Human Circulating Cancer Biomarker Magnetic Bead Panel 1

96 Well Plate Assay

Cat. # HCCBP1MAG-58K

MILLIPLEX® MAP

HUMAN CIRCULATING CANCER BIOMARKER PANEL 1 MAGNETIC BEAD KIT
96 Well Plate Assay

HCCBP1MAG-58K

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FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

INTRODUCTION

Cancer research has always focused on intracellular biomarkers, such as HER2 and B-Raf. However, in recent years more attention has shifted to include soluble cancer biomarkers. While the use of intracellular markers in research help clarify the process of oncogenesis, circulating cancer biomarkers give insight into how the body responds to the presence of a tumor, the dysregulation of homeostasis and the relationship between a tumor and its environment. Study of isolated biomarkers, whether intracellular or circulating, is often inadequate to analyze the complex relationship between tumor and non-tumor. Consequently, a large panel of cancer biomarkers better enables researchers to tease out these relationships and apply what they learn to understanding tumor biology.

Using Luminex® xMAP® technology, Millipore has developed the MILLIPLEX *MAP* Human
Circulating Cancer Biomarker Panel. Magnetic Beads can make the process of automation and high throughput screening easier with features such as walk-away washing. Advantages even outside automation include:

- More flexible plate and plate washer options
- Improved performance with turbid serum/plasma samples
- Assay results equivalent to non-magnetic beads
- Automated washing eliminates technical obstacles (e.g., clogging of wells that contain viscous samples) which may result during vacuum manifold/manual washing

Coupled with the Luminex xMAP® platform in a **magnetic bead** format, you receive the advantage of ideal speed and sensitivity, allowing quantitative multiplex detection of twenty six analytes, which can dramatically improve productivity.

- MILLIPLEX *MAP* offers you the ability to:
    - Choose combinations of analytes from our panel of 26 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 *MAP* Circulating Cancer Biomarker magnetic bead panel consists of 26 circulating cancer biomarkers that can be used to study a wide range of tumor types. Some biomarkers are tumor specific, such as PSA, while others, such as IL-8, have been detected in many cancers. The panel includes the following cancer biomarkers: AFP, CA125, CA15-3, CA19-9, CEA, CYFRA21-1, sFas, sFasL, FGF2, β-HCG, HE4, HGF, IL-6, IL-8, Leptin, MIF, Osteopontin, Prolactin, PSA (free), PSA (total), SCF, SDF-1 α+β/CXCL12, TGFα, TNFα, TRAIL and VEGF.

This kit may be used for the analysis of all or any combination of the above analytes in serum, plasma and tissue/cell lysate and culture supernatant samples with the following exceptions:

- **PSA (free) and PSA (total) cannot be plexed together.**
- **SDF-1 and CA19-9 cannot be plexed together.**

This kit is for research purposes only.

Please read entire protocol before use.

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

PRINCIPLE

MILLIPLEX 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 magnetic beads known as MagPlex ™-C 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-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 which 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 Circulating Cancer Biomarker Panel 1 Standard	HCC-8058	lyophilized	1 vial
Human Circulating Cancer Biomarker Panel 1 Quality Controls 1 and 2	HCC-6058	lyophilized	2 vials
Serum Matrix Note: Contains 0.08% Sodium Azide	HCP-SM	lyophilized	1 vial
Set of one 96-Well Plate with 2 Sealers	----------	----------	1 plate 2 sealers
Assay Buffer Note: Contains 0.08% Sodium Azide	L-AB	30 mL	2 bottles
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	30 mL	2 bottles
Human Circulating Cancer Biomarker Panel 1 Detection Antibodies	HCC-1058 or HCC-1058-2	3.2 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE 4	3.2 mL	1 bottle
Bead Diluent	LBD-4	3.5 mL	1 bottle
Mixing Bottle	----------	----------	1 bottle

Human Circulating Cancer Biomarker Panel 1 Antibody-Immobilized Magnetic Beads:

