Tips and Tricks

The Power of Multiplex Biomarker Analysis from the makers of MILLIPLEX® MAP

Why just multiplex when you can use MILLIPLEX®?

For over ten years, we have offered the benefits of MILLIPLEX® MAP multiplexed assay panels—containing all the components and reagents you need to detect multiple analytes simultaneously. The benefits of multiplex protein detection assays are endless, but navigating a protocol can be challenging. We're so confident in the benefits of MILLIPLEX® MAP kits that we've compiled this book of tips and tricks, straight from the experts, to eliminate any doubt in your ability to multiplex like a pro.

Every year, thousands of your colleagues experience the benefits of MILLIPLEX® MAP kits, publishing in scientific journals around the world. We hope this guide enhances the power of your research with multiplexing.

Note: Alternate methods presented in this guide may deviate from the protocol. These methods have either been tried by our scientists or end users working with our MILLIPLEX® MAP kits. We cannot guarantee methods presented will work in all cases. These procedures have not been validated.

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Introduction

The Luminex® xMAP® Technology:

- MILLIPLEX® MAP kits are based on the Luminex® xMAP® bead-based assay platform—one of the fastest-growing and most respected multiplex technologies, supporting applications throughout the life sciences. This platform is capable of performing a variety of bioassays, including immunoassays, on the surface of fluorescent-coded magnetic (MagPlex®) bead microspheres.
- Luminex® uses proprietary techniques to internally color-code microspheres with multiple fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets of 500 5.6 µm non-magnetic or 80 6.45 µm magnetic polystyrene microspheres are created, each of which is coated with a specific capture antibody.
- After the target protein 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.
- We provide three Luminex® instruments to acquire and analyze data using two detection methods (see **Figure 1**):
  - The Luminex® 200™ and FLEXMAP 3D® systems are flow cytometry-based instruments that integrate key xMAP® detection components, such as lasers, optics, advanced fluidics and high-speed digital signal processors.
  - The MAGPIX® analyzer is a LED/CCD-based instrument that integrates key xMAP® capture and detection components with the speed and efficiency of magnetic bead processing.
- Each individual microsphere is identified by its "bead signature," (or bead region) and the result of its bioassay is quantified based on fluorescent reporter signals. We combine the power of Luminex® xPONENT® acquisition software with sophisticated analysis capabilities of MILLIPLEX® Analyst 5.1 software, integrating data acquisition and analysis seamlessly on all Luminex® instruments.
- Use of MILLIPLEX® Analyst 5.1 affords the user enhanced features not available with most software packages, including the ability to select from various curve-fitting algorithms, batch uploading of sample names from text files and a detail report summarizing all data and conveniently exportable as an .xls file.

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

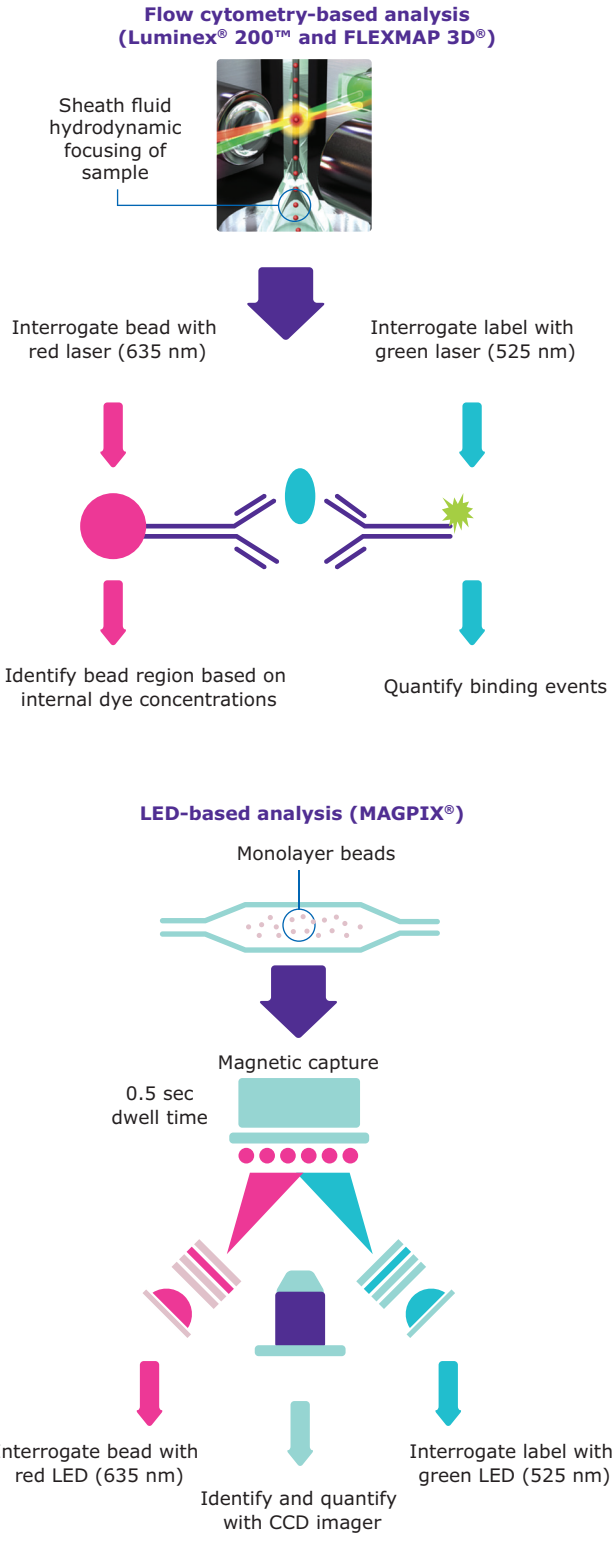
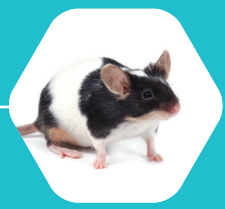


Figure 1. Two different fluorescence detection methods for acquiring and analyzing beadbased assay data. The corresponding Luminex® instrument for each method is highlighted above.

