Human Metabolic Hormone Magnetic Bead Panel

96-Well Plate Assay

Cat. # HMHMAG-34K

MILLIPLEX® MAP

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96 Well Plate Assay

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FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES

INTRODUCTION

Metabolic syndrome is a cluster of conditions that occur together, including increased blood pressure, elevated insulin levels, excess body fat around the waist and abnormal cholesterol levels. Key features of metabolic syndrome include insulin resistance, glucose intolerance, hypertension, dyslipidemia and central obesity—all of which are risk factors for atherosclerosis, coronary heart disease, type 2 diabetes, kidney disease, and even premature death. Adults with metabolic syndrome show a low-grade inflammation, whose link with obesity may be disregulated adipocyte production of pro- and anti-inflammatory factors. Consequently, research done in this area covers multi-faceted fields of cytokines, acute phase proteins, diabetes and obesity related hormones, as well as other cardiovascular disease biomarkers.

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. Consequently, using the Luminex xMAP technology, Millipore announces the launch of MILLIPLEX® MAP Human Metabolic Panels. This panel has been designed for the study of biomarkers that traditionally have only been found in our smaller metabolic and endocrine panels – now analytically validated and integrated into one panel. In addition, Millipore offers you the choice of a magnetic or non-magnetic format.

Both the magnetic and non-magnetic MILLIPLEX® MAP Human Metabolic Panels enable you to measure simultaneously either total or active amylin, C-peptide, active ghrelin, total GIP, active GLP-1, glucagon, IL-6, insulin, leptin, MCP-1, pancreatic polypeptide, PYY and TNFα. MILLIPLEX® MAP enables you to investigate the modulation and expression of multiple analytes simultaneously, giving you the advantage of speed and sensitivity, and dramatically improving productivity.

Millipore's MILLIPLEX® Human Metabolic Hormone Magnetic Bead Panel is the most versatile system available for metabolic hormone research.

- MILLIPLEX® MAP offers you the ability to:
    - Choose any combination* of analytes from our panel of 14 analytes to design a custom kit that better meets your needs.
- A convenient "all-in-one" box format gives you the assurance that you will have all the necessary reagents you need to run your assay.

Millipore's MILLIPLEX® Human Metabolic panel is to be used for the simultaneous quantification Amylin (active or total), C-peptide, Ghrelin, GIP, GLP-1,Glucagon, IL-6, Insulin, Leptin, MCP-1, PP, PYY, and TNFa, This kit may be used for the analysis of all or any combination of the above analytes in tissue/cell lysate and culture supernatant samples and serum or plasma samples.

***NOTE: Active Amylin and Total Amylin cannot be run together in the same assay.**

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 is capable of performing a variety of bioassays including immunoassays on the surface of fluorescent-coded magnetic beads known as MagPlex™-C microspheres.

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

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

STORAGE CONDITIONS UPON RECEIPT

- Recommended storage for kit components is 2 - 8°C.
- Once the standards and controls have been reconstituted, immediately transfer contents into polypropylene vials. **DO NOT STORE RECONSITUTED 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 Antibody, and Streptavidin-Phycoerythrin**.

REAGENTS SUPPLIED

Note: Store all reagents at 2 – 8 °C

Reagents Supplied	Catalog Number	Volume	Quantity
Human Metabolic Hormone Standard	HMH-8034	lyophilized	1 vial
Human Metabolic Quality Controls 1 and 2	HMH-6034	lyophilized	2 vials
Set of one 96-Well black Plate with 2 sealers	----------	----------	1 plate 2 sealers
Assay Buffer	LE-ABGLP	30 mL	1 bottle
Serum Matrix	LHGT-SM	1 mL	1 bottle
Bead Diluent	LHE-BD	3.5 mL	1 bottle
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	30 mL	2 bottles
Human Metabolic Hormone Detection Antibodies	HMH-1034 HMH-1034-2	5.5 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE12	5.5 mL	1 bottle
Mixing Bottle	----------	----------	1 bottle