Bead/Analyte Name	Luminex Magnetic Bead Region	Customizable Beads (20X Concentration, 200 µL)	
		Available	Cat. #
Anti – AFP Bead	12	✓	HC1AFP-MAG
Anti – Total PSA Bead	13	✓	HTPSA-MAG
Anti – CA 15-3 Bead	15	✓	HCA153-MAG
Anti – CA 19-9 Bead	18	✓	HCA199-MAG
Anti – MIF Bead	20	✓	HMIF-MAG
Anti – TRAIL Bead	27	✓	HTRAIL-MAG
Anti – Leptin Bead	28	✓	HCCLPTN-MAG
Anti – Free PSA Bead	29	✓	HFPSA-MAG
Anti – IL-6 Bead	34	✓	HIL6-MAG
Anti – sFasL Bead	37	✓	HSFASLG-MAG
Anti – CEA Bead	39	✓	HCEA-MAG
Anti – CA125 Bead	42	✓	HCA125-MAG
Anti – IL-8 Bead	44	✓	HIL8-MAG
Anti – HGF Bead	45	✓	HHGF-MAG
Anti – SDF1α+β Bead	48	✓	HSDF-MAG
Anti – sFas Bead	52	✓	HSFAS-MAG
Anti – TNFα Bead	55	✓	HTNFA-MAG
Anti – Prolactin Bead	56	✓	HCCPRLCTN-MAG
Anti – SCF Bead	61	✓	HSCF-MAG
Anti – CYFRA 21-1 Bead	63	✓	HCYFRA-MAG
Anti – OPN Bead	64	✓	H0PN-MAG
Anti – FGF2 Bead	67	✓	HFGF2-MAG
Anti – β HCG Bead	73	✓	BHCG-MAG
Anti – HE4 Bead	75	✓	HHE4-MAG
Anti – TGF α Bead	76	✓	HTGFA-MAG
Anti – VEGF Bead	78	✓	HVEGF-MAG

Note- 1. Total PSA and Free PSA cannot be run together in the same assay.

2. CA19-9 and SDF1α+β cannot be run together in the same assay.

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex Sheath Fluid (Luminex Catalogue #40-50000) or Luminex Drive Fluid (Luminex Catlogue # MPXDF-4PK)

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. Rubber Bands
7. Absorbent Pads
8. Laboratory Vortex Mixer
9. Sonicator (Branson Ultrasonic Cleaner Model #B200 or equivalent)
10. Titer Plate Shaker (Lab-Line Instruments Model #4625 or equivalent)
11. Luminex 200™, HTS, FLEXMAP 3D™, or MAGPIX® with xPONENT software by Luminex Corporation
12. Automatic Plate washer for magnetic beads (Bio-Tek ELx405, Millipore catalog #40-015 or equivalent) or Hand held Magnetic Separation Block (Millipore catalog # 40-285 or equivalent)

Note: If a plate washer or hand held magnetic separation block for magnetic beads is not available, one can use Microtiter filter plate (MX-PLATE) to run the assay with the use of Vacuum Filtration Unit (Millipore
Vacuum Manifold Catalog # MSVMHTS00, or equivalent. Millipore Vacuum Pump Catalog # WP6111560 or equivalent.)

SAFETY PRECAUTIONS

- All 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. On disposal, flush with a large volume of water to prevent azide buildup.

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 an 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.
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Wash Buffer provided.
- 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 ("Standard 7") which may be stored at ≤ -20°C for 1 month and at ≤ -80°C for greater than one month.
- If sample values fall outside the dynamic range of the assay, further dilute the samples with the appropriate diluent and repeat the assay.
- Any unused, mixed Antibody-Immobilized Beads may be stored in the Bead Mixing Bottle at 2-8°C for up to one month.
- During the preparation of the working standards, be certain to mix the higher concentration well before making the next dilution. In addition, use a new tip for 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 aluminum foil or an 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.
- 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.

- When reading the assay on Luminex 200™, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 4 alignment discs. When reading the assay on FLEXMAP 3D™, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 1 alignment disc. When reading the assay on MAGPIX, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 2 alignment disc.
- For cell culture supernatants or tissue extraction, use the culture or extraction medium as the matrix solution in background, standard curve and control wells.
- 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 30 minutes at room temperature then centrifuge the samples for 10 minutes at 1000 x g. Finally, collect the serum samples and use them immediately in the assay or aliquot and store them at ≤ -20°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.
- Serum samples should be diluted 1:6 using the Serum Matrix provided in the kit as serum diluent. For a 1:6 dilution, add 15 µL of sample to 75 µL of Serum Matrix.
- For serum samples that require further dilution beyond 1:6, use the Serum Matrix provided in the kit for further dilution. Note: Matrix volume provided in the kit may be insufficient. Additional matrix may be obtained from Millipore.
- For data analysis, multiply the final concentration of each sample by the dilution factor.

