MILLIPLEX® MAP

Mouse Immunoglobulin Isotyping Kit
96 Well Plate Assay

#MGAM-300

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

INTRODUCTION

Produced by plasma cells and lymphocytes, immunoglobulins (antibodies) are critically involved in immune response, attaching to antigens and playing a role in their destruction. Immunoglobulins (Ig) can be classified by isotype, classes that differ in function and antigen response due to structure variability. Five major isotypes have been identified in placental mammals: IgM, IgG, IgA, IgE and IgD (B-cell receptor) – all found in normal individuals. Immunoglobulin-deficiency disorders, such as autoimmune disease, some GI conditions and malignancies, are characterized by specific isotype deficiencies or varying concentrations of one or more isotypes. Disease states can range from the absence of one isotype class or subclass to a total deficiency of immunoglobulin classes. In addition, isotyping applications include analyzing hybridomas during antibody development.

Millipore recognizes the need to provide you with the ability to quantitate immunoglobulin classes and subclasses simultaneously. Therefore, we are proud to announce that the former Beadlyte Mouse Isotyping Kit has been re-launched in the MILLIPLEX MAP optimized format. In addition, our MILLIPLEX MAP Mouse Isotyping Kit has been designed to enable you to measure accurately murine IgG subclasses (1, 2a, 2b, and 3), IgM, and IgA – all in one well. The xMAP multiplex technology is ideal for measuring levels of these isotypes, which not only decreases the number of assays as well as the amount of sample required, but also greatly reduces the possible inaccuracies that result from performing multiple assays.

This kit is for research purposes only.

Please read entire protocol before use.

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

PRINCIPLE

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

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

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

STORAGE CONDITIONS UPON RECEIPT

- Recommended storage for kit components is 2 - 8°C.
- Once the standards and controls have been reconstituted, immediately transfer contents into polypropylene vials. **DO NOT STORE RECONSTITUTED STANDARDS OR CONTROLS IN GLASS VIALS**. For long-term storage, freeze reconstituted standards and controls at ≤ -20°C. Avoid multiple (>2) freeze/thaw cycles.
- **DO NOT FREEZE Antibody-Immobilized Beads or PE Antibodies**.

REAGENTS SUPPLIED

Note: Store all reagents at 2 – 8°C

REAGENTS SUPPLIED	CATALOG NUMBER	VOLUME	QUANTITY
MILLIPLEX™ MAP Anti-Mouse Multi-Ig Beads, 1X	MGAM-PMX6	3.5 mL	1 bottle
MILLIPLEX™ MAP Anti-Mouse κ Light Chain, PE	44-029	50 µL	1 tube
MILLIPLEX™ MAP Anti-Mouse λ Light Chain, PE	44-030	26 µL	1 tube
MILLIPLEX™ MAP Mouse Multi-Immunoglobulin Standard	47-300	0.5 mL	1 vial
MILLIPLEX™ MAP Mouse Immunoglobulin Positive Control	43-008L	0.25 mL	1 vial
MILLIPLEX™ MAP Assay Buffer	L-AB	30 mL	2 bottles
Set of one 96-Well Filter Plate with 2 Sealers	MX-PLATE	-------	1 plate

Ig	IgG1	IgG2a	IgG2b	IgG3	IgA	IgM
Bead #	20	35	37	32	51	19

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

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

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
9. Titer Plate Shaker
10. Vacuum Filtration Unit (Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with Millipore Vacuum Pump Catalog #WP6111560 or equivalent)
11. Luminex 100™ IS, 200™, or HTS by Luminex Corporation
12. Plate Stand (Millipore Catalog # MX-STAND)

SAFETY PRECAUTIONS

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium azide or Proclin has been added to some reagents as a preservative. Although the concentrations are low, sodium azide and Proclin may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.

TECHNICAL GUIDELINES

To obtain reliable and reproducible results, the operator should carefully read this entire manual and fully understand all aspects of each assay step before running the assay. The following notes should be reviewed and understood before the assay is set up.

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not use beyond the expiration date on the label.
- Do not mix or substitute reagents with those from other lots or sources.
- The Antibody-Immobilized Beads are light sensitive and must be protected from light at all times. Cover the assay plate containing beads with opaque plate lid or aluminum foil during all incubation steps.
- It is important to allow all reagents to warm to room temperature (20-25°C) before use in the assay.

