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MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A - Immunology Multiplex Assay

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We use this protocol and it's working

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Abstract

This protocol describes lab processing as well as data processing for the MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A - Immunology Multiplex Assay

Troubleshooting

Sample preparation

25m

1 Thaw plasma samples completely on ice. 00:15:00 — 15m

2 Mix well by vortexing.

3 Centrifuge thawed samples at 5000 x g @ 4°C for 10 minutes to remove particulates. 10m
5000 x g, 4°C, 00:10:00

Reagent preparation

4 Allow all reagents to warm to room temperature (20-25 °C) before use in the assay.
Room temperature

5 **Preparation of Antibody-Immobilized Beads**

5.1 For individual vials of beads, sonicate each antibody-bead vial for 30 seconds in an ultrasonic waterbath; vortex for 1 minute. Add 60 µL from each antibody-bead vial to the Mixing Bottle and bring final volume to 3.0 mL with Bead Diluent. Vortex the mixed beads well. Unused portion may be stored at 2-8 °C for up to one month.

5.2 Example 1: When using 24 antibody-immobilized beads, add 60 µL from each of the 24 bead vials to the Mixing Bottle. Then add 1.56 mL Bead Diluent.

6 **Preparation of Quality Controls**

6.1 Before use, reconstitute Quality Control 1 and Quality Control 2 with 250 µL deionized water. Invert the vial several times to mix and vortex. Allow the vial to sit for 5-10 minutes. Transfer the reconstituted Quality Control 1 and Quality Control 2 into two polypropylene microfuge tubes. Unused portion may be stored at -20 °C for up to one month. 00:10:00 — 10m

7 **Preparation of Wash Buffer**

7.1 Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution. Dilute 60 mL of 10X Wash Buffer with 540 mL deionized water. Store the unused portion at 2-8 °C for up to one month.

8 Preparation of Serum Matrix

8.1 Add 1.0 mL deionized water to the bottle containing lyophilized Serum Matrix. Mix well. Allow at least 10 minutes for complete reconstitution. Leftover reconstituted Serum Matrix should be stored at -20 °C for up to one month. 00:10:00 10m

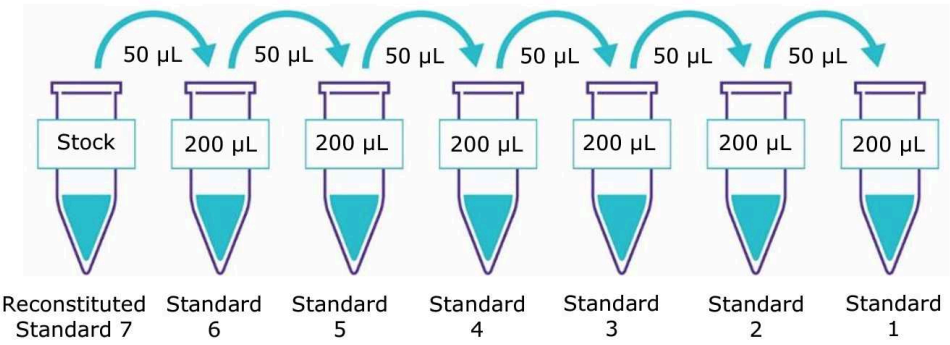
9 Preparation of Human Cytokine/Chemokine/Growth Factor Panel A Standard

9.1 Prior to use, reconstitute the Human Cytokine/Chemokine/Growth Factor Panel A Standard with 250 µL deionized water. Refer to table below for analyte concentrations. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes. Transfer the reconstituted Positive Control into a polypropylene microfuge tube. This will be used as Standard 7; the unused portion may be stored at -20 °C for up to one month. 00:10:00 10m

9.2 Preparation of Working Standards
Label 6 polypropylene microfuge tubes Standard 1 through Standard 6. Add 200 µL of Assay Buffer to each of the 6 tubes. Prepare serial dilutions by adding 50 µL of the reconstituted standard to the Standard 6 tube, mix well and transfer 50 µL of Standard 6 to the Standard 5 tube, mix well and transfer 50 µL of Standard 5 to the Standard 4 tube, mix well and transfer 50 µL of Standard 4 to the Standard 3 tube, mix well and transfer 50 µL of Standard 3 to the Standard 2 tube, mix well and transfer 50 µL of Standard 2 to the Standard 1 tube and mix well. The 0 pg/mL standard (Background) will be Assay Buffer.

