

**Human Soluble Cytokine
Receptor Panel**

96 Well Plate Assay

**Cat. # HSCR-32K or
HSCR-32K-PMX13**

MILLIPLEX® MAP

**HUMAN SOLUBLE CYTOKINE RECEPTOR PANEL KIT
96 Well Plate Assay**

**# HSCR-32K or
HSCR-32K-PMX13 (premixed)**

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For Research Use Only. Not for Use in Diagnostic Procedures.

By purchasing this product, which contains fluorescently labeled microsphere beads authorized by Luminex Corporation ("Luminex"), you, the customer, acquire the right under Luminex's patent rights, if any, to use this product or any portion of this product, including without limitation the microsphere beads contained herein, only with Luminex's laser based fluorescent analytical test instrumentation marketed under the name of Luminex 100™ IS, 200™, HTS.

INTRODUCTION

Cytokine receptors constitute an integral part of the cytokine biology. Like cytokines, cytokine receptors are involved in normal physiological and pathological processes of almost all disease states. Soluble cytokine receptors naturally arise from genes encoding membrane-bound receptors or are direct derivatives of the receptors themselves. The discovery that soluble cytokine receptors are involved in regulating excessive inflammatory responses and modulating immune events has stimulated significant research interest in their potential role as immunotherapeutic agents. Many of these soluble cytokine receptors have the ability to inhibit the binding and biological activity of their cytokine ligands, making them very specific cytokine antagonists.

Millipore is proud to announce the first multiplexed immunoassay panel for quantifying human soluble cytokine receptors in biological samples using the Luminex® xMAP® technology. Designed for the simultaneous analysis of multiple biomarkers, the Human Soluble Cytokine Receptor Panel provides important tools for the study of inflammatory and immune responses—evidence of Millipore's commitment to innovation and providing you with the best tools to do your best work.

Millipore's MILLIPLEX[®] Human Soluble Cytokine Receptor Panel is the most versatile system available for cytokine receptor research.

- MILLIPLEX MAP offers you the ability to:
 - Select a premixed kit (13-plex).
 - Choose any combination of analytes from our panel of 13 analytes to design a custom kit that better meets your needs.
- A convenient "all-in-one" box format gives you the assurance that you will have all the necessary reagents you need to run your assay.

Millipore's MILLIPLEX Human Soluble Cytokine Receptor kit is to be used for the simultaneous quantification of the following 13 human cytokine soluble receptors in any combination: soluble CD30 (sCD30, sTNFRSF8), soluble gp130 (sgp130), soluble Interleukin-1 Receptor Type I (sIL-1RI, sCD121a), soluble Interleukin-1 Receptor Type II (sIL-1RII, sCD121b), soluble Interleukin-2 Receptor alpha (sIL-2R α , CD25), soluble Interleukin-4 Receptor (sIL-4R, CD124), soluble Interleukin-6 Receptor (sIL-6R, CD126), soluble Receptor for Advanced Glycation Endproducts (sRAGE), soluble Tumor Necrosis Factor Receptor I (sTNFRI, TNFRSF1A), soluble Tumor Necrosis Factor Receptor II (sTNFRII, TNFRSF1B), soluble Vascular Endothelial Growth Factor Receptor 1 (sVEGFR1, sFlt-1), soluble Vascular Endothelial Growth Factor Receptor 2 (sVEGFR2, sFlk-1, sKDR), and soluble Vascular Endothelial Growth Factor Receptor 3 (sVEGFR3, sFlt-4).

This kit may be used for the analysis of all or any combination of the above soluble cytokine receptors in serum or plasma samples, tissue/cell lysate and tissue/cell culture supernatant samples.

For Research Use Only. Not for Use in Diagnostic Procedures.

Please read entire protocol before use.

It is important to use same assay incubation conditions throughout your study.

