Rat/Mouse Neuropeptide Magnetic Bead Panel

96-Well Plate Assay

Cat. # RMNPMAG-83K

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MILLIPLEX® MAP

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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, 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. EMD Millipore provides you with a multiplex kit for the study of neuropeptides. The Luminex® xMAP®-based MILLIPLEX® MAP Rat/Mouse Neuropeptide Magnetic Panel will enable you to better understand the complexities of the nervous system and the pathobiology of disease.

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

Each MILLIPLEX® MAP panel and kit includes:

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

In addition, each panel and kit meets stringent manufacturing criteria to ensure batch-to-batch reproducibility. The **MILLIPLEX® MAP** Rat/Mouse Neuropeptide Magnetic Bead Panel thus enables you to focus on the therapeutic potential of neurodegenerative disease research. Coupled with the Luminex® xMAP® platform in a **magnetic bead** format, you receive the advantage of ideal speed and sensitivity, allowing quantitative multiplex detection of dozens of analytes simultaneously, which can dramatically improve productivity.

EMD Millipore's MILLIPLEX® MAP Rat/Mouse Neuropeptide Magnetic Bead Panel is part of the most versatile system available for neuroscience research. From our single to multiplex biomarker solutions, we partner with you to design, develop, analytically validate and build the most comprehensive library available for protein detection and quantitation.

- MILLIPLEX® MAP offers you:
    - The ability to choose any combination of analytes from our panel of 5 analytes to design a custom kit that better meets your needs.
    - A convenient "all-in-one" box format that gives you the assurance that you will have all the necessary reagents you need to run your assay.

The MILLIPLEX® MAP Rat/Mouse Neuropeptide Magnetic Bead Panel is to be used for the simultaneous quantification of any or all of the following in rat and mouse serum, plasma, or tissue/cell lysate and culture supernatant samples: α-MSH, β-Endorphin, Neurotensin, Oxytocin and Substance P.

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

Please read entire protocol before use.

It is important to use same assay incubation conditions throughout your study.

PRINCIPLE

MILLIPLEX® MAP is based on the Luminex® xMAP® technology — one of the fastest growing and most respected multiplex technologies offering applications throughout the life-sciences and capable of performing a variety of bioassays including immunoassays on the surface of fluorescent-coded magnetic beads known as MagPlex®-C microspheres.

- Luminex® uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets of 500 5.6 µm polystyrene microspheres or 80 6.45 µm magnetic microspheres can be created, each of which is coated with a specific capture antibody.
- After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced.
- The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- EMD Millipore provides three Luminex® instruments to acquire and analyze data using two detection methods:
    - The Luminex® analyzers Luminex® 200™ and FLEXMAP 3D®, flow cytometry-based instruments that integrate key xMAP® detection components, such as lasers, optics, advanced fluidics and high-speed digital signal processors.
    - The Luminex® analyzer (MAGPIX®), a CCD-based instrument that integrates key xMAP® capture and detection components with the speed and efficiency of magnetic beads.
- Each individual microsphere is identified and the result of its bioassay is quantified based on fluorescent reporter signals. EMD Millipore combines the streamlined data acquisition power of Luminex® xPONENT® acquisition software with sophisticated analysis capabilities of the new MILLIPLEX® Analyst 5.1, integrating data acquisition and analysis seamlessly with all Luminex® instruments.

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

STORAGE CONDITIONS UPON RECEIPT

- Recommended storage for kit components is 2 - 8°C.
- For long-term storage, freeze reconstituted standards and controls at ≤ -20°C. Avoid multiple (>2) freeze thaw cycles.
- **DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin**.

REAGENTS SUPPLIED

Note: Store all reagents at 2 – 8 °C

Reagents Supplied	Catalog Number	Volume	Quantity
Rat/Mouse Neuropeptide Standard	RMNP-8083	Lyophilized	1 vial
Rat/Mouse Neuropeptide Quality Controls 1 and 2	RMNP-6083	Lyophilized	2 vial each
Set of one 96-Well Plate with 2 sealers	----------	----------	1 plate 2 sealers
Assay Buffer	LE-ABGLP	30 mL	2 bottles
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	60 mL	1 bottle
Rat/Mouse Neuropeptide Primary Antibodies	RMNP-1083P	3.5 mL	1 bottle
Rat/Mouse Neuropeptide Detection Antibodies	RMNP-1083D	5.5 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE13	5.5 mL	1 bottle
Mixing Bottle	----------	----------	1 bottle