Standard No.	Add Deionized Water (µL)	Add Standard (volume)
Standard 7	250	0

Standard No.	Add Assay Buffer (µL)	Add Standard (volume)
Standard 6	200	50 µL of Standard 7
Standard 5	200	50 µL of Standard 6
Standard 4	200	50 µL of Standard 5
Standard 3	200	50 µL of Standard 4
Standard 2	200	50 µL of Standard 3
Standard 1	200	50 µL of Standard 2



Standard	sCD40L, IL-22, PDGF-AA (pg/mL)	EGF, Eotaxin IL-12 (p70), IL-15, MCP-1, MIP-1α (pg/mL)	FGF-2 (pg/mL)	FLT-3L (pg/mL)
Standard 1	13	3	26	0.96
Standard 2	64	16	128	4.8
Standard 3	320	80	640	24
Standard 4	1,600	400	3,200	120
Standard 5	8,000	2,000	16,000	600
Standard 6	40,000	10,000	80,000	3,000
Standard 7	200,000	50,000	400,000	15,000

Standard	Fractalkine, IL-17F (pg/mL)	G-CSF, IL-1α (pg/mL)	GM-CSF, IL-10, IP-10, VEGF-A (pg/mL)	GROα, IFNγ, IL-3, IL-17A, RANTES, TGFα (pg/mL)
Standard 1	32	4.8	2.6	1.3
Standard 2	160	24	12.8	6.4
Standard 3	800	120	64	32
Standard 4	4,000	600	320	160
Standard 5	20,000	3,000	1,600	800
Standard 6	100,000	15,000	8,000	4,000
Standard 7	500,000	75,000	40,000	20,000

Standard	IFNα2, MCP-3 (pg/mL)	IL-1β, IL-1RA, TNFβ (pg/mL)	IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-18, MDC (pg/mL)	IL-12 (p40), IL-13, MIG, TNFα (pg/mL)
Standard 1	8	1.6	0.64	6.4
Standard 2	40	8	3.2	32
Standard 3	200	40	16	160
Standard 4	1,000	200	80	800
Standard 5	5,000	1,000	400	4,000
Standard 6	25,000	5,000	2,000	20,000
Standard 7	125,000	25,000	10,000	100,000

Standard	IL-17E/IL-25, M-CSF (pg/mL)	IL-27 (pg/mL)	MIP-1β (pg/mL)	PDGF-AB/BB (pg/mL)
Standard 1	40	16	0.38	9.6
Standard 2	200	80	1.9	48
Standard 3	1,000	400	9.6	240
Standard 4	5,000	2,000	48	1,200
Standard 5	25,000	10,000	240	6,000
Standard 6	125,000	50,000	1,200	30,000
Standard 7	625,000	250,000	6,000	150,000

Immunoassay Procedure

20h

10 Diagram the placement of Standards 0 (Background), Standard 1 through 7, Controls 1 and 2, and Samples on Well Map Worksheet in a vertical configuration. (Note: Most instruments will only read the 96-well plate vertically by default.) It is recommended to run the assay in duplicate.

11 Add 200 µL of Wash Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25 °C). 400 rpm, 24°C, 00:10:00 10m

12 Decant Wash Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.

13

14 Add 25 µL of Assay Buffer to the sample wells.

15 Add 25 µL of appropriate matrix solution to the background, standards, and control wells. When assaying serum or plasma, use the Serum Matrix provided in the kit.