Human Metabolic Hormone Antibody Immobilized Magnetic Beads:

Bead/Analyte Name	Luminex Magnetic Bead Region	Customizable 13 Analytes (20X concentration, 200µL) Available	Cat. #
Anti-Amylin (total) Beads	18	✓	HAMLNT-MAG
Anti- C-Peptide Beads	19	✓	HCP-MAG
Anti-Ghrelin Bead	20	✓	HGRLN-MAG
Anti-GIP Bead	21	✓	HGIP-MAG
Anti-GLP-1 Bead	22	✓	HGLP1-MAG
Anti-Glucagon Bead	33	✓	HGLU-MAG
Anti-IL-6 Bead	34	✓	HIL6-MAG
Anti-Insulin Bead	36	✓	HINS-MAG
Anti-Leptin Beads	39	✓	HLPTN-MAG
Anti – MCP-1 Beads	52	✓	HMCP1-MAG
Anti-PP Beads	53	✓	HPP-MAG
Anti-PYY Beads	54	✓	HPYY-MAG
Anti-TNFa Beads	55	✓	HTNFA-MAG
Anti-Amylin (active) Beads	78	✓	HAMLNA_MAG

Note that Active Amylin and Total Amylin cannot be run together in the same assay.

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

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

Instrumentation / Materials

1. Adjustable Pipettes with Tips capable of delivering 25 µL to 1000 µl
2. Multichannel Pipettes capable of delivering 5 µl to 50 µl or 25 µl to 200 µl
3. Reagent Reservoirs
4. Polypropylene Microfuge Tubes
5. Aluminum Foil
6. Absorbent Pads
7. Rubber Bands
8. Laboratory Vortex Mixer
9. Sonicator (Branson Ultrasonic Cleaner Model # B200 or equivalent)
10. Titer Plate Shaker (Lab-Line Instruments, Model #4625, or equivalent)
11. Luminex 200™, HTS, FLEXMAP 3D™, or MAGPIX® with xPONENT software by Luminex Corporation
12. Automatic Plate washer for magnetic beads (Bio-Tek ELx405, Millipore catalog #40-015 or equivalent) or Hand held Magnetic Separation Block (Millipore catalog # 40-285 or equivalent)

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

SAFETY PRECAUTIONS

- All 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.
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Wash Buffer provided.
- After hydration, all standards and controls must be transferred to polypropylene tubes.
- The standards prepared by serial dilution must be used within 1 hour of preparation. Discard any unused standards except the standard stock 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 Bead Mix 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.
- When reading the assay on Luminex 200™, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 4 alignment discs. When reading the assay on FLEXMAP 3D™, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using

1 alignment disc. When reading the assay on MAGPIX, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 2 alignment disc.

- For cell culture supernatants or tissue extraction, use the culture or extraction medium as the matrix solution in blank, standard curve and controls. 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 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 chunks. Centrifuge samples before use.
- Vortex all reagents well before adding to plate.

SAMPLE COLLECTION AND STORAGE

A. Preparation of Serum Samples:

- After collecting blood samples, invert tube several times to mix, immediately add DPPIV inhibitor (for GLP-1 measurement), Protease Inhibitor cocktail (for Amylin measurement), and Serine protease inhibitor (for active ghrelin measurement). We recommend using Millipore's DPPIV inhibitor (Cat.# DPP4), Sigma's Protease Inhibitor Cocktail, and Roche's Pefabloc SC (AEBSF). These should be used following manufactures' instructions.
- Allow the blood to clot for at least 30 minutes before centrifugation for 10 minutes at 1000 xg.
- Remove serum and assay immediately or aliquot and store samples at ≤ -20ºC.
- Avoid multiple (>2) freeze/thaw cycles.