A. 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 and assay immediately or aliquot and store samples at ≤ -20°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.

- Plasma samples should be diluted 1:6 using the Serum Matrix provided in the kit as sample diluent. For 1:6 dilution, add 15 µL of sample to 75 µL of serum matrix.
- For plasma samples that require further dilution beyond 1:6, use the Serum Matrix provided in the kit for further dilution. Note: Matrix volume provided in the kit may be insufficient. Additional matrix may be obtained from Millipore.
- For data analysis, multiply the final concentration of each sample by the dilution factor

B. Preparation of Tissue Culture Supernatant:

- Centrifuge the sample to remove debris and assay immediately or aliquot and store samples at ≤ -20°C.
- Avoid multiple (>2) freeze/thaw cycles.
- Tissue culture supernatant may require a dilution with an appropriate control medium prior to assay. Tissue/cell extracts should be done in neutral buffers containing reagents and conditions that do not interfere with assay performance. Excess concentrations of detergent, salt, denaturants, high or low pH, etc. will negatively affect the assay. Organic solvents should be avoided. The tissue/cell extract samples should be free of particles such as cells or tissue debris.

NOTE:

- A maximum of 25 µL per well of diluted serum or plasma can be used. Tissue culture or other media may also 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

Sonicate each individual antibody-bead vial for 30 seconds then vortex for 1 minute. Add 150 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. (Note:Due to the composition of magnetic beads, you may notice a slight color in the bead solution. This does not affect the performance of the beads or the kit.)

Example 1: When using 9 antibody-immobilized beads, add 150 µL from each of the 6 bead vials to the Mixing Bottle. Then add 1.65 mL Bead Diluent.

Example 2: When using 15 antibody-immobilized beads, add 150 µL from each of the 9 bead vials to the Mixing Bottle. Then add 0.75 mL Bead Diluent.

Example 3: When using ≥ 20 antibody-immoblilized beads, add 150 µL from each of the bead vials to the Mixing Bottle. No additional Bead Diluent should be added.

B. Preparation of Quality Controls

Before use, reconstitute Quality Control 1 and Quality Control 2 with 250 µL 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 ≤ -20°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°C for up to one month.

D. Preparation of Serum Matrix

This step is required for serum or plasma samples only.

Add 1.0 mL deionized water to vial containing lyophilized Serum Matrix. Mix well. Allow at least 10 minutes for complete reconstitution. Then add 5.0 ml of assay buffer in the same vial to get a final volume of 6 ml of Serum Matrix. Left-over reconstituted Serum Matrix can be stored at ≤ -20°C for up to one month.

E. Preparation of Human Circulating Cancer Biomarker Panel 1 Standard

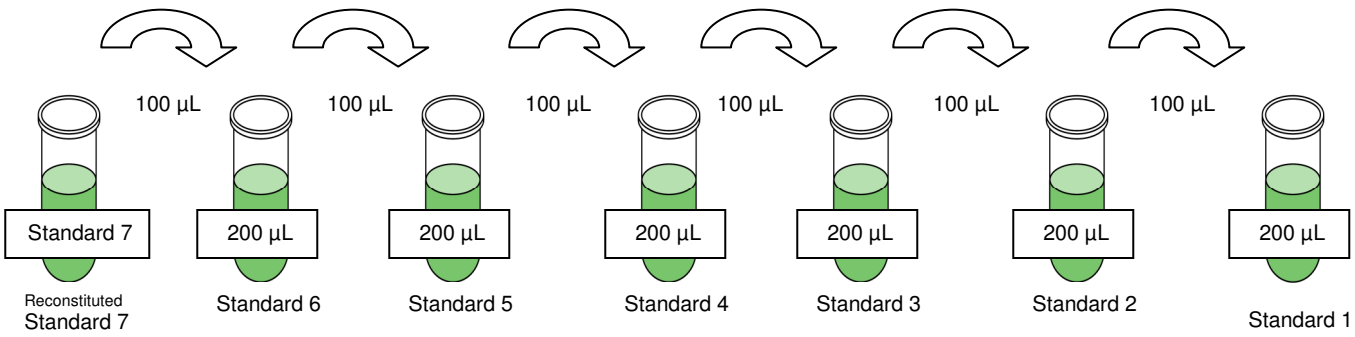
1.) Prior to use, reconstitute the Human Circulating Cancer Biomarker Panel 1 Standard with 250 µL deionized water (refer to table below for analyte concentrations). Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes and then transfer the standard to an appropriately labeled polypropylene microfuge tube. This will be used as Standard 7; the unused portion may be stored at ≤ -20°C for up to one month.

