HIGH SENSITIVITY HUMAN CYTOKINE PREMIXED LINCO*plex* KIT
96 Well Plate Assay (Cat. #HSCYTO-60SPMX13)

contact our technical service department Toll Free U.S. at 866-441-8400 or 636-441-8400 or email us at techserve_dd@millipore.com.

HIGH SENSITIVITY HUMAN CYTOKINE PREMIXED LINCO*plex* KIT

I. INTENDED USE

This high sensitivity multiplex assay kit manufactured by Millipore is intended for the simultaneous quantitative determination of the following human cytokines: GMCSF, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, and TNFα. This kit may be used for the analysis of all of the above cytokines in tissue or cell lysate, culture supernatant, neat serum or plasma, or other body fluid samples.

This kit is for research purposes only.

II. REAGENTS SUPPLIED

A. Premixed Antibody-Immobilized Beads:

The following beads are included in the premixed bead bottle. No dilution is necessary.

13 Plex Premix Beads
#01-Human IL-1β
#03-Human IL-2
#09-Human IL-4
#10-Human IL-5
#12-Human IL-6
#13-Human IL-7
#20-Human IL-8
#23-Human IL-10
#25-Human IL-12p70
#26-Human IL-13
#35-Human IFNγ
#39-Human GM-CSF
#40-Human TNFα

Quantity: 3.5 ml/bottle

B. High Sensitivity Human Cytokine Standard

Vials containing mixed cytokine cocktail, lyophilized

Quantity: 2 vials

C. High Sensitivity Human Cytokine Quality Controls
Control 1 – 2 vials containing mixed cytokine cocktail, lyophilized
Control 2 – 2 vials containing mixed cytokine cocktail, lyophilized

Quantity: 2 vials/Control

II. REAGENTS SUPPLIED (continued)

D. Serum Matrix, lyophilized (**optional – for serum/plasma samples**)

Serum containing 0.08% Sodium Azide

Quantity: 5 ml/vial

E. High Sensitivity Human Cytokine Detection Antibodies

1 bottle containing a cocktail of biotinylated detection antibodies in Assay Buffer

Quantity: 5.5 ml/bottle

F. Streptavidin-Phycoerythrin

1 bottle containing Streptavidin-Phycoerythrin prepared in Assay Buffer

Quantity: 5.5 ml/bottle

G. LINCOplex Assay Buffer

50 mM PBS with 25 mM EDTA, 0.08% Sodium Azide, 0.05% Tween 20, and 1% BSA, pH 7.4.

Quantity: 15 ml/bottle

H. 10X Wash Buffer

1:10 dilution required with deionized water to give 10 mM PBS with 0.05% Proclin, and 0.05% Tween 20, pH 7.4.

Quantity: 30 ml/bottle

I. Microtiter Filter Plate

Quantity: 1- 96 Well Filtration Plate

J. Plate Sealers

Quantity: 2 Plate Sealers

K. Mixing Bottle

III. STORAGE CONDITIONS UPON RECEIPT

Recommended storage for kit components is 2 - 8°C. See individual vials for long-term storage recommendations.

Once the standards and controls have been reconstituted following protocols described in Section VIII (B), immediately transfer contents into polypropylene vials. **DO NOT STORE RECONSITUTED STANDARDS OR CONTROLS IN GLASS VIALS**. After reconstitution, discard the remaining standards and controls after use. Avoid multiple (>2) freeze thaw cycles.

DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin.

IV. REAGENT PRECAUTIONS

Sodium Azide has been added to some reagents as a preservative. Although the concentrations are low, sodium azide may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.

