MILLIPLEX™ MAP

HUMAN ENDOCRINE KIT PROTOCOL
96 Well Plate Assay

HENDO-65K

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

INTRODUCTION

Obesity, diabetes and associated conditions under the umbrella of Metabolic Syndrome are urgent health issues. Hormones produced by various organs of the endocrine system, including adipocytes, the pancreas and the GI tract, play an integrated role in regulating energy and metabolism. These key hormones are linked to obesity and diabetes and may be involved in the pathogenesis of metabolic disorders.

Millipore recognizes that the availability of assays studying these analytes is limited and is, therefore, proud to announce that the former LINCO*plex* Human Endocrine Panel now has the MILLIPLEX MAP optimized format. While you will instantly recognize the quality and reproducibility that you have always trusted, you will also enjoy the enhancements that we have built into MILLIPLEX MAP.

Millipore's MILLIPLEX MAP Human Endocrine Panel is to be used for the simultaneous quantification of active or total amylin, C-Peptide, active glucagon-like peptide 1 (GLP-1), glucagon, insulin and leptin in human plasma, serum, and cell/tissue culture supernatant samples. This panel provides biomedical researchers quality tools for the study of obesity, diabetes and related disorders in human metabolic diseases.

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 beads known as microspheres.

- Luminex® uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, 100 distinctly colored bead sets can be created, each of which is coated with a specific capture antibody.
- After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced.
- The reaction mixture is then incubated with Streptavidin-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 Endocrine Standard	LHE-8065	lyophilized	1 vial
Human Endocrine Quality Controls 1 & 2	LHE-6065	lyophilized	2 vials
Serum Matrix Note: Contains 0.08% Sodium Azide	LHED-SD	lyophilized	1 vial (required for serum and plasma samples only)
Bead Diluent	LHE-BD	3.5 mL	1 bottle
Set of one 96-Well Filter Plate with 2 Sealers	MX-PLATE	----------	1 plate 2 sealers
Assay Buffer Note: Contains 0.08% Sodium Azide	LE-ABGLP	30 mL	1 bottle
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	30 mL	1 bottle
Human Endocrine Detection Antibodies	LHE-1065 LHE-1065-2	5.5 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE	5.5 mL	1 bottle
Mixing Bottle	----------	----------	1 bottle

Included Human Endocrine Antibody-Immobilized Beads are dependent on customizable selection of analytes within the panel (refer to next page).

Human Endocrine Antibody-Immobilized Beads:

Bead/Analyte Name	Luminex Bead Region	Customizable 7 Analytes (20X concentration, 200µL) Available	Cat. #
Anti-Human Leptin Beads	22	✓	HE-LPTN
Anti-Glucagon Beads	34	✓	HRM-GLU
Anti-GLP-1 (active) Beads	53	✓	HRE-GLP1
Anti-Amylin (active) Beads	15	✓	RME-AMLN
Anti-Amylin (total) Beads	55	✓	HE-AMLN
Anti-Human C-Peptide Beads	73	✓	HE-CP
Anti-Human Insulin Beads	82	✓	HE-INS

Note: Amylin (Total) Antibody-Immobilized Beads and Amylin (Active) Antibody-Immobilized Beads cannot be run together in the same assay.

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

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

Instrumentation / Materials

1. Adjustable Pipettes with Tips capable of delivering 5 µL to 1000 µL
2. Multichannel Pipettes capable of delivering 5 µL to 50 µL or 25 µL to 200 µL
3. Reagent Reservoirs
4. Polypropylene Microfuge Tubes
5. Rubber Bands
6. Absorbent Pads
7. Laboratory Vortex Mixer
8. Sonicator (Branson Ultrasonic Cleaner Model #B200 or equivalent)
9. Titer Plate Shaker (Lab-Line Instruments Model #4625 or equivalent)
10. Vacuum Filtration Unit (Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with Millipore Vacuum Pump Catalog #WP6111560 or equivalent)
11. Luminex 100™ IS, 200™, or HTS by Luminex Corporation
12. Plate Stand (Millipore Catalog # MX-STAND)

