Human Immuno-Oncology Checkpoint Protein Panel 2

96-Well Plate Assay

Cat. # HCKP2-11K

MILLIPLEX® MAP

HUMAN IMMUNO-ONCOLOGY CHECKPOINT PROTEIN PANEL 2
96-Well Plate Assay

 # HCKP2-11K

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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, FLEXMAP 3D®, MAGPIX®.

INTRODUCTION

Immune checkpoint inhibitors have been proven to be an effective method in improving antitumor immune response and saving lives. Many immune checkpoint proteins are expressed as soluble forms in circulation and in the tumor and tumor microenvironment, as putative immune-oncology biomarkers.

MILLIPLEX® MAP offers the broadest selection of analytes across a wide range of disease states and species. Once the analytes of interest have been identified, you can rely on the quality that we build into each kit to produce results you can trust. In addition to the assay characteristics listed in the protocol, other performance criteria evaluated during the validation process include: cross-reactivity, dilution linearity, kit stability, and sample behavior (e.g. detectability and stability).

Each MILLIPLEX® MAP panel and kit includes:

- Quality controls (QCs) provided to qualify assay performance
- Comparison of standard (calibrator) and QC lots to a reference lot to ensure lot-to-lot consistency
- Optimized serum matrix to mimic native analyte environment
- Detection antibody cocktails designed to yield consistent analyte profiles within panel

In addition each panel and kit meets stringent manufacturing criteria to ensure batch-to-batch reproducibility. The MILLIPLEX® MAP Human Immuno-Oncology Checkpoint Protein Panel 2 thus enables you to focus on the therapeutic potential of immuno-oncology checkpoint proteins. 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 dozens of analytes simultaneously, which can dramatically improve productivity.

EMD Millipore's MILLIPLEX® MAP Human Immuno-Oncology Checkpoint Protein Panel 2 is part of the most versatile system available for immuno-oncology research. From our single to multiplex biomarker solutions, we partner with you to design, develop, analytically validate and build the most comprehensive library available for protein detection and quantitation.

- MILLIPLEX® MAP offers you:
    - The ability to choose any combination of analytes from our panel of 31 analytes to design a custom kit that better meets your needs.
    - A convenient "all-in-one" box format that gives you the assurance that you will have all the necessary reagents you need to run your assay.

EMD Millipore's MILLIPLEX® MAP Human Immuno-Oncology Checkpoint Protein Panel 2 is a 31-plex kit to be used for the simultaneous quantification of any or all of the following analytes in serum, plasma and tissue culture samples: 4-1BBL/TNFSF9, 5'-NT/CD73 (5'-nucleotidase), APRIL (TNFSF13), Arginase-1, B7-H2/ICOSL (Inducible T cell costimulator ligand), B7-H3/CD276, B7-H4/VTCN1 (V-set domain-containing T-cell activation inhibitor 1), B7-H5/VISTA (V-set immunoregulatory receptor), B7-H6 (Natural killer cell cytotoxicity receptor 3 ligand), BAFF/BLyS (TNFSF13B), CD25/IL-2Rα (Interleukin-2 receptor alpha), CD30/TNFRSF8, CD40L (CD40 ligand), CD137/4-1BB (TNFRSF9), CD226/DNAM-1 (DNAX accessory molecule 1), E-Cadherin, FGL1/Hepassocin (Fibrinogen-like protein 1), Galectin-1 (Gal-1), Galectin-3 (Gal-3), Granulysin, Granzyme B, IDO1 (Indoleamine 2,3-dioxygenase 1), MICA (MHC class I polypeptide-related sequence A), MICB (MHC class I polypeptide-related sequence B), Nectin-2 (PVRL2, CD112), Nectin-4 (PVRL4), OX40/CD134 (TNFRSF4), Perforin, PVR/CD155 (Poliovirus receptor cell adhesion molecule), Siglec-7 (Sialic acid-binding Ig-like lectin 7, CD328), Siglec-9 (Sialic acid-binding Ig-like lectin 9, CD329).

