Miltenyi Biotec

MACSPlex Cytokine 12 Kit
human

For up to 100 tests

Order no. 130-099-169

Miltenyi Biotec

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2. Description

This product is for research use only.

Components	2 mL MACSPlex Cytokine 12 Capture Beads, human
	2 vials of MACSPlex Cytokine 12 Standard, human, lyophilized each containing a mixture of recombinant 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
	2 mL MACSPlex Cytokine 12 Detection Reagent, human
	2×100 mL MACSPlex Buffer
	0.35 mL MACSPlex Setup Beads
	1 MACSPlex Filter Plate
	2 adhesive foils
Size	up to 100 tests
Product format	MACSPlex Cytokine 12 Capture Beads, MACSPlex Cytokine 12 Detection Reagent, and MACSPlex Setup Beads are supplied in buffer containing stabilizer and 0.05% sodium azide. MACSPlex Cytokine 12 Standard contains stabilizer. MACSPlex Buffer contains stabilizer and 0.09% sodium azide.
Storage	Store MACSPlex Cytokine 12 Capture Beads, MACSPlex Cytokine 12 Detection Reagent, and MACSPlex Setup Beads protected from light at +2 to +8 °C. Do not freeze.

140-003-911.05

Store vials of MACSPlex Cytokine 12 Standard lyophilized at –20 °C.
Store MACSPlex Buffer, MACSPlex Filter Plate, and adhesive foils at room temperature.
The expiration dates are indicated on the labels.

2.1 Principle of MACSPlex Assays

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.

MACSPlex Capture Beads within a kit contain a cocktail of various fluorescently labeled bead populations, each coated with a specific antibody reacting with one of the respective analytes within the sample.

Samples containing unknown levels of analytes are incubated with the antibody-coated MACSPlex Capture Beads, and analytes bind to the specific antibody. A detection reagent, composed of a cocktail of APC-conjugated antibodies specific for the analytes, is added. Consequently, sandwich complexes are formed between the MACSPlex Capture Bead, the analyte and the detection reagent. These complexes can be analyzed based on the fluorescence characteristics of both the MACSPlex Capture Bead and the detection reagent. Standards of known quantities of given analytes are provided with the kit and are used for the quantification of the analytes within the unknown samples.

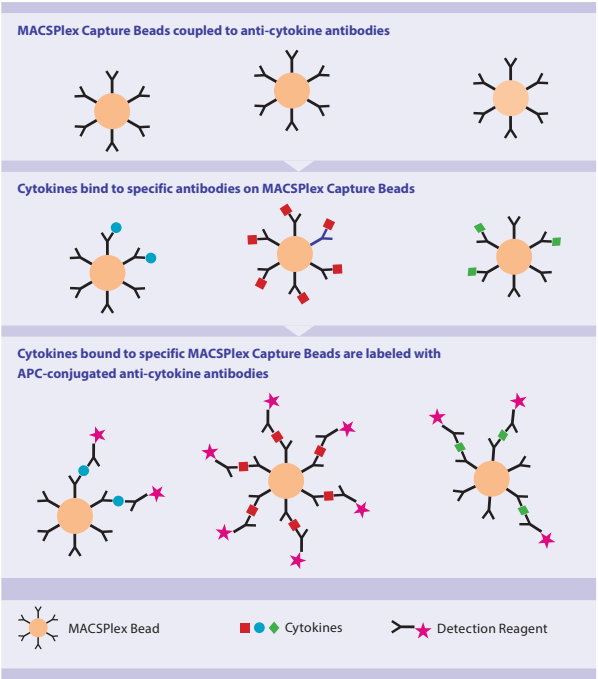


Figure 2.1: Principle of MACSPlex Assays.

2.2 Principle of MACSPlex Cytokine 12 Kit, human

The MACSPlex Cytokine 12 Capture Beads, human, consist of 12 bead populations that have been coated with capture antibodies specific for the following cytokines: 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. The 12 bead populations can be distinguished by different fluorescence intensities detected in the FITC (B1) and PE (B2) channel of the MACSQuant® Analyzer and MACSQuant Analyzer 10.