Cytokine/
Chemokine
Cytokine Receptors
Immunoglobulins
T Lymphocytes
MMPs, TIMPs
Complement
Myokine
Cardiovascular
Adipokine/
Adipocyte
Metabolic Hormone
IGFBPs
Cancer

Angiogenesis
Metastasis
Immuno Oncology
Autoimmune/
Autoantibody
Liver Injury
Pituitary
Thyroid
Bone Metabolism
Skin
Neurodegenerative
Neuropeptide
Kidney/Organ
Toxicity



Cytokine/Chemokine
T Lymphocytes
Immunoglobulins
MMPs
Adipokine/Adipocyte
Metabolic Hormone
Pituitary, Thyroid

Bone
Myokine
Cardiovascular
Angiogenesis
Neuropeptide
Kidney Toxicity

Cytokine/Chemokine
Metabolic Hormone
Pituitary

Cytokine/Chemokine

Cytokine/Chemokine

Cytokine/Chemokine
Adipokine/Adipocyte
Metabolic Hormone
Cardiovascular
Cardiac Injury
Vascular Injury
Pituitary

Stress Hormone
Thyroid
Bone
Myokine
Neurodegenerative
Neuropeptide
Kidney Toxicity

Y/S/T Phosphorylation
AKT, MAPK, STAT, SFK,
NF-κB, TGFβ, RTK,
Multi-Pathway, Apoptosis, DNA
Damage/Genotoxicity
Protein Translation

Cytokine/
Chemokine
Metabolic
Hormone

Pituitary
Kidney
Toxicity

Cytokine/Chemokine
Metabolic Hormone

We have the largest portfolio of kits, analytes and species compared to all other commercial suppliers:

- >100 kits to study circulating proteins
- >500 unique circulating analytes (not counting different species)
- >30 premixed kits and singleplex MAPmates™ to study intracellular proteins
- >100 intracellular analytes
- Multiple species
- 96- and 384-well formats

A broad portfolio means that you will:

- Find assays for analytes that you need
- Achieve greater consistency by purchasing assays from one vendor
- Retain the flexibility to meet your needs now and in the future
- Use one technology to quantify biomarkers in preclinical studies involving animal models and translational studies utilizing human samples
- Use one technology across multiple therapeutic areas

Let Industry Guidance Lead You to MILLIPLEX® MAP

From Academia to Contract Research to Big Pharma.
We meet the ever increasing demand for high quality assays for reproducible results.

- Detection and Sensitivity
- Performance in a Sample Matrix
- Specificity
- Selectivity
- Precision and Accuracy
- Linearity
- Stability
- Cross-talk
- Lot-to-Lot Variability/Reproducibility
- Vendor Support

We provide assay performance data upfront to you in every protocol.

Need more information? Contact:
EMDMillipore.com/techservice

Want to learn more about industry guidance on assay development and validation?
We recommend the following references:

1. Lee *et al.*, Pharmaceutical Research, Vol. 23, No. 2, February 2006 pp 317-328; Fit-for-Purpose Method Development and Validation for Successful Biomarker Measurement.
2. Jani *et al.*, The AAPS Journal, Vol. 18, No. 1, January 2016, pp 2-14; Recommendations for Use and Fit-for-Purpose Validation of Biomarker Multiplex Ligand Binding Assays in Drug Development.
3. Andreasson *et al.*, Frontiers in Neurology, Vol. 6, Article 179, August 2015, pp 1-8; A practical guide to immunoassay method validation.

Why Choose MILLIPLEX® MAP?

Our quality makes our assays stand out. From kit validation to manufacturing and QC, we give you confidence in your results.

Rely on the quality we build into each kit to produce results you trust. In addition to the assay specifications listed in the protocol, we evaluate other performance criteria during our validation process: cross-reactivity, dilutional linearity, kit stability, and sample behavior (e.g., detectability and stability).

Quality Controls

- We include Quality Controls (QCs) to qualify assay performance.
  - QC values are based on a minimum of six assays run by at least three different operators. +/- 35% of the high/low value (mean) are reported.
  - When a customer contacts Technical Support with a concern related to assay performance, the customer is usually first asked if the QC values are in a specific range. This tells the Technical Support Specialist whether or not the kit is performing correctly.
  - Use of a high and low QC value serve as an additional checkpoint in case there was user error associated with hydrating or diluting standard.
- Individual labs can qualify their own assay performance by including a high and low QC that may better reflect their unique experimental samples.
- QCs are important for translational studies that require more validation, ensuring that the data are reproducible across various kit lots.
- QCs are also important when comparing data for multi-site studies or assay results from multiple technicians.

Standards

- Each new lot of MILLIPLEX® standards is compared to previous lots AND a "reference" lot
  - All data are compiled in a single database and trend charts are maintained
  - Relative potency of analytes is maintained within tight specifications of the "reference" lot
- Other suppliers compare new standard (calibrator) lots to previous lots only
  - This may make it difficult to compare data from multiple lots since values may vary with each new lot
- Each lot of standards (calibrators) and quality controls (QCs) is compared to previous lots and a "reference" lot to ensure lot-to-lot consistency

Comparison of standard (calibrator) and quality control (QC) lots are compared to a reference lot to ensure lot-to-lot consistency

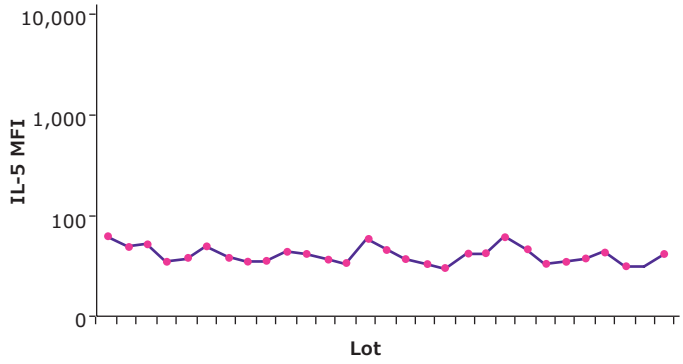


Figure 2. Trend chart shows consistent MFI values for IL-5 standard curve across 29 lots of a MILLIPLEX® map panel (Cat. No. HCYTOMAG-60K); ±10% of reference lot.

Don't see what you need?