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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, distinctly colored bead sets of 500 5.6 µm polystyrene microspheres or 80 6.45 µm magnetic microspheres 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.
- EMD Millipore provides three Luminex® instruments to acquire and analyze data using two detection methods:
    - The Luminex® analyzers Luminex® 200™ and FLEXMAP 3D®, flow cytometry-based instruments that integrate key xMAP® detection components, such as lasers, optics, advanced fluidics and high-speed digital signal processors.
    - The Luminex® analyzer (MAGPIX®), a CCD-based instrument that integrates key xMAP® capture and detection components with the speed and efficiency of magnetic beads.
- Each individual microsphere is identified and the result of its bioassay is quantified based on fluorescent reporter signals. EMD Millipore combines the streamlined data acquisition power of Luminex® xPONENT® acquisition software with sophisticated analysis capabilities of the new MILLIPLEX® Analyst 5.1, integrating data acquisition and analysis seamlessly with all Luminex® instruments.

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.
- 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 Antibody, and Streptavidin-Phycoerythrin**.

REAGENTS SUPPLIED

Note: Store all reagents at 2 – 8°C

Reagents Supplied	Catalog Number	Volume	Quantity
Human I-O Checkpoint Protein Panel 2 Standard	HCKP2-8011	Lyophilized	1 vial
Human I-O Checkpoint Protein Panel 2 Quality Controls 1 and 2	HCKP2-6011	Lyophilized	1 vial each
Serum Matrix Note: Contains 0.08% Sodium Azide	HCKP-SM	Lyophilized	1 vial
Set of one 96-Well Plate with 2 sealers	----------	----------	1 plate 2 sealers
Assay Buffer	L-AB	30 mL	2 bottles
Bead Diluent	LBD-4	3.5 mL	1 bottle
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	60 mL	1 bottle
Human I-O Checkpoint Protein Panel 2 Detection Antibodies	HCKP2-1011	3.2 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE4	3.2 mL	1 bottle
Mixing Bottle	----------	----------	1 bottle

Included Human Immuno-Oncology Checkpoint Protein Panel 2 Antibody-Immobilized Beads are dependent on customizable selection of analytes within the panel (see next page).

Human Immuno-Oncology Checkpoint Protein Panel 2 Antibody-Immobilized Magnetic Beads:

Bead/Analyte Name	Luminex® Magnetic Bead Region	Customizable 31 Analytes (50X concentration,90 µL) Available	Cat. #
Anti-Human CD40L Bead	12	✔	HCD40L-MG
Anti-Human 4-1BBL/TNFSF9 Bead	18	✔	H41BBL-MAG
Anti-Human Arginase-1 Bead	20	✔	HARG1-MAG
Anti-Human B7-H2/ICOSL Bead	22	✔	HB7H2-MAG
Anti-Human B7-H3/CD276 Bead	25	✔	HB7H3-MAG
Anti-Human 5'-NT/CD73 Bead	26	✔	HNT5E-MAG
Anti-Human B7-H4/VTCN1 Bead	27	✔	HB7H4-MAG
Anti-Human APRIL Bead	29	✔	HI0APRIL-MAG
Anti-Human B7-H5/VISTA Bead	30	✔	HB7H5-MAG
Anti-Human CD25/IL-2Rα Bead	33	✔	HSIL2RA-MAG
Anti-Human B7-H6 Bead	34	✔	HB7H6-MAG
Anti-Human CD137/4-1BB Bead	35	✔	HCD137-MAG
Anti-Human Granzyme B Bead	37	✔	HCDGRNZMB-MAG
Anti-Human CD226/DNAM-1 Bead	38	✔	HCD226-MAG
Anti-Human CD30/TNFRSF8 Bead	39	✔	HI0CD30-MAG
Anti-Human E-Cadherin Bead	42	✔	HECAD-MAG
Anti-Human FGL1/Hepassocin Bead	43	✔	HFGL1-MAG
Anti-Human Galectin-1 Bead	44	✔	HI0GAL1-MAG
Anti-Human Galectin-3 Bead	45	✔	HI0GAL3-MAG
Anti-Human Granulysin Bead	46	✔	HGNLY-MAG
Anti-Human IDO1 Bead	47	✔	HID01-MAG
Anti-Human MICA Bead	48	✔	HMICA-MAG
Anti-Human MICB Bead	51	✔	HMICB-MAG
Anti-Human Nectin-2 Bead	55	✔	HNTN2-MAG
Anti-Human BAFF/BLyS Bead	56	✔	HI0BAFF-MAG
Anti-Human Nectin-4 Bead	61	✔	HNTN4-MAG
Anti-Human OX40/CD134 Bead	65	✔	H0X40-MAG
Anti-Human PVR/CD155 Bead	66	✔	HPVR-MAG
Anti-Human Siglec-7 Bead	67	✔	HSGL7-MAG
Anti-Human Siglec-9 Bead	72	✔	HSGL9-MAG
Anti-Human Perforin Bead	78	✔	HPRFRN-MAG