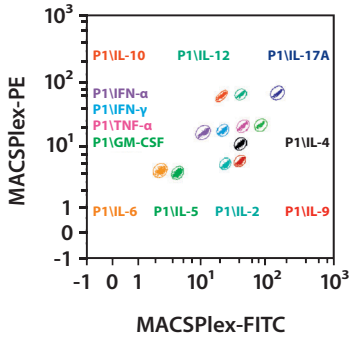


Figure 2.2: Detection of MACSPlex Cytokine 12 Capture Bead populations in a MACSPlex-FITC (B1) versus MACSPlex-PE (B2) dot plot.

MACSPlex Cytokine 12 Capture Beads are added to both, the unknown samples and to serial dilutions of the MACSPlex Cytokine 12 Standard.

During a 2-hour incubation period, the cytokines are captured by the MACSPlex Capture Beads. Subsequently, the MACSPlex Cytokine 12 Detection Reagent containing a mixture of 12 APC-conjugated anti-cytokine antibodies, is added in order to form sandwich complexes during a 1-hour incubation period. Standard curves for each of the 12 cytokines are generated. The median of the APC fluorescence of each capture bead population gives the concentration of each cytokine in the unknown samples.

2.3 Applications

The MACSPlex Cytokine 12 Kit, human has been developed for the simultaneous flow cytometric detection of the soluble human cytokines 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 in a single sample. The kit has been optimized for use with serum, plasma, and cell culture supernatants.

2.4 Reagent and instrument requirements

- Polypropylene or polystyrene reagent tubes for serial dilutions of the MACSPlex Cytokine 12 Standard as well as for preparation, dilution, and storage of unknown samples.
- Vacuum manifold or centrifuge with adapters to accommodate microtiter plates.
- Orbital shaker for microtiter plates or tubes (frequency 450–1400 rpm).

- MACSQuant Analyzer, MACSQuant Analyzer 10 (# 130-096-343), or other flow cytometers equipped with blue (488 nm) and red (635 nm) lasers able to discriminate FITC, PE, and APC fluorescence.
  ▲ **Note:** The MACSQuant VYB cannot be used.
- MACS® Chill 96 Rack (# 130-094-459), when using the MACSQuant Analyzer or MACSQuant Analyzer 10.
- MACSQuant Calibration Beads (# 130-093-607), when using the MACSQuant Analyzer or MACSQuant Analyzer 10.
- (Optional) Cell culture medium, e.g., TexMACS™ Medium (# 130-097-196) or RPMI 1640 supplemented with human AB serum.
- Disposable pipette tips or pipettes.

3. Protocols for assay performance

▲ Kit components should not be substituted or mixed with those from other kits or lots.

▲ Use of disposable pipette tips and/or pipettes is recommended to avoid any potential contamination or cross-contamination of reagents or samples, which might invalidate test results.

▲ Avoid air bubbles.

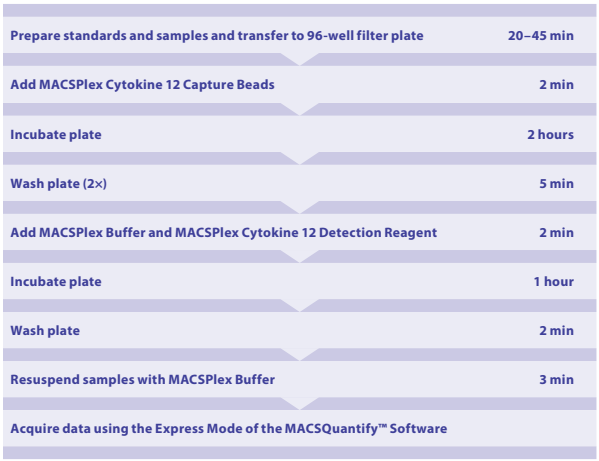


Figure 3.1: Experimental overview for the assay using a MACSPlex Filter Plate.

3.1 Preparation of the MACSPlex Cytokine 12 Standard

▲ Reconstitute and dilute MACSPlex Cytokine 12 Standard with MACSPlex Buffer, or use the same media as is used for the dilution of the unknown sample.

▲ Only use freshly prepared MACSPlex Cytokine 12 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 seven samples: six samples ranging from 3.2 to 10,000 pg/mL of the MACSPlex Cytokine 12 Standard, and one blank control. These samples will be measured as duplicates (refer to section 3.3).