Contact Custom Assay Development Services to:

- Combine analytes from 2 or more multiplex panels
- Develop custom assays on any of our 5 platforms

For more detailed information, visit
merckmillipore.com/customassay

For a complimentary project feasibility assessment, email
customassay@merckgroup.com

Effect of Serum Matrix

- If the recovery of analytes spiked into sample wells in an assay using a buffer standard curve falls outside our acceptance criteria (70-130%), this indicates that there is a nonspecific matrix effect from the samples.
  - To compensate for this effect, a native serum matrix with a similar effect is added to the standard curve wells to shift the actual curve so that it matches the recovery in the sample wells.
  - Serum matrix is usually a similar sample with all the endogenous and cross-reacting analytes extracted.
- Because blood is a complex matrix, which contains large numbers of proteins that may interfere with the accurate measurement of desired analytes, using an optimized serum matrix in the standard curve when measuring analytes secreted in serum/plasma:
  - Significantly improves accuracy of measurement.
  - More accurately simulates the conditions of the native analyte present in serum or plasma compared to a standard curve generated by spiking an analyte into a buffer solution.
  - Mimics the environment of native analytes in serum or plasma.
- Other commercial multiplex kits add a serum diluent buffer to sample wells. With some exceptions, we do not do this for the following reasons:
  - While this method does effectively show good recoveries, in most cases, adding serum matrix to sample wells can mask the matrix effect, likely affecting the sensitivity of the actual analyte measurement.
  - It is very difficult to predict the effect of mixing serum matrix with samples from a randomly sampled population.

Optimized serum matrix included in each appropriate kit ⟶ mimics native analyte environment ⟶ results in higher percent recovery for each analyte ⟶ improves accuracy of measurement

	Average Serum Sample Recovery			
	Sample dilution	IFNγ	IL-1	TNFα
Standards diluted in assay buffer	Neat	34%	40%	29%
	1:4	49%	63%	52%
	1:20	69%	81%	75%
Standards diluted in serum matrix	Neat	83%	117%	77%

Table 1. Comparison assay of three analytes interpolated against standard curves diluted into assay buffer vs. serum matrix.

Bead Diluent

- Approximately 10% of a normal population of samples, especially human serum or plasma, have heterophilic antibodies that can nonspecifically bind to the capture and detection antibodies simultaneously, thus generating a false positive signal.
  - Bead diluents contain a cocktail of proprietary reagents that significantly reduce this false signal without reducing the true analyte measurement.
  - Bead diluents may also contain factors for detection. For instance we have added the Insulin Detection antibody into the Bead Diluent of certain mouse panels. This ensures the best detection beginning from the initial incubation.

Detection Antibody Cocktail

- All MILLIPLEX® MAP panels include a detection antibody cocktail pre-hydrated in our proprietary buffer. Our detection antibodies are designed to yield consistent analyte profiles within the panel, lot-to-lot and regardless of the plex size.

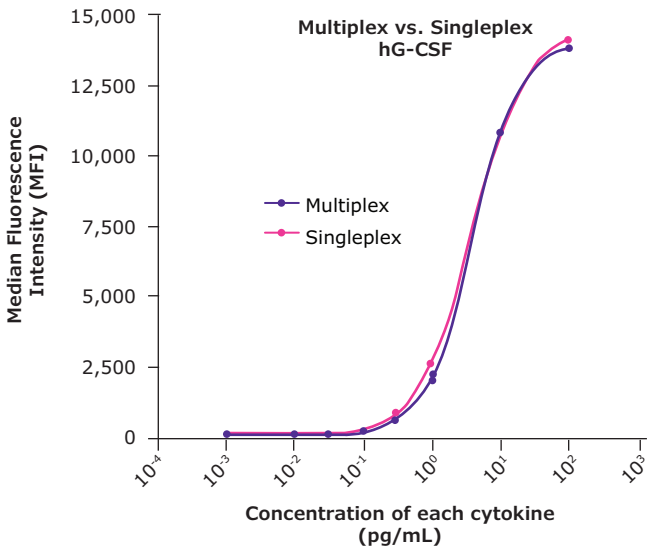


Figure 3. Consistent analyte profiles are seen when comparing Multiplex and Singleplex assays from the same MILLIPLEX® Panel, as in this example of the analyte hG-CSF.

Deciding Which MILLIPLEX® Assays are Best for Your Research

- All kits are for Research Use Only.
- Our multiplex and singleplex assays for the same analyte commonly use the same antibody pairs and conditions.
  - In method comparison tests, while the absolute values may not be exactly the same, the results do correlate. Hence when switching from one assay platform to another, a correlation factor may often be used when comparing with other platform data.
  - Please contact Technical Support for more information on correlation factors.
- To locate protocols and technical documents for a specific panel:
  - Search the website using the catalog number.
  - Click on "Protocols" on the right side.
    - This will take you to a documentation page
  - Or click on the linked header, which will take you to the Product Detail Page. There you will be able to view:
    1. Linked documentation
    2. Product details and data
    3. Design and Price your kit button
- There are three easy methods to find your analytes of interest:
  - The MILLIPLEX® MAP Analyte Kit Finder located on the MILLIPLEX® MAP home page.
  - Search the latest edition of the Analyte Quarterly.
  - Contact Technical Support.
- To find publications citing a specific panel or analyte, contact Technical Support or your Sales Specialist
- To determine cross-reactivity for other species for a panel or analyte:
  - See the Species Cross-reactivity Tables in Appendix 1.
  - Contact Technical Support.
  - For intracellular assay kits, we analytically validate the assay with human cell/tissue culture samples. However, we provide the species homology for each analyte in a table on the product detail page on our website. We have also compiled a list of this information on our website:
    - Kits: Kit Species Cross-reactivity: Merckmillipre.com/kits_species
    - MAPmate™ assays: MAPmate™ Species Cross-reactivity Table Merckmillipre.com/mapmates_sp
  - Search the latest edition of the Analyte Quarterly.
- How to design a "customizable" kit:
  - Select your panel of interest: for example, Human Cytokine/Chemokine Panel 1 (Cat. No. HCYTOMAG-60K).
  - Choose only the analytes you want from that panel: for example, you may need only five analytes: IL-2, IL-6, IL-10, GM-CSF, VEGF-A.
  - Add the number of analytes you chose to the catalog number: HCYTOMAG-60K-05 and list the specific analytes.
- How to design and order a customizable kit online:
  - From the Product Description page:
    - Click "Design And Price Your Kit."
    - Select your analytes; add to the cart and/or save to your favorites and go to "Checkout."
  - From the MILLIPLEX® MAP website: emdmillipore.com/milliplex
    - Click "Design & Purchase Your Own Kit".
    - Some kits require you to choose your sample type before you choose your analytes.
    - Select your analytes, add to the cart and/or save to your favorites and go to "Checkout."
  - In "Quick Purchase":
    - Click on the "Begin" icon located within "Design and Price your MILLIPLEX® MAP Kits."
    - Select your analytes, add to the cart and/or save to your favorites and go to "Checkout."