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex® Sheath Fluid (EMD Millipore Catalog # 40-50018 (1L) or 40-50015 (20L)) or Luminex® Drive Fluid (EMD Millipore Catalog # 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. Rubber Bands
6. Aluminum Foil
7. Absorbent Pads
8. Laboratory Vortex Mixer
9. Sonicator (Branson Ultrasonic Cleaner Model # B200 or equivalent)
10. Titer Plate Shaker (VWR® Microplate Shaker Cat # 12620-926 or equivalent)
11. Luminex® 200™, HTS, FLEXMAP 3D®, or MAGPIX® with xPONENT® software by Luminex® Corporation
12. Automatic Plate Washer for magnetic beads (BioTek® 405 LS and 405 TS, EMD Millipore Catalog #40-094, # 40-095, # 40-096, # 40-097 or equivalent) or Handheld Magnetic Separation Block (EMD Millipore Catalog # 40-285 or equivalent).

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

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.
- Full Hazard Label- see next page.

Full Hazard Label:

Ingredient, Cat #		Full Label	
10X Wash Buffer	L-WB		**Warning.** May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water.
Bead Diluent	LBD-4		**Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Streptavidin-Phycoerythrin	L-SAPE4		**Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Hu I-O Checkpoint Protein Panel 2 Standard/Hu I-O Checkpoint Panel 2 QC1 & QC2	HCKP2-8011/HCKP2-6011	  	**Danger**. Harmful if swallowed or if inhaled. Toxic in contact with skin. Causes serious eye damage. May cause damage to brain through prolonged or repeated exposure. Do not breathe dust/ fume/ gas/ mist/ vapours/ spray. Wash skin thoroughly after handling. Do not eat, drink or smoke when using this product. Use only outdoors or in a well-ventilated area. Wear protective gloves/ eye protection/ face protection. IF SWALLOWED: Call a POISON CENTER/doctor if you feel unwell. IF ON SKIN: Wash with plenty of soap and water. IF INHALED: Remove person to fresh air and keep comfortable for breathing. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a POISON CENTER/doctor. Specific measures (see supplemental first aid instructions on this label). Rinse mouth. Remove/Take off immediately all contaminated clothing. Wash contaminated clothing before reuse. Store locked up. Dispose of contents/ container to an approved waste disposal plant.

Hu I-O Checkpoint Protein Panel 2 Detection	HCKP2-1011		**Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Serum Matrix	HCKP-SM	no symbol required.	Harmful to aquatic life with long lasting effects. Avoid release to the environment.

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℃) before use in the assay.
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Wash Buffer provided.
- 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 ≤ -20℃ for 1 month and at ≤ -80℃ 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.
- Any unused mixed Antibody-Immobilized Beads may be stored in the Mixing Bottle at 2-8℃ for up to one month.
- 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 aluminum foil or an opaque lid, and store the plate at 2-8℃ 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.

TECHNICAL GUIDELINES (continued)

- When reading the assay on Luminex® 200™, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate or to the recommended EMD Millipore filter plates using 2 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.

  For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid in each well and 75 µL should be aspirated.
- 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 that require further dilution beyond 1:2 use the Assay Buffer 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. <u>Preparation of Serum Samples:</u>
   - 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 ≤ -20℃.
   - 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 1:2 in theAssay Buffer provided in the kit. For example, in a tube, 30 µL of serum may be combined with 30 µL of Assay Buffer When further dilution beyond 1:2 is required, use Assay Buffer as the diluent.

