-076-303)
- Phosphate-buffered saline (PBS), pH 7.2
- Human AB serum

T cell transduction

- CAR vector/gene construct (e.g. CD19 CAR)
- Individual chosen CAR Detection Reagent (CAR DR)

T cell activation and expansion

- T Cell TransAct™, human (# 130-111-160)
- TexMACS Medium (# 130-097-196)

Antibodies

Table 1: Antibodies used for analysis of immune cell composition and transduction efficiency.

Antigen	Clone	Fluorochrome
CD45	REA747	VioBlue®
CD3	REA613	FITC
CD4	REA623	VioGreen™
CD8	REA734	APC-Vio® 770
CD16	REA423	PE
CD56	REA196	PE
CD14	REA599	APC
CD20	REA780	PE-Vio 770
CAR DR	-	PE
7-AAD	-	-

▲ **Note:** Antibodies can be replaced/included according to the respective needs. For additional antibodies refer to www.miltenyibiotec.com/antibodies.

▲ **Note:** For additional CAR T cell phenotyping panels, e.g., to assess activation, differentiation, proliferation, and exhaustion of T cells, consult our experts at macstec@miltenyi.com. All panels are compatible with MACSQuant® Express Modes.

- MACSQuant Analyzer, MACSQuant Analyzer 10 (# 130-096-343), or any flow cytometer equipped with violet (405 nm), blue (488 nm), and red (635 nm) laser and able to discriminate FITC, PE, PerCP, and APC.
  ▲ **Note:** The MACSQuant VYB cannot be used.
- MACS Chill 96 Rack (# 130-094-459), when using MACSQuant Analyzer or MACSQuant Analyzer 10
- MACSQuant Calibration Beads (# 130-093-607), when using the MACSQuant Analyzer or MACSQuant Analyzer 10

GFP-based analysis of target cell killing

- GFP^+ target cell line that expresses the specific antigen (e.g. $CD19^+$ JeKo-1 B cell lymphoma target cell line) and control cell line which is negative for the respective antigen (e.g. CD19 knockout)
- If not working with GFP^+ target cells: target cell labeling can be performed using the CellTrace™ Violet Cell Proliferation Kit (ThermoFisher Scientific, # C34557)

Cytokine analysis

- MACSPlex Cytokine 12 Kit, human (# 130-099-169)
- Polypropylene or polystyrene reagent tubes for serial dilutions of the MACSPlex Cytokine 12 Standard and for preparation, dilution, and storage of samples
- (Optional) Vacuum manifold or centrifuge with adapters to accommodate microtiter plates
- Orbital shaker for microtiter plates or tubes (frequency 450–1400 rpm)
- MACSQuant Analyzer, MACS Chill 96 Rack, and MACSQuant Calibration Beads

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	Clone	Applications	Size	Price
130-110-637 **CD45 Antibody, anti-human, VioBlue®, REAfinity™** For research use only	REA747	FC	100 tests in 200 µL **Quantity** One test corresponds to labeling of 10^6 cells in a final volume of 100 µL (applies only to flow cytometry).	**EUR 249,00**

	Clone	Applications	Size	Price
130-110-681 **CD8 Antibody, anti-human, APC-Vio® 770, REAfinity™** For research use only	REA734	FC	100 tests in 200 µL **Quantity** One test corresponds to labeling of 10^6 cells in a final volume of 100 µL (applies only to flow cytometry).	**EUR 259,00**

	Clone	Applications	Size	Price
130-110-520 **CD14 Antibody, anti-human, APC, REAfinity™** For research use only	REA599	FC, MICS, IF, IHC	100 tests in 200 µL	**EUR 211,00**

Quantity

One test corresponds to labeling of 10^6 cells in a final volume of 100 µL (applies only to flow cytometry).

	Clone	Applications	Size	Price
130-111-341 **CD20 Antibody, anti-human, APC-Vio® 770, REAfinity™** For research use only	REA780	FC	100 tests in 200 µL	**EUR 259,00**

Quantity

One test corresponds to labeling of 10^6 cells in a final volume of 100 µL (applies only to flow cytometry).

	Size	Price
130-111-160 **T Cell TransAct™, human** For research use only	2×2 mL	**EUR 642,00**

	Size	Price
130-097-196 **TexMACS™ Medium** For research use only	500 mL	**EUR 62,00**

	Applications	Size	Price
130-099-169 **MACSPlex Cytokine 12 Kit, human** For research use only	FC	for 100 tests	**EUR 1.798,00**

HIGHLIGHT

130-096-343
MACSQuant® Analyzer 10
For research use only

Size: 1 unit

Price: **On request**

130-093-607
MACSQuant® Calibration Beads
For research use only

Size: 3.5 mL

Price: **EUR 192,00**

130-095-362
Human IL-7, premium grade
For research use only

Source: *E. coli*

Size: 25 µg

Price: **EUR 389,00**

130-095-764
Human IL-15, premium grade
For research use only

Source: *E. coli*

Size: 25 µg

Price: **EUR 389,00**

HIGHLIGHT

130-092-545
autoMACS® Pro Separator - Starter Kit
For research use only

Price: **On request**

130-094-459
Chill 96 Rack
For research use only

Size: 1 rack

Price: **EUR 390,00**

130-091-222
autoMACS® Rinsing Solution
For research use only

Size: 6×1.45 L

Price: **EUR 120,00**

130-091-376
MACS® BSA Stock Solution
For research use only

Size: 6×75 mL

Price: **EUR 219,00**

130-021-101 **autoMACS® Columns** For research use only	Size 5×2 columns	Price **EUR 599,00**

130-041-407 **Pre-Separation Filters (30 µm)** For research use only	Size 50 filters	Price **EUR 152,00**

130-045-101 **CD4 MicroBeads, human** For research use only	Size for 1×10^9 total cells	Price **EUR 545,00**

130-045-201 **CD8 MicroBeads, human** For research use only	Size for 1×10^9 total cells	Price **EUR 545,00**

130-111-568 **7-AAD Staining Solution** For research use only	Applications FC	Size 1 mL	Price **EUR 52,00**

130-113-138 **CD3 Antibody, anti-human, FITC, REAfinity™** For research use only	Clone REA613	Applications FC, MICS, IF, IHC	Size 100 tests in 200 µL	Price **EUR 156,00**

Quantity

One test corresponds to labeling of 10^6 cells in a final volume of 100 µL (applies only to flow cytometry).

	Clone	Applications	Size	Price
130-113-230 **CD4 Antibody, anti-human, VioGreen™, REAfinity™** For research use only	REA623	FC	100 tests in 200 µL **Quantity** One test corresponds to labeling of 10^6 cells in a final volume of 100 µL (applies only to flow cytometry).	**EUR 249,00**

	Clone	Applications	Size	Price
130-113-312 **CD56 Antibody, anti-human, PE, REAfinity™** For research use only	REA196	FC	100 tests in 200 µL **Quantity** One test corresponds to labeling of 10^6 cells in a final volume of 100 µL (applies only to flow cytometry).	**EUR 211,00**

	Clone	Applications	Size	Price
130-113-393 **CD16 Antibody, anti-human, PE, REAfinity™** For research use only	REA423	FC, MICS, IF, IHC	100 tests in 200 µL **Quantity** One test corresponds to labeling of 10^6 cells in a final volume of 100 µL (applies only to flow cytometry).	**EUR 211,00**

	Size	Price
150-000-362 **Gas-permeable Culture Plate (24 well)** For research use only	4 pieces	**EUR 47,00**