PRINCIPLE

MILLIPIX MAP is based on the Luminex® xMAP® technology — one of the fastest growing and most respected multiplex technologies offering applications throughout the life sciences and capable of performing a variety of bioassays including immunoassays on the surface of fluorescent-coded beads known as microspheres.

- Luminex uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, 100 distinctly colored bead sets can be created, each of which is coated with a specific capture antibody.
- After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced.
- The reaction mixture is then incubated with Streptavidin-Phycoerythrin (SA-PE) conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- The microspheres are allowed to pass rapidly through a laser that excites the internal dyes marking the microsphere set. A second laser excites PE, the fluorescent dye on the reporter molecule.
- Finally, high-speed digital-signal processors identify each individual microsphere and quantify the result of its bioassay based on fluorescent reporter signals.

The capability of adding multiple conjugated beads to each sample results in the ability to obtain multiple results from each sample. Open-architecture xMAP® technology enables multiplexing of many types of bioassays reducing time, labor and costs over traditional methods.

STORAGE CONDITIONS UPON RECEIPT

- Recommended storage for kit components is 2 - 8°C.
- Once the standards and controls have been reconstituted, immediately transfer contents into polypropylene vials. **DO NOT STORE RECONSTITUTED STANDARDS OR CONTROLS IN GLASS VIALS.** For long-term storage, freeze reconstituted standards and controls at ≤ -20°C. Avoid multiple (>2) freeze/thaw cycles.
- **DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibodies, and Streptavidin-Phycoerythrin.**

REAGENTS SUPPLIED

Note: Store all reagents at 2 – 8°C

REAGENTS SUPPLIED	CATALOG NUMBER	VOLUME	QUANTITY
Human Soluble Cytokine Receptor Standard	HSCR-8032	lyophilized	1 vial
Human Soluble Cytokine Receptor Quality Controls 1 and 2	HSCR-6032	lyophilized	2 vials
Serum Matrix Note: Contains 0.08% Sodium Azide	HSCR-SM	lyophilized	1 vial
Set of one 96-Well Microtiter Filter Plate with 2 Sealers	MX-PLATE	-----	1 plate 2 sealers
Assay Buffer Note: Contains 0.08% Sodium Azide	L-AB	30 mL	1 bottle
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	30 mL	1 bottle
Human Soluble Cytokine Receptor Detection Antibodies	HSCR-1032	3.2 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE3	3.2 mL	1 bottle
Bead Diluent (not provided with premixed panel)	LBD	3.5 mL	1 bottle
Mixing Bottle (not provided with premixed panel)	-----	-----	1 bottle

Human Soluble Cytokine Receptor Antibody-Immobilized Premixed Beads:

Premixed 13-plex Beads	HSCR-PMX13	3.5 mL	1 bottle
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Included Human Soluble Cytokine Receptor Antibody-Immobilized Beads are dependent on customizable selection of analytes within the panel (see following table on page 5).

Human Soluble Cytokine Receptor Antibody-Immobilized Beads:

Bead/Analyte Name	Luminex Bead Region	Customizable Beads (50X, 90 µL)		13-Plex Premixed Beads
		Available	Cat. #	
Anti – sCD30 Bead	57	✓	HCD30	✓
Anti – sgp130 Bead	63	✓	HGP130	✓
Anti – sIL-1RI Bead	65	✓	HIL-1R1	✓
Anti – sIL-1RII Bead	69	✓	HIL-1R2	✓
Anti – sIL-2R α Bead	76	✓	HIL-2RA	✓
Anti – sIL-4R Bead	77	✓	HIL-4R	✓
Anti – sIL-6R Bead	79	✓	HIL-6R	✓
Anti – sRAGE Bead	81	✓	HRAGE	✓
Anti – sTNFRI Bead	83	✓	HTNF-R1	✓
Anti – sTNFRII Bead	85	✓	HTNF-R2	✓
Anti – sVEGFR1 Bead	88	✓	HVEGF-R1	✓
Anti – sVEGFR2 Bead	90	✓	HVEGF-R2	✓
Anti – sVEGFR3 Bead	92	✓	HVEGF-R3	✓