2.) Preparation of Working Standards

Label six polypropylene microfuge tubes as Standard 1, Standard 2, Standard 3, Standard 4, Standard 5, and Standard 6. Add 200 µL of Assay Buffer to each of the six tubes. Prepare 1:3 serial dilutions by adding 100 µL of the reconstituted Standard 7 to the Standard 6 tube, mix well and transfer 100 µL of the Standard 6 to the Standard 5 tube, mix well and transfer 100 µL of the Standard 5 to the Standard 4 tube, mix well and transfer 100 µL of the Standard 4 to Standard 3 tube, mix well and transfer 100 µL of the Standard 3 to the Standard 2 tube, mix well and transfer 100 µL of the Standard 2 to the Standard 1 tube and mix well. The 0 pg/mL standard (Background) will be the 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	200 µL	100 µL of Standard 7
Standard 5	200 µL	100 µL of Standard 6
Standard 4	200 µL	100 µL of Standard 5
Standard 3	200 µL	100 µL of Standard 4
Standard 2	200 µL	100 µL of Standard 3
Standard 1	200 µL	100 µL of Standard 2



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

Tube Number	Standard dilution	HE4 (pg/mL)	OPN (pg/mL)	AFP (pg/mL)	CYFRA 21-1 (pg/mL)	Leptin , Prolactin, SDF1α+β (pg/mL)	sFas (pg/mL)	MIF, CEA, HGF (pg/mL)	Total PSA, sFasL, FGF-2, VEGF (pg/mL)
1	1:729	685.8	548.6	342.9	205.7	137.1	34.3	27.4	13.7
2	1:243	2057.8	1646.1	1028.8	617.2	411.5	102.9	82.3	41.1
3	1:81	6172.8	4938.2	3086.4	1851.8	1234.5	308.7	246.9	123.4
4	1:27	18518.5	14814.8	9259.2	5555.5	3703.7	925.9	740.7	370.3
5	1:9	55555.5	44444.4	27777.7	16666.6	11111.1	2777.8	2222.2	1111.1
6	1:3	166666.6	133333.3	83333.3	50000	33333.3	8333.3	6666.6	3333.3
7	Original	500000	400000	250000	150000	100000	25000	20000	10000

Tube Number	Standard dilution	Free PSA (pg/mL)	SCF (pg/mL)	TRAIL, TGF-α (pg/mL)	IL-8, TNFα (pg/mL)	CA125 (U/mL)	CA19-9 (U/mL) IL-6 (pg/mL)	CA15-3 (U/mL)	β-HCG (mU/mL)
1	1:729	Refer to QC analysis sheet for exact concentration	6.9	2.7	1.3	0.8	0.68	0.13	0.09
2	1:243		20.6	8.2	4.1	2.5	2.05	0.4	0.27
3	1:81		61.8	24.6	12.3	7.7	6.2	1.2	0.8
4	1:27		185.2	74.1	37	23.1	18.5	3.7	2.5
5	1:9		555.5	222.2	111.1	69.4	55.5	11.1	7.4
6	1:3		1666.6	666.6	333.3	208.3	166.6	33.3	22.3
7	Original		5000	2000	1000	625	500	100	67

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.
- If using a filter plate, 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. Add 200 µL of Assay Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
2. Decant Assay Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
3. Add 25 µL of Assay Buffer to Background wells.
4. Add 25 µL of each Standard or Control into the appropriate wells.
5. Add 25 µL of appropriate matrix to Background, Standard 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 culture medium as the matrix solution.
6. Add 25 µL of Assay Buffer to sample wells.
7. Add 25 µL of 1:6 diluted Sample into the appropriate wells. When assaying serum or plasma, use the Serum Matrix provided in the kit to dilute the sample. When assaying tissue culture or other supernatant, use proper control culture medium as the diluent.
8. Vortex Mixing Bottle and add 25 µL of the Mixed Beads to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.)

