Miltenyi Biotec

Commercial success starts with a fit-for-purpose flow cytometer

Make precision, consistency, and reliability your competitive edge

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Introduction

The success of cell-based therapies depends on accurate, consistent analytical data. Flow cytometry plays a key role, but variability in instruments and reagents can impact results. Using standardized, high-quality reagents and harmonized, automated instruments supports efficient assay development and validation. This approach improves consistency, saves time, and increases your confidence that the data is accurate. Below are key strategies and considerations to help achieve this level of reliability and standardization.

Non-compendial methods or assays require full validation

The level of validation required for your assays will depend on if it is an established assay (compendial method) or a unique assay that requires full validation (non-compendial method).

The majority of assays derived from cell manufacturing will be non-compendial and therefore require in-house assay development and validation.

Most of these assays will need four to six fluorescence channels. Using more channels than required can make it harder to set clear specifications.

The biggest challenge in developing fit-for-purpose assays is variability. These sources of variance can arise from:

- The flow cytometer used
- The reagents selected
- Analysis and reporting (human)
- The specific protocols
- The reference materials and controls

Manufacturers, not users, typically have the most control over the instrumentation or flow analyzer. In this scenario, what do you need from your flow cytometer to have a robust, validated assay?

From chaos to control with CQAs

Cell manufacturing analysis involves identifying critical quality attributes (CQAs) and understanding how they relate to process parameters. These CQAs are key physical, chemical, biological, or microbiological properties that affect the product's identity, potency, purity, or safety.

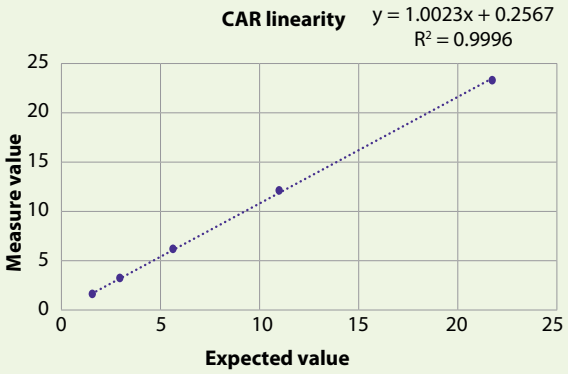
Linearity

Linearity is one of the most important criteria to consider when designing a flow cytometer assay.

The linearity of an analytical technique refers to its ability (within a specified range) to produce test findings that are directly proportional to the concentration (quantity) of analyte in the sample.

The performance of a flow cytometer plays a critical role in accurately measuring a product's CQAs, making it essential for maintaining assay reliability.

To assess linearity, CAR-transduced T cells were mixed with non-transduced T cells in seven different ratios. Each ratio was analyzed in five replicates via flow cytometry with the Panel B CAR T Express Mode Panel on the MACSQuant® Analyzer 16 Flow Cytometer. For each ratio, the average CAR$^+$% (of live CD3$^+$ cells) among replicates was measured and plotted as the measured % CAR. The expected % CAR was calculated using the measured CAR+% of the CAR-transduced T cell sample, the non-transduced T cell sample alone, and the quantity of each cellular population mixed in each ratio. Results are shown in figure 1 and figure 2 shows a representative dot plot.



Sample	Replicates	CAR$^+$ volume (µL)	CAR$^-$ volume (µL)	Expected CAR$^+$ (%)
L-1	5	1,000	0	X
L-2	5	800	200	0.8X + 0.2Y
L-3	5	400	600	0.4X + 0.6Y
L-4	5	200	800	0.2X + 0.8Y
L-5	5	100	900	0.1X + 0.9X
L-6	5	50	950	0.05X + 0.95Y
L-7	5	0	1,000	Y

Figure 1: Linearity is evaluated using R^2, a statistical measurement of how close the data points are to the fitted regression line, and shows how well the model predicts the outcome. R^2 values: range from 0 to 1. Values closer to 1 indicate that the fitted regression line can better predict the dependent variable based on the independent variable.