For questions or issues with Luminex® instruments, contact Luminex® at:

All Regions	merckmillipore.com/lmx_contact
Technical Support	Phone: 512-381-4397
	Toll-free: 1-877-785-2323
	Fax: 512-219-5114
Email:	support@luminexcorp.com

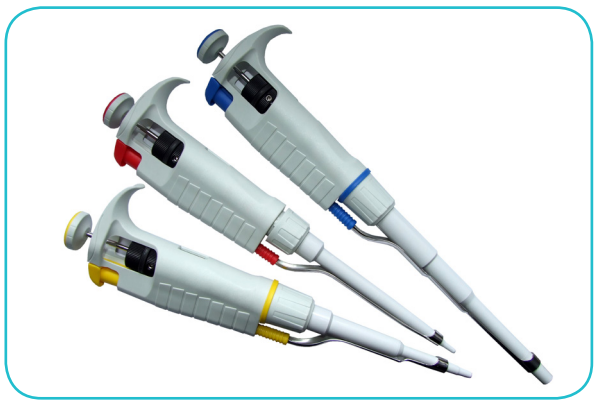
For questions or issues with BioTek® washers, contact BioTek® at:

All Regions	merckmillipore.com/biotek_contact
Technical Support:	
In North America	(800) 242-4685
Outside the U.S.	(802) 655-4740
Email	TAC@biotek.com

please visit MerckMillipore.com/contact to find your regional Technical Support Team or contact your Sales Specialist.

Materials Required But Not Provided

- Adjustable pipettes with tips capable of delivering 25 µL to 1000 µL



- Multichannel pipettes capable of delivering 5 µL to 50 µL or 25 µL to 200 µL

- Laboratory vortex mixer

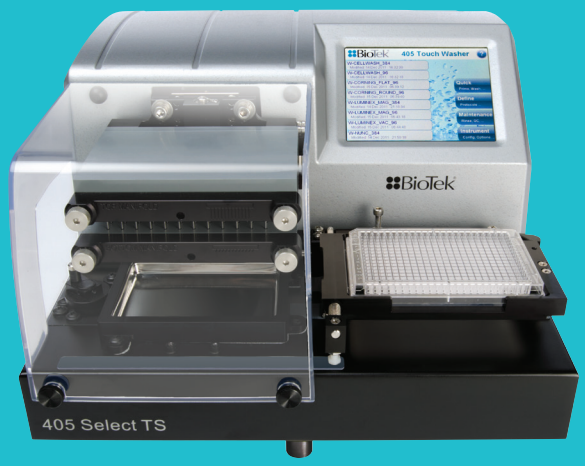
- Water bath sonicator (Branson Ultrasonic Cleaner Model #B200 or equivalent)
  - Sonicator probes are not recommended

- Orbital titer plate shaker (VWR® Microplate Shaker Cat # 12620-926 or equivalent).

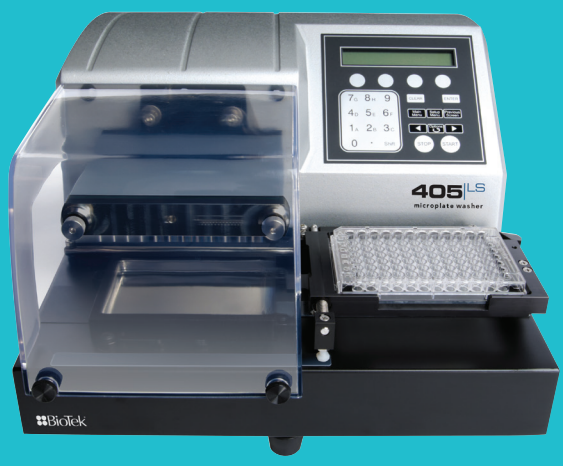
- Luminex® 200™, MAGPIX®, or FLEXMAP 3D® instruments, with analysis software.
- Sheath fluid (Luminex® 200™ or FLEXMAP 3D® systems) or drive fluid (MAGPIX® instrument).
  - Sheath fluid or drive fluid can be reordered directly from us:
    - Sheath Fluid, 20L (Cat. No. 40-50015)
    - MAGPIX® Drive Fluid 4PK , 750mL each (Cat. No. 40-50014)
- Before you open a MILLIPLEX® MAP kit, check your instrument to be sure it has been properly calibrated and maintained.
  All Luminex® instruments using xMAP® technology, operating on xPONENT® software, require regular calibration and performance verification testing to ensure that the system is operating correctly and maintaining data accuracy.
- Maintenance Kits for Luminex® Instruments are available directly from us:
  - Luminex® 200 (xPONENT®)
    - Calibration Kit (Cat. No. LX2R-CAL-K25)
    - Performance Verification Kit (Cat. No. LX200-CON-K25)
  - MAGPIX®
    - Calibration Kit (Cat. No. MPX-CAL-K25)
    - Performance Verification Kit (Cat. No. MPX-PVER-K25)
  - FLEXMAP 3D®
    - Calibration Kit (Cat. No. F3DIVD-CAL-K25)
    - Performance Verification Kit (Cat. No. F3DIVD-PVER-K25)
- Bead washer (either automated or manual):
  - Automated magnetic bead plate washers:
    - BioTek® 405 LS Magnetic 96-well Washer (Cat. No. 40-094).
    - BioTek® 405 LS Magnetic/Vacuum Filtration 96-well Washer (Cat. No. 40-095).
    - BioTek® 405 TS Magnetic 96-well Washer Complete with Touch Screen and Ultrasonic Cleaning (Cat. No. 40-096).
    - BioTek® 405 TS Magnetic/Vacuum Filtration 96-well Washer Complete with Touch Screen and Ultrasonic Cleaning (Cat. No. 40-097).
    - BioTek® ELx50 Magnetic 96-well Strip Washer (Cat. No. 40-062).
    - BioTek® Multiflow Automated Washer Fully automated, optimized for both 384- and 96-well plates (Cat. No. 40-099).
  - Handheld Magnetic Separator Block for 96-well Flat Bottom or Conical Well Plates (Cat. No. 40-285).

For more information, please see our Analyte Quarterly.