B. <u>Preparation of Plasma Samples:</u>
   - Plasma collection using EDTA as an anti-coagulant 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℃.
   - 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 1:2 in the Assay Buffer provided in the kit. For example, in a tube, 30 µL of plasma may be combined with 30 µL of Assay Buffer. When further dilution beyond 1:2 is required, use Assay Buffer as the diluent.

SAMPLE COLLECTION AND STORAGE (continued)

C. 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 1:2 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 anti-coagulant 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

For individual vials of beads, sonicate each antibody-bead vial for 30 seconds; vortex for 1 minute. Add 60 µL from each antibody-bead vial to the Mixing Bottle and bring final volume to 3.0 mL with Bead Diluent. Vortex the mixed beads well. Unused portion may be stored at 2-8°C for up to one month. (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 31 antibody-immobilized beads, add 60 µL from each of the 31 bead vials to the Mixing Bottle. Then add 1.14 mL Bead Diluent.

Example 2: When using 15 antibody-immobilized beads, add 60 µL from each of the 15 bead vials to the Mixing Bottle. Then add 2.1 mL Bead diluent.

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 and vortex. Allow the vial to sit for 5-10 minutes. Unused portion 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 60 mL of 10X Wash Buffer with 540 mL deionized water. Store the 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 mL deionized water to the bottle containing lyophilized Serum Matrix. Mix well. Allow at least 10 minutes for complete reconstitution then add 1 mL Assay Buffer. Leftover reconstituted Serum Matrix should be stored at ≤ -20℃ for up to one month.

E. Preparation of Human I-O Checkpoint Protein Panel 2 Standard

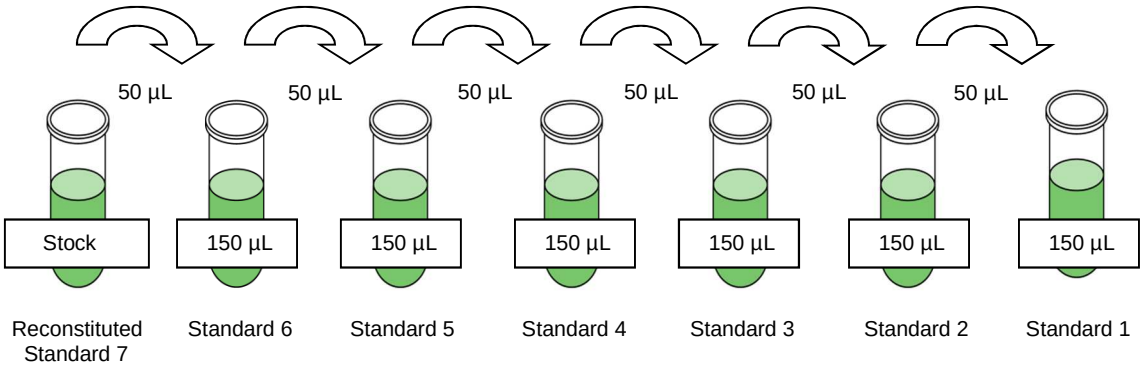
1.) Prior to use, reconstitute the Human I-O Checkpoint Protein Panel 2 Standard with 250 µL deionized water. Refer to table below for analyte concentrations. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes. This will be used as "Standard 7"; the unused portion may be stored at ≤ -20℃ for up to one month.

2). Preparation of Working Standards

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

Standard #	Volume of Deionized Water to Add	Volume of Standard to Add
Standard 7	250 µL	0

Standard #	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



Standard	Arginase-1 (pg/mL)	B7-H3/ CD276, CD226/ DNAM-1 (pg/mL)	B7-H4/ VTCN1 (pg/mL)	MICB (pg/mL)	PVR/ CD155 (pg/mL)	Granulysin, CD40L, Siglec-7 (pg/mL)
Standard 1	29.3	195	391	104	85.4	3.66
Standard 2	117	781	1,563	415	342	14.6
Standard 3	469	3,125	6,250	1,660	1,367	58.6
Standard 4	1,875	12,500	25,000	6,641	5,469	234
Standard 5	7,500	50,000	100,000	26,563	21,875	938
Standard 6	30,000	200,000	400,000	106,250	87,500	3,750
Standard 7	120,000	800,000	1,600,000	425,000	350,000	15,000

