# Human Sepsis Panel 3
# Magnetic Bead Panel

## 96-Well Plate Assay

## Cat. # HSP3MAG-63K

# MILLIPLEX® MAP

# HUMAN SEPSIS PANEL 3 MAGNETIC BEAD PANEL KIT
# 96-Well Plate Assay

# HSP3MAG-63K

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

## Human Sepsis Panel 3 Magnetic Bead Panel

## INTRODUCTION

Sepsis or SIRS (systemic inflammatory response syndrome) results from infection, whether bacterial, viral, fungal or parasitic. According to the CDC, sepsis is the second leading cause of death in non-coronary ICU patients in the U.S. This hyper-reaction to infection interrupts homeostasis through an uncontrolled inflammatory response, including glucocorticoids and catecholamines, mediators of the humoral immune response, as well as pro-inflammatory cytokines. Severe sepsis occurs when hypotension or hypoperfusion to one or more organs leads to organ dysfunction, which, in turn, can cause septic shock, multiple organ dysfunction syndrome (MODS) and death.
Apoptosis plays a critical role in the development of severe sepsis and MODS. Studies involving critically ill patients have demonstrated that increased apoptosis of lymphoid organs and other organ tissues contributes to immune suppression, the body's failure to respond to an antigen and organ dysfunction. The same pro-inflammatory cytokines and humoral mediators also contribute to the development of acquired immune defects. During sepsis, lymphocyte apoptosis can be initiated by the release of glucocorticoids and "death" cytokines. Understanding this has caused a shift in interest toward therapies aimed at inhibiting apoptosis and reversing immune suppression.

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
- Automated washing eliminates technical obstacles (i.e., 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 dozens of analytes simultaneously, which can dramatically improve productivity.
Millipore's MILLIPLEX Human Sepsis Panel 3 Magnetic Bead panel is the most versatile system available for systemic inflammatory response syndrome research.

- MILLIPLEX MAP offers you the ability to:
    - Select a 5-plex
    - Choose any combination of analytes from our panel of 5 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 Human Sepsis Panel 3 Magnetic Bead kit is to be used for the simultaneous quantification from the following: Elastase 2, Lactoferrin, NGAL, Resistin, and Thrombospondin-1.

***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

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.
- 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 Sepsis Panel 3 Standard | HSP3-8063-3 | lyophilized | 1 vial |
| Human Sepsis Panel 3 Quality Controls 1 and 2 | HSP3-6063-3 | lyophilized | 2 vials |
| Set of one 96-Well Plate with 2 sealers | ---------- | ---------- | 1 plate<br>2 sealers |
| Assay Buffer | L-AB | 30 mL | 2 bottles |
| 10X Wash Buffer<br>Note: Contains 0.05% Proclin | L-WB | 30 mL | 2 bottles |
| Human Sepsis Panel 3 Detection Antibodies | HSP3-1063-3 | 3.2 mL | 1 bottle |
| Streptavidin-Phycoerythrin | L-SAPE9 | 3.2 mL | 1 bottle |
| Mixing Bottle<br>(not provided with premixed panel) | ---------- | ---------- | 1 bottle |
| Bead Diluent | LBD | 3.5 mL | 1 bottle |

**Included Human Sepsis Panel 3 Antibody-Immobilized Magnetic Beads are dependent on customizable selection of analytes within the panel.**

**Human Sepsis Panel 3 Antibody-Immobilized Magnetic Beads:**

| Bead/Analyte Name | Luminex Magnetic Bead Region | Customizable 5 Analytes (20X concentration, 200 µL) Cat. # |
|---|---|---|
| Anti-Human Elastase 2 Bead | 45 | HELA2-MAG |
| Anti-Human Lactoferrin Bead | 51 | HLTF-MAG |
| Anti-Human NGAL Bead | 56 | HNGAL-MAG |
| Anti-Human Resistin Bead | 64 | HRES-MAG |
| Anti-Human Thrombospondin-1 Bead | 66 | HTSP1-MAG |

## MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex Sheath Fluid (Luminex Catalogue #40-50000) or Luminex Drive Fluid (Luminex Catalogue #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 (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 a microtiter filter plate (Millipore Catalog #MX-PLATE) to run the assay using a Vacuum Filtration Unit (Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with 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. On disposal, flush with a large volume of water to prevent azide build up.