Add 200 µL Assay Buffer per well

Shake 10 min, RT

Decant

- Add 25 µL Assay Buffer to Background wells
- Add 25 µL Standard or Control to appropriate wells
- Add 25 µL appropriate matrix to Background, Standard and Control wells
- Add 25 µL Assay Buffer to sample wells
- Add 25 µL 1:6 diluted Sample to sample wells
- Add 25 µL Beads to each well

9. Seal the plate with a plate sealer.. Wrap the plate with foil and incubate with agitation on a plate shaker overnight (16-18 hr) at 4°C
10. Gently remove well contents and wash plate 3 times following instructions listed in the **PLATE WASHING** section .
11. Add 25 µL of Detection Antibodies into each well.
12. Seal, cover with foil, and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C). **DO NOT ASPIRATE AFTER INCUBATION.**
13. Add 25 µL Streptavidin-Phycoerythrin to each well containing the 25 µL of Detection Antibodies.
14. Seal, cover with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).
15. Gently remove well contents and wash plate 3 times following instructions listed in the **PLATE WASHING** section.

Incubate overnight at 4°C with shaking (16 – 18 hours)

Remove well contents and wash 3X with 200 µL Wash Buffer

Add 25 µL Detection Antibodies per well

Incubate 1 hour at RT

Do not aspirate

Add 25 µL Streptavidin-Phycoerythrin per well

Incubate for 30 minutes at RT

Remove well contents and wash 3X with 200µL Wash Buffer

16. Add 100 µL of Sheath Fluid (or Drive Fluid if using MAGPIX®) to all wells. Resuspend the beads on a plate shaker for 5 minutes.
17. Run plate on Luminex 200™ , HTS, FLEXMAP 3D™ or MAGPIX® with xPONENT software.
18. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating analyte concentrations in samples. (Note: Multiply the calculated concentration of the samples by the dilution factor, which is 6.)

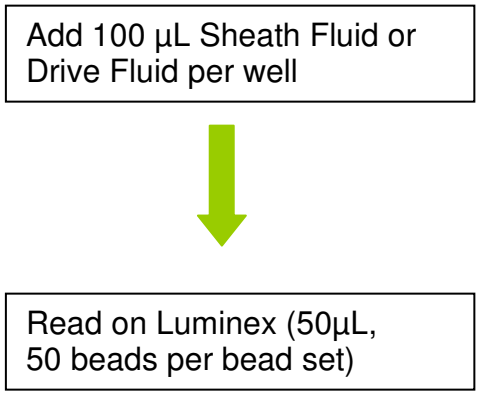


PLATE WASHING

1.) Solid Plate
If using a solid plate, use either a hand-held magnet or magnetic plate washer.

A.) For hand-held magnet, rest plate on magnet for 60 seconds to allow complete settling of magnetic beads. Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid. Wash plate with 200 uL of Wash Buffer by removing plate from magnet, adding Wash Buffer, shaking for 30 seconds, reattaching to magnet, letting beads settle for 60 seconds and removing well contents as previously described after each wash. Repeat wash steps as recommended in Assay Procedure.

B.) For magnetic plate washer, let plate "soak" on magnet for 60 seconds to allow complete settling of the magnetic beads. Remove well contents by aspiration. Wash plate with 200 µL/well of Wash Buffer, letting beads "soak" for 60 seconds and removing Wash Buffer by aspiration after each wash. Repeat wash steps as recommended in Assay Procedure. **Note:** If using the recommended plate washer for magnetic beads (Bio-Tek ELx405) follow the appropriate equipment settings outlined in **EQUIPMENT SETTINGS.**

2.) Filter Plate (Millipore Cat# MX-PLATE)
If using a filter plate, use a vacuum filtration manifold to remove well contents. Wash plate 200 µL/well of Wash Buffer, removing Wash Buffer by vacuum filtration after each wash Repeat wash steps as recommended in the Assay Procedure.

EQUIPMENT SETTINGS

Bio-Tek ELx405:

The general recommended wash protocol (Link Protocol) is as follows:

Soak Program:	Wash Program:
Soak →	Aspirate→Dispense→Soak→Aspirate→Dispense→Soak→Aspirate →Dispense→Soak→Aspirate

1.) Soak program:
   1. Soak duration: 60 sec
   2. Shake before soak?: NO

2.) Wash program:

Method:
   1. Number of cycles: 3
   2. soak/shake: YES
   3. Soak duration: 60 sec
   4. Shake before soak: NO
   5. Prime after soak: NO

Dispense:
   1. Dispense volume: 200 µL/well
   2. Dispense flow rate: 5
   3. Dispense height: 130 (16.51 mm)
   4. Horizontal disp pos: 00 (0 mm)
   5. Bottom Wash first?: NO
   6. Prime before start?: NO

Aspiration:
   1. Aspirate height: 35 (4.445 mm)
   2. Horizontal Asp Pos: 30 (1.372 mm)
   3. Aspiration rate: 06 (15.0 mm/sec)
   4. Aspiration delay: 0
   5. Crosswise Aspir: NO
   6. Final Aspir: YES
   7. Final Aspir delay: 0 (0 msec)

3.) Link program: (**Note:** this is the program to use during actual plate washing). Link together the Soak and Wash programs outlined above.