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

Rat/Mouse Neuropeptide Antibody Immobilized Magnetic Beads:

Bead/Analyte Name	Luminex® Magnetic Bead Region	Customizable 5 Analytes (20X concentration, 200 µL) Available	Cat. #
Anti-α-MSH Bead	15	✓	AMSH-MAG
Anti-Rat β-Endorphin Bead	20	✓	RMBEND-MAG
Anti-Neurotensin Bead	48	✓	NRTNSN-MAG
Anti-Oxytocin Bead	65	✓	0XYTCN-MAG
Anti-Substance P Bead	75	✓	SBTNCP-MAG

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex® Sheath Fluid (EMD Millipore Catalog #SHEATHFLUID) or Luminex® Drive Fluid (EMD Millipore Catalog #MPXDF-4PK)
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. Aluminum Foil
7. Absorbent Pads
8. Laboratory Vortex Mixer
9. Sonicator (Branson Ultrasonic Cleaner Model #B200 or equivalent)
10. Titer Plate Shaker (Lab-Line Instruments Model #4625 or equivalent)
11. Luminex® 200™, HTS, FLEXMAP 3D®, or MAGPIX® with xPONENT® software by Luminex® Corporation
12. Automatic Plate Washer for magnetic beads (BioTek® 405 LS and 405 TS, EMD Millipore Catalog #40-094, #40-095, #40-096, #40-097 or equivalent) or Handheld Magnetic Separation Block (EMD Millipore Catalog #40-285 or equivalent).

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

SAFETY PRECAUTIONS

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium Azide or Proclin has been added to some reagents as a preservative. Although the concentrations are low, Sodium Azide and Proclin may react with lead and copper plumbing to form highly explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

Note: See Full Labels of Hazardous components on next page.

Full Labels of Hazardous Components:

Ingredient, Cat #		Full Label	
Rat & Mouse Neruopeptide Quality Controls 1 & 2	RMNP-6083		**Warning.** Harmful if swallowed. Causes serious eye irritation. Toxic to aquatic life with long lasting effects. Avoid release to the environment. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Rat & Mouse Neuropeptide Standard	RMNP-8083		**Warning.** Harmful if swallowed. Causes serious eye irritation. Toxic to aquatic life with long lasting effects. Avoid release to the environment. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Streptavidin-Phycoerythrin	L-SAPE13		**Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
10X Wash Buffer	L-WB		**Warning.** May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water.

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.
- 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.
- When reading the assay on Luminex® 200™, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate or to the recommended EMD Millipore filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate using 1 alignment disc.

  For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid in each well and 75 µL should be aspirated.

TECHNICAL GUIDELINES (continued)

- For cell culture supernatants use the culture 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 extracted serum/plasma samples, use the Assay Buffer provided in the kit as the matrix solution in blank, standard curve and controls.
- 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 Protocols 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 Protocols or aliquot and store samples at ≤ -20 ºC.
- Avoid multiple (>2) freeze/thaw cycles.

C. Preparation of Cell Culture Supernatant:

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

NOTE:

- A maximum of 50 µL per well of cell culture supernatant, CSF or extracted serum or plasma may 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 PROTOCOLS

Use one of the following:

OPTION 1. 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) by vacuum at setting of Hg 2-5.
- Dry the samples by using Speed Vac at highest vacuum setting. Dried samples can be covered and stored at ≤ -20 ºC.
- Reconstitute samples with 250 µL Assay Buffer. Shake for 10 minutes. Assay immediately or store at ≤ -20 ºC.

OPTION 2. Acetonitrile Precipitation (recommended option for Oxytocin and Substance P)

- Put 250 µL sample into a microfuge tube.
- Add 375 µL acetonitrile to the sample, vortex 5 seconds, and let sit for 10 minutes at room temperature.
- Vortex again for 5 seconds, then centrifuge at 17,000 x g for 5 minutes.
- Carefully remove 500 µL of supernatant into a 96-well collecting plate.
- Dry the samples by using Speed Vac at highest vacuum setting. Dried samples can be covered and stored at ≤ -20 ºC.
- Reconstitute samples with 200 µL Assay Buffer. Shake for 10 minutes. Assay immediately or store at ≤ -20 ºC.