## 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.

**TECHNICAL GUIDELINES (continued)**

- 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.
- 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°C for 1 month and at ≤ -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.
- Any unused mixed Antibody-Immobilized Beads may be stored in the Mixing Bottle at 2-8°C for up to 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°C for up to 24 hours. Prior to reading, agitate the plate on the plate shaker at room temperature for 5 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.
- 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 discs.
- 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 Assay Buffer provided in the kit for further dilution.
- 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 ≤ -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.
- It is recommended to test samples diluted 100-fold in Assay Buffer. For example: combine 5 µL sample and 495 µL Assay Buffer. Use Assay Buffer if further dilution is required.

B. Preparation of Plasma Samples:

- 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°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.
- It is recommended to test samples diluted 100-fold in Assay Buffer. For example: combine 5 µL sample and 495 µL Assay Buffer. Use Assay Buffer if further dilution is required.

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 1:100 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 antibody-bead vial for 30 seconds; 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 1 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 3 antibody-immobilized beads, add 150 µL from each of the 3 bead vials to the Mixing Bottle. Then add 2.55 mL Bead Diluent.

Example 2: When using 5 antibody-immobilized beads, add 150 µL from each of the 5 bead vials to the Mixing Bottle. Then add 2.25 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 (two bottles) with 540 mL deionized water. Store unused portion at 2-8°C for up to one month.

D. Preparation of Human Sepsis Panel 3 Standard

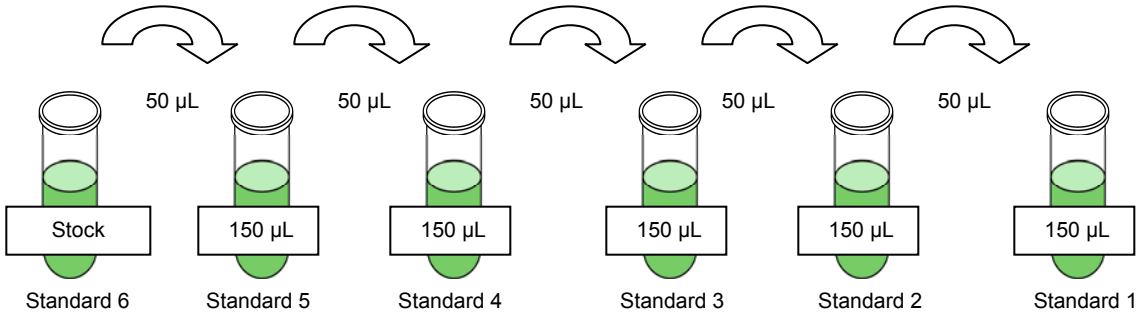
1.) Prior to use, reconstitute the Human Sepsis Panel 3 Standard with 250 µL deionized water. Invert the vial several times to mix. Allow the vial to sit for 5-10 minutes to make sure that the standards are completely reconstituted. This will be used as the stock standard (Standard 6); the unused portions of this stock may be stored at ≤ -20°C for up to one month.

2.) Preparation of Working Standards

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

| Standard | Volume of Deionized Water to Add | Volume of Standard to Add |
|---|---|---|
| Standard 6 | 250 µL | 0 |

| Standard Number | Volume of Assay Buffer to Add | Volume of Standard to Add |
|---|---|---|
| Standard 5 | 150 µL | 50 µL of Standard Tube 6 |
| Standard 4 | 150 µL | 50 µL of Standard Tube 5 |
| Standard 3 | 150 µL | 50 µL of Standard Tube 4 |
| Standard 2 | 150 µL | 50 µL of Standard Tube 3 |
| Standard 1 | 150 µL | 50 µL of Standard Tube 2 |