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. Mix gently. This is the stock solution (1:5^0; 10,000 pg/mL).
3. Label five reagent tubes and arrange them in the following order: 1:5 (1:5^1; 2,000 pg/mL), 1:25 (1:5^2; 400 pg/mL), 1:125 (1:5^3; 80 pg/mL), 1:625 (1:5^4; 16 pg/mL), and 1:3125 (1:5^5; 3.2 pg/mL)
4. Pipette 200 µL of MACSPlex Buffer or media 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 1:3125. Mix each dilution before performing the next transfer.

6. Keep 200 µL MACSPlex Buffer or media as blank control (0 pg/mL).

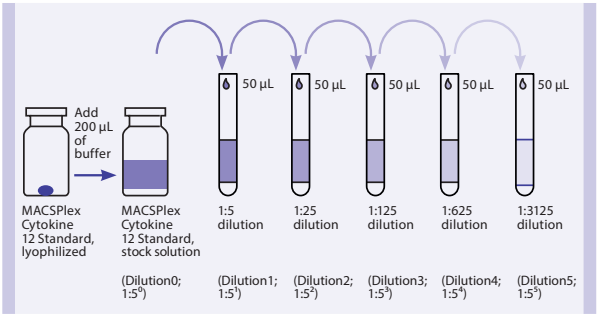


Figure 3.2: Serial dilution of the MACSPlex Cytokine 12 Standard.

3.2 Sample preparation

▲ Handle all blood components and biological material as potentially hazardous.

▲ If unknown samples are expected or known to contain levels >2000 pg/mL of a certain cytokine, it is recommended to dilute the samples to make sure the fluorescence values are within the dynamic range of the standard curve.

▲ Use polypropylene or polystyrene reagent tubes. Do not use glass vials for sample preparation, dilution, or storage.

Preparation of serum samples

1. Allow the blood to clot for at least 30 minutes.
2. Centrifuge at 10,000×g for 10 minutes at 4 °C.
3. Transfer serum into a new tube and dilute at least 1:8 with MACSPlex Buffer, i.e., add 25 µL of the undiluted sample to 175 µL of MACSPlex Buffer.
4. Proceed to section 3.3.

Preparation of plasma samples

▲ Use freshly drawn blood samples supplemented with EDTA as anticoagulant. Do not store blood samples longer than 30 minutes before performing the assay.

1. Centrifuge peripheral blood at 10,000×g for 10 minutes at 4 °C.
2. Transfer plasma into a new tube and dilute at least 1:8 with MACSPlex Buffer, i.e., add 25 µL of the undiluted sample to 175 µL of MACSPlex Buffer.
3. Proceed to section 3.3.

Preparation of cell culture supernatant samples

1. Centrifuge cell culture supernatant at 10,000×g for 10 minutes at 4 °C.
2. Transfer the supernatant into a new tube.
3. (Optional) Dilute with cell culture medium or MACSPlex Buffer.
4. Proceed to section 3.3.

Frozen samples of serum, plasma, or cell culture supernatant

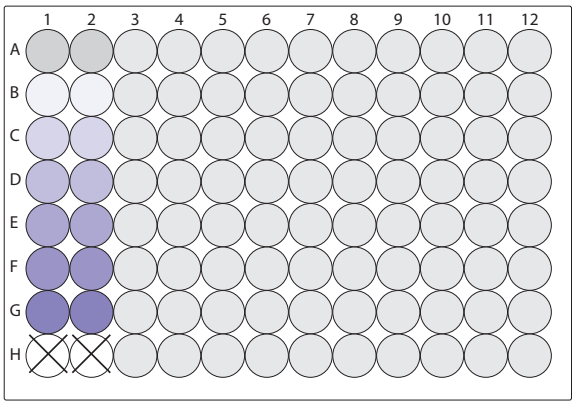
1. Thaw the samples completely and mix well by vortexing.
2. Centrifuge at 10,000×g for 10 minutes at 4 °C to remove particulates.
3. Transfer serum or plasma into a new tube and dilute at least 1:8 with MACSPlex Buffer, i.e., add 25 µL of the undiluted sample to 175 µL of MACSPlex Buffer. Cell culture supernatants can be diluted optionally with cell culture medium or MACSPlex Buffer.
4. Proceed to section 3.3.