Note: 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.

If using an automatic plate washer other than BioTek ELx405, please refer to the manufacturers's recommendations for programming instructions.

These specifications are for the Luminex 200™ xPONENT™, FlexMAP 3D™, MAGPIX® and Luminex HTS. Luminex instruments with other software (e.g. MasterPlex, StarStation, LiquiChip, Bio-Plex, LABScan100) would need to follow instrument instructions for gate settings and additional specifications from the vendors for reading Luminex Magnetic Beads.

For magnetic bead assays, the Luminex 200™and HTS instruments must be calibrated with the xPonent 3.1 compatible Calibration Kit (Millipore Cat.# 40-275) and performance verified by Verification Kit (Millipore Cat. # 40-276). The Luminex FlexMAP 3D™instrument must be calibrated with the FlexMAP 3D™ Calibration Kit (Millipore cat# 40-028) and performance verified with the FlexMAP 3D™ Performance Verification Kit (Millipore cat# 40-029). The Luminex MAGPIX® instrument must be calibrated with the MAGPIX® Calibration Kit (Millipore cat# 40-049 and performance verified with the MAGPIX® Performance Verification Kit (Millipore cat# 40-050).

Note: These assays cannot be run on the Luminex IS 2.3 or Luminex 1.7 instruments.

The Luminex probe height must be adjusted to the plate provided in the kit. Please use Cat.# MAG-PLATE, if additional plates are required for this purpose.

Events:	50 per bead	
Sample Size:	50 µL	
Gate Settings	8,000 to 15,000	
Reporter Gain:	Default (low PMT)	
Time Out	60 seconds	
Bead Set:	Bead regions	
	AFP	12
	Total PSA	13
	CA15-3	15
	CA19-9	18
	MIF	20
	TRAIL	27
	Leptin	28
	Free PSA	29
	IL-6	34
	sFasL	37
	CEA	39
	CA125	42
	IL-8	44
	HGF	45
	SDF1α+β	48
	sFas	52
	TNFα	55
	Prolactin	56
	SCF	61
	CYFRA 21-1	63
	OPN	64
	FGF2	67
	β-HCG	73
	HE4	75
	TGFα	76
	VEGF	78

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

Cross-Reactivity

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

Assay Sensitivities (minimum detectable concentrations, pg/mL)

MinDC: Minimum Detectable Concentration is calculated using 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	***MinDC + 2SD (pg/ml)***	***MinDC + 2SD* (U/ml)**	***MinDC + 2SD* (mU/ml)**
AFP	83.4		
Total PSA	2.0		
MIF	7.6		
TRAIL	0.5		
Leptin	42.8		
Free PSA	1.4		
IL-6	0.2		
sFasL	3.7		
CEA	5.2		
IL-8	0.3		
HGF	6.8		
SDF1α+β	33.9		
sFas	8.4		
TNFα	0.3		
Prolactin	30.2		
SCF	2.0		
CYFRA 21-1	59.3		
OPN	285.3		
FGF2	3.6		
HE4	193.5		
TGFα	0.5		
VEGF	6.4		

CA15-3		0.03	
CA19-9		0.3	
CA125		0.2	
β-HCG			29.5

N=7 assays

Precision

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

Analyte	***Intra- assay %CV***	***Inter- assay %CV***
AFP	6	6.7
Total PSA	9	5.6
CA15-3	15	8.2
CA19-9	10.8	7.8
MIF	7.7	10.3
TRAIL	6.7	4.1
Leptin	4.9	6.7
Free PSA	8.9	7.6
IL-6	9.3	5.5
sFasL	7.0	6.4
CEA	11.3	4.6
CA125	6.5	5.3
IL-8	6.8	5.5
HGF	8.7	8.8
SDF1α+β	12.2	15.4
sFas	6.5	7.1
TNFα	7.9	7.5
Prolactin	7.6	5.5
SCF	10.4	7.5
CYFRA 21-1	6.7	16.2
OPN	8.9	6.0
FGF2	7.9	6.3
β-HCG	7.5	5.3
HE4	9.3	6.4
TGFα	8.7	4.9
VEGF	12	10.2

Accuracy

Spike Recovery: The data represent mean percent recovery of 3 levels of spiked standards in serum samples (n=5).