BioTek® 405™ plate washer models:



BioTek® 405 TS Washer with Touch Screen and Ultrasonic Cleaning



BioTek® 405 LS Washer

Sample Collection and Preparation

General Assay Information

- All kits are for Research Use Only.
- Always read the entire protocol before proceeding.
- Do not use a kit beyond its expiration date.
- The expiration date for a kit is that of the component with the shortest expiration date. This date is printed on the box label.
- Kits will ship with a minimum of 3 months until expiration.
- Longer expiration dates can be requested. Please contact your Sales Specialist.

General Information

- Protocol procedures are optimized for best data results; consequently, protocols can vary from kit to kit.
- Proper and consistent pipetting technique is key to accurate data, especially if multiple users will be generating data in collaboration. Improper or inconsistent technique can affect delivery volumes and impact data reliability. Training on best practices for pipetting and maintaining properly calibrated pipettors can substantially increase pipetting precision.
- For tips on proper pipetting techniques visit: www.emdmillipore.com/mlo ("Ten tips to improve pipetting technique").
- **Before you collect samples** to run an assay, it's important to read the entire protocol. If you have any questions, contact Technical Support or your Sales Specialist.
- If the protocol states that the kit can be used in either serum or plasma, and you have the option, choose serum because it tends to be cleaner. However, always consider the biology of the biomarkers under consideration to determine the appropriate sample type for your study/experiment.
- If you are trying to decide whether to collect serum or plasma samples, ask yourself what you have observed from preliminary data, publications or collaborators.
- Be consistent with the use of sample types within a study/project.
  - Still unsure? Contact Technical Support.
- Freeze/thaw limits:
  - Multiple freeze/thaw cycles may reduce the stability of the analytes, however this may be analyte dependent. When aliquoting samples to freeze, carefully determine what volume to aliquot. If in doubt, freeze single-use aliquots.
- Vortexing samples:
  - Vortexing is recommended for a homogeneous sample prep, especially after a sample has been centrifuged and supernatant separated.
- Tips on using tissue culture media as assay buffer:
  - If cell culture medium is used as assay matrix, be certain there are no active proteases, phosphatases or supplements present which may interfere with the assay or generate inaccurate results. (e.g. cytokines, human serum, fetal bovine serum, etc.)
- Some kits for metabolism biomarkers require an addition of a variety of protease and/or phosphatase inhibitors to samples. Others may require a sample extraction or acidification.
  - Consult the protocol of the appropriate kit.
  - See Sample Preparation outlines for kits, required serum matrix (if needed), dilutions and sample type in Appendix 2.

Preparation of Serum/Plasma Samples

- For serum or plasma samples that require a dilution instead of "Neat," use the serum matrix provided in the kit as the diluent.
- Hemolysis can result in increased proteolytic activity and analyte degradation primarily due to enzymes released from lysed cells.
- Trace hemolysis in samples collected with protease inhibitors may be acceptable, but gross hemolysis will probably interfere with assay performance.
- Hemoglobin (at >10 mg/mL) is known to interfere with antigen/antibody interactions.

What if I have other sample types?

If you want to run a MILLIPLEX® MAP kit using samples other than serum or plasma, we have protocols to address tissue lysates, urine, blood spots, gingival fluid, nasal lavage fluid, tears, cerebrospinal fluid (CSF), bronchoalveolar fluid, saliva, cervical/vaginal secretions, and many other sample types, as well as protocols that are modified for use with small volume samples.

Please refer to Appendix 3 or contact Technical Support.

Preparation of Tissue Culture Supernatants

- For cell culture supernatants, use fresh culture medium as the matrix solution in the blank, standard curves and controls.
  - If samples are diluted in assay buffer, use the assay buffer as the matrix.

Preparation of Cell Lysates for Cell Signaling Assays in 96-well Plates

- For adherent cell lines: Seed ~40,000 cells/well and allow growth for 48 hours. Remove cell supernatant prior to cell lysis.
- For suspension cell lines: Seed ~250,000 cells/well and collect at desired time. Gently centrifuge, remove supernatant and lyse the cell pellet.
- For cell lysates: Add 30 µL lysis buffer per well and pipet up and down thoroughly without creating too many bubbles. For a more detailed protocol, request information from Technical Support.

Peripheral Blood Mononuclear Cell (PBMC) Sample Prep

Note: PBMC sample prep is the most critical step for obtaining reproducible results.

- Strong detergents are used in lysis buffer. Enough detergent in the lysis buffer is required to solubilize proteins and must not exceed total protein concentrations of 5 to 6 mg/mL. A drop in signal has been observed for several analytes using PBMC samples at greater than 6 mg/mL total protein (not enough lysis buffer was added to solubilize proteins).
- Because strong detergents are used in the lysis buffer, samples must be diluted sufficiently in Assay Buffer 1. This means that you must not exceed total protein concentrations below 2 mg/mL. If protein concentration is below 2 mg/mL, then more of the sample volume which is in lysis buffer (with strong detergents) will be added for the assay, and this will decrease signal. If protein concentration below 2 mg/mL is unavoidable, then it is recommended to run less of the sample, thus minimizing the volume of lysis buffer used for the assay.
- The optimal total protein concentration is 2 to 6 mg/mL. Using PBMCs purchased from Bioreclamation, we determined that 10 µL of Lysis Buffer per 1 million PBMC cells yields approximately 2 mg/mL. Adding 10 µL of this 2 mg/mL sample plus 15 µL of Assay Buffer yielded good results. As a starting point, it is recommended to add 10 µL of Lysis Buffer per 2 million PBMC cells. Never dilute samples in Lysis Buffer, rather dilute in Assay Buffer 1 which lacks strong detergents.

Short Protocol for PBMC cell lysis:

- If PBMC cells are from frozen stock, it is recommended to allow cells to recover 24 hours in complete media. (Less than 24h recovery leads to decrease in signal.)
- After 24 hours of recovery, count cells using an appropriate cell counter.
- Pellet the PBMC cells at 1000 x g using a table top centrifuge for 5 minutes at room temperature.
- Remove supernatant and wash cells with PBS.
- Pellet the PBMC cells at 1000 x g using a table top centrifuge for 5 minutes at room temperature.
- Remove wash buffer and add 10 µL Lysis Buffer (with 2x concentrated protease inhibitors added just prior to use) per 2 million cells.
- Gently vortex for 30 seconds before transferring cell lysate into a centrifuge tube.
- Gently rock cell lysate for 10 minutes at 4°C.
- Pellet unbroken cells and organelles at 12,000 x g for 10 minutes at 4°C.
- Transfer clear supernatant into a new centrifuge tube.
- It is recommended, at least for the first time, to determine total protein concentration. If not, then it is recommended to run a lysate titration starting at 10 µL sample + 15 µL of Assay Buffer 1 and performing a 1:1 serial dilution in Assay Buffer 1.
- Add protease inhibitors (such as Protease Inhibitor Cocktail I, Cat. No. 20-201; AEBSF Cat. No. 101500) and/or phosphatase inhibitors to “home-brew” lysis buffers.