3.3 MACSPlex Cytokine 12 Assay

▲ 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.

3.3.1 Protocol for the assay using the MACSPlex Filter 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	

Figure 3.3: Setup of the assay using a 96-well plate.

Design your 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). Start with the unknown sample in the next column of the plate. For details, see figure 3.3.

▲ Place the MACSPlex Filter Plate on a non-absorbent surface during loading steps and incubation, i.e. remove any tissues from the surface, to prevent the wells from running dry. Ensure that residual drops under the plate are completely removed to prevent liquid transfer, by placing the plate briefly on a tissue.

▲ Cover unused wells of the filter plate for later use with the adhesive foil provided with the kit.

▲ Washing steps are described for the use of a vaccum manifold. Alternatively, a centrifuge with an adapter for microtiter plates can be used: Put the MACSPlex Filter Plate on top of a conventional 96-flat-bottom microtiter plate without lid and place both into the adapter. Centrifuge at 300×g for 3 minutes at room temperature.

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 protect 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 protect from light on an orbital shaker (450 rpm).
13. Repeat washing 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.

3.3.2 Protocol for the assay using 1.5 mL reagent tubes

▲ Use polypropylene or polystyrene reagent tubes. Do not use glass vials.

▲ Standards should be run as duplicates. The order starts from the blank control (0 pg/mL) moving to the highest concentration (stock solution 10,000 pg/mL).

1. Label reagent tubes for the blank control, each dilution and the stock solution of the MACSPlex Cytokine 12 Standard, and unknown samples.
2. Pipette 50 µL of MACSPlex Buffer or media as blank control, 50 µL of each dilution and the stock solution of the MACSPlex Cytokine 12 Standard into the corresponding reagent tubes. Pipette 50 µL of each unknown sample into the corresponding reagent tube.
3. Resuspend MACSPlex Cytokine 12 Capture Beads by mixing for at least 30 seconds and transfer 20 µL of MACSPlex Cytokine 12 Capture Beads to each tube.
4. Incubate for 2 hours protect from light on an orbital shaker (1400 rpm).
5. Add 0.5 mL of MACSPlex Buffer to each tube.
6. Centrifuge at 3000×g for 5 minutes.
7. Carefully aspirate off the supernatant, leave 20 µL in the tube.
8. Resuspend the MACSPlex Capture Bead pellet in each tube by adding 0.5 mL of MACSPlex Buffer and pipetting up and down.

9. Repeat steps 6 and 7.
10. Resuspend the MACSPlex Capture Bead pellet in each tube with MACSPlex Buffer to a total volume of 80 µL by pipetting up and down, e.g., add 60 µL of MACSPlex Buffer to the remaining 20 µL of supernatant (see step 7).
11. Add 20 µL of MACSPlex Cytokine 12 Detection Reagent to each tube.
12. Incubate for 1 hour protect from light on an orbital shaker (1400 rpm).
13. Add 0.5 mL of MACSPlex Buffer to each tube.
14. Centrifuge at 3000×g for 5 minutes.
15. Carefully aspirate off the supernatant, leave 20 µL in the tube.
16. Resuspend the pellet in each tube by adding 0.5 mL of MACSPlex Buffer and pipetting up and down.
17. Repeat steps 14 and 15.
18. Resuspend the pellet in each tube with 200 µL of MACSPlex Buffer.
19. For sample acquisition with MACSQuant Instruments using the Express Mode, transfer samples to a 96-well microtiter plate. Place the microtiter plate onto a Chill 96 Rack.

▲ **Note:** Acquire cytokine standards first, beginning with the standard samples of the first dilution series in order from the blank control to the highest concentration. Then process the standard samples of the second dilution series in the same order (see also figure 3.3). Afterwards acquire the unknown samples.

▲ **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.

4. Flow cytometer set up

The kit includes MACSPlex Setup Beads for flow cytometer set up. MACSPlex Setup Beads are not required when using the MACSQuant Analyzer or MACSQuant Analyzer 10 but for all other instruments.

The kits is not suitable for use with the MACSQuant VYB.