- The bottom of the Microtiter Filter Plate should not be in direct contact with any surface during assay set-up or incubation times. The plate can be set on a plate stand or on the non-flat side of the plate cover or any other plate holder to raise the plate from the surface. A plate stand can be purchased separately from Millipore (Millipore Catalog #MX-STAND).
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Assay 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).
- 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 transferred to polypropylene tubes and stored at ≤ -20°C for 1 month and at ≤ -80°C for greater than one month.
- If samples fall above the dynamic range of the assay, further dilute the samples with the appropriate diluent and repeat the assay.
- 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 Luminex 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, use the culture medium as the diluent in background, standard curve, and control wells. In assays using serum samples, all samples, standards, and controls should be diluted in Assay Buffer. In all cases, use Assay Buffer for wash and resuspension steps.
- Vortex all reagents well before adding to plate.

SAMPLE COLLECTION AND STORAGE

A. Preparation of Serum Samples:

- Allow the blood to clot for at least 30 minutes before centrifugation for 10 minutes at 1000xg. Remove serum and assay immediately or aliquot and store samples at ≤ -20°C.
- Avoid multiple (>2) freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.
- Serum samples should be diluted 1:25,000 in the Assay Buffer and a standard curve diluted in Assay Buffer should be used.
- To achieve a 1:25,000 dilution, dilute 5µL of sample into 1245µL of ultrapure water (1:250). Subsequently, dilute 5µL of the 1:250 dilution into 495µL of Assay Buffer (1:100) dilution.

B. Preparation of Tissue Culture Supernatant:

- Centrifuge the sample to remove debris and assay immediately or aliquot and store samples at ≤ -20°C.
- Avoid multiple (>2) freeze/thaw cycles.
- Dilute the sample to approximately 1 ug/mL Ig in Assay Buffer. [Cell culture supernatants samples approximately (1:5); bioreactor supernatants (1:100). Note: Cell culture supernatant concentrations are cell-line dependent and range from 5-50 ug/mL. Bioreactor supernatants may be as concentrated as 1 mg/mL.

NOTE:

- A maximum of 50µL per well of diluted serum or supernatant can be used.
- All samples must be stored in polypropylene tubes. **DO NOT STORE SAMPLES IN GLASS.**
- Avoid debris, lipids and cells when using samples with gross hemolysis or lipemia.
- Care must be taken when using heparin as an anticoagulant since an excess of heparin will provide falsely high values. Use no more than 10 IU heparin per mL of blood collected.

PREPARATION OF REAGENTS FOR IMMUNOASSAY

A. Preparation of Antibody-Immobilized Beads

Sonicate the pre-mixed bead bottle 30 seconds and then vortex for 1 minute before use.

B. Preparation of Standards

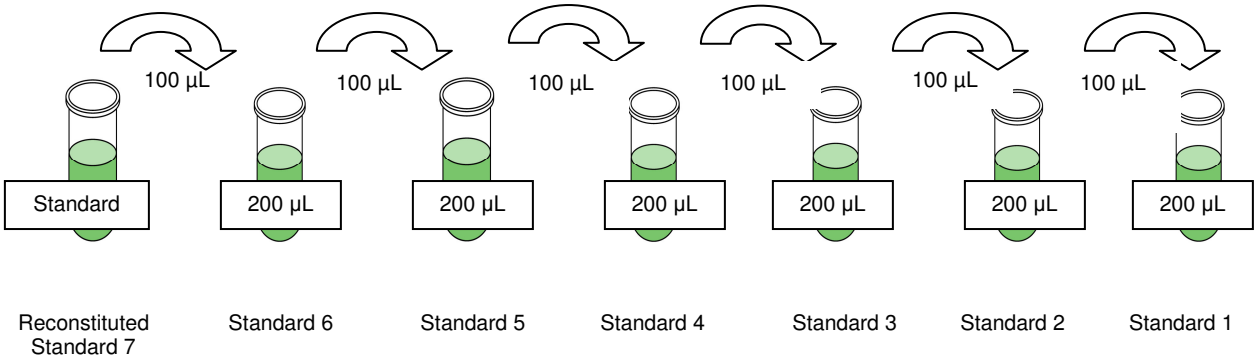
Resuspend MILLIPLEX MAP Mouse Multi-Immunoglobulin (IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM) Standard (Catalog # 47-300) in 0.5 mL Assay Buffer (Catalog # L-AB). Vortex at high speed for 15 seconds. Place on ice for 15 minutes. This is Standard **A**. Note: Standards are of kappa light chain isotype and therefore will react only with anti-Mouse Kappa-PE detection reagent.