V. MATERIALS REQUIRED BUT NOT PROVIDED

A. Reagents

Luminex Sheath Fluid (Luminex Catalogue #40-50000)

B. Instrumentation/Materials

1. Adjustable Pipettes with Tips capable of delivering 25 µl to 1000 µl
2. Multichannel Pipettes capable of delivering 5 µl to 50 µl or 25 µl to 200 µl
3. Reagent Reservoirs
4. Polypropylene Microfuge Tubes
5. Aluminum Foil
6. Absorbent Pads
7. Laboratory Vortex
8. Sonicator (Branson Ultrasonic Cleaner Model # B200 or equivalent)
9. Titer Plate Shaker (Lab-Line Instruments, Model #4625, or equivalent)
10. Vacuum Filtration Unit (Millipore Vacuum Manifold Catalogue #MAVM0960R, or equivalent)
11. Luminex Instrument

VI. SPECIMEN COLLECTION AND STORAGE

A. A maximum of 50 µl of serum or plasma can be used per sample well. Tissue culture or other media may also be used.

B. Preparation of Tissue Culture Supernatant:

Centrifuge the sample to remove debris and assay immediately or aliquot and store samples at ≤ -20ºC. Avoid multiple (>2) freeze/thaw cycles. Tissue Culture Supernatant may require a dilution prior to assay with the appropriate control medium consistent with samples in term of composition, ionic strength and pH.

C. Preparation of Serum Samples:

Allow the blood to clot for at least 30 minutes. Then centrifuge the blood tubes for 10 minutes at 1000 xg. Remove serum and assay immediately or aliquot and store samples at ≤ -20ºC. Avoid multiple (>2) freeze/thaw cycles. If dilution is required for serum or plasma samples, use Serum Matrix provided in the kit as the sample diluent.

D. Preparation of Plasma Samples:

Plasma collection using EDTA as an anti-coagulant is recommended. Centrifuge for 10 minutes at 1000 xg within 30 minutes of blood collection. Remove plasma and assay immediately or aliquot and store samples at ≤ -20ºC. Avoid multiple (>2) freeze/thaw cycles. It is recommended to centrifuge samples again prior to assay setup. Serum Matrix should be used as the sample diluent if dilution is required for serum or plasma samples.

E. All samples must be stored in polypropylene tubes. **DO NOT STORE SAMPLES IN GLASS.**

F. Avoid using samples with gross hemolysis or lipemia.

G. All samples should be free of particles, excess lipids and precipitates. Particles in the samples may clog the sample probe. It is good practice to centrifuge samples before addition to sample wells.

VII. TECHNICAL GUIDELINES

To obtain reliable and reproducible results, the operator should carefully read this entire manual and fully understand all aspects of each assay step before running the assay. The following notes should be reviewed and understood before the assay is set-up.

A. The Antibody-Immobilized Beads are light sensitive and must be covered with aluminum foil at all times. Cover the assay plate containing beads with aluminum foil during all incubation steps.

B. It is critical to allow all reagents to warm to room temperature (20-25°C) before use in the assay.

C. The bottom of the Microtiter Filter Plate should not be in direct contact with any absorbent material during assay set-up or incubation times. The plate can be set on the non-flat side of the plate cover or any other plate holder to raise the plate from the surface.

D. After the wash steps, keep the bottom of the Microtiter Filter Plate clean by blotting on paper towels or absorbent pads to prevent any leakage due to capillary action.

E. Keep the vacuum suction on the plate as low as possible. It is recommended to have a vacuum setting that will remove 200 µl of buffer in $\geq$ 5 seconds (equivalent to < 100 mmHg).

F. After hydration, all standards and controls must be transferred to polypropylene tubes.

G. The standards prepared by serial dilution must be used within 1 hour of preparation. Discard any unused standards once reconstituted.

H. Any unused Antibody-Immobilized Beads may be stored in the bead mix bottle at 2-8°C for up to one month.

I. During the preparation of the standard curve, make certain to vortex the higher concentration well before making the next dilution. Use a fresh tip with each dilution.

J. The plate should be read immediately after the assay is finished. If, however, the plate cannot be read immediately, seal the plate, cover with aluminum foil, and store at 2-8°C for up to 24 hours. Prior to reading, agitate the plate on the plate shaker at room temperature for 10 minutes. Delay in reading a plate may result in decreased sensitivity for some cytokines.