4.1 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 successful 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.

4.2 Setup of other flow cytometers

The analysis of MACSPlex Cytokine 12 Kit requires flow cytometers with a blue (e.g. 488 nm) and a red (e.g. 635 nm) lasers, which are capable of detecting FITC, PE, and APC. For setting up these cytometers, MACSPlex Setup Beads are 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's webpage at www.miltenyibiotec.com/130-099-169.

5. 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 under the Library tab. 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 your Express Mode package available on your MACSQuant Instrument please select Help > Info within the MACSQuantify™ Software (refer to figure 5.1). 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.

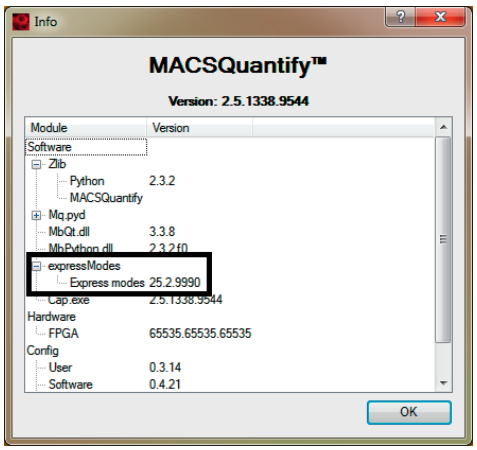


Figure 5.1: Identification of version number of Express Mode package.

Overview

Relevant parameter for Express Mode experiments	
Maximum number of standard positions (including blank) within the serial standard dilution	7
Dilution factor of serial standard dilution	5

6. Performance

6.1 Theoretical limit of detection

The assay sensitivity, or theoretical limit of detection, was determined as the concentration corresponding to a median fluorescence intensity (MFI), which is two standard deviations above the mean of MFIs of 23 replicates of negative controls (0 pg/mL). Standard curves were calculated from duplicate standards with a five parameter logistic curve fit.

Cytokine	Limit of detection [pg/mL]
GM-CSF	0.20
IFN-α	10.4
IFN-γ	6.26
IL-2	1.52
IL-4	34.2
IL-5	0.27
IL-6	0.077
IL-9	32.7
IL-10	2.76
IL-12	3.44
IL-17A	0.7
TNF-α	2.17

6.2 Intra-assay precision

To confirm the reproducibility of the MACSPlex Cytokine 12 Assay within one assay four replicates of three different concentrations of each cytokine were tested. The assay was carried out including two standard curves. The table below shows the mean, the standard deviation, and the coefficient of variation for each sample.

Cytokine	Sample	Mean (pg/mL)	Standard Deviation	%CV
GM-CSF	Sample 1	74.6	1.8	2.4
	Sample 2	369.8	20.7	5.6
	Sample 3	1841.7	42.1	2.3
IFN-α	Sample 1	76.4	6.4	8.3
	Sample 2	373.3	18.2	4.9
	Sample 3	1742.3	80.8	4.6
IFN-γ	Sample 1	67.3	3.6	5.4
	Sample 2	351	41.5	11.8
	Sample 3	1805.2	51.8	2.9
IL-2	Sample 1	75.1	3.8	5.1
	Sample 2	379.5	19.8	5.2
	Sample 3	1783.3	54.7	3.1
IL-4	Sample 1	368.8	44.9	12.2
	Sample 2	1867.7	96.3	5.2
	Sample 3	9343.4	410.3	4.4
IL-5	Sample 1	60	5.2	8.6
	Sample 2	358	14.1	3.9
	Sample 3	1806.6	93.6	5.2
IL-6	Sample 1	74.1	0.9	1.2
	Sample 2	372.3	11.6	3.1
	Sample 3	1829.5	43.7	2.4
IL-9	Sample 1	84.9	4.5	5.3
	Sample 2	365.4	21.5	5.9
	Sample 3	1837.1	84.2	4.6
IL-10	Sample 1	66.4	3.9	5.9
	Sample 2	345.6	23.4	6.8
	Sample 3	1802	38.5	2.1
IL-12	Sample 1	70.7	5.2	7.3
	Sample 2	380.1	32.7	8.6
	Sample 3	1909.2	42.5	2.2