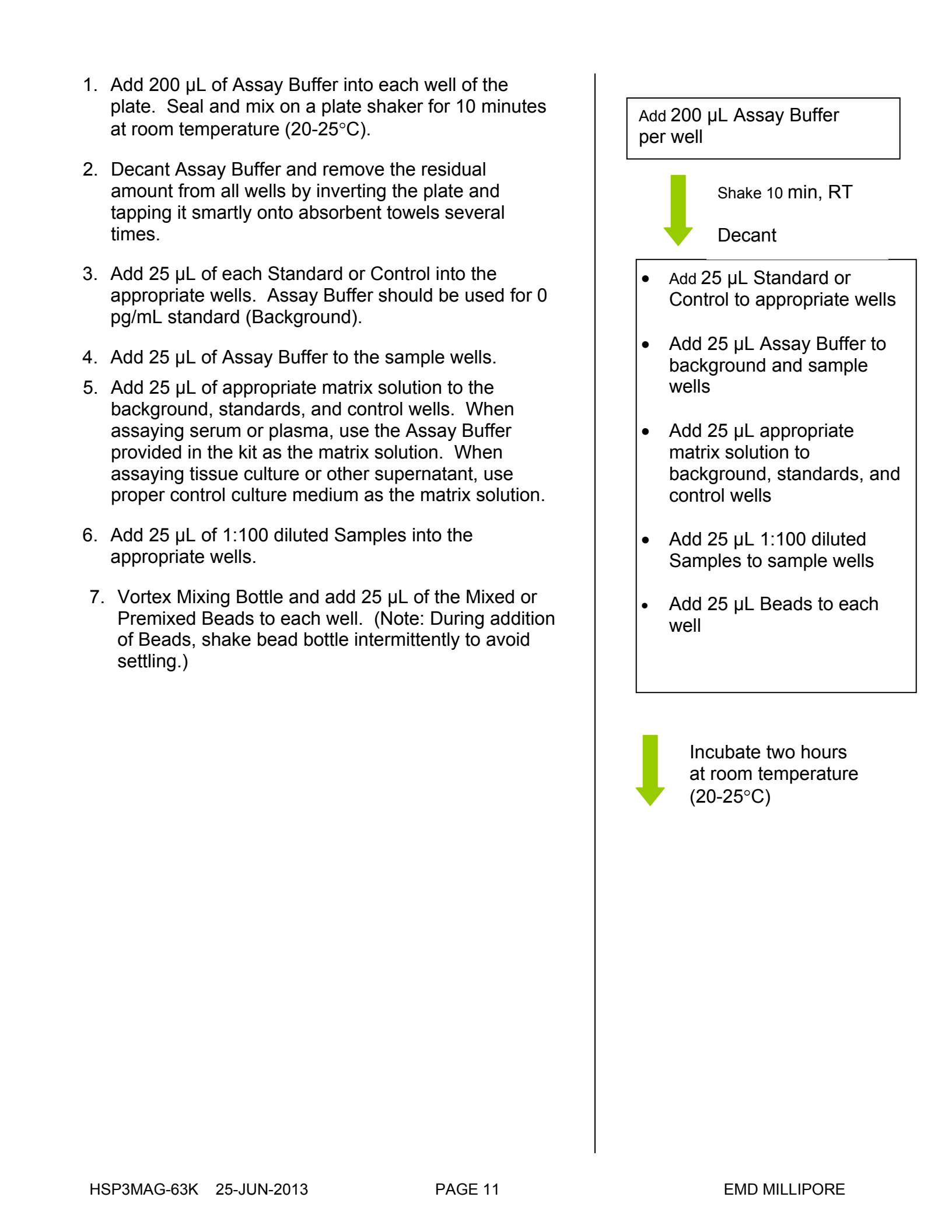

The serial dilutions result in the following concentrations of standards.

| Standard Dilution | Elastase 2 (pg/mL) | Lactoferrin & Thrombospondin-1 (pg/mL) | NGAL (pg/mL) | Resistin (pg/mL) |
|---|---|---|---|---|
| Standard 6 | 20,000 | 200,000 | 30,000 | 10,000 |
| Standard 5 | 5,000 | 50,000 | 7,500 | 2,500 |
| Standard 4 | 1,250 | 12,500 | 1,875 | 625 |
| Standard 3 | 313 | 3,125 | 469 | 156 |
| Standard 2 | 78 | 781 | 117 | 39 |
| Standard 1 | 20 | 195 | 29 | 10 |

## 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 Standards [0 (Background), Standard 1 through 6], 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°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 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 Assay Buffer provided in the kit as the matrix solution. When assaying tissue culture or other supernatant, use proper control culture medium as the matrix solution.
6. Add 25 µL of 1:100 diluted Samples 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 two hours at room temperature (20-25°C).

9. Gently remove well contents and wash plate two 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°C). **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°C).

14. Gently remove well contents and wash plate two 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, multiply the calculated concentration by the dilution factor.)

Remove well contents and wash 2X 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 2X 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

### 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 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.) 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 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

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

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

2.) Wash program:

Method:
1. Number of cycles: 2
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/wel
2. Dispense flow rate: 5
3. Dispense height: 130 (16.51 mm)
4. Horizontal disp pos: 00 (0 mm)
5. Disable Aspirate: YES
6. Bottom Wash first?: NO
7. 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 manufacturer's recommendations for programming instructions.**

## EQUIPMENT SETTINGS (continued)

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, 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 catalog #40-275) and performance verified with the Performance Verification Kit (Millipore catalog #40-276). The Luminex FLEXMAP 3D™ instrument must be calibrated with the FLEXMAP 3D™ Calibrator Kit (Millipore catalog #40-028) and performance verified with the FLEXMAP 3D™ Performance Verification Kit (Millipore catalog #40-029). The Luminex MAGPIX® instrument must be calibrated with the MAGPIX® Calibration Kit (Millipore catalog #40-049) and performance verified with the MAGPIX® Performance Verification Kit (Millipore catalog #40-050).