Lysis Buffers

- Lysis buffer selection:
  - Lysis buffer can be found in the Cell Signaling Buffer & Detection Kit (Cat. No. 48-602MAG) or sold separately (Cat. No. 43-040).
  - Non-ionic detergents (NP40, Tergitol, IPEGAL) are recommended in lysis buffers for solubilizing cytoplasmic proteins.
  - Partially ionic detergents (Triton® X-100) are recommended in lysis buffers for cytoplasmic or membrane-bound proteins.
  - Ionic detergents (sodium dodecyl sulfate, SDS) are recommended in lysis buffers for membrane-bound, nuclear or mitochondrial proteins. If using SDS in the lysis buffer (i.e., Radioimmunoprecipitation assay (RIPA) buffer), then cell lysate must be diluted to less than 0.05% SDS for assays to detect intracellular proteins, such as cell signaling proteins.

  - NOTE: to solubilize nuclear/mitochondrial proteins, you must use either SDS or another method (such as ultrasonication) to puncture the tough nuclear/mitochondrial membranes.
  - Reducing agents, like β-mercaptoethanol or dithiothreitol, are not recommended.

- For more information about the compatibility of buffers with MILLIPLEX® MAP Cell Signaling kits, contact Technical Support.
- A selection of pre-made lysis buffers and protease/phosphatase inhibitors are available from SigmaAldrich.com
- Perform all dilutions with lysis buffer (not assay buffer or phosphate-buffered saline (PBS)).

Total Protein Concentration

Total protein concentration limits:

- Do not collect lysates at greater than 5 mg/mL protein concentration.
  - At protein concentrations higher than 5 mg/mL, not all proteins will be solubilized equally by the lysis buffer. Some proteins can be solubilized at a given detergent concentration, while other proteins are not as affected.
  - For example, β-tubulin signal decreases with increasing total protein concentration (signal decrease occurs at 5 to 6 mg/mL for Jurkat cell and peripheral blood mononuclear cell (PBMC) lysates).
- Total protein concentrations should be within a specific range, which is outlined in each protocol. In the following example the protocol requires a final protein concentration of 0.4 µg/µL added to each well.
  - A starting protein amount of 10 µg per well (10 µg protein in the final 25 µL that is loaded into each assay well) is recommended.
  - 10 µg/25 µL = 0.4 µg/µL (mg/mL).
  - All samples must first be brought to a protein concentration of 0.8 µg/µL in lysis buffer.
  - Then dilute the cell/tissue lysates 1:1 in the assay buffer provided in the Cell Signaling kit as recommended.
  - For example, 30 µL of a 0.8 µg/µL lysate sample added to 30 µL of assay buffer, dilutes the protein down to a final concentration of 0.4 µg/µL.
  - Then load 25 µL of this diluted sample into each well (duplicate wells are recommended).

Type of detergents	Protein localization	Maximum allowed protein concentration	MILLIPLEX® assay compatibility
Non-ionic detergents	Cytoplasm	5 mg/mL	Yes
Partially ionic detergents	Cytoplasm, Membrane-bound	5 mg/mL	Yes
Ionic detergents	Membrane-bound, Nucleus, Mitochondria	5 mg/mL	Requires dilution

Table 2. Detergent compatibility with MILLIPLEX® MAP intracellular assays.

Preparation of Reagents

General Information

- All kits are for Research Use Only.
- Protocol procedures are optimized for best data results; consequently, protocols can vary from kit to kit.
- It is important to read the entire protocol before proceeding.
- Deliver extremely precise volumes of solvent when reconstituting lyophilized products. Variations of even a few microliters will significantly affect quantitation.
- Do not mix or substitute assay reagents with those from other lots or sources.
- If leftover reagent lots match and the reagents have been kept at the appropriate storage conditions, they can be used in combination until the expiration dates.
- Serum matrix, bead diluents and wash and assay buffers from other kits can be used/combined if the catalog numbers of these components match in the protocols for the kits in question.

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 portion at 2-8°C for up to one month.
- If more wash buffer is required, see Protocol sections “Reagents Supplied” or “Replacement Reagents” for the appropriate catalog number.

Quality Controls

- Quality Controls (QCs) are included to qualify assay performance.
  - Before use, reconstitute Quality Control 1 and Quality Control 2 with 250 µL deionized water.
  - Invert the vial several times to mix and vortex.
  - Allow the vial to sit for 5-10 minutes and then transfer the controls to appropriately labeled polypropylene microfuge tubes.
  - Unused portion may be stored at ≤ -20°C for up to one month.

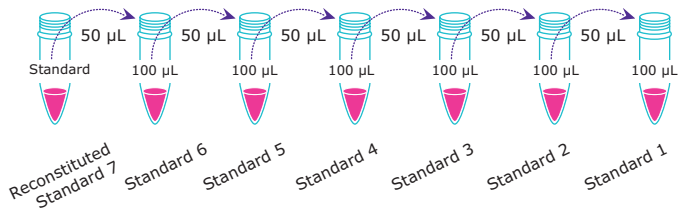
Standards/Calibrators

- After hydration/reconstitution, all standards and controls must be transferred to polypropylene tubes.
- During the preparation of standard curves, thoroughly mix each higher concentration before making the next dilution.
- Use a new pipette tip with each dilution.
- The standards prepared by serial dilution must be used within one hour of preparation.
  - Discard any unused standards except the standard stock.
  - The standard stock can be stored at ≤-20°C for one month or at ≤-80°C for more than one month.
- The quality of the standard curves can be determined by the % recovery of the standards and the QC values.

Serum Matrix

- This step is required for serum or plasma samples only.