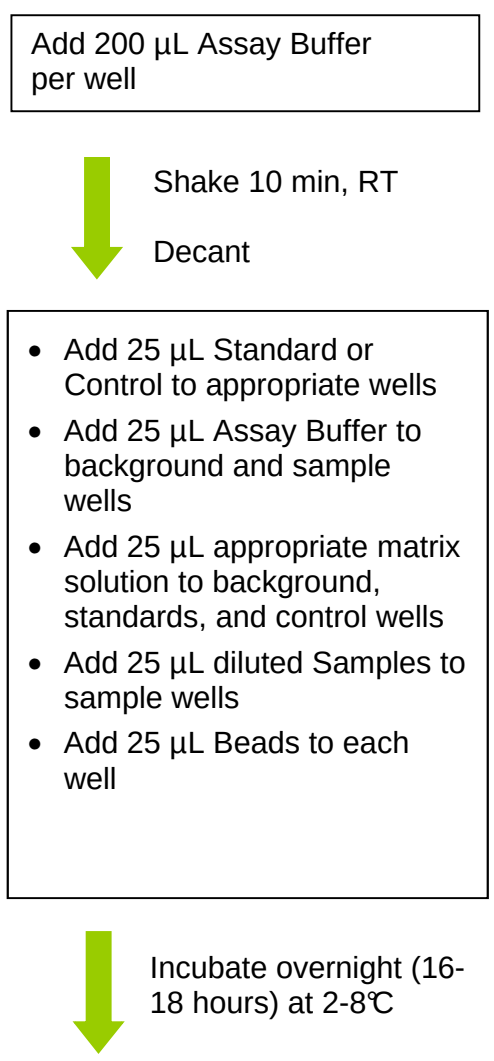
Standard	B7-H5/ VISTA (pg/mL)	Granzyme B (pg/mL)	B7-H6 (pg/mL)	IDO1 (pg/mL)	E-Cadherin (pg/mL)	Galectin-1 (pg/mL)	APRIL, Siglec-9, CD137/4-1BB (pg/mL)
Standard 1	18.3	1.22	6.10	36.6	366	61.0	2.44
Standard 2	73.2	5	24.4	146.5	1465	244	9.77
Standard 3	293	20	97.7	586	5859	977	39.1
Standard 4	1,172	78	391	2,344	23,438	3,906	156
Standard 5	4,688	313	1,563	9,375	93,750	15,625	625
Standard 6	18,750	1,250	6,250	37,500	375,000	62,500	2,500
Standard 7	75,000	5,000	25,000	150,000	1,500,000	250,000	10,000

Standard	5'-NT/CD73, Nectin-2, Nectin-4, Perforin, CD30/ TNFRSF8, CD25/ IL-2Rα (pg/mL)	B7-H2/ ICOSL, MICA, BAFF/ BLyS, OX40/ CD134 (pg/mL)	4-1BBL/ TNFSF9, FGL1/ Hepassocin, Galectin-3 (pg/mL)
Standard 1	24.4	12.2	48.8
Standard 2	97.7	48.8	195
Standard 3	391	195	781
Standard 4	1,563	781	3,125
Standard 5	6,250	3,125	12,500
Standard 6	25,000	12,500	50,000
Standard 7	100,000	50,000	200,000

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℃) before use in the assay.
- Diagram the placement of Standards [0 (Background), Standard 1 through 7, Controls 1 and 2, and Samples on Well Map Worksheet in a vertical configuration. (Note: Most instruments will only read the 96-well plate vertically by default.) It is recommended to run the assay in duplicate.
- 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℃).
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 each Standard or Control into the appropriate wells. Assay Buffer should be used for 0 pg/mL standard (Background).
4. Add 25 µL of Assay Buffer to the sample wells.
5. Add 25 µL of appropriate matrix solution to the background, standards, and control wells. When assaying serum or plasma, use the Serum Matrix. When assaying tissue culture or other supernatant, use proper control culture medium as the matrix solution.
6. Add 25 µL of Sample (diluted) into the appropriate wells.
7. Vortex Mixing Bottle and add 25 µL of the Mixed or Premixed Beads to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.)
8. Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker overnight (16-18 hours) at 2-8℃.