Analyte	***Spike Recovery in Serum***
AFP	86
Total PSA	59
CA15-3	77
CA19-9	58
MIF	50
TRAIL	91
Leptin	81
Free PSA	59
IL-6	89
sFasL	63
CEA	74
CA125	68
IL-8	51
HGF	77
SDF1α+β	35
sFas	69
TNFα	70
Prolactin	108
SCF	68
CYFRA 21-1	97
OPN	71
FGF2	85
β-HCG	69
HE4	77
TGFα	76
VEGF	74

Cell Culture Analysis

The following human cell lines were cultured according to the recommendations of the ATCC to around 80% confluence; then the respective media was centrifuged and run in the assay.

- A431– Epidermoid Carcinoma
- Daudi – Burkitt's lymphoma
- HeLa – Cervical Adenocarcinoma
- HepG2 – Hepatocellular Carcinoma
- HuVec – Umbilical vein endothelial cell
- Jurkat – Lymphoblast
- LnCap – Prostate Adenocarcinoma
- PC-3 – Prostate Adenocarcinoma
- SW116 – Colon Carcinoma
- ZR75-1– Mammary gland Carcinoma

	A431	Daudi	HeLa	HepG2	HuVec	Jurkat	LnCap	PC-3	SW116	ZR75-1
AFP				H						L
CA125	L	L	M						L	L
CA15-3										L
CA19-9	L	L		L					M	M
CEA	L							L	H	M
CYFRA21	L		L	H	L			M	H	H
FGF2					M					
β-HCG	M		L						L	L
HE4										L
HGF										
IL-6	L		M		H			M	L	L
IL-8	L		L	M	H		L	M	M	L
Leptin										L
MIF		H			H	M	M		M	H
OPN	L	L	L	H		L			L	L
Prolactin		L								L
Total PSA							H			L
sFas				L	L		L		L	L
sFasL									L	M
SCF										L
TGFα				L				L	L	L
TNFα		L							L	L
TRAIL	L				L					L
VEGF	M	L	L	H			H	L	L	M

Blank- Not detected

L - Values in the lower third of the respective analyte standard curve

M - Values in the mid third of the respective analyte standard curve

H - Values in the upper third of the respective analyte standard curve

TROUBLESHOOTING GUIDE

Problem	Probable Cause	Solution
Insufficient Bead Count	Plate Washer aspirate height set too low	Adjust aspiration height according to manufacturers instructions.
	Bead mix prepared inappropriately	Sonicate bead vials and vortex just prior to adding to bead mix bottle according to protocol. Agitate bead mix intermittently in reservoir while pipetting this into the plate.
	Samples cause interference due to particulate matter or viscosity	See above. Also sample probe may need to be cleaned with Alcohol flush, Back flush and washes; or if needed probe should be removed and sonicated.
	Probe height not adjusted correctly	When reading the assay on Luminex 200™, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 4 alignment discs. When reading the assay on FLEXMAP 3D™, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 1 alignment disc. When reading the assay on MAGPIX, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 2 alignment disc.
Background is too high	Background wells were contaminated	Avoid cross-well contamination by using sealer appropriately, and pipeting with Multichannel pipets without touching reagent in plate.
	Matrix used has endogenous analyte or interference	Check matrix ingredients for cross reacting components (e.g. interleukin modified tissue culture medium).
	Insufficient washes	Increase number of washes.
Beads not in region or gate	Luminex not calibrated correctly or recently	Calibrate Luminex based on Instrument Manufacturer's instructions, at least once a week or if temperature has changed by >3^{o}C.
	Gate Settings not adjusted correctly	Some Luminex instruments (e.g. Bioplex) require different gate settings than those described in the Kit protocol. Use Instrument default settings.
	Wrong bead regions in protocol template	Check kit protocol for correct bead regions or analyte selection.
	Incorrect sample type used	Samples containing organic solvents or if highly viscous should be diluted or dialyzed as required.
	Instrument not washed or primed	Prime the Luminex 4 times to rid of air bubbles, wash 4 times with sheath fluid or water if there is any remnant alcohol or sanitizing liquid.
	Beads were exposed to	Keep plate and bead mix covered with dark