K. The titer plate shaker should be set at a speed to provide maximum agitation without splashing of liquid outside the wells. For the recommended plate shaker, this would be a setting of 5-7, which is approximately 500-800 rpm.

L. Ensure the needle probe is clean. Following the protocols recommended in the Luminex manual for needle probe cleaning and de-clogging. If necessary, adjust probe height to the filter plate prior to reading an assay.

M. For cell culture supernatants or tissue extraction, use the culture or extraction medium as matrix in blank, standard curve and controls. If samples require dilutions, use the control medium as sample diluent.

VIII. PREPARATION OF REAGENTS FOR IMMUNOASSAY

A. Preparation of Antibody-Immobilized Beads

Sonicate the premixed antibody-bead bottle for 30 seconds and then vortex for 1 minute prior to use. No dilution is necessary. Unused portions may be stored at 2-8°C for up to one month.

B. Preparation of High Sensitivity Human Cytokine Standard

1.) Prior to use, reconstitute one vial of the High Sensitivity Human Cytokine Standard with 250 µl Deionized Water to give a 2,000 pg/ml concentration of standard for each analyte. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to set for 5-10 minutes and then transfer the standard to appropriately labeled polypropylene microfuge tube. This will be used as the 2,000 pg/ml standard; the unused portions of the reconstituted standard should be discarded.

2). Preparation of Working Standards

The following description and the Plate Map illustrate 1:5 dilution of standards. However, the end user has an option to select 1:4 or 1:3 dilution of standards with lowest concentration point at ~0.1 pg/ml. Label six polypropylene microfuge tubes 400, 80, 16, 3.2, 0.64, and 0.13 pg/ml. Add 200 µl of LINCOplex Assay Buffer to each of the six tubes. Prepare serial dilutions by adding 50 µl of the 2,000 pg/ml reconstituted standard to the 400 pg/ml tube, mix well and transfer 50 µl of the 400 pg/ml standard to the 80 pg/ml tube, mix well and transfer 50 µl of the 80 pg/ml standard to the 16 pg/ml tube, mix well and transfer 50 µl of the 16 pg/ml standard to 3.2 pg/ml tube, mix well and transfer 50 µl of the 3.2 pg/ml standard to the 0.64 pg/ml tube, mix well and transfer 50 µl of the 0.64 pg/ml standard to the 0.13 pg/ml tube and mix well. The 0 pg/ml standard (Background) will be LINCOplex Assay Buffer.

Standard Concentration (pg/ml)	Volume of Deionized Water to Add	Volume of Standard to Add
2,000	250 µl	0

Standard Concentration (pg/ml)	Volume of LINCOplex Assay Buffer to Add	Volume of Standard to Add
400	200 µl	50 µl of 2,000 pg/ml
80	200 µl	50 µl of 400 pg/ml
16	200 µl	50 µl of 80 pg/ml
3.2	200 µl	50 µl of 16 pg/ml
0.64	200 µl	50 µl of 3.2 pg/ml
0.13	200 µl	50 µ of 0.64 pg/ml

VIII. PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

C. Preparation of High Sensitivity Human Cytokine Quality Controls

Before use, reconstitute one vial of High Sensitivity Human Cytokine Control 1 and High Sensitivity Human Cytokine Control 2 each with 250 µl Deionized Water. Invert the vials several times to mix and vortex. Allow the vial to set for 5-10 minutes and then transfer the controls to an appropriately labeled polypropylene microfuge tube. Unused portions of reconstituted Quality Controls should be discarded.

D. Preparation of Wash Buffer

Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution. Dilute 30 ml of 10X Wash Buffer with 270 ml deionized water. Store unused portions at 2-8°C for up to one month.

E. Preparation of Serum Matrix

This step is required for serum or plasma samples only.

Add 5.0 mL deionized water to the bottle containing lyophilized Serum Matrix. Mix well. Allow at least 10 minutes for complete reconstitution. Left-over reconstituted Serum Matrix should be stored at ≤ -20°C for up to one month.