9. Gently remove well contents and wash plate 3 times following instructions listed in the **PLATE WASHING** section.
10. Add 25 µL of Detection Antibodies into each well. (Note: Allow the Detection Antibodies to warm to room temperature prior to addition.)
11. Seal, cover with foil and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25℃). **DO NOT ASPIRATE AFTER INCUBATION.**
12. Add 25 µL Streptavidin-Phycoerythrin to each well containing the 25 µL of Detection Antibodies.
13. Seal, cover with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25℃).
14. Gently remove well contents and wash plate 3 times following instructions listed in the **PLATE WASHING** section.
15. 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.
16. Run plate on Luminex® 200™, HTS, FLEXMAP 3D® or MAGPIX® with xPONENT® software.
17. 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: For diluted samples, final sample concentrations should be multiplied by the dilution factor. For samples diluted as per protocol instructions, multiply by 2. If using another dilution factor, multiple by the appropriate dilution factor.)

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 200 µL Wash Buffer

Add 100 µL Sheath Fluid or Drive Fluid per well

Read on Luminex® (50 µL, 50 beads per bead set)

PLATE WASHING

If using a solid plate, use either a handheld magnet or magnetic plate washer.

1.) Solid Plate

A.) Handheld magnet **(EMD Millipore Catalog #40-285)** - 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 µL 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.) Magnetic plate washer **(EMD Millipore Catalog #40-094, #40-095, #40-096 and #40-097)** - Please refer to specific automatic plate washer manual for appropriate equipment settings. Please note that after the final aspiration, there will be approximately 25 µL of residual wash buffer in each well. This is expected when using the BioTek® plate washer and this volume does not need to be aspirated from the plate.

If using an automatic plate washer other than BioTek® 405 LS or 405 TS, please refer to the manufacturer's recommendations for programming instructions.

2.) Filter Plate (EMD Millipore Catalog #MX-PLATE)

If using a filter plate, use a vacuum filtration manifold to remove well contents. Wash plate with 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

Luminex® 200™, HTS, FLEXMAP 3D®, and MAGPIX® with xPONENT® software:

These specifications are for the Luminex® 200™, Luminex® HTS, Luminex® FLEXMAP 3D®, and Luminex® MAGPIX® with xPONENT® software. Luminex® instruments with other software (e.g. MasterPlex®, StarStation, LiquiChip, Bio-Plex® Manager™, LABScan™100) 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 (EMD Millipore Catalog # LX2R-CAL-K25)) and performance verified with the Performance Verification Kit (EMD Millipore Catalog # LX2R-PVER-K25). The Luminex® FLEXMAP 3D® instrument must be calibrated with the FLEXMAP 3D® Calibrator Kit (EMD Millipore Catalog # F3D-CAL-K25) and performance verified with the FLEXMAP 3D® Performance Verification Kit (EMD Millipore Catalog # F3D-PVER-K25). The Luminex® MAGPIX® instrument must be calibrated with the MAGPIX® Calibration Kit (EMD Millipore Catalog # MPX-CAL-K25) and performance verified with the MAGPIX® Performance Verification Kit (EMD Millipore Catalog # MPX-PVER-K25).

NOTE: When setting up a Protocol using the xPONENT® software, you must select MagPlex® as the Bead Type in the Acquisition settings.

EQUIPMENT SETTINGS (continued)
NOTE: These assays cannot be run on any instruments using Luminex® IS 2.3 or Luminex® 1.7 software.
EQUIPMENT SETTINGS (continued)

The Luminex® probe height must be adjusted to the plate provided in the kit. Please use Catalog #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:	Customizable 31-plex Beads	
	CD40L	12
	4-1BBL/TNFSF9	18
	Arginase-1	20
	B7-H2/ICOSL	22
	B7-H3/CD276	25
	5'-NT/CD73	26
	B7-H4/VTCN1	27
	APRIL	29
	B7-H5/VISTA	30
	CD25/IL-2Rα	33
	B7-H6	34
	CD137/4-1BB	35
	Granzyme B	37
	CD226/DNAM-1	38
	CD30/TNFRSF8	39
	E-Cadherin	42
	FGL1/Hepassocin	43
	Galectin-1	44
	Galectin-3	45
	Granulysin	46
	IDO1	47
	MICA	48
	MICB	51
	Nectin-2	55
	BAFF/BLyS	56
	Nectin-4	61
	OX40/CD134	65
	PVR/CD155	66
	Siglec-7	67
	Siglec-9	72
	Perforin	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 EMD Millipore website emdmillipore.com 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)