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex Sheath Fluid (Luminex Catalogue #40-50000)

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. Rubber Bands
6. Absorbent Pads
7. Laboratory Vortex Mixer
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 Catalog #MSVMHTS00 or equivalent with Millipore Vacuum Pump Catalog #WVP6111560 or equivalent)
11. Luminex 100™ IS, 200™, HTS by Luminex Corporation
12. Plate Holder (Millipore Catalog # MX-STAND)

SAFETY PRECAUTIONS

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium Azide or Proclin has been added to some reagents as a preservative. Although the concentrations are low, Sodium Azide and Proclin may react with lead and copper plumbing to form highly explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

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.

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not use beyond the expiration date on the label.
- Do not mix or substitute reagents with those from other lots or sources.
- The Antibody-Immobilized Beads are light sensitive and must be protected from light at all times. Cover the assay plate containing beads with opaque plate lid or aluminum foil during all incubation steps.
- It is important to allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- The bottom of the Microtiter Filter Plate should not be in direct contact with any surface during assay setup or incubation times. The plate can be set on a plate holder or on the non-flat side of the plate cover or any other plate holder to raise the plate from the surface. A plate holder can be purchased separately from Millipore (Millipore Catalog #MX-STAND).
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Wash Buffer provided.
- 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.
- Keep the vacuum suction on the plate as low as possible. It is recommended to have a vacuum setting that will remove 200 μ L of buffer in \geq 5 seconds (equivalent to $<$ 100 mmHg).
- After hydration, all Standards and Controls must be transferred to polypropylene tubes.
- The Standards prepared by serial dilution must be used within 1 hour of preparation. Discard any unused standards except the standard stock, which may be stored at \leq -20°C for 1 month and at \leq -80°C for greater than one month.
- If samples fall outside the dynamic range of the assay, further dilute the samples with the appropriate diluent and repeat the assay.

- Mix only required amount of beads prior to assay setup. Discard any unused premixed beads.
- During the preparation of the standard curve, make certain to mix the higher concentration well before making the next dilution. Use a new tip with each dilution.
- The plate should be read immediately after the assay is finished. If, however, the plate cannot be read immediately, seal the plate, cover with opaque lid and store the plate 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 analytes.
- The titer plate shaker should be set at a speed to provide maximum orbital mixing 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.
- Ensure that the needle probe is clean. This may be achieved by sonication and/or alcohol flushes. Adjust probe height according to the protocols recommended by Luminex to the kit filter plate using 3 alignment discs prior to reading an assay.
- For cell culture supernatants or tissue extraction, use the culture or extraction medium as the matrix solution in background, standard curve and control wells. If samples are diluted in Assay Buffer, use the Assay Buffer as matrix.
- For serum/plasma samples, use the Serum Matrix provided in the kit.
- For cell/tissue homogenate, the final cell or tissue homogenate should be prepared in a buffer that has a neutral pH, contains minimal detergents or strong denaturing detergents, and has an ionic strength close to physiological concentration. Avoid debris, lipids, and cell/tissue aggregates. Centrifuge samples before use.
- Vortex all reagents well before adding to plate.

SAMPLE COLLECTION AND STORAGE

A. Preparation of Serum Samples:

- Allow the blood to clot for at least 30 minutes before centrifugation for 10 minutes at 1000xg. Remove serum and assay immediately or aliquot and store samples at $\leq -20^{\circ}\text{C}$.
- Avoid multiple (>2) freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.
- Customers need to determine the optimal dilution factor for their samples. Generally, serum samples from normal subjects should be diluted 1:5 using the Serum Matrix provided in the kit as the sample diluent (20 μL sample mixed with 80 μL Serum Matrix). If samples require dilution beyond 1:5, continue to use the Serum Matrix as the sample diluent.