IX. IMMUNOASSAY PROCEDURE

Prior to beginning this assay, it is imperative to read this protocol completely and to thoroughly understand the Technical Guidelines outlined in Section VII.

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

1. Diagram placement of Standards, 0 (Background), 0.13, 0.64, 3.2, 16, 80, 400, and 2,000 pg/ml, Controls I and II, and samples on Well Map Worksheet in a vertical configuration. (Note: the instrument will only read the 96-well plate vertically). It is recommended to run the assay in duplicate.
2. Pre-wet the filter plate by pipetting 200 µL of **1X Wash Buffer** into each well of the microtiter plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
3. Remove Wash Buffer by vacuum. Remove any excess Wash Buffer from the bottom of the plate by blotting on an absorbent pad or paper towels.
4. Sonicate Bead Bottle for 30 seconds, then vortex for 1 minute and add 25 µL of the Premixed Beads to each well. (Note: during addition of Premixed Beads, shake bead mix intermittently to avoid settling).
5. Remove liquid from the wells by vacuum. (**NOTE: DO NOT INVERT PLATE**). Remove any excess liquid from the bottom of the plate by blotting on an absorbent pad or paper towels.
6. Add 50 µL of LINCOplex Assay Buffer to the 0 Standard (Background).
7. Add 50 µL of LINCOplex Assay Buffer to the Sample wells.
8. Add 50 µL of each Standard or Control into the appropriate wells.

IX. IMMUNOASSAY PROCEDURE (continued)

9. Add 50 µL of appropriate matrix solution to the Background, Standards, and Control wells. When assaying serum or plasma, use Serum Matrix provided in the kit. When assaying Tissue Culture supernatant samples, use proper control culture medium as the matrix solution.
10. Add 50 µL of Sample into the appropriate sample wells. Before addition to wells, the samples should be centrifuged to remove any precipitates or denatured proteins occurred during storage and handling.
11. Seal, cover with aluminum foil, and incubate with agitation on a plate shaker overnight (16-18 hr) at 4°C.
12. Gently remove fluid by vacuum. **(NOTE: DO NOT INVERT PLATE)**.
13. Wash plate 2 times with 200 µL/well of Wash Buffer, removing Wash Buffer by vacuum filtration after each wash. Remove any excess Wash Buffer from the bottom of the plate by blotting on an absorbent pad or paper towels.
14. Add 50 µL of Detection Antibody Cocktail into each well. (Note: allow the Detection Antibody to warm to room temperature prior to addition.)
15. Seal, cover with aluminum foil, and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C). **DO NOT VACUUM AFTER INCUBATION.**
16. Add 50 µL Streptavidin-Phycoerythrin to each well containing the 50 µL of Detection Antibody Cocktail.
17. Seal, cover with aluminum foil, and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).
18. Gently remove all contents by vacuum. **(NOTE: DO NOT INVERT PLATE)**.
19. Wash plate 2 times with 200 µL/well Wash Buffer, removing Wash Buffer by vacuum filtration after each wash. Wipe any excess buffer on the bottom of the plate with a tissue.
20. Add 100 µL of Sheath Fluid to all wells. Seal, cover with aluminum foil and resuspend the beads on a plate shaker for 5 minutes.
21. Read plate on Luminex Instrument.
22. Save and evaluate the Median Fluorescence Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating cytokine/chemokines concentrations in samples.

X. EQUIPMENT SETTINGS

Select the following equipment settings:

Events:	100, per bead
Sample Size:	50 µl
Bead Set:	01 for IL-1β
	03 for IL-2
	09 for IL-4
	10 for IL-5
	12 for IL-6
	13 for IL-7
	20 for IL-8
	23 for IL-10
	25 for IL-12(p70)
	26 for IL-13
	35 for IFNγ
	39 for GM-CSF
	40 for TNFα

**Gate (for IS System):	7,500 to 15,000
**Gate (for 1.7 System):	8,060 to 13,000

**These specifications are for the Luminex100 or Luminex200 with software v. 1.7 or IS. Luminex instruments with other software (e.g. Masterplex, Starstation, LiquiChip, Bioplex, LABScan100) would need to follow instrument instructions for gate settings and additional specifications.