	light	lid or aluminum foil during all incubation steps.
Signal for whole plate is same as background	Incorrect or no Detection Antibody was added	Add appropriate Detection Antibody and continue.
	Streptavidin-Phycoerythrin was not added	Add Streptavidin-Phycoerythrin according to protocol. If Detection Antibody has already been removed, sensitivity may be low.
Low signal for standard curve	Detection Antibody may have been removed prior to adding Streptavidin Phycoerythrin	May need to repeat assay if desired sensitivity not achieved.
	Incubations done at inappropriate temperatures, timings or agitation	Assay conditions need to be checked.
Signals too high, standard curves are saturated	Calibration target value set too high	With some Luminex Instrument (e.g. Bio-plex) Default target setting for RP1 calibrator is set at High PMT. Use low target value for calibration and reanalyze plate.
	Plate incubation was too long with standard curve and samples	Use shorter incubation time.
Sample readings are out of range	Samples contain no or below detectable levels of analyte	If below detectable levels, it may be possible to use higher sample volume. Check with tech support for appropriate protocol modifications.
	Samples contain analyte concentrations higher than highest standard point.	Samples may require dilution and reanalysis for just that particular analyte.
	Standard curve was saturated at higher end of curve.	See above.
High Variation in samples and/or standards	Multichannel pipet may not be calibrated	Calibrate pipets.
	Plate washing was not uniform	Confirm all reagents are removed completely in all wash steps.
	Samples may have high particulate matter or other interfering substances	See above.
	Plate agitation was insufficient	Plate should be agitated during all incubation steps using a vertical plate shaker at a speed where beads are in constant motion without causing splashing.
	Cross well contamination	Check when reusing plate sealer that no reagent has touched sealer. Care should be taken when using same pipet tips that are used for reagent additions and that pipet tip does not touch reagent in plate.
FOR FILTER PLATES ONLY		
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.
	High lipid concentration	After centrifugation, remove lipid layer and

		use supernatant.
Plate leaked	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 (blocked) plate and continue.
	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 plate.
	Probe height not adjusted correctly	Adjust probe to 3 alignment discs in well H6.
	Sample too viscous	May need to dilute sample.

REPLACEMENT REAGENTS

Components	Cat #
Human Circulating Cancer Biomarker Panel 1 Standard	HCC-8058
Human Circulating Cancer Biomarker Panel 1 Quality Controls 1 and 2	HCC-6058
Serum Matrix	HCP-SM
Human Circulating Cancer Biomarker Panel 1 Detection Antibodies	HCC-1058 or HCC-1058-2
Streptavidin-Phycoerythrin	L-SAPE 4
Assay Buffer	L-AB
Bead Diluent	LBD-4
96-Well Plate with two Sealers	MAG-PLATE
10X Wash Buffer	L-WB

Antibody-Immobilized Beads

Analyte	Bead #	Cat. #
AFP	12	HC1AFP-MAG
Total PSA	13	HTPSA-MAG
CA15-3	15	HCA153-MAG
CA19-9	18	HCA199-MAG
MIF	20	HMIF-MAG
TRAIL	27	HTRAIL-MAG
Leptin	28	HCCLPTN-MAG
Free PSA	29	HFPSA-MAG
IL-6	34	HIL6-MAG
sFasL	37	HSFASLG-MAG
CEA	39	HCEA-MAG
CA125	42	HCA125-MAG
IL-8	44	HIL8-MAG
HGF	45	HHGF-MAG
SDF1α+β	48	HSDF-MAG
sFas	52	HSFAS-MAG
TNFα	55	HTNFA-MAG
Prolactin	56	HCCPRLCTN-MAG
SCF	61	HSCF-MAG
CYFRA 21-1	63	HCYFRA-MAG
OPN	64	H0PN-MAG
FGF2	67	HFGF2-MAG
β-HCG	73	BHCG-MAG
HE4	75	HHE4-MAG
TGFα	76	HTGFA-MAG
VEGF	78	HVEGF-MAG

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Material Safety Data Sheets (MSDS)

Material Safety Data Sheets for Millipore products may be ordered by fax or phone or through our website at www.millipore.com/techlibrary/index.do

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	Etc.								
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	Sample 1									
F	Standard 2	Standard 6	Sample 1									
G	Standard 3	Standard 7	Sample 2									
H	Standard 3	Standard 7	Sample 2									