**NOTE: These assays cannot be run on any instruments using Luminex IS 2.3 or Luminex 1.7 software.**

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 5-Plex Beads | |
| | Elastase 2 | 45 |
| | Lactoferrin | 51 |
| | NGAL | 56 |
| | Resistin | 64 |
| | Thrombospondin-1 | 66 |

## 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)

Minimum Detectable Concentration (MinDC) is calculated using the Milliplex Analyst software. 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 (n = 9 Assays) | |
|---|---|---|
| | MinDC pg/ml | MinDC+2SD pg/ml |
| Elastase 2 | 3 | 6.2 |
| Lactoferrin | 195 | 255 |
| NGAL | 15 | 23 |
| Resistin | 4.3 | 8.5 |
| Thrombospondin-1 | 69.4 | 133.4 |

### 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 6 different assays.

| Analyte | 2 Hour Protocol | |
|---|---|---|
| | Intra-assay %CV | Inter-assay %CV |
| Elastase 2 | 6 | 7 |
| Lactoferrin | 6 | 8 |
| NGAL | 6 | 4 |
| Resistin | 6 | 9 |
| Thrombospondin-1 | 6 | 9 |

## Accuracy

Spike Recovery: The data represent mean percent recovery of spiked standards ranging from low, medium, and high concentration in serum matrices (n=5).

| Analyte | 2 Hour Protocol |
|---|---|
| | % Recovery in Serum Matrix |
| **Elastase 2** | 76 |
| **Lactoferrin** | 99 |
| **NGAL** | 96 |
| **Resistin** | 71 |
| **Thrombospondin-1** | 70 |

## 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 discs. |
| 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. |

| Problem | Probable Cause | Solution |
|---|---|---|
| Beads not in region or gate (continued) | Beads were exposed to light | Keep plate and bead mix covered with dark lid or aluminum foil during all incubation steps. |
| Signal for whole plate is same as background | Incorrect or no Detection Antibody was added<br><br>Streptavidin-Phycoerythrin was not added | Add appropriate Detection Antibody and continue.<br><br>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<br><br>Incubations done at inappropriate temperatures, timings or agitation | May need to repeat assay if desired sensitivity not achieved.<br><br>Assay conditions need to be checked. |
| Signals too high, standard curves are saturated | Calibration target value set too high<br><br>Plate incubation was too long with standard curve and samples | 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.<br><br>Use shorter incubation time. |
| Sample readings are out of range | Samples contain no or below detectable levels of analyte<br><br>Samples contain analyte concentrations higher than highest standard point.<br><br>Standard curve was saturated at higher end of curve. | If below detectable levels, it may be possible to use higher sample volume. Check with tech support for appropriate protocol modifications.<br><br>Samples may require dilution and reanalysis for just that particular analyte.<br><br>See above. |
| High Variation in samples and/or standards | Multichannel pipet may not be calibrated<br><br>Plate washing was not uniform<br><br>Samples may have high particulate matter or other interfering substances<br><br>Plate agitation was insufficient<br><br>Cross well contamination | Calibrate pipets.<br><br>Confirm all reagents are removed completely in all wash steps.<br><br>See above.<br><br>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.<br><br>Check when reusing plate sealer that no reagent has touched sealer. |

| Problem | Probable Cause | Solution |
|---|---|---|
| | | 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 0.2 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 Sepsis Panel 3 Standard | HSP3-8063-3 |
| Human Sepsis Panel 3 Quality Controls 1 & 2 | HSP3-6063-3 |
| Human Sepsis Panel 3 Detection Antibodies | HSP3-1063-3 |
| Streptavidin-Phycoerythrin | L-SAPE9 |
| Bead Diluent | LBD |
| Assay Buffer | L-AB |
| 10X Wash Buffer | L-WB |
| Set of two 96-Well plates with sealers | MAG-PLATE |

**Antibody-Immobilized Magnetic Beads**

| Analyte | Bead # | Cat. # |
|---|---|---|
| Elastase 2 | 45 | HELA2-MAG |
| Lactoferrin | 51 | HLTF-MAG |
| NGAL Bead | 56 | HNGAL-MAG |
| Resistin Bead | 64 | HRES-MAG |
| Thrombospondin-1 | 66 | HTSP1-MAG |

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## WELL MAP

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