Note: Smaller sample volumes may be used with either extraction protocol. For example: If 150 µL sample is used in Option 1, the samples are acidified with 150 µL of 1% TFA, loaded, washed and eluted as above. Reconstitute dried samples in 150 µL Assay Buffer. If 150 µL sample is used in Option 2, add 225 µL acetonitrile, vortex and centrifuge as above, remove 300 µL supernatant. Reconstitute dried samples in 120 µL 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. (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: When using 5 antibody-immobilized beads, add 150 µL from each of the 5 bead sets to the Mixing Bottle. Then add 2.25 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 60 mL of 10X Wash Buffer with 540 mL deionized water. Store unused portions at 2-8°C for up to one month.

D. Preparation of Rat/Mouse Neuropeptide Standard

1.) Prior to use, reconstitute the Rat/Mouse 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

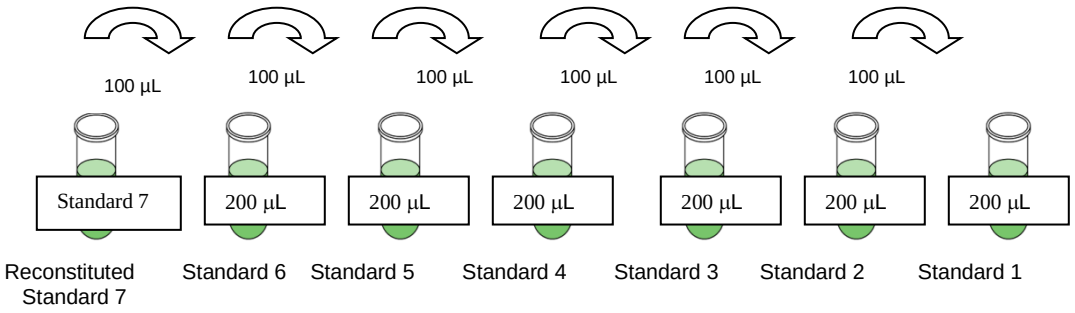
Label 6 polypropylene microfuge tubes "Standard 6," "Standard 5," "Standard 4," "Standard 3," "Standard 2," and "Standard 1." Add 200 µL Assay Buffer to each of the six tubes. Prepare serial dilutions by adding 100 µL of "Standard 7" to the "Standard 6" tube, mix well and transfer 100 µL of "Standard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

PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

Standard	Volume of Deionized Water to Add	Volume of Standard to Add
Standard 7 (Reconstituted Standard)	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 Standards



Note: Standard Concentrations

Standard Tube #	α-MSH (pg/mL)	β-Endorphin (pg/mL)	Neurotensin (pg/mL)	Oxytocin (pg/mL)	Substance P (pg/mL)
1	41.2	68.6	27	13.7	2.7
2	123.5	205.8	82	41.2	8.2
3	370.4	617.3	246	123.5	24.7
4	1111.1	1,851.9	740	370.4	74.1
5	3,333.3	5,555.6	2,222	1,111.1	222.2
6	10,000	16,666.7	6,666	3,333.3	666.7
7	30,000	50,000	20,000	10,000	2,000

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.
- If using a filter plate, set the filter plate on a plate holder at all times during reagent dispensing and incubation steps so that the bottom of the plate does not touch any surface.

1. Add 200 µL of Assay Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
2. Decant Assay Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
3. Add 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 (**RMNP-1083P**) to each well.
8. Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker 2 hr at room temperature (20-25°C).
9. Vortex Mixing Bottle and add 25 µL of the prepared Beads to each well. (Note: during addition of Beads, shake beads intermittently to avoid settling.)
10. Seal, the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker overnight at 4°C *(16-18 hr).*

Add 200 µL Assay Buffer to each well

↓ Shake 10 min, RT

↓ Decant

- Add 50 µL Standard or Control to appropriate wells
- Add 50 µL Assay Buffer to Background and Sample wells
- Add 50 µL samples to sample wells
- Add 50 µL appropriate matrix to Background, Standard and Control wells
- Add 25 µL Primary antibody (**RMNP-1083P**) to each well

↓ Incubate for 2 hours at room temperature

11. Gently remove well contents and wash plate 3 times following instructions listed in the **PLATE WASHING** section. **(NOTE: DO NOT INVERT PLATE)**.