C. Preparation of Working Standards (Std):

Label five polypropylene microfuge tubes Std 6, Std 5, Std 4, Std 3, Std 2 and Std 1. Add 200 µL of Assay Buffer to each of the six tubes. Prepare 3-fold serial dilutions by adding 100 µL of the reconstituted Standard 7 to the Std 6 tube, mix well and transfer 100 µL of the Std 6 to the Std 5 tube, mix well and transfer 100 µL of the Std 5 to the Std 4 tube, mix well and transfer 100 µL of the Std 4 to Std 3 tube, mix well and transfer 100 µL of the Std 3 to the Std 2 tube and mix well and transfer 100 µL of the Std 2 to the Std 1 tube and mix well . The Standard 0 (Background) will be Assay Buffer.

Standard	Volume of Assay Buffer to Add	Volume of Standard to Add
Original (Standard 7)	500 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 dilutions, the standard tubes should have the following concentrations for the isotypes, as noted below:

Standard Tube #	IgG1 (ng/mL)	IgG2a (ng/mL)	IgG2b (ng/mL)	IgG3 (ng/mL)	IgA (ng/mL)	IgM (ng/mL)
1	2	3	1	2	6	2
2	6	8	4	6	19	6
3	19	25	12	19	56	19
4	56	74	37	56	167	56
5	167	222	111	167	500	167
6	500	667	333	500	1500	500
7	1500	2000	1000	1500	4500	1500

D. Preparation of Positive Control

Rususpend MILLIPLEX MAP Mouse Multi-Ig Positive Control (Catalog # 43-008L) in 0.25 mL Assay Buffer (Catalog # L-AB) (or cell culture medium if running with cell culture supernatants). Vortex this at high speed for 15 seconds. Place this on ice for 15 minutes. Use 50µL per well.

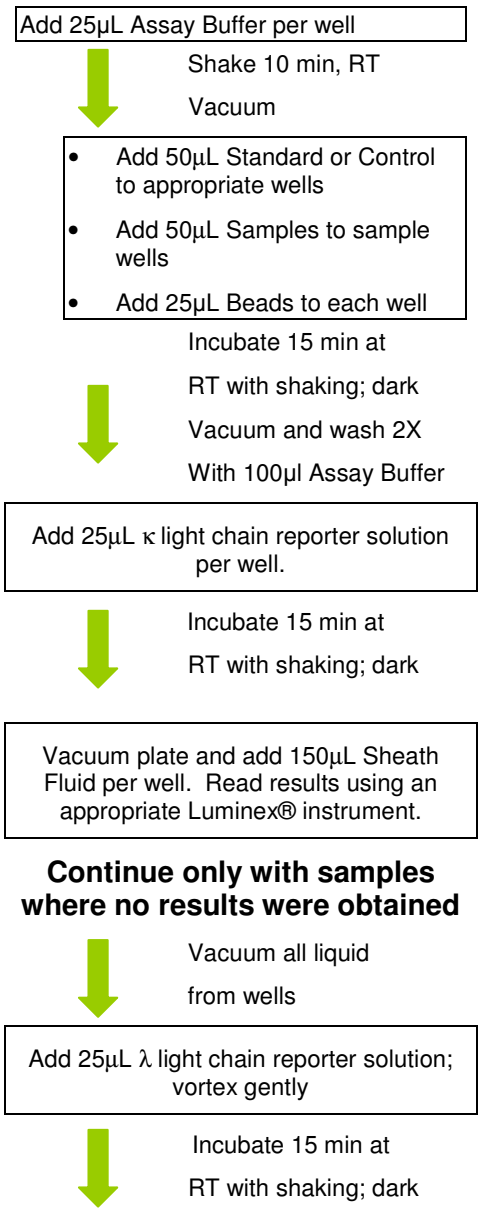
E. Preparation of Detection Reagent

To prepare 100X detection reagent, dilute anti-Mouse Kappa-PE to working solution (1:100) with Assay Buffer (for a full plate, use 25µL of the 100X anti-Mouse kappa-PE in 2.475 mL assay buffer). [Note: 95% of mouse antibodies have κ light chains, so in most instances, use of only the κ Light Chain Reporter will be necessary. If determination of light chain is desired or samples show no signal with the kappa light chain detection, vacuum plate and prepare anti-Mouse Lambda-PE the same as above. Repeat detection step with anti-mouse Lambda-PE and reread plate.