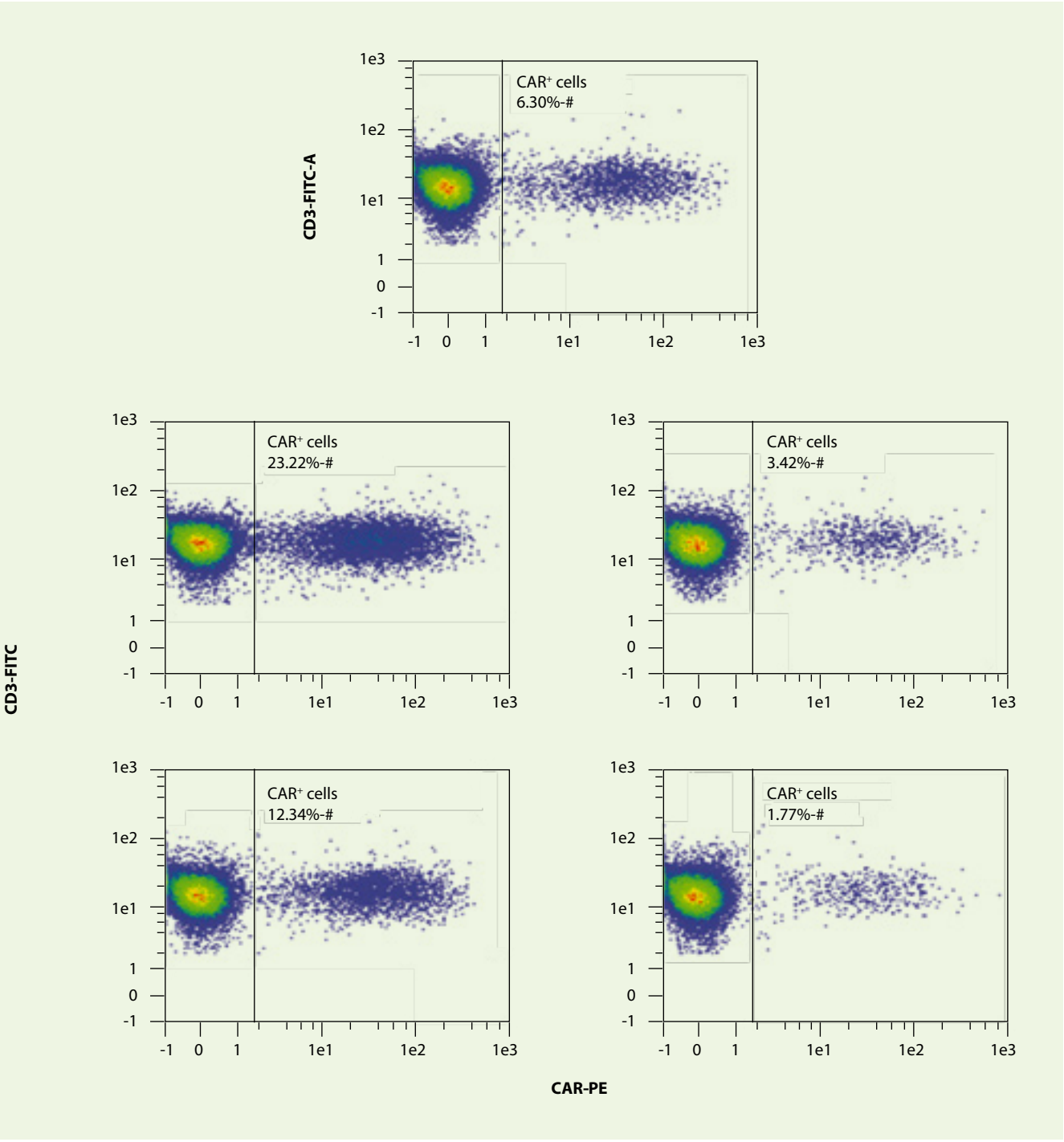


Figure 2: Representative dot plot of CAR-transduced T cells and non-transduced T cells ratios: CAR-transduced T cells were mixed with non-transduced T cells in seven different ratios. Each ratio was analyzed in five replicates via flow cytometry with the Panel B CAR T Cell Express Mode Panel on the MACSQuant Analyzer 16 Flow Cytometer. . For each ratio, the average CAR^+% (of live $CD3^+$ cells) among replicates was measured and plotted as the measured % CAR. The expected % CAR was calculated using the measured CAR^+% of the CAR-transduced T cell sample and the non-transduced T cell sample alone and the quantity of each cellular population mixed in each ratio, as shown in figure 1.