SAFETY PRECAUTIONS

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium Azide or Proclin has been added to some reagents as a preservative. Although the concentrations are low, Sodium Azide and Proclin may react with lead and copper plumbing to form highly explosive metal azides. 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.
- Do not mix or substitute reagents with those from other lots or sources.
- The Antibody-Immobilized Beads are light sensitive and must be protected from light at all times. Cover the assay plate containing beads with opaque plate lid or aluminum foil during all incubation steps.
- It is important to allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- The bottom of the Microtiter Filter Plate should not be in direct contact with any surface during assay set-up or incubation times. The plate can be set on a plate stand or on the non-flat side of the plate cover or any other plate holder to raise the plate from the surface. A plate stand can be purchased separately from Millipore (Millipore Catalog #MX-STAND).
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Wash Buffer provided.
- After the wash steps, keep the bottom of the Microtiter Filter Plate clean by blotting on paper towels or absorbent pads to prevent any leakage due to capillary action.
- Keep the vacuum suction on the plate as low as possible. It is recommended to have a vacuum setting that will remove 200 µL of buffer in ≥ 5 seconds (equivalent to < 100 mmHg).
- After hydration, all Standards and Controls must be transferred to polypropylene tubes.
- The Standards prepared by serial dilution must be used within 1 hour of preparation. Discard any unused standards except the standard stock which may be stored at ≤ -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 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 10 minutes. Delay in reading a plate may result in decreased sensitivity for some analytes.

- The titer plate shaker should be set at a speed to provide maximum orbital mixing without splashing of liquid outside the wells. For the recommended plate shaker, this would be a setting of 5-7 which is approximately 500-800 rpm.
- Ensure that the needle probe is clean. This may be achieved by sonication and/or alcohol flushes. Adjust probe height according to the protocols recommended by Luminex to the kit filter plate using 3 alignment discs prior to reading an assay.
- For cell culture supernatants or tissue extraction, use the culture or extraction medium as the matrix solution in background, standard curve and control wells. If samples are diluted in Assay Buffer, use the Assay Buffer as matrix.
- For serum/plasma samples, use the Serum Matrix provided in the kit.
- For cell/tissue homogenate, the final cell or tissue homogenate should be prepared in a buffer that has a neutral pH, contains minimal detergents or strong denaturing detergents, and has an ionic strength close to physiological concentration. Avoid debris, lipids, and cell/tissue aggregates. Centrifuge samples before use.
- Vortex all reagents well before adding to plate.

SAMPLE COLLECTION AND STORAGE

A. Preparation of Serum Samples:

- Note: Plasma samples are preferred for GLP-1 and Amylin measurement.
- For GLP-1 (Active) samples, after collecting blood samples, immediately add the appropriate amount of a DPPIV inhibitor according to the manufacturer's instruction. Invert tube several times to mix (if using Millipore Catalogue #DPP4, add 10 µL DPP4 per milliliter of blood).
- For C-Peptide samples, after collecting blood samples, immediately add Trasylol (Aprotinin) at a concentration of 500 KIU per mL of serum to protect from proteolysis. Invert tube several times to mix.
- For Amylin (Total and Active) samples, after collecting blood samples, immediately add an appropriate amount of a general protease inhibitor cocktail according to the manufacturer's instruction. Invert tube several times to mix.
- If all three analytes (GLP-1, C-Peptide and/or Amylin) need to be tested, then both the DPPIV Inhibitor and the general protease inhibitor cocktail may be added to the same blood sample.
- 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.
- Use Serum Matrix as the diluent if certain samples require dilution prior to assay.

B. Preparation of Plasma Samples:

- Plasma collection using EDTA as an anticoagulant is recommended.
- For GLP-1 (Active) samples, after collecting blood samples, immediately add the appropriate amount of a DPPIV inhibitor according to manufacturer's instruction. Invert tube several times to mix (if using Millipore Catalogue #DPP4, add 10 µL DPP4 per milliliter of blood).
- For C-Peptide samples, after collecting blood samples, immediately add Trasylol (Aprotinin) at a concentration of 500 KIU per mL of serum to protect from proteolysis. Invert tube several times to mix.
- For Amylin (Total and Active) samples, after collecting blood samples, immediately add an appropriate amount of a general protease inhibitor cocktail according to manufacturer's instruction. Invert tube several times to mix.
- If all three analytes (GLP-1, C-Peptide and/or Amylin) need to be tested, then both the DPPIV Inhibitor and the general protease inhibitor cocktail may be added to the same blood sample.
- 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.
- Use Serum Matrix as the diluent if certain samples require dilution prior to assay.