12. Add 50 µL of Detection Antibodies (**RMNP-1083D**) into each well. (Note: allow the Detection Antibodies to warm to room temperature prior to addition.)

13. Seal, cover with foil, and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C) **DO NOT ASPIRATE AFTER INCUBATION.**

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

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

16. Gently remove well contents and wash plate 3 times following instructions listed in the **PLATE WASHING** section. **(NOTE: DO NOT INVERT PLATE)**.

17. 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 at room temperature.

18. Run plate on Luminex® 200™, HTS, FLEXMAP 3D® or MAGPIX® with xPONENT® software.

19. 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.

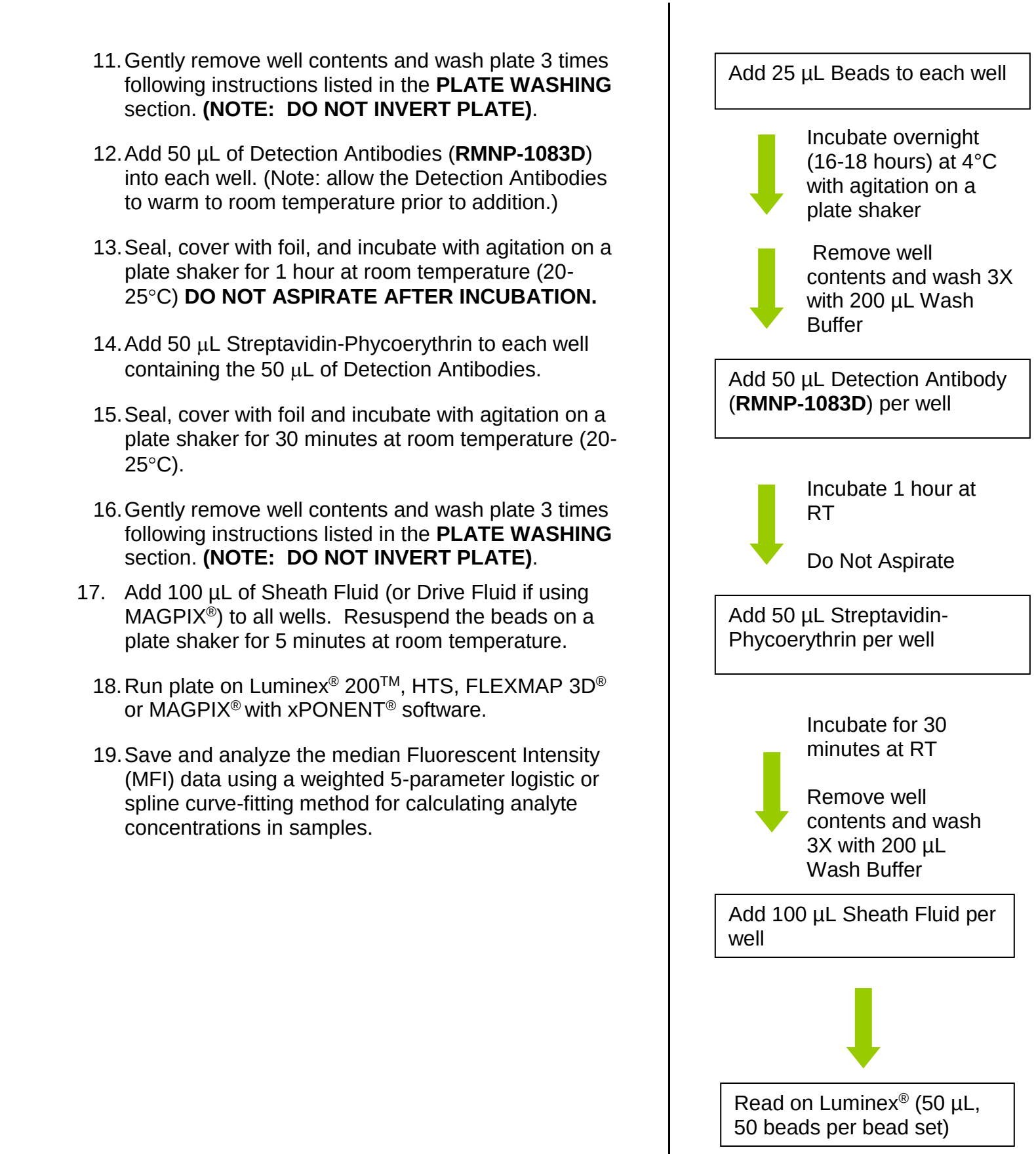


PLATE WASHING

1.) Solid Plate

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

A.) Handheld magnet **(EMD Millipore Catalog #40-285)** - Rest plate on magnet for 60 seconds to allow complete settling of magnetic beads. Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid. Wash plate with 200 µL of Wash Buffer by removing plate from magnet, adding Wash Buffer, shaking for 30 seconds, reattaching to magnet, letting beads settle for 60 seconds and removing well contents as previously described after each wash. Repeat wash steps as recommended in Assay Procedure.

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

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

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

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

EQUIPMENT SETTINGS

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

These specifications are for the Luminex® 200™, Luminex® HTS, Luminex® FLEXMAP 3D®, and Luminex® MAGPIX® with xPONENT® software. Luminex® instruments with other software (e.g. MasterPlex®, STarStation, LiquiChip, Bio-Plex Manager™, LABScan™ 100) would need to follow instrument instructions for gate settings and additional specifications from the vendors for reading Luminex® magnetic beads.

For magnetic bead assays, the Luminex® 200™ and HTS instruments must be calibrated with the xPONENT® 3.1 compatible Calibration Kit (EMD Millipore Catalog #40-275) and performance verified with the Performance Verification Kit (EMD Millipore Catalog #40-276). The Luminex® FLEXMAP 3D® instrument must be calibrated with the FLEXMAP 3D® Calibrator Kit (EMD Millipore Catalog #40-028) and performance verified with the FLEXMAP 3D® Performance Verification Kit (EMD Millipore Catalog #40-029). The Luminex® MAGPIX® instrument must be calibrated with the MAGPIX® Calibration Kit (EMD Millipore Catalog #40-049) and performance verified with the MAGPIX® Performance Verification Kit (EMD Millipore Catalog #40-050).

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

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

EQUIPMENT SETTINGS (continued)