B. Preparation of Plasma Samples:

- For Plasma collection, EDTA as an anticoagulant is recommended.
- After collecting blood, immediately add DPPIV inhibitor (for GLP-1 measurement), Protease Inhibitor cocktail (for Amylin measurement), and Serine protease inhibitor (for active ghrelin measurement). We recommend using Millipore's DPPIV inhibitor (Cat.# DPP4)), Sigma's Protease Inhibitor Cocktail, and Roche's Pefabloc SC (AEBSF). These should be used following manufactures' instructions.
- Invert tube several times to mix. Centrifuge for 10 minutes at 1000 xg within 30 minutes of blood collection.
- Remove plasma and assay immediately or aliquot and store samples at ≤ -20ºC.
- Avoid multiple (>2) freeze/thaw cycles.
- It is recommended to centrifuge plasma samples again at 3000 xg for five minutes prior to assay set up.

C. Preparation of Tissue Culture Supernatant:

- Centrifuge the sample to remove debris and assay immediately or aliquot and store samples at ≤ -20ºC.
- Avoid multiple (>2) freeze/thaw cycles.
- Tissue Culture Supernatant may require a dilution with an appropriate control medium prior to assay.

Note:

- 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 portions 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 13 antibody-immobilized beads, add 150 µL from each of the 13 bead sets to the Mixing Bottle. Then add 1.05 mL Bead Diluent.

Example 2: when using 3 antibody-immobilized beads, add 150 µL from each of the 3 bead sets to the Mixing Bottle. Then add 2.55 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 and then transfer the controls to appropriately labeled polypropylene microfuge tubes. Unused portions may be stored at ≤ -20°C for up to one month.

C. Preparation of Wash Buffer

Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution. Dilute 30 mL of 10X Wash Buffer with 270 mL deionized water. Store unused portions 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 Assay Buffer to the bottle containing lyophilized Serum Matrix. Mix well. Allow at least 10 minutes for complete reconstitution. Leftover reconstituted Serum Matrix should be stored at ≤ -20°C for up to one month.

E. Preparation of Human Metabolic Hormone Standard

1.) Prior to use, reconstitute the Human Metabolic Hormone Standard with 250 µL Deionized Water. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes and then transfer the standard to appropriately labeled polypropylene microfuge tube. This will be used as Standard 7.

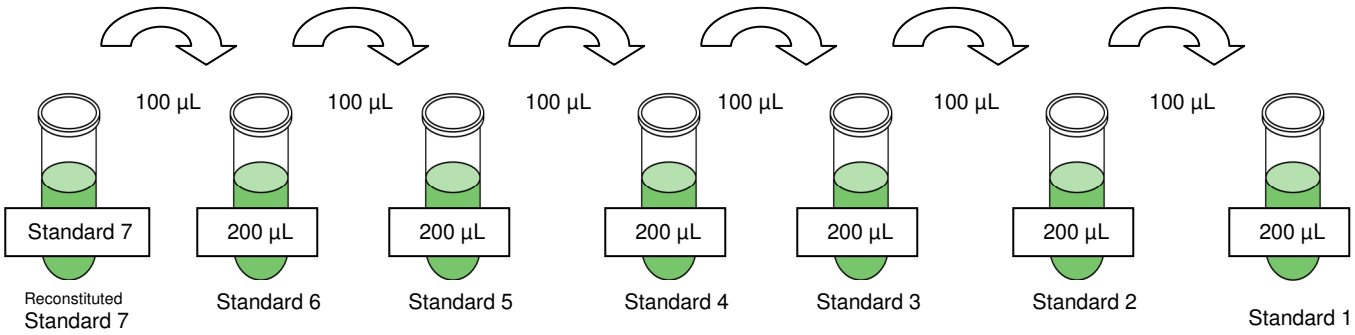
2.) Preparation of Working Standards

Label 6 polypropylene microfuge tubes tubes "Standard 6," "Standard 5," "Standard 4," "Standard 3," "Standard 2," and "Standard 1." Add 200 µL Assay Buffer to each of the six tubes. Perform 3 times serial dilutions by adding 100 µL of the "Standard 7" to the "Standard 6" tube, mix well and transfer 100 µL of the "Standard 6" to the "Standard 5" tube, mix well and transfer 100 µL of the "Standard 5" to "Standard 4" tube, mix well and transfer 100 µL of the "Standard 4" to the "Standard 3", mix well and transfer 100 µL of the "Standard 3" to the "Standard 2" tube, mix well and transfer 100 µL of the "Standard 2" to the "Standard 1". The 0 Standard (background) will be assay buffer.