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.

NOTE:

- A maximum of 25 µL per well of 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 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; 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.

Example 1: When using 6 endocrine antibody-immobilized beads, add 150 µL from each of the 6 bead sets to the Mixing Bottle. Then add 2.10 mL Bead Diluent.

Example 2: When using 3 endocrine antibody-immobilized beads, add 150 µL from each of the 3 bead sets to the Mixing Bottle. Then add 2.55 mL Bead Diluent.

Note: Amylin (Total) Antibody-Immobilized Beads and Amylin (Active) Antibody-Immobilized Beads cannot be run together in the same assay.

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 and then transfer the controls to appropriately labeled polypropylene microfuge tubes. 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 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 the bottle containing lyophilized Serum Matrix. Mix well. Allow the bottle to sit for 5-10 minutes for complete reconstitution. Serum Matrix should be used for background, standard and control wells.

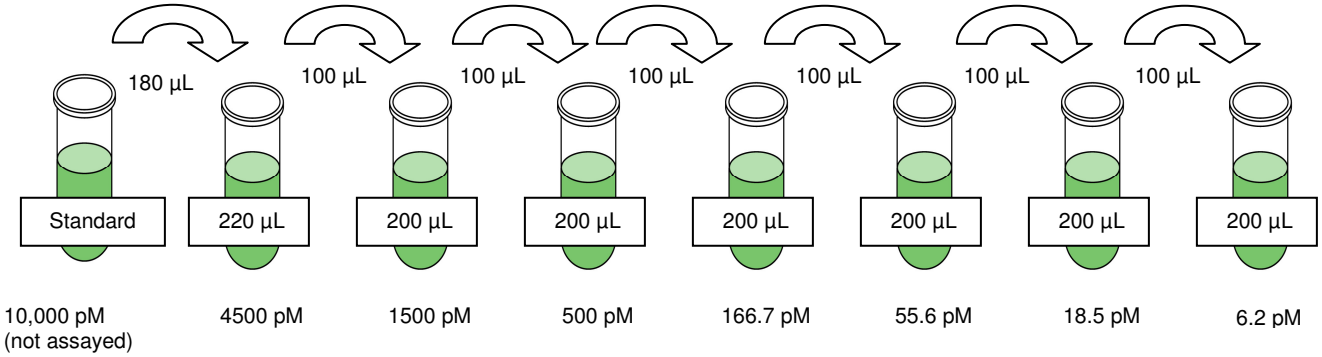
E. Preparation of Human Endocrine Standard

1.) Prior to use, reconstitute the Human Endocrine Standard Cocktail with 250 µL Deionized Water to give a 10,000 pM concentration of standard. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to set for 5-10 minutes. Add 220 µL Assay Buffer into a polypropylene microfuge tube and take 180 µL standard (10,000 pM) to prepare the 4500 pM concentration of standard. This will be used as the 4500 pM standard; the unused portions may be stored at ≤ -20°C for up to one month.

2.) Preparation of Working Standards

Label six polypropylene microfuge tubes 1500, 500, 166.7, 55.6, 18.5 and 6.2 pM. Add 200 µL of Assay Buffer to each of the six tubes. Prepare serial dilutions by adding 100 µL of the 4500 pM reconstituted standard to the 1500 pM tube. Mix well and transfer 100 µL of the 1500 pM standard to the 500 pM tube. Mix well and transfer 100 µL of the 500 pM standard to the 166.7 pM tube. Mix well and transfer 100 µL of the 166.7 pM standard to 55.6 pM tube. Mix well and transfer 100 µL of the 55.6 pM standard to the 18.5 pM tube. Mix well and transfer 100 µL of the 18.5 pM standard to the 6.2 pM tube. Mix well. The 0 pM standard (Background) will be Assay Buffer.

