Analytical performance of IFN-γ quantification for T cell functional characterization using a cytokine bead assay

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Introduction

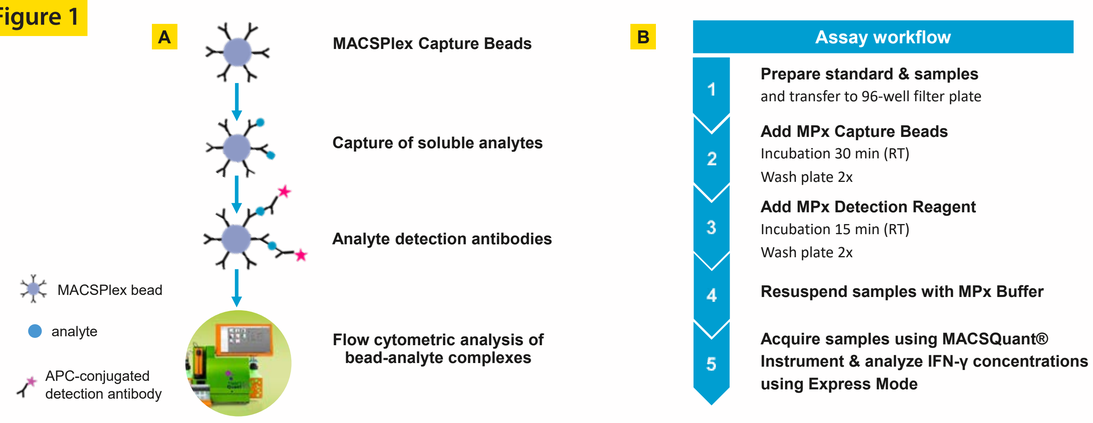
T cell–based therapies, including CAR T cells, are expanding beyond oncology into autoimmune disease indications, increasing the need for analytical methods that reliably characterize T cell functional activity across development stages. IFN-γ secretion is widely used as a functional readout of T cell activation and potency, but reproducible quantification requires systematic evaluation of analytical performance.

The aim of this study was to assess the analytical performance of a MACSPlex cytokine bead assay for reliable IFN-γ quantification in accordance with current regulatory guidance.

Methods

1 MACSPlex assay principle and workflow

Figure 1

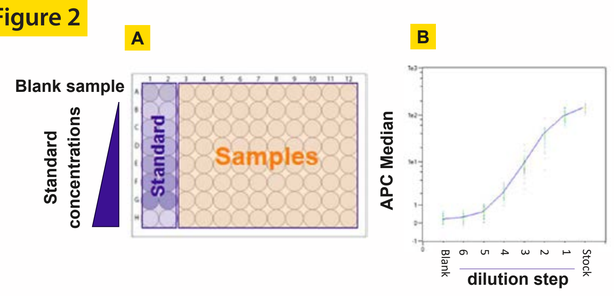


The MACSPlex (MPx) assay principle for singleplex direct analyte detection is shown in Figure 1A. MPx is a bead-based ligand-binding assay (LBA) for quantitative cytokine analysis. Fluorescently coded capture beads, each coupled to target-specific antibodies, bind analytes present in the sample. After incubation with fluorescent detection antibodies, bead–analyte complexes are quantified by flow cytometry. The MACSQuant® Analyzer Express Mode automatically calculates cytokine concentrations, enabling rapid and standardized analysis. All experiments conducted in this study were performed according to the standardized assay workflow (Figure 1B). IFN-γ quantification performance of the MPx assay was evaluated in alignment with regulatory expectations for LBA characterization (1). Accuracy, precision, and linearity were analyzed using recombinant IFN-γ spike-and-recovery samples across eight nominal concentrations (0-100,000 pg/mL). Dilutional integrity was evaluated by spiking samples at 150,000 pg/mL IFN-γ and quantifying serial dilutions. Matrix effects were assessed by preparing matched spike concentrations in two different diluents and comparing resulting accuracy and precision. All samples were acquired on a MACSQuant® Analyzer and quantified using automated Express Mode analysis, which supports MACSQuant data (MQD) files. To assess the IFN-γ concentration, an automated MPx cytokine analysis tool, MACSPlex InspectoR compatible with both MQD files and standard Flow Cytometry Standard (FCS) files, was evaluated and compared to Express Mode analysis.