The Luminex® probe height must be adjusted to the plate provided in the kit. Please use Catalog # MAG-PLATE, if additional plates are required for this purpose.

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Events:	50, per bead	
Sample Size:	50 µL	
Gate Settings:	8,000 to 15,000	
Reporter Gain:	Default (low PMT)	
Time Out:	60 seconds	
Bead Set:	Customizable 5-Plex Beads	
	α-MSH	15
	β-Endorphin	20
	Neurotensin	48
	Oxytocin	65
	Substance P	75

QUALITY CONTROLS

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert or can be located at the EMD Millipore website emdmillipore.com using the catalog number as the keyword.

ASSAY CHARACTERISTICS

Assay Sensitivities (minimum detectable concentrations, pg/mL)

Minimum Detectable Concentration (MinDC) is calculated using MILLIPLEX® Analyst 5.1. It measures the true limits of detection for an assay by mathematically determining what the empirical MinDC would be if an infinite number of standard concentrations were run for the assay under the same conditions.

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Analyte	Overnight Protocol (N = 8 assays)	
	Mean MinDC	Mean MinDC + 2SD
α-MSH	86	142
β-Endorphin	70	98
Neurotensin	32	48
Oxytocin	7	12
Substance P	2	3

Precision

Intra-assay precision is generated from the mean of the %CV's from 8 reportable results across two different concentrations of analytes in a single assay. Inter-assay precision is generated from the mean of the %CV's across two different concentrations of analytes across 4 different experiments.

Analyte	Intra-Assay CV %	Inter-Assay CV %
α MSH	<15	<20
β-Endorphin	<10	<15
Neurotensin	<10	<15
Oxytocin	<10	<15
Substance P	<10	<15

Accuracy

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

	Spike and Recovery %	
Analyte	**Rat**	**Mouse**
α-MSH	132	125
β-Endorphin	135	127
Neurotensin	115	120
Oxytocin	116	113
Substance P	107	113

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 to the kit solid plate or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height to the kit solid plate or to the recommended EMD Millipore filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height to the kit solid plate using 1 alignment disc. For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid in each well and 75 µL should be aspirated.
Background is too high	Background wells were contaminated	Avoid cross-well contamination by using sealer appropriately, and pipetting with multichannel pipettes without touching reagent in plate.
	Matrix used has endogenous analyte or interference	Check matrix ingredients for cross reacting components (e.g. interleukin modified tissue culture medium).
	Insufficient washes	Increase number of washes.
Beads not in region or gate	Luminex® instrument not calibrated correctly or recently	Calibrate Luminex® instrument based on manufacturer's instructions, at least once a week or if temperature has changed by >3°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® instrument 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.

