Human Neuropeptide Panel

96 Well Plate Assay

Cat. # HNP-35K

MILLIPLEX® MAP

Human Neuropeptide Panel
96 Well Plate Assay

HNP-35K

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FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES

INTRODUCTION

The central nervous system is a complex environment consisting of billions of neurons as well as glial support cells. These neurons use many different chemical signals to communicate information, including neurotransmitters, cannabinoids, gases such as nitric oxide, and peptides. Neuropeptides are secreted primarily from the central and peripheral nervous systems, exerting a broad spectrum of biological functions that includes the regulation of metabolism, reproduction and immunity. In addition many neuropeptides can be associated with specific behaviors. For example, orexin activity has been linked to sleep and wakefulness, feeding and appetite and with the reward pathway, playing a role in addiction. Oxytocin is best known for the role it plays in pregnancy and maternal behaviors, but is also involved in social recognition, bonding, trust and anxiety.

Understanding neurobiology is fundamental in determining the pathogenesis of devastating neurodegenerative diseases such as Alzheimer's Disease, Parkinson's Disease, and Lewy Body Dementia. Accurate measurement of these neuropeptides is important. However, conventional methods, including RIAs and ELISAs, are not able to measure simultaneously multiple neuropeptides with small sample volume. Millipore is the first to provide you with a multiplex kit for the study of neuropeptides. The Luminex® xMAP®-based MILLIPLEX® MAP Human Neuropeptide Panel will enable you to understand better the complexities of the nervous system and the pathobiology of disease.

Millipore's MILLIPLEX® Human Neuropeptide Panel is to be used for the simultaneous quantification of β-Endorphin, Cortisol, Neurotensin, Orexin A, Substance P, Melatonin, Oxytocin, and α MSH. 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.

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 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 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 ℃

Reagents Supplied	Catalog Number	Volume	Quantity
Human Neuropeptide Standard	HNP-8035	lyophilized	1 vial
Human Neuropeptide Quality Controls 1 and 2	HNP-6035	lyophilized	2 vials
Set of one 96-Well Filter Plate with 2 sealers	MX-PLATE	----------	1 plate 2 sealers
Assay Buffer	LE-ABGLP	30 mL	2 bottles
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	30 mL	1 bottle
Human Neuropeptide Primary Antibody	HNP-1035P	3.5 mL	1 bottle
Human Neuropeptide Detection Antibody	HNP-1035D	5.5 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE	5.5 mL	1 bottle
Mixing Bottle	----------	----------	1 bottle

Included Human Neuropeptide Panel Antibody-Immobilized Beads are dependent on customizable selection of analytes within the panel.

Human Neuropeptide Antibody Immobilized Beads:

Bead/Analyte Name	Luminex Bead Region	Customizable 8 Analytes (20X concentration, 200µL) Available	Cat. #
Anti- β-Endorphin Bead	1	✓	HBEND
Anti-Cortisol Bead	2	✓	HC0RT
Anti-Neurotensin Bead	7	✓	HNRTNSN
Anti-Orexin A Bead	17	✓	H0RXNA
Anti-Substance P Bead	36	✓	HSBTNCP
Anti-Melatonin Bead	54	✓	RMLT
Anti-Oxytocin Bead	60	✓	H0XYTCN
Anti-α MSH	64	✓	HAMSH

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex Sheath Fluid (Luminex Catalogue #40-50000)
2. Acetonitrile
3. Trifluoroacetic Acid

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. 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. Millipore Vacuum Pump Catalog #WP6111560 or equivalent.)
11. Luminex$^{100,\ 200,\ HTS}$ by Luminex Corporation
12. Plate Stand (Millipore Catalog # MX-STAND)
13. Extraction plate (Waters Oasis HLB Extraction Plate), Recommended.
14. Extraction Plate Manifold (Waters), Recommended
15. Speed Vacuum

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 holder or on the non-flat side of the plate cover or any other plate holder to raise the plate from the surface. The plate holder 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 $\geq$ 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 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. 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 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:

- Allow the blood to clot for at least 30 minutes before centrifugation. Centrifuge samples for 10 minutes at 1000 x g. Remove serum and proceed to sample extraction or aliquot and store samples at ≤ -20 ºC.
- Avoid multiple (>2) freeze/thaw cycles.