Results

1 Calibration curve for the MACSPlex IFN-γ assay

Figure 2



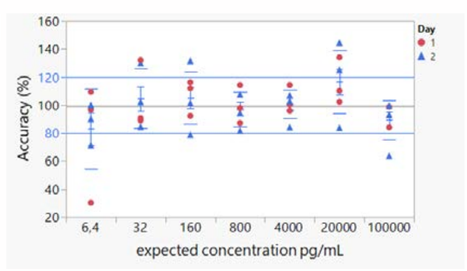
C

Sample/ dilution step	nominal concentration pg/mL	APC Median
blank	0	0.18
dilution 6	6.4	0.27
dilution 5	32	0.61
dilution 4	160	2.08
dilution 3	800	8.54
dilution 2	4000	36.66
dilution 1	20000	89.13
Stock solution	100000	121.97

Schematic plate layout showing the distribution of blank, serially diluted standards and experimental samples (Figure 2A). To generate the calibration curves, the standard of known quantities of IFN-γ was reconstituted in MPx Buffer to an initial concentration of 100,000 pg/mL, followed by six serial dilutions by a factor of 5, including one blank sample as control (0 pg/mL). Each sample was analyzed in duplicate using the MACSQuant® Analyzer and the APC median fluorescence values for each calibrator concentration were recorded and plotted to create the calibration curve using the Express Mode function (Figure 2B). Nominal standard concentrations (pg/mL) and corresponding APC median values for calibrator samples are shown (Figure 2C).

2 Assessing accuracy and precision

Figure 3



B

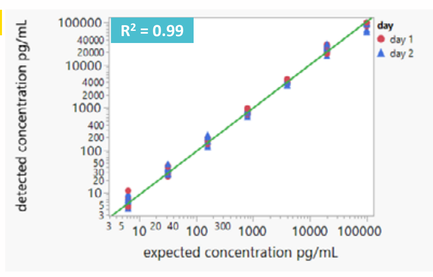
Target concentration (pg/mL)	Overall Mean (pg/mL)	Overall Std Dev	Overall Precision (%CV)	Overall Accuracy (%)
6.4	5.3	2.8	52.3	83.0
32	33.5	7	20.9	104.8
160	168.6	29.6	17.6	105.4
800	777	99.9	12.9	97.1
4000	4027.6	406.3	10.1	100.7
20000	23330.8	4938.1	21.2	116.7
100000	89661	13389.2	14.9	89.7

Intra-Assay accuracy and precision were assessed using spike/recovery samples with target concentrations of 6.4, 32, 160, 800, 4000, 20,000 and 100,000 pg/mL. Each sample was analyzed in triplicate, on three independent assay plates and on two separate days.

The mean detected concentration, standard deviation (SD), accuracy (%) and precision (%CV) were calculated for each tested concentration level within one plate (totaling 3 replicates per concentration per plate) (Figure 3A). To assess between-run (inter-assay) accuracy and precision, all measured values across both days, each consisting of three plates per day, and each plate containing 3 replicates per concentration (totaling 18 replicates per concentration), were combined. For each concentration level, the overall mean, SD, precision (%CV), and overall accuracy (%) were calculated (Figure 3B). All values above 100,000 pg/mL are indicated by the Express Mode as ">100,000 pg/mL!" and were used for calculations as 100,000 pg/mL. Specifications for accuracy and intra/inter-assay precision were set at ±20% of expected concentrations, or ±25% at lower and upper limits of quantification (LLOQ and ULOQ), in accordance with ICH M10 guidelines. Values outside the acceptance criteria are marked in orange (Figure 3B). Accuracy and precision consistently met predefined acceptance criteria within the range of 32- 100,000 pg/mL, with inter-assay precision and overall accuracy well within ±20%.

3 Assay linearity

Figure 4



B

dilution factor	expected concentration (pg/mL)	measured concentration (pg/mL)	back-calculated concentration (pg/mL)	Accuracy (%)	Precision (%)
1	150000	97029	97029	64.7	3.3
16	9375	11103	177649	118.4	2.2
64	2344	2546	162948	108.6	3.5
256	586	654	173359	115.6	5.0
1024	146	169	172920	115.3	3.2
4096	37	38	154351	102.9	6.7

The mean of detected concentration (pg/mL) calculated for spike/recovery replicates assayed on three plates on two days was plotted against expected concentrations of 6.4, 32, 160, 800, 4000, 20,000, 100,000 pg/mL to create the assay linearity graph. Linearity was maintained across the calibration range with log-log regression with R^2=0.9918, indicating proportional response over more than 4 orders of magnitude (Figure 4A). Dilution linearity was assessed by spiking three independent matrix samples with 150,000 pg/mL of recombinant IFN-γ and performing serial dilutions by a factor of 16, 64, 256, 1024, and 4096, down to 36.62 pg/mL. Samples were measured in duplicates. Values outside the acceptance criteria are marked in orange (Figure 4B). All dilutions up to 1:4096 met accuracy and precision acceptance limits, demonstrating that samples above the ULOQ can be reliably quantified following appropriate dilution (Figure 4B).