Repeatability

A key element of flow assay validation repeatability, ensures consistent analytical results within a single run.

The ability of the MACSQuant Analyzer 16 Flow Cytometer to produce consistent results for identical samples was measured by:

1. Examining five replicates under the same settings.
2. Analyzing the device versus device precision in two different instruments.
3. Harmonizing them though Smart Gains technology.

Effortless assay transfer and scaling with Smart Gain technology

Smart Gain technology ensures consistent results by automatically changing PMT voltages to predefined fluorescent intensity target values during daily calibration with MACSQuant Calibration Beads (fig. 3).

This flow cytometry software feature eliminates the need for manual adjustments when transferring settings between instruments.

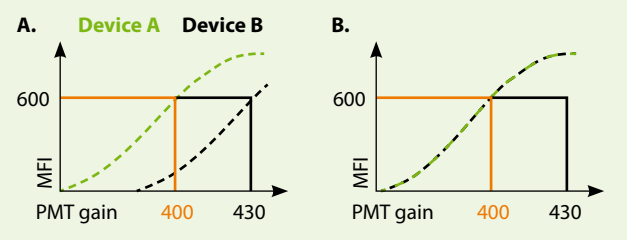


Figure 3: A. Different PMT gains on device A and B required to establish an MFI of 600. **B.** Automatic gain adjustment of both instruments using Smart Gain technology. **Note:** Scatter channels and compensation settings should be checked after the transfer of instrument settings between devices.

Achieving consistent results with instrument harmonization

To assess Smart Gain technology, we standardized instrument settings across eight MACSQuant Analyzers. Lysed whole-blood samples, stained with our 8-Color Immunophenotyping Kit, anti-human, REAfinity™, were analyzed in triplicate on each device. The results demonstrated exceptional reproducibility (fig. 4).

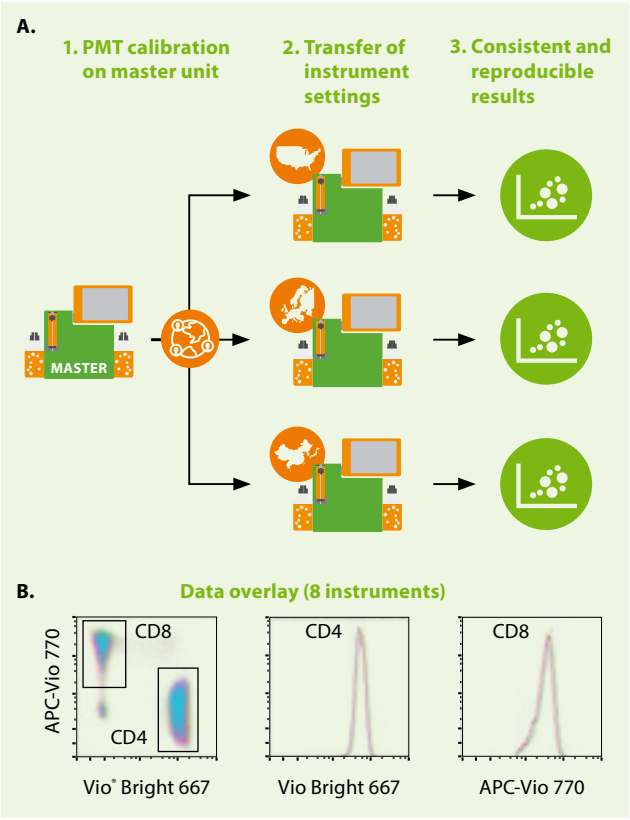


Figure 4: A. Schematic overview of multi-instrument harmonization: Instrument settings are transferred from a single-master unit to multiple MACSQuant Analyzers in different locations. **B.** Data overlays from eight individual MACSQuant Analyzers including CVs of fluorescence intensity. Signal intensities are highly comparable between instruments due to Smart Gain technology.