B. Preparation of Plasma Samples:

- Plasma collection using EDTA as an anticoagulant is recommended. Centrifuge for 10 minutes at 1000xg within 30 minutes of blood collection. Remove plasma from the tube and assay immediately or aliquot and store samples at $\leq -20^{\circ}\text{C}$.
- Avoid multiple (>2) freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.
- Customers need to determine the optimal dilution factor for their samples. Generally, plasma samples from normal subjects should be diluted 1:5 using the Serum Matrix provided in the kit as the sample diluent (20 μL sample mixed with 80 μL Serum Matrix). If samples require dilution beyond 1:5, continue to use the Serum Matrix as the sample diluent.

C. Preparation of Tissue Culture Supernatant:

- Centrifuge the sample to remove debris and assay immediately or aliquot and store samples at $\leq -20^{\circ}\text{C}$.
- Avoid multiple (>2) freeze/thaw cycles.
- Tissue culture supernatant samples may require a dilution with an appropriate control medium prior to assay.

NOTE:

- A maximum of 25 μL per well of tissue extract, cell/tissue culture supernatant sample, or 1:5 diluted serum or plasma sample can be used.
- All samples must be stored in polypropylene tubes. **DO NOT STORE SAMPLES IN GLASS.**
- Avoid debris, lipids and cells when using samples with gross hemolysis or lipemia.
- Care must be taken when using heparin as an anticoagulant since an excess of heparin will provide falsely high values. Use no more than 10 IU heparin per mL of blood collected.

PREPARATION OF REAGENTS FOR IMMUNOASSAY

A. Preparation of Antibody-Immobilized Beads

If premixed beads are used, sonicate the premixed bead bottle 30 seconds then vortex for 1 minute before use.

For individual vials of beads, sonicate each antibody-bead vial for 30 seconds then vortex for 1 minute. Add 60 μ L 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. Discard any unused premixed beads.

Example 1: When using 3 antibody-immobilized beads, add 60 μ L from each of the 3 bead sets to the Mixing Bottle. Then add 2.82 mL Bead Diluent.

Example 2: When using 11 antibody-immobilized beads, add 60 μ L from each of the 11 bead sets to the Mixing Bottle. Then add 2.34 mL Bead Diluent.

B. Preparation of Quality Controls

Before use, reconstitute Quality Control 1 and Quality Control 2 with 250 μ L deionized water. Invert the vial several times to mix then vortex briefly. Allow the vial to sit for 5-10 minutes and then transfer the controls to appropriately labeled polypropylene microfuge tubes. Unused portions may be stored at $\leq -20^{\circ}\text{C}$ for up to one month.

C. 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 portion at 2-8 $^{\circ}\text{C}$ for up to one month.

D. Preparation of Serum Matrix

This step is required for serum or plasma samples only.

Add 5.0 mL Assay Buffer 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 $\leq -20^{\circ}\text{C}$ for up to one month.

E. Preparation of Human Soluble Cytokine Receptor Standard

1.) Prior to use, reconstitute the Human Soluble Cytokine Receptor Panel Standard with 250 μL deionized water (refer to table on page 11 for exact analyte concentrations). Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes and then transfer the standard to appropriately labeled polypropylene microfuge tube. This will be used as Standard 7; the unused portion may be stored at $\leq -20^{\circ}\text{C}$ for up to one month.