4 Evaluation of matrix effects

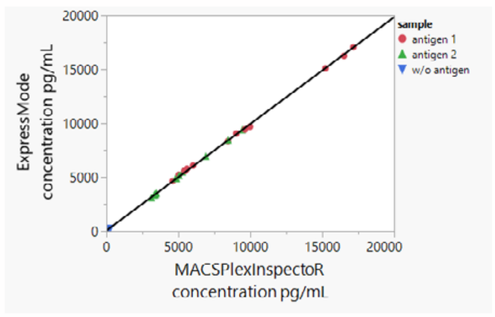
Figure 5

Buffer	Target concentration (pg/mL)	Overall Mean (pg/mL)	Overall Std Dev	Overall Precision (%CV)	Overall Accuracy (%)
MPx Buffer	6.4	5.3	2.8	52.3	83
MPx Buffer	32	33.5	7	20.9	104.8
MPx Buffer	160	168.6	29.6	17.6	105.4
MPx Buffer	800	777	99.9	12.9	97.1
MPx Buffer	4000	4027.6	406.3	10.1	100.7
MPx Buffer	20000	23330.8	4938.1	21.2	116.7
MPx Buffer	100000	89661	13389.2	14.9	89.7
Buffer B	6.4	86.3	129.1	149.5	1349
Buffer B	32	164.7	354.5	215.3	514.5
Buffer B	160	343.9	145.3	42.3	214.9
Buffer B	800	997.4	206.4	20.7	124.7
Buffer B	4000	4535.3	727.1	16	113.4
Buffer B	20000	22425.3	2580.1	11.5	112.1
Buffer B	100000	99735.8	818.1	0.8	99.7

To evaluate the impact of matrix components on assay performance, we compared two sample diluents: MACSPlex Buffer (standard MPx Buffer) and Buffer B (a commercial culture medium). Overall accuracy (%) and precision (%CV) were assessed across the full range of analyte concentrations. Values outside the acceptance criteria are marked in orange (Figure 5). The matrix comparison defined matrix-specific reportable ranges: 32-100,000 pg/mL for MPx Buffer and 800-100,000 pg/mL for Buffer B. These results demonstrate that the assay remains accurate and precise across a broader range in MPx Buffer, while supporting a narrower but still valid quantification range in Buffer B (Figure 5).

5 Automated MPx Cytokine Analysis Tool comparison: Express Mode and MACSPlex InspectoR Tool

Figure 6



Engineered Chimeric Antigen Receptor (CAR) T cells were co-cultured overnight with Jeko-1 tumor cells, either expressing or lacking the respective antigen, on three independent days. After 16 hours, cell culture supernatants were collected, centrifuged to remove particulates, and diluted samples were analyzed using the MPx IFN-γ Assay. IFN-γ concentrations were determined using two automated analysis tools: Express Mode and the new web-based MACSPlex InspectoR tool. The calculated concentration values (pg/mL) from Express Mode were plotted against those from MACSPlex InspectoR Tool (Figure 6). Linear correlation was observed across concentrations (R^2 = 0.99), indicating strong agreement between the two analysis methods.

Conclusion

This study demonstrates robust analytical performance of IFN-γ quantification using a MACSPlex cytokine bead assay, including accuracy, precision, linearity, and dilutional integrity within the validated reportable range of 32-100,000 pg/mL in MPx Buffer, and demonstrated acceptable analytical performance between 800 and 100,000 pg/mL in Buffer B. When used within these matrix-specific ranges, the assay reliably quantified IFN-γ across multiple sample types. Both automated MPx Cytokine analysis tools produced closely matched concentration values. These results support the use of this assay for reliable quantification of T cell functional activity in both exploratory and standardized immunomonitoring applications.

References

1. ICH M10, Bioanalytical method validation and study sample analysis – Guidance for Industry, 2022

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