Standard Concentration (pM)	Volume of Assay Buffer to Add	Volume of Standard to Add
4500	220 µL	180 µL of 10,000 pM
1500	200 µL	100 µL of 4500 pM
500	200 µL	100 µL of 1500 pM
166.7	200 µL	100 µL of 500 pM
55.6	200 µL	100 µL of 166.7 pM
18.5	200 µL	100 µL of 55.6 pM
6.2	200 µL	100 µL of 18.5 pM



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), 6.2, 18.5, 55.6, 166.7, 500, 1500, 4500 pM], 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.
- Set the filter plate on a plate holder at all times during reagent dispensing and incubation steps so that the bottom of the plate does not touch any surface.

1. Prewet the filter plate by pipetting 200 µL of 1X Assay Buffer into each well of the Microtiter Filter Plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
2. Remove Assay Buffer by vacuum. **(NOTE: DO NOT INVERT PLATE.)** Blot excess Assay Buffer from the bottom of the plate with an absorbent pad or paper towels.
3. Add 25 µL of each Standard or Control into the appropriate wells. Assay Buffer should be used for the 0 pM 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 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 Sample into the appropriate wells.
7. 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.)
8. Seal the plate with a plate sealer, cover it with the lid. Wrap a rubber band around the plate holder, plate and lid and incubate with agitation on a plate shaker overnight (16-18 hours) at 4°C.

Add 200 µL Assay Buffer per well

↓ Shake 10 min, RT

↓ Vacuum

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

↓ Incubate overnight at 4°C with shaking

9. Gently remove fluid by vacuum. **(NOTE: DO NOT INVERT PLATE.)**

10. Wash plate 3 times with 200 µL/well of Wash Buffer, removing Wash Buffer by vacuum filtration between each wash. Blot excess Wash Buffer from the bottom of the plate with an absorbent pad or paper towels.

11. Add 50 µL of Detection Antibodies into each well. (Note: Allow the Detection Antibodies to warm to room temperature prior to addition.)

12. Seal, cover with lid, and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C). **DO NOT VACUUM AFTER INCUBATION.**

13. Add 50 µL Streptavidin-Phycoerythrin to each well containing the 50 µL of Detection Antibodies.

14. Seal, cover with lid and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).

15. Gently remove all contents by vacuum. **(NOTE: DO NOT INVERT PLATE.)**

16. Wash plate 3 times with 200 µL/well Wash Buffer, removing Wash Buffer by vacuum filtration between each wash. Wipe any excess buffer on the bottom of the plate with a tissue.

17. Add 100 µL of Sheath Fluid to all wells. Resuspend the beads on a plate shaker for 5 minutes.

18. Run plate on Luminex 100™ IS, 200™, or HTS.

19. Save and analyze the Median Fluorescent Intensity (MFI) data using a weighted 5-parameter logistic or spline curve-fitting method for calculating concentrations in samples.

Vacuum and wash 3X with 200 µL Wash Buffer

Add 50 µL Detection Antibodies per well

Incubate 30 minutes at RT

Do Not Vacuum

Add 50 µL Streptavidin-Phycoerythrin per well

Incubate for 30 minutes at RT

Vacuum and wash 3X with 200 µL Wash Buffer

Add 100 µL Sheath Fluid per well

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

EQUIPMENT SETTINGS

These specifications are for the Luminex 100™ IS v.1.7 or Luminex 100™ IS v2.1/2.2, Luminex 200™ v2.3, xPONENT, and Luminex HTS. Luminex instruments with other software (e.g. MasterPlex, StarStation, LiquiChip, Bio-Plex, LABScan100) would need to follow instrument instructions for gate settings and additional specifications from the vendors.

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:	7-Plex Beads	
	Amylin (active)	15
	Leptin	22
	Glucagon	34
	GLP-1 (active)	53
	Amylin (total)	55
	C-Peptide	73
	Insulin	82

QUALITY CONTROLS

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert or can be located at the MILLIPORE website www.millipore.com/techlibrary/index.do using the catalog number as the keyword.