Problem	Probable Cause	Solution
Signal for whole plate is same as background	Incorrect or no Detection Antibody was added	Add appropriate Detection Antibody and continue.
	Streptavidin-Phycoerythrin was not added	Add Streptavidin-Phycoerythrin according to protocol. If Detection Antibody has already been removed, sensitivity may be low.
Low signal for standard curve	Detection Antibody may have been removed prior to adding Streptavidin-Phycoerythrin	May need to repeat assay if desired sensitivity not achieved.
	Incubations done at inappropriate temperatures, timings or agitation	Assay conditions need to be checked.
Signals too high, standard curves are saturated	Calibration target value set too high	With some Luminex® instruments (e.g. Bio-Plex®) default target setting for RP1 calibrator is set at high PMT. Use low target value for calibration and reanalyze plate.
	Plate incubation was too long with standard curve and samples	Use shorter incubation time.
Sample readings are out of range	Samples contain no or below detectable levels of analyte	If below detectable levels, it may be possible to use higher sample volume. Check with technical support for appropriate protocol modifications.
	Samples contain analyte concentrations higher than highest standard point	Samples may require dilution and reanalysis for just that particular analyte.
	Standard curve was saturated at higher end of curve	See above.
High Variation in samples and/or standards	Multichannel pipette may not be calibrated	Calibrate pipettes.
	Plate washing was not uniform	Confirm all reagents are removed completely in all wash steps.
	Samples may have high particulate matter or other interfering substances	See above.
	Plate agitation was insufficient	Plate should be agitated during all incubation steps using an orbital plate shaker at a speed where beads are in constant motion without causing splashing.
	Cross well contamination	Check when reusing plate sealer that no reagent has touched sealer. Care should be taken when using same pipet tips that are used for reagent additions and that pipet tip does not touch reagent in plate.

FOR FILTER PLATES ONLY		
Problem	**Probable Cause**	**Solution**
Filter plate will not vacuum	Vacuum pressure is insufficient	Increase vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds.
	Samples have insoluble particles	Centrifuge samples just prior to assay 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 0.2 mL buffer can be suctioned in 3-5 seconds. May need to transfer contents to a new (blocked) plate and continue.
	Plate set directly on table or absorbent towels during incubations or reagent additions	Set plate on plate holder or raised edge so bottom of filter is not touching any surface.
	Insufficient blotting of filter plate bottom causing wicking	Blot the bottom of the filter plate well with absorbent towels after each wash step.
	Pipette touching plate filter during additions	Pipette to the side of plate.
	Probe height not adjusted correctly	Adjust probe to 3 alignment discs in well H6.
	Sample too viscous	May need to dilute sample.

REPLACEMENT REAGENTS	**Cat #**
Rat/Mouse Neuropeptide Panel Standard	RMNP-8083
Rat/Mouse Neuropeptide Panel Quality Controls	RMNP-6083
Rat/Mouse Neuropeptide Primary Antibodies	RMNP-1083P
Rat/Mouse Neuropeptide Detection Antibodies	RMNP-1083D
Streptavidin-Phycoerythrin	L-SAPE13
Assay Buffer (2 bottles)	LE-ABGLP
Set of two 96-Well Filter Plates with sealers	MAG-PLATE
10X Wash Buffer	L-WB

Antibody-Immobilized Magnetic Beads

Analyte	*Bead #*	*Cat. #*
α MSH	15	AMSH-MAG
β-Endorphin	20	RMBEND-MAG
Neurotensin	48	NRTNSN-MAG
Oxytocin	65	0XYTCN-MAG
Substance P	75	SBTNCP-MAG

ORDERING INFORMATION

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

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

emdmillipore.com/contact

Conditions of Sale

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

Safety Data Sheets (SDS)

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

WELL MAP

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