Intermediate precision device versus device experiment (MACSQuant 16 vs. MACSQuant 16)

The CAR-transduced T cells were mixed with $CD45^+$ $CD3^+$ cells in three different ratios (100% CAR, 66.7% CAR, and 33.3% CAR cells). Each ratio was analyzed in five replicates via flow cytometry with the CAR T Cell Express Mode Package Immune Cell Composition on two different MACSQuant 16 Instruments. For each ratio, the average viability of the CD45, CD3, CD4, CD8, and natural killer (NK) T cell populations was measured among replicates (fig. 5)

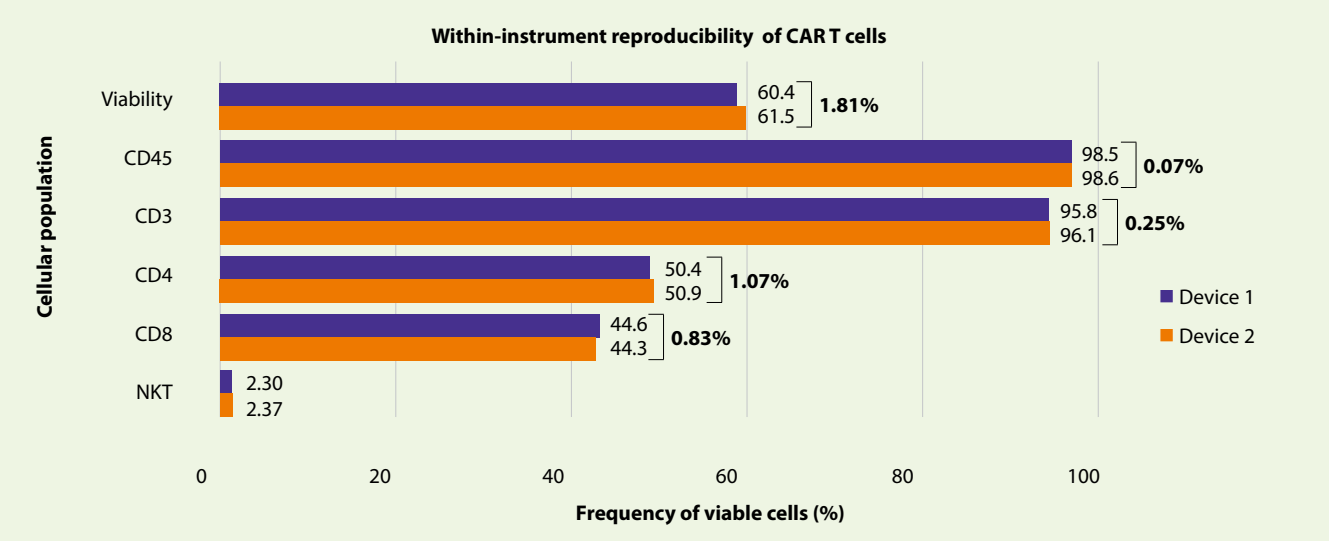


Figure 5: Immune cell markers that the CAR T cells will exhibit and the close agreement between replicates on separate devices.

Specificity

Specificity refers to the instrument's ability to accurately distinguish the analyzed target from non-target populations — for example, differentiating $CD3^+$ from $CD3^-$ cells.

Reliable detection at low frequencies is essential for preclinical characterization and IND-enabling studies.

To evaluate specificity, we analyzed the % of $CD3^+$ T cells within live $CD45^+$ leukocytes across three defined T cell-to-K562 ratios (100%, 66.7%, and 33.3%). The K562 cells lack CD3 expression and serve as a negative background. All samples were stained with the CAR T Panel A: Immune Cell Composition Panel, and each ratio was run in five replicates. Both cell percentages and median fluorescent intensity (MFI) are shown in the (fig. 6).

Data were acquired on two different MACSQuant Analyzers, calibrated with the same MACSQuant Calibration Beads, demonstrating instrument-to-instrument reproducibility, a key factor for standardizing quality control.

Frequently, flow cytometers require individual optimization due to variances in optical components and detection channels. Manual adjustment is a multi-step technique that relies heavily on the operator to achieve equal performance between instruments.