Example of Standards Preparation



- Add 1.0 mL deionized water 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.
- Kits designed for non-serum/plasma samples (e.g., urine, CSF) or samples that require a significant dilution (at least 1:20) do not require serum matrix.
- For non-serum/plasma samples, the appropriate medium (e.g., cell culture medium) should be added instead of serum matrix.
- In the absence of appropriate medium or when using a blank, assay buffer can be used.
  - For cell/tissue homogenates, the final cell or tissue homogenate should be prepared in a buffer that has a neutral pH, contains minimal detergents or strongly denaturing agents and has an ionic strength close to physiological concentrations.

- Normalize the sample protein concentration with lysis buffer according to the protocol. For example, dilute sample to 0.8 µg/µL with lysis buffer. Then dilute the 0.8 µg/µL sample 1:1 with kit Assay Buffer. The matrix here is then a 1:1 dilution of lysis buffer with kit assay buffer and the protein concentration is now 0.4 µg/µL.

Antibody-Immobilized Beads

- For individual vials of beads, sonicate each antibody-bead vial for 30 seconds; vortex for 1 minute.
- Follow the protocol to prepare each antibody bead vial and add antibody beads to the Mixing Bottle and bring final volume to 3.0 mL with Bead Diluent.
- Vortex the mixed beads well.
- The antibody-immobilized beads are light-sensitive and must be protected from light at all times.
  - Cover the assay plate containing beads with an opaque plate lid or aluminum foil during all incubation steps.
- Any unused mixed antibody-immobilized beads may be stored in the Mixing Bottle at 2-8 °C for up to one month.

Don't want to mix beads?

Contact your technical sales representative about receiving premixed beads for select kits. Each vial of premixed beads are background tested and assessed for the appropriate bead regions.

Immunoassay Procedure

General Information

- All kits are for Research Use Only.
- Protocol procedures are optimized for best data results; consequently, protocols can vary from kit to kit.
- It is important to read the entire protocol before running an assay.

Tips for Reducing Variability

- Ensure proper sample collection.
- To avoid low bead counts, thaw, vortex and centrifuge all samples for 5-10 minutes at a minimum of 10,000 x g. Avoid or remove any fat layers that may develop.
- Centrifuge samples after thawing or if they appear turbid. This is especially recommended for plasma samples, cell/tissue lysates, or other sample types that are viscous or contain lipid/debris. For some sample types, the centrifugation could be repeated 2 or 3 times to completely clarify the supernatants.
- Ensure the proper mixing of samples and controls.
- Use appropriate pipetting technique:
  - Hold the pipette at the same angle each time.
  - Use pipettes calibrated for values in the middle range (not extremes).
- Warm reagents to room temperature (20–25 °C) before mixing. For assays requiring overnight incubation in a cold room, warm reagents to room temperature on the second day as well.
- Cover the plate with a plate sealer before shaking.
- The plate shaker speed should be increased to agitate the plate at the highest speed that does not lead to splashing on the sealer.

Did you know?

We can help you with your higher-throughput assay needs. Contact your sales representative for product availability. To design a custom 384-well formatted assay email your request to: **customassay@merckmillipore.com**

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

96-well Plate Map. Sample map showing placement of standards, QCs, background and 38 samples in duplicate..

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	Standard 0	QC1																						
B	Standard 0	QC1																						
C	Standard 1	QC2																						
D	Standard 1	QC2																						
E	Standard 2	Sample 1																						
F	Standard 2	Sample 1																						
G	Standard 3	Sample 2																						
H	Standard 3	Sample 2																						
I	Standard 4	Etc.																						
J	Standard 4																							
K	Standard 5																							
L	Standard 5																							
M	Standard 6																							
N	Standard 6																							
O	Standard 7																							
P	Standard 7																							

384-well Plate Map. Sample map showing placement of standards, QCs, and 182 samples in duplicate.

Immunoassay Procedure

- The day before running an assay, check the instrument.
  - Is the instrument calibrated?
  - Has it been maintained?
  - Have fresh water prepared, calibrated and accurate pipettes, multichannel pipettes and an orbital shaker or alternative.
  - Confirm availability of a cold room or refrigerator with power access for the orbital shaker.
- When running samples in duplicate, a maximum of 38 samples can be run per 96-well kit. If using a validated MILLIPLEX® MAP 384-well kit, a maximum of 182 samples may be run in duplicate.
- To pre-wet the plate, use 150 µL wash buffer or assay buffer.
- If you accidentally use wash buffer instead of assay buffer for your assay, and if sample has not yet been loaded, remove wash buffer and replace with assay buffer.
  - If sample has been added to the plate with wash buffer, there is a potential for low recovery as it may not have the required protein concentration or protease inhibitors.
- Vortex all reagents well before adding them to the plate.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing at high setting and centrifuge at a minimum of 10,000 x g prior to use in the assay to remove particulates.
- Be precise when adding samples, standards and QCs to the plate.
  - Pipette onto the sides of the wells.
  - Be sure all fluid is expelled from the pipette tips. Use fresh tips for each addition.
- For incubating assays overnight, a power supply must be available for the orbital shaker in a refrigerator or cold room.
  - The plate shaker should be a shaker designed to hold a 96-well plate firmly, and it should reach at least 500 rpm. Also it should not be a gentle rocker or slow orbital mixer.
  - If the plate shaker has been turned off during the night, shake again at room temperature for one hour before proceeding with the assay protocol.
- After overnight incubation of assays, remember to allow all reagents to warm to room temperature (20-25 °C) before use in the assay.
- Detection antibody cocktail and SAPE incubation times are critical. Do NOT exceed the dictated times as this will result in higher background signals. Also, do not under-incubate, as loss of signal dynamic range may occur.
- If the detection antibody has been accidentally aspirated off or poured off before adding SAPE to the well, it is possible to recover the assay:
  - Add 20 µL to 50 µL (follow the recommended volume stipulated in the protocol) of detection antibody and continue to follow the protocol.
  - Replace the detection antibody cocktail volume with assay buffer, add SAPE and continue to follow the protocol. If no detection antibody is available, add SAPE and continue to follow the protocol, keeping in mind that the signal may be lower.
- Use the Sheath Fluid, Drive Fluid (if using the MAGPIX®), or if your samples are very "sticky," use 1X Wash Buffer for the final resuspension before reading the plate.
- The plate should be read immediately (within 4 hours) after the assay is finished. If 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 72 hours on an orbital plate shaker, with samples brought up in sheath/drive fluid. There may be a loss of sensitivity after 24 hours.
- Before reading the plate, agitate the plate on the plate shaker at room temperature for 10 minutes.
- Do not store processed samples in wash buffer.
- It is possible to run a portion of a plate initially, then reuse the plate with other samples later.
  - Cover the wells that are not being used.
  - Use precise volumes of reagents to ensure that enough remains to run the remaining wells at a later time.
  - Store reagents at appropriate conditions quickly after the first use (e.g., stock standard at -20°C or lower). For components to be stored frozen, for consistency, aliquot and freeze all aliquots prior to use, including the one to be used on the day of preparation.
  - Remake standards for subsequent batches. Be sure to run a standard curve for each batch.
  - When running subsequent batches, cover the previously used wells.
  - The mix of beads may be used for one month if stored at 2-8 °C; stock standards should be stored at ≤ -20 °C for one month and at -80 °C for more than one month.
  - If using the same plate, keep the plate very clean. Alternatively, use a second plate for the remaining samples (for extra 96-well plates, use Cat. No. MAG-PLATE; for extra 384-well plates, use Cat. No. MAG384-PLATE).