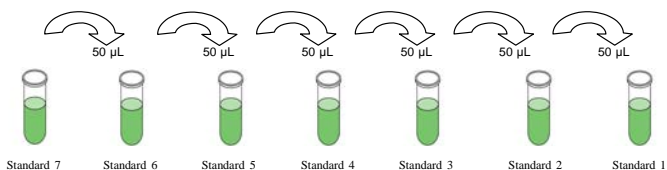
2.) Preparation of Working Standards

Label six polypropylene microfuge tubes Standard 6, Standard 5, Standard 4, Standard 3, Standard 2 and Standard 1. Add 150 μL of Assay Buffer to each of the six tubes. Prepare serial dilutions by adding 50 μL of the reconstituted standard (Standard 7) to the Standard 6 tube, mix well and transfer 50 μL of the Standard 6 tube to the Standard 5 tube, mix well and transfer 50 μL of the Standard 5 tube to the Standard 4 tube, mix well and transfer 50 μL of the Standard 4 tube to the Standard 3 tube, mix well and transfer 50 μL of the Standard 3 tube to the Standard 2 tube, mix well and transfer 50 μL of the Standard 2 tube to the Standard 1 tube and mix well. The 0 pg/mL Standard (Background) will be Assay Buffer.

Standard (Tube #)	Volume of Deionized Water to Add	Volume of Standard to Add
Standard 7 (reconstituted standard)	250 μL	0

Standard (Tube #)	Volume of Assay Buffer to Add	Volume of Standard to Add
Standard 6	150 μL	50 μL of Standard 7
Standard 5	150 μL	50 μL of Standard 6
Standard 4	150 μL	50 μL of Standard 5
Standard 3	150 μL	50 μL of Standard 4
Standard 2	150 μL	50 μL of Standard 3
Standard 1	150 μL	50 μL of Standard 2

Preparation of Standards



After dilution, each tube has the following concentrations for each analyte:

Standard	sIL-4R, sIL-6R, sRAGE, sTNFRI, sTNFRII (pg/mL)	sCD30, sgp130, sIL-1RI, sIL-2R α (pg/mL)	sIL-1RII, sVEGFR1, sVEGFR2, sVEGFR3 (pg/mL)
Standard 7	50,000	100,000	500,000
Standard 6	12,500	25,000	125,000
Standard 5	3,125	6,250	31,250
Standard 4	781.3	1,562.5	7,812.5
Standard 3	195.3	390.6	1,953.1
Standard 2	48.8	97.7	488.3
Standard 1	12.2	24.4	122.1

IMMUNOASSAY PROCEDURE

- Prior to beginning this assay, it is imperative to read this protocol completely and to thoroughly understand the Technical Guidelines.
- Allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- Diagram the placement of Background, Standards 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 samples in duplicate.
- Set the filter plate on a plate holder at all times during reagent dispensing and incubation steps so that the bottom of the plate does not touch any surface.

1. Prewet the filter plate by pipetting 200 μ L of 1X Wash Buffer into each well of the Microtiter Filter Plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
2. Remove Wash Buffer by vacuum. (**NOTE: DO NOT INVERT PLATE.**) Blot excess Wash Buffer from the bottom of the plate with an absorbent pad or paper towels.
3. Add 25 μ L of each Standard or Control into the appropriate wells. Assay Buffer should be used for 0 pg/mL Standard (Background).
4. Add 25 μ L of Assay Buffer to the sample wells.
5. Add 25 μ L of appropriate matrix solution to the background, standards, and control wells. When assaying serum or plasma, use the Serum Matrix provided in the kit. When assaying tissue culture or other supernatant, use proper control medium as the matrix solution.
6. Add 25 μ L of Sample into the appropriate wells.
7. Vortex Mixing Bottle and add 25 μ L of the Mixed or Premixed Beads to each well. (Note: During addition of Mixed Beads, shake Mixing Bottle intermittently to avoid settling.)
8. Seal the plate with a plate sealer then cover it with the opaque lid. Wrap a rubber band around the plate holder, plate and lid and incubate with agitation on a plate shaker overnight at 4°C or 2 hours at room temperature (20-25°C). *An overnight incubation (16-18 hr) may improve assay sensitivity for some analytes.*