Cytokine	Sample	Mean (pg/mL)	Standard Deviation	%CV
IL-17A	Sample 1	72.3	1.9	2.7
	Sample 2	389.8	17.2	4.4
	Sample 3	1756.4	161.4	9.2
TNF-α	Sample 1	72.6	2.3	3.2
	Sample 2	353.2	35.8	10.1
	Sample 3	1801.5	67.9	3.8

6.3 Inter-assay precision

To assess the assay-to-assay reproducibility two different concentrations of each cytokine were tested in five independent experiments. Each assay was carried out including two standard curves on each plate and two replicates of samples. The table below shows the mean, the standard deviation, and the coefficient of variation for each sample.

Cytokine	Sample	Mean (pg/mL)	Standard Deviation	%CV
GM-CSF	Sample 1	413.5	14.8	3.6
	Sample 2	2080.9	122.4	5.9
IFN-α	Sample 1	206.4	20.1	9.7
	Sample 2	1324.1	123.4	9.3
IFN-γ	Sample 1	110.2	10.6	9.6
	Sample 2	591.4	79	13.4
IL-2	Sample 1	362.4	10.8	3
	Sample 2	1835.3	43	2.3
IL-4	Sample 1	433.3	71.3	16.5
	Sample 2	2259.6	239.7	10.6
IL-5	Sample 1	104.3	17.1	16.4
	Sample 2	590.6	38	6.4
IL-6	Sample 1	373.2	16.3	4.4
	Sample 2	1817.1	37.9	2.1

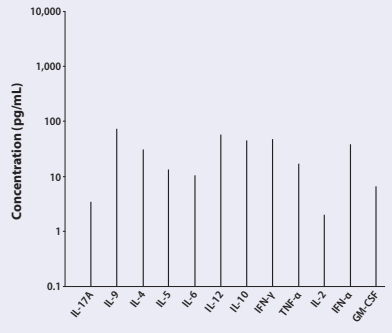
Cytokine	Sample	Mean (pg/mL)	Standard Deviation	%CV
IL-9	Sample 1	503.2	85.1	16.9
	Sample 2	3040.4	373.6	12.3
IL-10	Sample 1	130.7	6.5	5
	Sample 2	703.1	22.8	3.2
IL-12	Sample 1	261.9	13.9	5.3
	Sample 2	1271.1	40.9	3.2
IL-17A	Sample 1	219.8	11.8	5.4
	Sample 2	1414.3	125.2	8.9
TNF-α	Sample 1	268.5	11.8	4.4
	Sample 2	1362.9	45	3.3

Specificity

Cross-reactivity of the antibodies against the analytes was tested. No cross-reactivity was observed.

6.4 Exemplary results of the analysis using the Express Mode

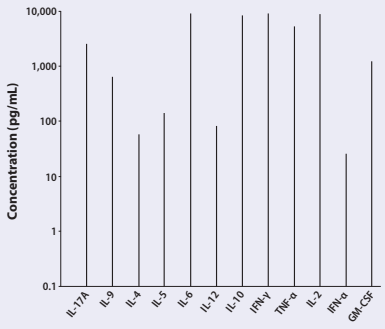
A: Unstimulated sample



MACSPlex Cytokine 12 Kit, human
Rack position A6

GM-CSF	6.6 pg/mL
IFN-α	38.4 pg/mL
IFN-γ	47.3 pg/mL
IL-10	44.7 pg/mL
IL-12	57.8 pg/mL
IL-17A	3.3 pg/mL
IL-2	2.0 pg/mL
IL-4	31.5 pg/mL
IL-5	13.0 pg/mL
IL-6	10.3 pg/mL
IL-9	70.4 pg/mL
TNF-α	16.9 pg/mL

B: Stimulated sample



MACSPlex Cytokine 12 Kit, human
Rack position A7

GM-CSF	1365.6 pg/mL
IFN-α	27.9 pg/mL
IFN-γ	> 10000.0 pg/mL
IL-10	9265.4 pg/mL
IL-12	86.4 pg/mL
IL-17A	2863.4 pg/mL
IL-2	9748.8 pg/mL
IL-4	61.1 pg/mL
IL-5	146.0 pg/mL
IL-6	> 10000.0 pg/mL
IL-9	711.3 pg/mL
TNF-α	5783.7 pg/mL

Figure 6.1: Peripheral blood mononuclear cells (PBMCs) were cultivated over night in RPMI with 5% AB serum without (A) or with (B) stimulation with CytoStim (# 130-092-172). 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 all 12 analytes were determined using MACSQuant Express Mode.