Minimum Detectable Concentration (MinDC) is calculated using MILLIPLEX® Analyst 5.1. 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	Overnight Protocol (n = 8 Assays)	
	MinDC (pg/mL)	MinDC+2SD (pg/mL)
CD40L	1.95	3.86
4-1BBL/TNFSF9	30.36	57.79
Arginase-1	14.75	30.25
B7-H2/ICOSL	10.40	12.12
B7-H3/CD276	183.06	196.87
5'-NT/CD73	17.84	29.56
B7-H4/VTCN1	150.90	243.69
APRIL	1.31	2.09
B7-H5/VISTA	8.49	14.11
CD25/IL-2Rα	21.96	23.74
B7-H6	2.17	3.99
CD137/4-1BB	1.04	1.98
Granzyme B	0.30	0.45
CD226/DNAM-1	314.49	753.35
CD30/TNFRSF8	9.88	20.71
E-Cadherin	203.80	377.18
FGL1/Hepassocin	27.53	53.10
Galectin-1	40.26	69.78
Galectin-3	78.88	297.98
Granulysin	3.24	3.44
IDO1	14.01	31.69
MICA	3.34	4.51
MICB	49.13	98.82
Nectin-2	9.99	19.31
BAFF/BLyS	9.52	13.44
Nectin-4	19.80	39.03
OX40/CD134	5.73	9.78
PVR/CD155	120.12	176.72
Siglec-7	1.08	1.89
Siglec-9	0.94	1.84
Perforin	17.00	25.33

ASSAY CHARACTERISTICS (continued)

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 assay. Inter-assay precision is generated from the mean of the %CV's across two different concentrations of analytes across 7 different assays.

Analyte	Overnight Protocol	
	Intra-assay %CV	Inter-assay %CV
CD40L	<5	<10
4-1BBL/TNFSF9	<5	<10
Arginase-1	<5	<10
B7-H2/ICOSL	<5	<10
B7-H3/CD276	<10	<15
5'-NT/CD73	<5	<10
B7-H4/VTCN1	<5	<10
APRIL	<5	<10
B7-H5/VISTA	<5	<10
CD25/IL-2Rα	<5	<10
B7-H6	<5	<10
CD137/4-1BB	<5	<10
Granzyme B	<5	<10
CD226/DNAM-1	<5	<15
CD30/TNFRSF8	<5	<10
E-Cadherin	<5	<15
FGL1/Hepassocin	<5	<10
Galectin-1	<5	<10
Galectin-3	<5	<10
Granulysin	<5	<10
IDO1	<5	<10
MICA	<5	<10
MICB	<5	<10
Nectin-2	<5	<10
BAFF/BLyS	<5	<15
Nectin-4	<5	<10
OX40/CD134	<5	<10
PVR/CD155	<5	<10
Siglec-7	<5	<10
Siglec-9	<5	<10
Perforin	<5	<10

ASSAY CHARACTERISTICS (continued)

Accuracy

Spike Recovery: The data represent mean percent recovery of spiked standards ranging from low, medium, and high concentration in serum matrices (n=6).

Analyte	Overnight Protocol
	% Recovery in Serum Matrix
CD40L	87
4-1BBL/TNFSF9	89
Arginase-1	87
B7-H2/ICOSL	87
B7-H3/CD276	73
5'-NT/CD73	89
B7-H4/VTCN1	93
APRIL	84
B7-H5/VISTA	91
CD25/IL-2Rα	86
B7-H6	88
CD137/4-1BB	88
Granzyme B	90
CD226/DNAM-1	82
CD30/TNFRSF8	94
E-Cadherin	90
FGL1/Hepassocin	87
Galectin-1	89
Galectin-3	90
Granulysin	85
IDO1	89
MICA	94
MICB	92
Nectin-2	83
BAFF/BLyS	91
Nectin-4	91
OX40/CD134	92
PVR/CD155	89
Siglec-7	91
Siglec-9	90
Perforin	88