Add 200 μ L Wash Buffer per well



Shake 10 min, RT
Vacuum

- Add 25 μ L Standard or Control to appropriate wells
- Add 25 μ L Assay Buffer to background and sample wells
- Add 25 μ L Samples to sample wells
- Add 25 μ L Matrix to background, standards and control wells
- Add 25 μ L Beads to each well



Incubate overnight at 4°C or 2 hours at RT with shaking

9. Gently remove fluid by vacuum. (**NOTE: DO NOT INVERT PLATE.**)
10. Wash plate 2 times with 200 μ L/well of Wash Buffer, removing Wash Buffer by vacuum filtration between each wash. Blot excess Wash Buffer from the bottom the plate by with an absorbent pad or paper towels.
11. Add 25 μ L of Detection Antibodies into each well. (Note: Allow the Detection Antibodies to warm to room temperature prior to addition.)
12. Seal, cover with lid, and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C). **DO NOT VACUUM AFTER INCUBATION.**
13. Add 25 μ L Streptavidin-Phycoerythrin to each well containing the 25 μ L of Detection Antibodies.
14. Seal, cover with lid and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).
15. Gently remove all contents by vacuum. (**NOTE: DO NOT INVERT PLATE.**)
16. Wash plate 2 times with 200 μ L/well Wash Buffer, removing Wash Buffer by vacuum filtration between each wash. Wipe any excess buffer on the bottom of the plate with an absorbent pad or paper towel.
17. Add 150 μ L of Sheath Fluid to all wells. Resuspend the beads by incubating with agitation on a plate shaker for 5 minutes at room temperature.
18. Run plate on Luminex 100™ IS, 200™, HTS.
19. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method to calculate analyte concentrations in samples.



Vacuum and wash
2X with 200 μ L
Wash Buffer

Add 25 μ L Detection
Antibodies per well



Incubate 1 hour at
RT

Do not vacuum

Add 25 μ L Streptavidin-
Phycoerythrin per well



Incubate for 30
minutes at RT

Vacuum and
wash 2X with
200 μ L Wash
Buffer

Add 150 μ L Sheath Fluid per
well

Read on Luminex (100 μ L,
50 beads per bead set)

EQUIPMENT SETTINGS

These specifications are for the Luminex 100™ IS v.1.7, Luminex 100™ IS v2.1/2.2, Luminex 200™ v2.3, xPONENT®, and Luminex HTS. Luminex instruments with other software (e.g. MasterPlex®, StarStation®, LiquiChip®, Bio-Plex®, LABScan®100) would need to follow instrument instructions for gate settings and additional specifications from the vendors.

Events:	50 per bead	
Sample Size:	100 µL	
Gate Settings	8,000 to 15,000	
Time Out	60 seconds	
Bead Set:	13-plex Customizable Beads	
	sCD30	57
	sgp130	63
	sIL-1RI	65
	sIL-1RII	69
	sIL-2Rα	76
	sIL-4R	77
	sIL-6R	79
	sRAGE	81
	sTNFR1	83
	sTNFR2	85
	sVEGFR1	88
	sVEGFR2	90
	sVEGFR3	92

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/techlibrary/index.do using the catalog number as the keyword.

ASSAY CHARACTERISTICS

Assay Sensitivities (minimum detectable concentrations, pg/mL)

MinDC: Minimum Detectable Concentration is calculated by the StatLIA® Immunoassay Analysis Software from Brendan Technologies. It measures the true limits of detection for an assay by mathematically determining what the empirical MinDC would be if an infinite number of standard concentrations were run for the assay under the same conditions.

Analyte	2 Hour Protocol*	Overnight Protocol**
sCD30	31.5	17.9
sgp130	18.8	7.2
sIL-1RI	7.9	4.0
sIL-1RII	69.9	45.9
sIL-2R α	26.1	15.0
sIL-4R	20.2	16.9
sIL-6R	5.8	3.6
sRAGE	7.8	4.7
sTNFR1	25.0	9.6
sTNFR2	23.4	16.9
sVEGFR1	209.9	164.5
sVEGFR2	642.8	497.8
sVEGFR3	672.0	706.9

*mean minDC + 2 standard deviations, N=6 assays

**mean minDC + 2 standard deviations, N=10 assays

Precision

Intra-assay precision is generated from the mean of the %CV's from two to four reportable results across two different concentrations of analytes in nine different assays. Inter-assay precision is generated from the mean of the %CV's from two to four reportable results across two different concentrations of analytes across nine different assays.