Preparation of Working Standards

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	200 µL	100 µL of Standard 7
Standard 5	200 µL	100 µL of Standard 6
Standard 4	200 µL	100 µL of Standard 5
Standard 3	200 µL	100 µL of Standard 4
Standard 2	200 µL	100 µL of Standard 3
Standard 1	200 µL	100 µL of Standard 2



After serial dilution, the tubes should have the following concentrations for constructing standard curves.

Standard Tube #	GIP, TNFa (pg/ml)	Ghrelin, GLP-1, Glucagon, PP, PYY, IL-6, MCP-1 (pg/ml)	Amylin (pg/ml)	C-Peptide, (pg/ml)	Insulin, Leptin (pg/ml)
1	2.7	13.7	27.4	68.6	137.2
2	8.2	41.2	82.3	205.8	411.5
3	24.6	123.5	246.9	617.3	1,234.6
4	74	370.3	740.7	1,851.9	3,703.7
5	222.2	1111.1	2,222	5,555	11,111
6	666.6	3,333	6,666	16,666	33,333
7	2,000	10,000	20,000	50,000	100,000

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°C) before use in the assay.
- Diagram the placement of Standards, 0 (Background), Std 1, Std 2, Std 3, Std 4, Std 5, Std 6, and Std 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 on a plate holder at all times during reagent dispensing and incubation steps so that the bottom of the plate does not touch any surface.

1. Add 200 µL of Assay Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
2. Decant Assay Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
3. Add 25 µL of Assay Buffer to the Background wells. Add 25 µL of each Standard or Control into the appropriate wells.
4. Add 25 µl Assay buffer in Sample wells
5. Add 25 µL of samples to the Sample wells.
6. Add 25 µL of Matrix Solution (when measuring serum or plasma samples) or appropriate culture media (when measuring culture samples) in Background, Standards, and Quality Control wells.
7. Vortex Bead Bottle and add 25 µL of the prepared Beads to each well. (Note: during addition of the Beads, shake beads 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 for overnight incubation at 4°C *(16-18 hr).*

Add 200µL Assay Buffer per well

↓ Shake 10 min, RT

↓ Decant

- Add 25µL Assay Buffer to background wells and 25µl Standard, and 25µl Controls to Standard and Control wells, respectively.
- Add 25µl Assay Buffer to Sample wells.
- Add 25µL samples to Sample Wells.
- Add 25µL Matrix Solution (or appropriate media) to Background, Standard and Quality Controls.
- Add 25 µL Beads to each well

↓ Incubate overnight at 4°C with agitation on a plate shaker

9. Gently remove well contents and wash plate 3 times following instructions listed in the **PLATE WASHING** section.
10. Add 50 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 30 minutes at room temperature (20-25°C) **DO NOT ASPIRATE AFTER INCUBATION.**
12. Add 50 µL Streptavidin-Phycoerythrin to each well containing the 50 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 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 weighted 5-parameter logistic or spline curve-fitting method for calculating analyte concentrations in samples.

Remove well contents Wash 3X with 200µL Wash Buffer

Add 50µL Detection Antibody per well

Incubate 30 min at RT

Do Not Aspirate

Add 50µL Streptavidin-Phycoerythrin per well

Incubate for 30 minute at RT

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

Add 100µL Sheath Fluid or Drive Fluid per well

Read on Luminex (50µL, 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 uL of Wash Buffer by removing plate from magnet, adding Wash Buffer, shaking for 30 seconds, reattaching to magnet, letting beads settle for 60 seconds and removing well contents as previously described after each wash. Repeat wash steps as recommended in Assay Procedure.