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 [A though H (Background)] and Positive Control, and Samples on Well Map Worksheet in a vertical configuration. (Note: Most instruments will only read the 96-well plate vertically by default.) It is recommended to run the assay in duplicate.
- Set the filter plate on a plate holder at all times during reagent dispensing and incubation steps so that the bottom of the plate does not touch any surface.

1. Pre-wet filter plate by pipetting 25µL of Assay Buffer into each well of the Microtiter Filter Plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25 °C). 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.
2. Add 50µL of control, standard, or diluted sample to each well.
3. Vortex the MILLIPLEX MAP Anti-Mouse Multi-Immunoglobulin Beads at medium speed for 15 seconds, and then sonicate for 15 seconds using a sonication bath. Add 25µL of bead solution to each well.
4. Cover with opaque plate cover and incubate 15 minutes with agitation on plate shaker at room temperature.
5. Remove fluid by vacuum. Wash plate 2 times with 100µL of Assay Buffer. Remove Assay Buffer by vacuum filtration between each wash. Blot bottom of plate on paper towel. **Do not over-dry.**
6. Dilute MILLIPLEX MAP Anti-Mouse **κ** Light Chain, PE 1:100 in Assay Buffer (for a full plate, dilute 25µl in 2.475mL Assay Buffer). [Note: 95% of mouse antibodies have κ light chains, so in most instances, use of only the κ Light Chain Reporter will be necessary.] Add 25µL per well.
7. Cover with opaque plate cover and incubate 15 minutes with agitation on plate shaker at room temperature.
8. Apply vacuum manifold to bottom of the filter plate to remove the liquid and resuspend in 150µL of Sheath Fluid.
9. Proceed to reading results on an appropriate Luminex® instrument.
10. **OPTIONAL**: If no results are seen or if determination of light chain is desired, apply vacuum manifold to bottom of the filter plate to remove the liquid and re-assay with MILLIPLEX MAP Anti-Mouse λ Light Chain, PE.
11. Dilute MILLIPLEX MAP Anti-Mouse λ Light Chain, PE 1:100 in Assay buffer (for a full plate, dilute 25µL in 2.475mL Assay Buffer). Add 25µL per well.
12. Cover and incubate 15 minutes with agitation on plate shaker in the dark at room temperature.



13. Apply vacuum manifold to bottom of the filter plate to remove the liquid and resuspend in 150µL of Sheath Fluid.
14. Proceed to reading results on an appropriate Luminex® Instrument
15. Proceed to reading results on Luminex® 100 Instrument

Vacuum and Add 150µL Sheath Fluid per well. Read results using an appropriate Luminex® instrument.

EQUIPMENT SETTINGS

These specifications are for the Luminex100 v.1.7 or Luminex100IS v2.1/2.2, Luminex200 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	100 µL
Gate Settings	8,000 to 15,000
Reporter Gain	Default (Low PMT)
Time Out	60 seconds
Bead Set	Bead #
IgG1	20
IgG2a	35
IgG2b	37
IgG3	32
IgA	51
IgM	19

QUALITY CONTROLS

The ranges for each analyte in the Mouse Multi-Ig Positive Control 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, ng/mL)

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

Analyte	*MinDC*	*MinDC + 2SD*
IgM	0.3	0.45
IgG1	0.3	0.65
IgG3	0.4	0.80
IgG2a	0.4	0.40
IgG2b	0.4	0.59
IgA	0.7	1.06

N=5 assays

Precision (%CV)

Intra-assay precision is generated from the mean of the %CV's from four reportable results across 1 concentration of analytes in 4 different assays. Inter-assay precision is generated from the mean of the %CV's from four reportable results across 1 concentration of analytes across 5 different assays.