XI. QUALITY CONTROLS

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert or can be located at the Millipore website www.millipore.com/bmia.

XII. ASSAY CHARACTERISTICS

A. Assay Sensitivities (minimum detectable concentrations, pg/ml)

Cytokine	MinDC
IL-1β	0.06
IL-2	0.16
IL-4	0.13
IL-5	0.01
IL-6	0.10
IL-7	0.12
IL-8	0.11
IL-10	0.15
IL-12(p70)	0.11
IL-13	0.48
IFN-γ	0.29
GM-CSF	0.46
TNF-α	0.05

B. Precision

Cytokine	Intra-assay (%CV)	Inter-assay (%CV)
IL-1β	3.11	2.16
IL-2	4.27	7.48
IL-4	4.16	9.12
IL-5	4.50	14.27
IL-6	3.51	4.48
IL-7	4.75	6.24
IL-8	3.26	6.48
IL-10	3.31	11.84
IL-12(p70)	4.31	5.08
IL-13	5.86	11.99
IFNγ	4.88	7.79
GM-CSF	4.14	5.03
TNFα	3.49	3.78

Intra-assay precision is generated from the mean of the %CV's from 8 reportable results across two different concentrations of cytokines in a single assay. Inter-assay precision is generated from the mean of the %CV's from two different concentrations of cytokines across 8 different assays.

XII. ASSAY CHARACTERISTICS (continued)

C. Accuracy

Cytokine	%Recovery
IL-1β	102.9
IL-2	98.8
IL-4	98.9
IL-5	93.0
IL-6	100.7
IL-7	112.0
IL-8	103.1
IL-10	93.1
IL-12(p70)	100.2
IL-13	93.2
IFNγ	98.4
GM-CSF	100.4
TNFα	103.6

Accuracy is calculated from the %Recovery of seven different levels of cytokine (ranging from 3.13 to 400 pg/ml) spiked into human serum matrix with known low or no measurable cytokine levels.

XII. ASSAY CHARACTERISTICS (continued)

D. Linearity of Dilution (% Neat Serum Sample Concentrations)

Cytokine	1:2 Dilution	1:4 Dilution	1:8 Dilution
IL-1β	122.3	131.2	130.4
IL-2	95.3	97.7	118.0
IL-4	129.9	151.6	-
IL-5	95.8	70.0	90.7
IL-6	93.0	102.5	113.7
IL-7	99.7	115.7	130.3
IL-8	118.9	156.1	-
IL-10	90.5	102.7	113.7
IL-12(p70)	88.6	92.1	84.8
IL-13	108.6	117.2	124.5
IFN-γ	83.9	92.6	89.9
GM-CSF	104.3	112.5	124.0
TNF-α	93.6	101.7	123.5

E. Cross-Reactivity

There was no significant cross-reactivity between the antibodies and any of the other analytes in this panel.

XIII. REPLACEMENT REAGENTS

REAGENTS	Cat #
High Sensitivity Human Cytokine Standard	L-8060SEN
High Sensitivity Human Cytokine Quality Controls	L-6060SEN
Serum Matrix	LHHS-SM (optional)
High Sensitivity Human Cytokine Detection Antibodies	L-1060SEN
Streptavidin-Phycoerythrin	L-SAPE7
Assay Buffer	L-ABIR
Set of two 96-Well Filter Plates with sealers	L-PLATE
10X Wash Buffer	L-WB
Premixed Human Cytokine Beads	HCB-PMX13

XIV. ORDERING INFORMATION

A. To place an order:

To assure the clarity of your custom cytokine kit order, please FAX the following information to our customer service department:

1. Your name, telephone and/or fax number
2. Customer account number
3. Shipping and billing address
4. Purchase order number
5. Catalog number and description of product
6. Quantity of kits
7. Selection of LINCO*plex* Cytokine Analytes/Serum Matrix Requirements

FAX: (636) 441-8050
Toll Free US: (866) 441-8400
(636) 441-8400

MAIL ORDERS: Millipore
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

For International Customers:
To best serve our international customers, it is Millipore's policy to sell our products through a network of distributors. To place an order or to obtain additional information about Millipore products, please contact your local distributor.