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

2.) Filter Plate (Millipore Cat# MX-PLATE)

If using a filter plate, use a vacuum filtration manifold to remove well contents. Wash plate 200 µL/well of Wash Buffer, removing Wash Buffer by vacuum filtration after each wash Repeat wash steps as recommended in the Assay Procedure.

EQUIPMENT SETTINGS

Bio-Tek ELx405:

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

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

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

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

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

Note: After the final aspiration, there will be approximately 25 µl of residual Wash Buffer in each well. This is expected when using the BioTek Plate washer and this volume does not need to be aspirated from the plate.

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

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

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

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

NOTE: These assays cannot be run on Luminex 100™ instruments or any instruments using the 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 Cat# MAG-PLATE, if additional plates are required for this purpose.

Events:	50, per bead	
Sample Size:	50 µL	
Gate Settings:	8,000 to 15,000	
Reporter Gain:	Default (low PMT)	
Time Out:	60 seconds	
Bead Set:	13-Plex Beads	
	Amylin (total) Beads	18
	C-Peptide Beads	19
	Ghrelin Bead	20
	GIP Bead	21
	GLP-1 Bead	22
	Glucagon Bead	33
	IL-6 Bead	34
	Insulin Bead	36
	Leptin Beads	39
	MCP-1 Beads	52
	PP Beads	53
	PYY Beads	54
	TNFa Beads	55
	Amylin (active) Beads	78

QUALITY CONTROLS

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

ASSAY CHARACTERISTICS

Assay Sensitivities (minimum detectable concentrations, pg/mL)

Minimum Detectable Concentration (MinDC) defines the reportable range of the assay. It is a measure of the true limits of detection for an assay and is mathematically determined.

Analyte	Overnight Protocol (N = 8 assays)	
	Mean MinDC	Mean MinDc + 2SD
Amylin	14	23
C-Peptide	24	34
Ghrelin	2	4
GIP	0.6	1.2
GLP-1	7	10
Glucagon	6	22
IL-6	3	6
Insulin	58	117
Leptin	27	65
MCP-1	6	8
PP	2	6
PYY	8	21
TNFα	0.3	0.8

Precision

Intra-assay precision is generated from the mean of the %CV's from 8 reportable results across two different concentration of analytes in one experiment. Inter-assay precision is generated from the mean of the %CV's from two reportable results each for two different concentrations of cytokine different experiments.

Analyte	Intra-Assay CV %	Inter-Assay CV %
Amylin	2%	33%
C-Peptide	2%	6%
Ghrelin	2%	8%
GIP	3%	5%
GLP-1	7%	10%
Glucagon	3%	7%
IL-6	3%	7%
Insulin	3%	6%
Leptin	3%	4%
MCP-1	2%	2%
PP	4%	7%
PYY	2%	11%
TNFα	3%	6%

Accuracy

Spike Recovery: The data represents mean recovery of 3 levels of spiked standards using 5 independent matrix samples.

Analyte	Spike and Recovery %
Amylin	108%
C-Peptide	101%
Ghrelin	96%
GIP	99%
GLP-1	123%
Glucagon	98%
IL-6	99%
Insulin	101%
Leptin	102%
MCP-1	91%
PP	103%
PYY	98%
TNFα	98%

Cross-Reactivity

The antibody pairs in the panel are specific only to the desired analyte and exhibit no or negligible (<2%) cross-reactivity with other analytes in the panel.