B. Preparation of Plasma Samples:

- For plasma collection, EDTA as an anti-coagulant is recommended. After collecting blood, mix well and centrifuge samples for 10 minutes at 1000 x g, within 30 minutes of blood collection. Remove plasma and proceed to sample extraction or aliquot and store samples at ≤ -20 ºC.
- Avoid multiple (>2) freeze/thaw cycles.

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:

- A maximum of 50 µl per well of extracted 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.

SAMPLE EXTRACTION PROTOCOL (Using Waters 96-well HLB extraction plate)

- Allow 1 mL acetonitrile to pass through the extraction wells using gravity.
- Equilibrate with 2 x 1 mL 0.1% Triflouroacetic acid (TFA) in water. **(Solvent A)**
- Acidify 250 µl serum or plasma samples by adding 250 µl 1% TFA. Mix well.
- Load the acidified sample in the wells and pull through at a vacuum setting of Hg 2-5.
- Wash each well 3 times with 1 ml Solvent A by vacuum at setting of Hg 2-5.
- Elute the samples in 96-well collecting plate with 0.5 mL acetonitrile/water/TFA (60%/40%/0.1%, v/v/v).
- Dry the samples by using Speed Vac at highest vacuum setting.
- Reconstitute using assay buffer.

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 Assay Buffer. Vortex the mixed beads well. Unused portions may be stored at 2-8°C for up to one month.

Example 1: when using 8 antibody-immobilized beads, add 150 µL from each of the 8 bead sets to the Mixing Bottle. Then add 1.8 mL Assay Buffer

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 Assay Buffer.

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.

PREPARATION OF REAGENTS FOR IMMUNOASSAY

D. Preparation of Human Neuropeptide Standard

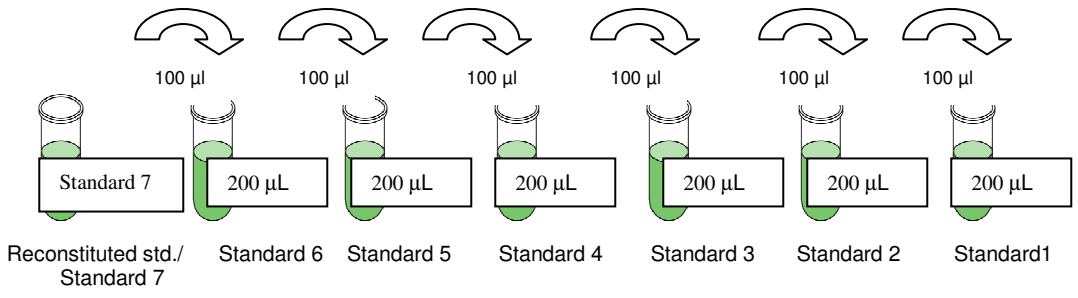
1.) Prior to use, reconstitute the Human Neuropeptide Standard with 250 µL Deionized Water. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes and then transfer the standard to appropriately labeled polypropylene microfuge tube. This will be used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

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

Standard Tube	Volume of Assay Buffer to Add	Volume of Standard to Add
Standard 6	200 µL	100 µL of Standard 7
Standard 5	200 µL	100 µL of Standard 6
Standard 4	200 µL	100 µL of Standard 5
Standard 3	200 µL	100 µL of Standard 4
Standard 2	200 µL	100 µL of Standard 3
Standard 1	200 µL	100 µL of Standard 2



PREPARATION OF REAGENTS FOR IMMUNOASSAY

Note: Standard Concentrations

Standard Tube #	Substance P α-MSH (pg/mL)	Neurotensin (pg/mL)	Oxytocin (pg/mL)	β-Endorphin Orexin A (pg/mL)	Melatonin (pg/mL)	Cortisol (pg/mL)
1	13	27	68	137	274	685
2	41	82	205	412	823	2,057
3	123	246	617	1,235	2,469	6,172
4	370	740	1,851	3,704	7,407	18,518
5	1111	2,222	5,555	11,111	22,222	55,555
6	3,333	6,666	16,666	33,333	66,666	166,666
7	10,000	20,000	50,000	100,000	200,000	500,000

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), Standard 1, Standard 2, Standard 3, Standard 4, Standard 5, Standard 6, and Standard 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.
- 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.

IMMUNOASSAY PROCEDURE

1. Block the filter plate by pipetting 200 µL of Assay Buffer into each well of the microtiter 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 50 µL of Assay Buffer to the Background wells. Add 50 µL of each Standard or Control into the appropriate wells.