Analyte	Intra-Assay (%CV)	Inter-Assay (%CV)
sCD30	4.5	6.8
sgp130	4.3	7.5
sIL-1RI	5.1	8.2
sIL-1RII	4.0	6.3
sIL-2R α	3.8	6.5
sIL-4R	4.3	7.1
sIL-6R	5.6	8.2
sRAGE	5.2	8.5
sTNFRI	4.1	6.6
sTNFRII	3.7	5.8
sVEGFR1	6.5	15.8
sVEGFR2	9.0	14.6
sVEGFR3	7.1	13.3

Accuracy

Spike Recovery: The data represent mean percent recovery of 3 levels of spiked standards in diluted serum samples.

Analyte	Spike Recovery in Serum
sCD30	137.4
sgp130	102.0
sIL-1RI	117.5
sIL-1RII	114.7
sIL-2R α	113.3
sIL-4R	96.9
sIL-6R	110.3
sRAGE	120.3
sTNFRI	116.0
sTNFRII	114.1
sVEGFR1	108.1
sVEGFR2	99.8
sVEGFR3	103.8

Sample Linearity of Dilution (% Recovery):

Dilution Linearity, defined as the analyte levels measured in diluted samples as a percentage of the 1:5 diluted serum samples, was determined by measuring analyte concentrations in 5 independent samples. The samples were diluted 1:5 in the kit Serum Matrix then further diluted to 1:10, 1:20 and 1:40 in the kit Serum Matrix. These samples were analyzed in duplicate. The average percent recovery is reported.

Analyte	Dilution 1:10	Dilution 1:20	Dilution 1:40
sCD30	99.7	97.0	104.6
sgp130	104.4	108.9	112.1
sIL-1RI	105.0	109.6	103.3
sIL-1RII	95.2	94.6	89.6
sIL-2R α	95.3	91.7	89.9
sIL-4R	104.0	116.3	154.0
sIL-6R	99.1	105.5	100.3
sRAGE	103.8	107.5	113.3
sTNFR1	97.5	102.6	101.1
sTNFR2	96.5	95.3	89.5
sVEGFR1	106.2	114.6	114.4
sVEGFR2	103.6	139.9	149.2
sVEGFR3	101.4	129.1	122.2

Cross-Reactivity

There was no or negligible cross-reactivity between the antibodies for an analyte and any of the other analytes in this panel.

TROUBLESHOOTING GUIDE

Problem	Probable Cause	Solution
Filter plate will not vacuum	Vacuum pressure is insufficient	Increase vacuum pressure such that 0.2mL buffer can be suctioned in 3-5 seconds.
	Samples have insoluble particles	Centrifuge samples just prior to assay setup and use supernatant. If high lipid concentration, after centrifugation, remove lipid layer and use supernatant.
Insufficient bead count	Sample too viscous	May need to dilute sample.
	Vacuum pressure too high	Adjust vacuum pressure such that 0.2mL buffer can be suctioned in 3-5 seconds.
	Bead mix prepared incorrectly	Sonicate bead vials and vortex just prior to adding to Mixing Bottle according to protocol. Agitate bead mix intermittently in reservoir while pipetting into the plate.
Plate leaked	Samples cause interference due to particulate matter or viscosity	See above. Also sample probe may need to be cleaned with alcohol flush, backflush and washes; or, if needed, probe should be removed and sonicated.
	Probe height not adjusted correctly	Adjust probe to 3 alignment discs in well H6.
	Vacuum pressure too high	Adjust vacuum pressure such that 0.2mL buffer can be suctioned in 3-5 seconds. May need to transfer contents to a new (prewetted) plate and continue.
Background is too high	Plate set directly on table or absorbent towels during incubations or reagent additions	Set plate on plate holder or raised edge so bottom of filter is not touching any surface.
	Insufficient blotting of filter plate bottom causing wicking	Blot the bottom of the filter plate well with absorbent towels after each wash step.
	Pipette touching plate filter during additions	Pipette to the side of well.
	Probe height not adjusted correctly	Adjust probe to 3 alignment discs in well H6.
Background wells were contaminated	Background wells were contaminated	Avoid cross-well contamination by using sealer appropriately and by pipetting with multichannel pipets without touching reagent in plate.
	Matrix used has endogenous analyte or interference	Check matrix ingredients for crossreacting components (e.g. interleukin modified tissue culture medium).
	Insufficient washes	Increase number of washes.