7. Troubleshooting

The following section offers solutions for problems that might be encountered when using MACSPlex Assays.

- **Variation between replicate samples:**
  MACSPlex Cytokine 12 Capture Beads can aggregate. Mix MACSPlex Cytokine 12 Capture Beads at least 30 seconds before pipetting.
- **Low bead number in samples:**
  Mix MACSPlex Cytokine 12 Capture Beads sufficiently before pipetting.
  Ensure that the instrument is calibrated for the relevant 96-well plate to avoid aspiration of air.
  Avoid aspiration of beads during washing steps. Do not wash or resuspend beads in volumes higher than recommended.
- **High background:**
  Avoid cross-well contamination. Ensure pipetting with multichannel pipettes and avoid touching reagent in the plate.
  The sample may be too concentrated. Test various sample dilutions.
  The background may be due to non-specific binding. Increase number of washes to remove excess of MACSPlex Cytokine 12 Detection Reagent.
- **Little or no detection of cytokine in sample:**
  Sample may be too dilute. Test various sample dilutions (refer to figure 3.2). Use positive and negative control samples.

- **MACSPlex Cytokine 12 Standard Samples show low fluorescence or result in poor standard curves:**
  Check that all kit components are prepared and stored properly.
  Use a new vial of MACSPlex Cytokine 12 Standard for each experiment. Adhere to incubation times as indicated in the protocol.
  Assay was exposed to light. Keep the plate and beads covered with aluminum foil or a dark lid during all incubation steps.
  Use the protection lid during the sample acquisition with the MACSQuant Instrument.
- **All samples are positive or above the high standard mean fluorescence value:**
  The samples may be too concentrated. Dilute the samples further.
- **Beads not in region or gate:**
  Ensure proper calibration of the MACSQuant Instrument.
  Samples containing organic solvents or samples of high viscosity should be diluted or dialyzed, respectively
- **Signal for whole plate is same as background:**
  Incorrect amount or no MACSPlex Cytokine 12 Detection Reagent was added.
- **Low signal for standard curve:**
  Incubation steps were done at inappropriate temperatures, timings, or agitation.

- **Signals too high, standard curves are saturated:**
  Incubation time was too long. Adhere to incubation times as indicated in the protocol.
- **Sample readings are out of range –**
  if below detection limit:
  Samples may be too diluted. Adjust dilutions accordingly.
  Samples may contain no analyte. Analyte concentration may be below the detection limit. It may be possible to use higher sample volumes. Please contact Milteny Biotec's Technical Support for appropriate protocol modifications.

  If above detection limit:
  Samples contain analyte concentrations above 10 ng/mL. Dilute the samples further.
- **High variation in samples and/or standards:**
  Multichannel pipette may not be calibrated.
  Plate washing was not uniform.
  Samples may have contained high particulate matter or other interfering substances.
  Plate agitation was insufficient.
  Cross-well contamination. Ensure pipetting with multichannel pipettes and avoid touching reagent in the plate. Change pipette tips for each well when touching the reagent.

- **Filter plate will not vacuum:**
  Vacuum pressure is insufficient. Increase vacuum pressure.
  Samples contain insoluble particles. Centrifuge samples just prior to performing the assay and use supernatant.
  High lipid concentration. Centrifuge samples, remove lipid layer, and use supernatant to perform the assay.
- **Plate leakage:**
  Vacuum pressure is too high. Adjust vacuum pressure to maximal –300 mbar.
  Place the MACSPlex Filter Plate on a non-absorbent surface during filling steps and incubation, i.e., remove any tissues from the surface, to prevent the wells from running dry.
  Ensure that residual drops under the plate are completely removed to prevent leakiness of the wells, by placing the plate briefly on a tissue, after each washing step.
  Avoid touching the plate filter with the tip of the pipette when adding reagents to the wells.

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