4. Add 50 µl Assay buffer in Sample wells

5. Add 50 µL of extracted samples to the Sample wells.

6. Add 50 µL of Assay buffer (when measuring extracted samples) or appropriate culture media (when measuring culture samples) in Background, Standards, and Quality Control wells.

7. Add 25 µL of Primary Antibody (**HNP-1035P**) to each well.

8. Seal and mix on a plate shaker for 2 hours at room temperature (20-25°C).

9. 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)

10. 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 for overnight incubation at 4°C *(16-18 hr).*

Add 200µL Assay Buffer per well

↓ Shake 10 min, RT

↓ Vacuum

- Add 50µL Standard or Control to appropriate wells
- Add 50µl Assay Buffer to Background and Sample wells.
- Add 50µL samples to sample wells.
- Add 50µL appropriate media to Background, Standard and Control wells.
- Add 25µL Primary antibody (**HNP-1035P**) to each well

↓ Incubate for 2 hours at room temperature

Add 25 µL Beads to each well

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

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

12. 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 the plate by with an absorbent pad or paper towels.

13. Add 50 µL of Detection Antibodies (**HNP-1035D**) into each well. (Note: allow the Detection Antibodies to warm to room temperature prior to addition.)

14. 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**

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

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

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

18. 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.

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

20. Run plate on Luminex$^{100, 200, HTS}$.

21. 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.

Vacuum. Wash 3X with 200µL Wash Buffer

Add 50µL Detection Antibody (**HNP-1035D**) per well

Incubate 30 min at RT

Do Not Vacuum

Add 50µL Streptavidin-Phycoerythrin per well

Incubate for 30 minute at RT

Vacuum and Wash 3X with 200µL Wash Buffer

Add 100µL Sheath Fluid per well

Read on Luminex (50µL, 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:	8-Plex Beads	
	β-Endorphin	01
	Cortisol	02
	Neurotensin	07
	Orexin A	17
	Substance P	36
	Melatonin	54
	Oxytocin	60
	α MSH	64

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 (IMinDC) 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
β-Endorphin	91	137
Cortisol	298	622
Neurotensin	22	26
Orexin A	49	195
Substance P	10	12
Melatonin	177	273
Oxytocin	59	83
αMSH	31	39

Precision

Intra-assay precision is generated from the mean of the %CV's from 8 reportable results across two different concentration of cytokines 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 across 4 different experiments.

Analyte	Intra-Assay CV %	Inter-Assay CV %
β-Endorphin	9	12
Cortisol	7	8
Neurotensin	10	9
Orexin A	19	21
Substance P	11	10
Melatonin	11	13
Oxytocin	10	10
αMSH	12	10

Accuracy

Spike Recovery: The data represents mean recovery of 3 levels of spiked standards using 8 independent samples.

Analyte	Spike and Recovery %
β-Endorphin	123
Cortisol	108
Neurotensin	123
Orexin A	232
Substance P	129
Melatonin	125
Oxytocin	118
αMSH	120

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
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 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 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	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 (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.

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 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 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 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 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.

REPLACEMENT REAGENTS **Cat #**

Human Neuropeptide Panel Standard	HNP-8035
Human Neuropeptide Panel Quality Controls	HNP-6035
Human Neuropeptide Primary Antibodies	HNP-1035P
Human Neuropeptide Detection Antibodies	HNP-1035D
Streptavidin-Phycoerythrin	L-SAPE
Assay Buffer (2 bottles)	LE-ABGLP
Set of two 96-Well Filter Plates with sealers	MX-PLATE
10X Wash Buffer	L-WB

Antibody-Immobilized Beads

Analyte	Bead #	Cat. #
Anti- β-Endorphin	01	HBEND
Anti- Cortisol	02	HC0RT
Anti- Neurotensin	07	HNRTNSN
Anti- Orexin A	17	H0RXNA
Anti- Substance P	36	HSBTNCP
Anti- Melatonin	54	RMLT
Anti- Oxytocin	60	H0XYTCN
Anti- αMSH	64	HAMSH

ORDERING INFORMATION

To place an order:

To assure the clarity of your custom cytokine 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

FAX: (636) 441-8050
Toll Free US: (800) MILLIPORE
MAIL ORDERS: Millipore Corp.
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

For International Customers:

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 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									