ASSAY CHARACTERISTICS

Assay Sensitivities (minimum detectable concentrations, pg/mL)

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

Hormone	MinDC (pM)
Leptin	8.68
GLP-1 (Active)	3.37
C-Peptide	2.65
Insulin	2.98
Glucagon	2.17
Amylin (Total)	2.32
Amylin (Active)	1.53

Precision

Intra-assay precision is generated from the mean of the %CV's from 8 reportable results across three different concentrations of hormone in one experiment. Inter-assay precision is generated from the mean of the %CV's from a single reportable result from two different concentrations of hormone across six different experiments.

Hormone	Intra-Assay Precision (%CV)	Inter-assay Precision (%CV)
Leptin	5.1	7.4
GLP-1 (Active)	5.1	7.4
C-Peptide	7.3	4.2
Insulin	2.6	7.2
Glucagon	4.9	7.1
Amylin (Total)	4.7	9.3
Amylin (Active)	3.8	8.4

Accuracy

Spike Recovery: The data represent mean percent recovery of multiple levels of spiked standards in serum matrices in several independent experiments.

Hormone	Average Recovery
Leptin	103%
GLP-1 (Active)	136%
C-Peptide	84%
Insulin	83%
Glucagon	93%
Amylin (Total)	94%
Amylin (Active)	80%

Cross-Reactivity

To test cross-reactivity between any of the assays in the panel, each endocrine standard was prepared at 3.2-10,000 pM and tested in the 6-plex assay as well as individually. There was no significant cross-reactivity between the antibodies and any of the other analytes in this panel.

TROUBLESHOOTING GUIDE

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

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°C.
	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 4 times to eliminate 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.
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 vacuumed out, sensitivity may be low.
Low signal for standard curve	Detection Antibody may have been vacuumed out prior to adding Streptavidin Phycoerythrin	May need to repeat assay if desired sensitivity not achieved.
	Incubations done at incorrect 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 tech support for appropriate protocol modifications.
	Samples contain analyte concentrations higher than highest standard point	Samples may require dilution and reanalysis for 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 vacuumed out 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 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.

REPLACEMENT REAGENTS	**Catalog #**
Human Endocrine Standard	LHE-8065
Human Endocrine Quality Controls	LHE-6065
Serum Matrix	LHED-SD
Human Endocrine Detection Antibodies	LHE-1065 LHE-1065-2
Streptavidin-Phycoerythrin	L-SAPE
Assay Buffer	LE-ABGLP
Set of two 96-Well Filter Plates with Sealers	MX-PLATE
10X Wash Buffer	L-WB
Bead Diluent	LHE-BD

Antibody-Immobilized Beads

Hormone	Bead #	Cat. #
Leptin	22	HE-LPTN
GLP-1 (active)	53	HRE-GLP1
C-Peptide	73	HE-CP
Insulin	82	HE-INS
Glucagon	34	HRM-GLU
Amylin (active)	15	RME-AMLN
Amylin (total)	55	HE-AMLN

ORDERING INFORMATION

To place an order:

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

- Your name, telephone and/or fax number
- Customer account number
- Shipping and billing address
- Purchase order number
- Catalog number and description of product
- Quantity of kits
- Selection of MILLIPLEX™ Analytes/Serum Matrix Requirements

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

Mail Orders: Millipore Corp.
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

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To best serve our international customers in placing an order or obtaining additional information about MILLIPLEX™ MAP products, please contact your multiplex specialist or sales representative or email our European Customer Service at customerserviceEU@Millipore.com.

Conditions of Sale

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

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 pM Standard (Background)	166.7 pM Standard	QC-I Control	Etc.								
B	0 pM Standard (Background)	166.7 pM Standard	QC-I Control									
C	6.2 pM Standard	500 pM Standard	QC-II Control									
D	6.2 pM Standard	500 pM Standard	QC-II Control									
E	18.5 pM Standard	1500 pM Standard	Sample 1									
F	18.5 pM Standard	1500 pM Standard	Sample 1									
G	55.6 pM Standard	4500 pM Standard	Sample 2									
H	55.6 pM Standard	4500 pM Standard	Sample 2									