TROUBLESHOOTING GUIDE

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

	light	lid or aluminum foil during all incubation steps.
Signal for whole plate is same as background	Incorrect or no Detection Antibody was added	Add appropriate Detection Antibody and continue.
	Streptavidin-Phycoerythrin was not added	Add Streptavidin-Phycoerythrin according to protocol. If Detection Antibody has already been removed, sensitivity may be low.
Low signal for standard curve	Detection Antibody may have been removed prior to adding Streptavidin Phycoerythrin	May need to repeat assay if desired sensitivity not achieved.
	Incubations done at inappropriate temperatures, timings or agitation	Assay conditions need to be checked.
Signals too high, standard curves are saturated	Calibration target value set too high	With some Luminex Instrument (e.g. Bio-plex) Default target setting for RP1 calibrator is set at High PMT. Use low target value for calibration and reanalyze plate.
	Plate incubation was too long with standard curve and samples	Use shorter incubation time.
Sample readings are out of range	Samples contain no or below detectable levels of analyte	If below detectable levels, it may be possible to use higher sample volume. Check with tech support for appropriate protocol modifications.
	Samples contain analyte concentrations higher than highest standard point.	Samples may require dilution and reanalysis for just that particular analyte.
	Standard curve was saturated at higher end of curve.	See above.
High Variation in samples and/or standards	Multichannel pipet may not be calibrated	Calibrate pipets.
	Plate washing was not uniform	Confirm all reagents are removed completely in all wash steps.
	Samples may have high particulate matter or other interfering substances	See above.
	Plate agitation was insufficient	Plate should be agitated during all incubation steps using a vertical plate shaker at a speed where beads are in constant motion without causing splashing.
	Cross well contamination	Check when reusing plate sealer that no reagent has touched sealer. Care should be taken when using same pipet tips that are used for reagent additions and that pipet tip does not touch reagent in plate.
FOR FILTER PLATES ONLY		
Filter plate will not vacuum	Vacuum pressure is insufficient	Increase vacuum pressure such that 0.2mL buffer can be suctioned in 3-5 seconds.
	Samples have insoluble particles	Centrifuge samples just prior to assay setup and use supernatant.
	High lipid concentration	After centrifugation, remove lipid layer and

		use supernatant.
Plate leaked	Vacuum Pressure too high	Adjust vacuum pressure such that 0.2mL buffer can be suctioned in 3-5 seconds. May need to transfer contents to a new (blocked) plate and continue.
	Plate set directly on table or absorbent towels during incubations or reagent additions	Set plate on plate holder or raised edge so bottom of filter is not touching any surface.
	Insufficient blotting of filter plate bottom causing wicking	Blot the bottom of the filter plate well with absorbent towels after each wash step.
	Pipette touching plate filter during additions	Pipette to the side of plate.
	Probe height not adjusted correctly	Adjust probe to 3 alignment discs in well H6.
	Sample too viscous	May need to dilute sample.

REPLACEMENT REAGENTS	**Cat #**
Human Metabolic Standard	HMH-8034
Human Metabolic Quality Controls	HMH-6034
Human Metabolic Detection Antibodies	HMH-1034
	HMH-1034-2
Serum Matrix	LHGT-SM
Bead Diluent	LHE-BD
Assay Buffer	LE-ABGLP
Streptavidin-Phycoerythrin	L-SAPE12
Set of two 96-Well Black plates with sealers	MAG-PLATE
10X Wash Buffer	L-WB

Antibody-Immobilized Beads

Analyte	Bead #	Cat. #
Amylin Beads	18	HAMLNT-MAG
C-Peptide Beads	19	HCP-MAG
Ghrelin Bead	20	HGRLN-MAG
GIP Bead	21	HGIP-MAG
GLP-1 Bead	22	HGLP1-MAG
Glucagon Bead	33	HGLU-MAG
IL-6 Bead	34	HIL6-MAG
Insulin Bead	36	HINS-MAG
Leptin Beads	39	HLPTN-MAG
MCP-1 Beads	52	HMCP1-MAG
PP Beads	53	HPP-MAG
PYY Beads	54	HPYY-MAG
TNFa Beads	55	HTNFA-MAG
Amylin (active) Beads	78	HAMLNA-MAG

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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 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									