16 Add 25 µL of Sample (neat) into the appropriate wells.

17 Vortex Mixing Bottle and add 25 µL of the Mixed or Premixed Beads to each well using a multichannel pipet.

18 Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker 400 rpm, 4°C overnight (18 hours) at 2-8 °C. Alternatively, incubate for 2 hours at room temperature (20-25 °C). Overnight 18h

19 Gently remove well contents and wash plate 3 times following instructions below: 2m 30s

Magnetic plate washer (Cat. No. 40-094, 40-095, 40-096 and 40-097)
Rest plate on magnet for 60 seconds to allow complete settling of magnetic beads.
00:01:00
Remove well contents by aspiration. Wash plate with 200 µL of Wash Buffer by adding Wash Buffer, shaking for 30 seconds 00:00:30 , letting beads settle for 60 seconds 00:01:00 and removing well contents as previously described after each wash.
Please note that after the final aspiration, there will be approximately 25 µL of residual wash buffer in each well. This is expected when using the BioTek® plate washer and this volume does not need to be aspirated
from the plate.

20 Add 25 µL of Detection Antibodies into each well using a multichannel pipet.

Note

Allow the Detection Antibodies to warm to room temperature prior to addition.
Room temperature

21 Seal, cover with foil and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25 °C). 400 rpm, 24°C, 01:00:00 1h

Note

DO NOT ASPIRATE AFTER INCUBATION.

22 Add 25 µL Streptavidin-Phycoerythrin to each well containing the 25 µL of Detection Antibodies.

23 Seal, cover with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25 °C). 400 rpm, 24°C, 00:30:00 30m

24 Gently remove well contents and wash plate 3 times following instructions below: 7m 30s

Magnetic plate washer (Cat. No. 40-094, 40-095, 40-096 and 40-097)
Rest plate on magnet for 60 seconds to allow complete settling of magnetic beads.
00:01:00
Remove well contents by aspiration. Wash plate with 200 µL of Wash Buffer by adding Wash Buffer, shaking for 30 seconds 00:00:30 , letting beads settle for 60 seconds 00:01:00 and removing well contents as previously described after each wash.
Please note that after the final aspiration, there will be approximately 25 µL of residual wash buffer in each well. This is expected when using the BioTek® plate washer and this volume does not need to be aspirated
from the plate.

25 Add 150 µL of Wash Buffer to all wells. Resuspend the beads on a plate shaker for 5 minutes. 400 rpm, 24°C, 00:05:00 5m

26 Run plate on Luminex® 200™ instrument with xPONENT® software.

27 Save and analyze the Median Fluorescent Intensity (MFI) data using a 4-parameter logistic curve-fitting method for calculating analyte concentrations in samples (Belysa 1.2.1 Software - Millipore).

Expected result

The Millipore kit includes two Quality Controls (QC1 - lower concentration & QC2 - higher concentration) for each analyte. It is essential that, for every assay, these quality controls align with the specified range set by Millipore for each analyte. Failure to meet the specified ranges for any analyte during a run will result in the exclusion of that analyte from the report. The QC ranges can be found at the top of the raw data report for each analyte.

Data Transfer

28 **Raw data**

Analyte concentrations from each well are measured twice (since the assay is ran in duplicate). The raw data Excell file has the following tabs.

- **Summaries tab:** This tab contains the mean analyte concentrations from the two measurements.
- **Analyte-Specific Tabs:** Each of these tabs is dedicated to a single analyte and displays the individual concentration results for the two measurements from each well.

29 **Processed Data**

The raw data is first processed by core G, followed by an additional processing round by the data science core.

29.1 **Core G Processing**

The analyte concentration summaries from the "Summaries" tab of the raw data are imputed with

- "ND": If the analyte concentration falls below the limit of detection (LOD).
- "%MFIe": The analytes CV% of the MFI is above 30%.
- "QCe": The quality controls (QC1 and QC2) fall outside of the range specified by Millipore for the specific analyte.

Then, the summaries with the imputed values are split into the Sample Data tab, QC Data Runs tab, and the Internal Cntrl data tab. Two additional tabs are included to explain the imputed values (Data key & explanation) and the minimum detectable concentrations for each metabolite (Imputed ND values).

29.2 **Data Science Core Processing**

Analyte concentrations for each sample are taken from the Sample data tab of the core G processed data and matched with the HML_ID for the participant. Then the following transformations were made on the data.

- Analytes labeled "ND" were replaced with 1/2 of the LOD. Millipore provided the LOD for each analyte.
- Analytes labeled "%MFIe" were replaced with the batch average concentration for that analyte.
- Analytes that were imputed with "QCe" are left missing (NA).

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