B. Conditions of Sale

All products are for research use only. They are not intended for use in clinical diagnosis or for administration to humans or animals. All products are intended for *in vitro* use only.

C. Material Safety Data Sheets (MSDS)

Material Safety Data Sheets for Millipore products may be ordered by fax or phone.

WELL MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 pg/ml Standard (Background)	16 pg/ml Standard	QC-1 Control									
B	0 pg/ml Standard (Background)	16 pg/ml Standard	QC-1 Control									
C	0.13 pg/ml Standard	80 pg/ml Standard	QC-2 Control									
D	0.13 pg/ml Standard	80 pg/ml Standard	QC-2 Control									
E	0.64 pg/ml Standard	400 pg/ml Standard										
F	0.64 pg/ml Standard	400 pg/ml Standard										
G	3.2 pg/ml Standard	2,000 pg/ml Standard										
H	3.2 pg/ml Standard	2,000 pg/ml Standard										

Assay Method for High Sensitivity Human Cytokine Linco*plex* Kit (HSCYTO-60SK)

Procedure Step:		Step 2-3		Step 4	Step 5	Step 6-7	Step 8	Step 9	Step 10	Step 11-13	Step 14	Step 15	Step 16	Step 17-19	Step 20
Well #	Well Identification	1X Wash Buffer		Premixed Beads		Assay Buffer	Standard/ Control	Serum Matrix/TC Medium*	Sample		Detection Antibody		SA-PE		Sheath Fluid
1A, 1B	0 pg/ml Standard (Background)	200 µl	**Seal, Agitate, Incubate 10 minutes at Room Temperature. Remove Wash Buffer by Vacuum**	25 µl	**Remove liquid in wells by vacuum. Wipe the bottom of plate with paper towels.**	50 µl	-	50 µl	-	**Seal, Agitate, Incubate Overnight at 4°C . Remove liquid by Vacuum. Wash 2X with 200 µl Wash Buffer, Vacuum, Wipe the bottom of plate with paper towels.**	50 µl	**Seal, Agitate, Incubate 1 hour at Room Temperature.**	50 µl	**Seal, Agitate, Incubate 30 minutes at Room Temperature. Remove liquid by Vacuum. Wash 2X with 200 µl Wash Buffer, Vacuum, Wipe the bottom of plate with paper towels.**	100 µl
1C, 1D	0.13 pg/ml Standard					-	50 µl	50 µl	-						
1E, 1F	0.64 pg/ml Standard					-	50 µl	50 µl	-						
1G, 1H	3.2 pg/ml Standard					-	50 µl	50 µl	-						
2A, 2B	16 pg/ml Standard					-	50 µl	50 µl	-						
2C, 2D	80 pg/ml Standard					-	50 µl	50 µl	-						
2E, 2F	400 pg/ml Standard					-	50 µl	50 µl	-						
2G, 2H	2000 pg/ml Standard					-	50 µl	50 µl	-						
3A, 3B	QC1					-	50 µl	50 µl	-						
3C, 3D	QC2					-	50 µl	50 µl	-						
3E, 3F	Sample					50 µl	-	-	50 µl						
3G, 3H	Sample					50 µl	-	-	50 µl						
4A, 4B ↓	Sample					50 µl	-	-	50 µl						
Final Sample	Sample					50 µl	-	-	50 µl						

* Use Linco*plex* Serum Matrix, tissue culture medium, or tissue extraction buffer, as appropriate to sample type. See Section VII (M).