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 flushes, back flushes 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 to the kit solid plate or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height to the kit solid plate or to the recommended EMD Millipore filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height to the kit solid plate using 1 alignment disc. For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid in each well and 75 µL should be aspirated.
Background is too high	Background wells were contaminated	Avoid cross-well contamination by using sealer appropriately and pipetting with multichannel pipettes 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® instrument not calibrated correctly or recently	Calibrate Luminex® instrument based on manufacturer's instructions, at least once a week or if temperature has changed by >3℃.
	Gate settings not adjusted correctly	Some Luminex® instruments (e.g. Bio-Plex®) 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® instrument 4 times to rid it of air bubbles, wash 4 times with sheath fluid or water if there is any remnant alcohol or sanitizing liquid.
	Beads were exposed to light	Keep plate and bead mix covered with dark lid or aluminum foil during all incubation steps.

Problem	Probable Cause	Solution
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® instruments (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 technical 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 pipette may not be calibrated	Calibrate pipettes.
	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 an orbital 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 pipette tips that are used for reagent additions and that pipette tip does not touch reagent in plate.

FOR FILTER PLATES ONLY		
Problem	**Probable Cause**	**Solution**
Filter plate will not vacuum	Vacuum pressure is insufficient	Increase vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds.
	Samples have insoluble particles	Centrifuge samples just prior to assay set-up 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 0.2 mL 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	**Catalog #**
Human I-O Checkpoint Protein Panel 2 Standard	HCKP2-8011
Human I-O Checkpoint Protein Panel 2 Quality Control 1 & 2	HCKP2-6011
Serum Matrix	HCKP-SM
Bead Diluent	LBD-4
Human I-O Checkpoint Protein Panel 2 Detection Antibodies	HCKP2-1011
Streptavidin-Phycoerythrin	L-SAPE4
Assay Buffer	L-AB
Set of two 96-Well plates with sealers	MAG-PLATE
10X Wash Buffer	L-WB

Antibody-Immobilized Magnetic Beads

Analyte	*Bead #*	*Cat. #*
CD40L	12	HCD40L-MG
4-1BBL/TNFSF9	18	H41BBL-MAG
Arginase-1	20	HARG1-MAG
B7-H2/ICOSL	22	HB7H2-MAG
B7-H3/CD276	25	HB7H3-MAG
5’-NT/CD73	26	HNT5E-MAG
B7-H4/VTCN1	27	HB7H4-MAG
APRIL	29	HI0APRIL-MAG
B7-H5/VISTA	30	HB7H5-MAG
CD25/IL-2Rα	33	HSIL2RA-MAG
B7-H6	34	HB7H6-MAG
CD137/4-1BB	35	HCD137-MAG
Granzyme	37	HCDGRNZMB-MAG
CD226/DNAM-1	38	HCD226-MAG
CD30/TNFRSF8	39	HI0CD30-MAG
E-Cadherin	42	HECAD-MAG
FGL1/Hepassocin	43	HFGL1-MAG
Galectin-1	44	HI0GAL1-MAG
Galectin-3	45	HI0GAL3-MAG
Granulysin	46	HGNLY-MAG
IDO1	47	HID01-MAG
MICA	48	HMICA-MAG
MICB	51	HMICB-MAG
Nectin-2	55	HNTN2-MAG
BAFF/BLyS	56	HI0BAFF-MAG
Nectin-4	61	HNTN4-MAG
OX40/CD134	65	H0X40-MAG
PVR/CD155	66	HPVR-MAG
Siglec-7	67	HSGL7-MAG
Siglec-9	72	HSGL9-MAG
Perforin	78	HPRFRN-MAG

ORDERING INFORMATION

To place an order or to obtain additional information about our immunoassay products, please contact your Customer Service or Technical Support Specialist.

Contact information for each region can be found on our website:

emdmillipore.com/contact

Conditions of Sale

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

Safety Data Sheets (SDS)

Safety Data Sheets for EMD Millipore products may be downloaded through our website at emdmillipore.com/msds.

WELL MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 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									