The MACSQuant Analyzer automatically harmonizes the performance between instruments using Smart Gain technology during daily PMT calibration.

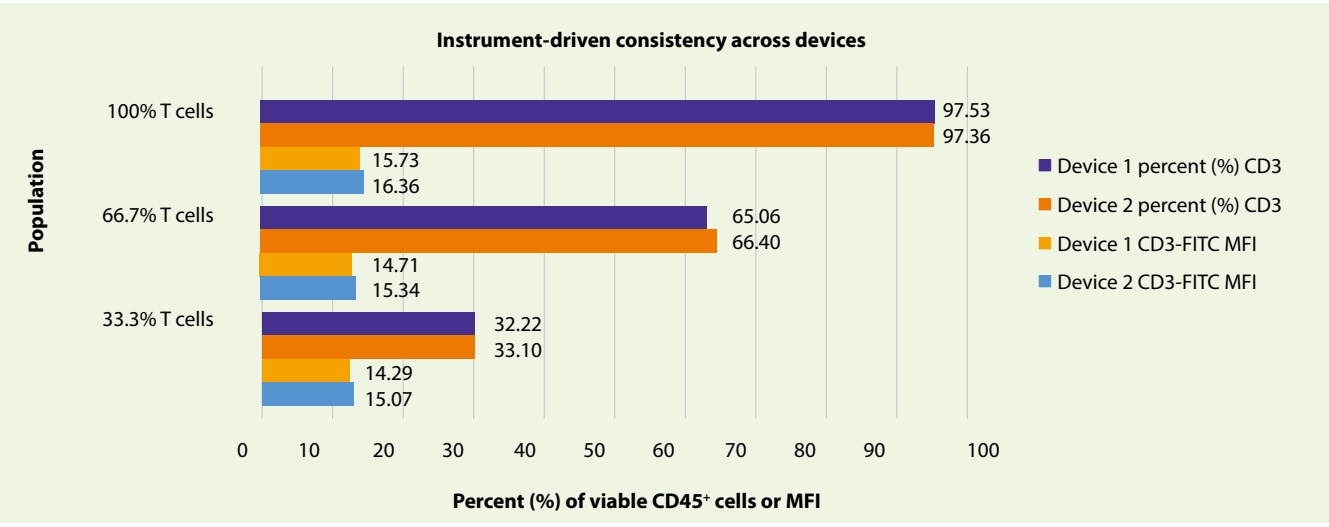


Figure 6: Percent (%) CD3-FITC of live $CD45^+$ leukocytes (identified by upstream gating of 7-AAD and CD45) from three ratios of T cells mixed with K562 cells which do not express CD3. 100% T cells, 66.7% T cells, and 33.3 % T cell ratios that were each analyzed in five replicates with our CAR T Panel A Immune Cell Composition Panel. CD3% was determined using direct cell counts, with %CV ranging from 0.1 to 2.4 across the three different cell ratios. Additionally, we measured the CD3-FITC MFI, which remained consistent across all three ratios, with %CV values between 0.34 and 0.43. The results were highly comparable between two different MACSQuant Analyzers, both of which were calibrated using the same MACSQuant Calibration Beads.

Are you ready for a fit-for-purpose instrument?

Throughout the preclinical development and cell and gene therapy commercialization, analytical assays are essential to produce safe and potent drug products. Despite their importance, conventional analytical assays often consist of complex manual handling steps. Therefore, it can be unclear whether variations measured come from the drug product or if they have been unwittingly introduced by the operator or technology used.

Miltenyi Biotec, with over 25 years of pioneering partnerships in CGT, has been at the forefront of innovation, contributing to over 100,000 cell therapy procedures worldwide. Our platforms have played a vital role in the treatment of more than 6,000 patients with life-saving cell therapies.



200+

Pioneering more than **200 cell therapy trials** with point-of-care manufacturing and analytics*



7/8

Approved engineered T cell therapies are manufactured using Miltenyi Biotec solutions**



25+

Over **25 years of CGT expertise** and relentlessly pushing boundaries

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**Approved cell therapies and gene-modified cell therapies in EU and USA, excluding minimally manipulated cell therapies, cord blood products, and tissue-engineered therapies.

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Notes:



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