Analyte	*Intra-Assay (%CV)*	*Inter-Assay (%CV)*
IgM	4	17
IgG1	4	18
IgG3	4	15
IgG2a	6	14
IgG2b	4	20
IgA	15	21

Accuracy (% Recovery)

Spike Recovery: The data represent mean percent recovery of 3 levels of spiked standards in diluted serum from 4 different mouse samples.

Analyte	***Spike Recovery in Serum***			
	Sample 1	*Sample 2*	*Sample 3*	*Sample 4*
IgM	100	102	97	92
IgG1	95	101	99	90
IgG3	79	87	75	62
IgG2a	103	106	97	87
IgG2b	88	89	83	80
IgA	105	102	94	98

TROUBLESHOOTING GUIDE

Situation	Possible Problem	Solution
Data acquisition time exceeds 30 seconds per well and/or "Sample Empty" occurs	**Mechanical** 1. Needle height is not correct (common problem due to variation in well depth between plate types). 2. Sample needle is clogged. 3. Air in the system. 4. Low pressure in the system. **Sample Related** 1. Lysate concentration too high. 2. Particulate matter in lysate. 3. No buffer in well.	**Mechanical** 1. Adjust needle height using 2 disks using "options; XY setup function". 2. Remove needle (see user's manual) and sonicate it to remove obstruction. 3. Perform alcohol flush and then wash with alcohol; followed by a wash with sheath fluid and a prime. 4. Tighten tops on the ***sheath fluid*** container. 5. Loosen the top on the ***waste*** container. **Sample Related** 1. Refer to lysate preparation protocol. 2. Centrifuge or filter lysate to remove particulate matter.
Readings are lower than expected	1. Gate is not set properly. 2. Calibration is not correct. 3. Cells responded poorly to stimulation.	1. Right click within the "doublet discriminator" and create gate. Move lines to 8,000 and 13,500 2. Run a machine calibration with calibration beads available from Luminex® Corp. 3. Check stimulation conditions.

Bead pattern is diffuse and missing the bead target (white oval)	1. Precipitate buildup in system. 2. Calibration is not correct. 3. Incompatible buffer used to resuspend beads for Luminex® analysis.	1. Drain the system, followed by a backflush, proceed with solution for air in the system. 2. Run a machine calibration with calibration beads available from Luminex® Corp. 3. Vacuum plate and resuspend beads in Sheath Fluid.
Applying vacuum to filter plate does not remove liquid from wells	1. Wells not in use are empty. 2. Particulate matter in lysate.	1. Place tape over the top of empty wells that are not in use. 2. Pre-filter lysate before use.
Aggregation of beads during analysis	1. Particulate matter in lysate. 2. Incompatible lysis buffer. 3. Beads not sonicated for step 3 of main protocol.	1. Pre-filter the lysate. 2. Use recommended lysis buffer. 3. Sonicate ***resuspended*** 1X beads prior to adding to filter plate.
Liquid wicking out from 96-well filter plate	1. Plate was not blotted on paper towel prior to adding next reagent. 2. The bottom of the plate is in contact with absorbent material such as paper towel or bench paper.	1. After vacuum step, gently blot the bottom of the plate using a paper towel to remove excess liquid. 2. Place the plate on a flat, non-absorbent surface during loading steps.

REPLACEMENT REAGENTS	Cat #
MILLIPLEX™ MAP Anti-Mouse Multi-Ig Beads, 1X	MGAM-PMX6
MILLIPLEX™ MAP Anti-Mouse κ Light Chain, PE	44-029
MILLIPLEX™ MAP Anti-Mouse λ Light Chain, PE	44-030
MILLIPLEX™ MAP Mouse Multi-Immunoglobulin Standard	47-300
MILLIPLEX™ MAP Mouse Immunoglobulin Positive Control	43-008L
MILLIPLEX™ MAP Assay Buffer	L-AB
Set of two 96-Well Filter Plate with sealers	MX-PLATE

ORDERING INFORMATION

To place an order:

To assure the clarity of your 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

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	Standard 0 Background	Standard 4	Pos Control									
B	Standard 0 Background	Standard 4	Pos Control									
C	Standard 1	Standard 5										
D	Standard 1	Standard 5										
E	Standard 2	Standard 6										
F	Standard 2	Standard 6										
G	Standard 3	Standard 7										
H	Standard 3	Standard 7										