<p>Beads not in region or gate</p>	<p>Luminex not calibrated correctly or recently</p> <p>Gate settings not adjusted correctly</p> <p>Wrong bead regions in protocol template</p> <p>Incorrect sample type used</p> <p>Instrument not washed or primed</p> <p>Beads were exposed to light</p>	<p>Calibrate Luminex based on instrument manufacturer's instructions, at least once a week or if temperature has changed by >3°C.</p> <p>Some Luminex instruments (e.g. Bioplex) require different gate settings than those described in the kit protocol. Use instrument default settings.</p> <p>Check kit protocol for correct bead regions or analyte selection.</p> <p>Samples containing organic solvents or if highly viscous should be diluted or dialyzed as required.</p> <p>Prime the Luminex 4 times to eliminate air bubbles. Wash 4 times with sheath fluid or water if there is any remnant alcohol or sanitizing liquid.</p> <p>Keep plate and bead mix covered with dark lid or aluminum foil during all incubation steps.</p>
<p>Signal for whole plate is same as background</p>	<p>Incorrect or no Detection Antibody was added</p> <p>Streptavidin-Phycoerythrin was not added</p>	<p>Add appropriate Detection Antibody and continue.</p> <p>Add Streptavidin-Phycoerythrin according to protocol. If Detection Antibody has already been vacuumed out, sensitivity may be low.</p>
<p>Low signal for standard curve</p>	<p>Detection Antibody may have been vacuumed out prior to adding Streptavidin Phycoerythrin</p> <p>Incubations done at incorrect temperatures, timings or agitation</p>	<p>May need to repeat assay if desired sensitivity not achieved.</p> <p>Assay conditions need to be checked.</p>
<p>Signals too high, standard curves are saturated</p>	<p>Calibration target value set too high</p> <p>Plate incubation was too long with standard curve and samples</p>	<p>With some Luminex instruments (e.g. Bioplex) default target setting for RP1 calibrator is set at High PMT. Use low target value for calibration and reanalyze plate.</p> <p>Use shorter incubation time.</p>
<p>Sample readings are out of range</p>	<p>Samples contain no or below detectable levels of analyte</p> <p>Samples contain analyte concentrations higher than highest standard point</p> <p>Standard curve was saturated at higher end of curve</p>	<p>If below detectable levels, it may be possible to use higher sample volume. Check with tech support for appropriate protocol modifications.</p> <p>Samples may require dilution and reanalysis for that particular analyte.</p> <p>See above.</p>

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WELL MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 pg/mL Standard (Background)	Standard 4	QC-1 Control									
B	0 pg/mL Standard (Background)	Standard 4	QC-1 Control									
C	Standard 1	Standard 5	QC-2 Control									
D	Standard 1	Standard 5	QC-2 Control									
E	Standard 2	Standard 6										
F	Standard 2	Standard 6										
G	Standard 3	Standard 7										
H	Standard 3	Standard 7										