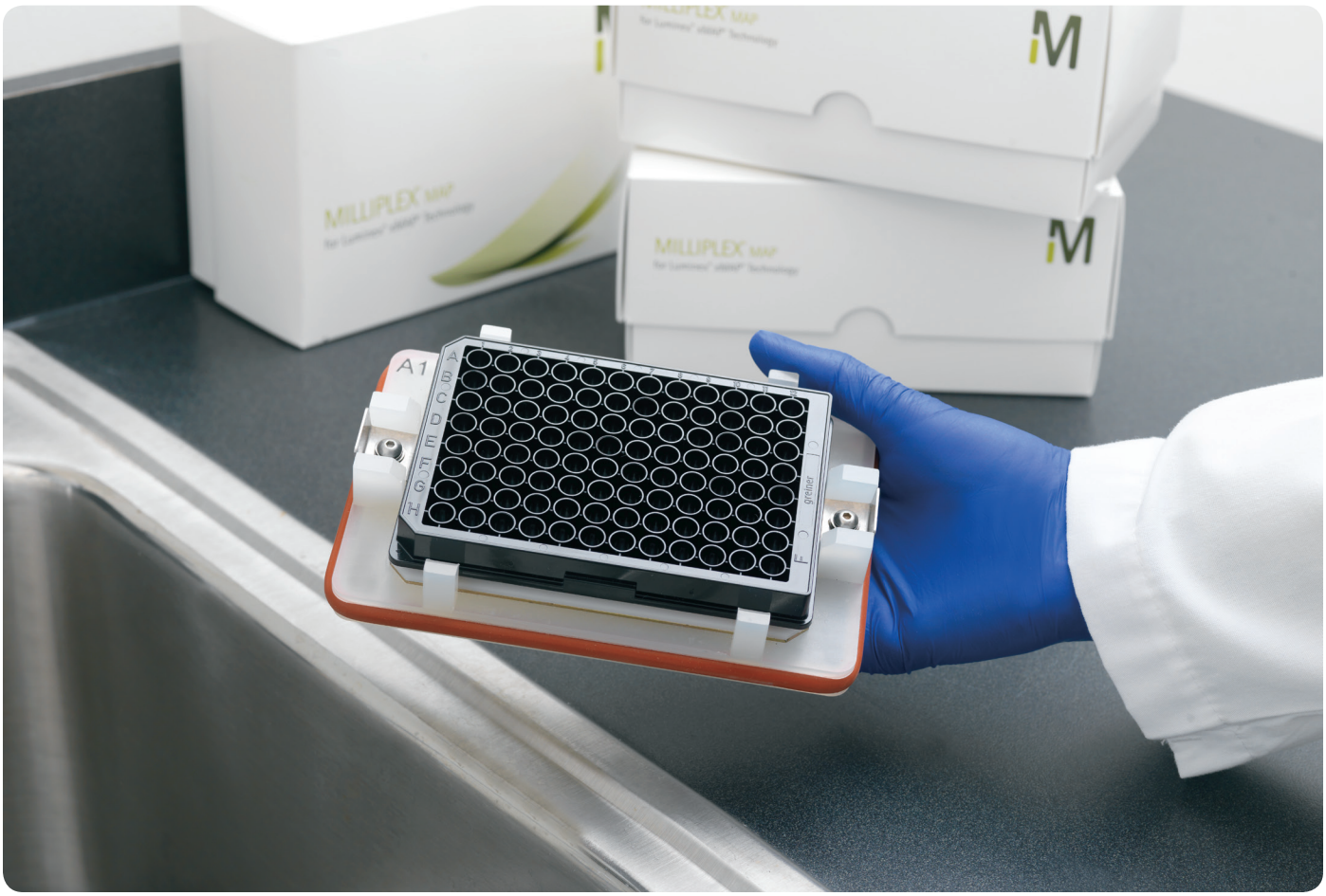
What if I have sticky samples?

If your samples are particularly sticky, it can help to resuspend the beads in 1X Wash Buffer before reading the plate on the instrument. The detergents in this buffer can help with any aggregation that may occur. Note that the plate must be read within four hours.

Plate Washing

Tips for Reducing Variability

- Orbital Titer Plate Shaker (VWR® Microplate Shaker Cat # 12620-926 or equivalent)
  - Very important: For incubating assays overnight, a power supply must be available for the orbital shaker in a refrigerator or cold room.
  - The orbital 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.
  - However, orbital shakers vary. Your shaker can be calibrated by pre-wetting the plate with buffer and slowly increasing the speed until splashing occurs. Then lower the speed slightly. The shaker should be set at the highest speed allowable without splashing of the liquid.
- Handheld Magnetic Separation Block (Cat. No. 40-285)
  - When ready to decant the liquid from the plate, the plate MUST be firmly attached to the magnet. To determine that the plate is attached firmly, listen for the click of the clasps.
  - Grip the handheld separation block firmly.
  - During the wash steps while using a hand magnet, decant the liquid, then gently blot the plate.
  - When using a new magnet, check for space between the plate and magnet. Adjustments require a US Allen (hex) key to adjust the screws (not provided).
- Incomplete washing can adversely affect the assay outcome.
- All washing must be performed with the wash buffer provided.



Equipment Settings

FLEXMAP 3D® System

Luminex® 100/200™ System

MAGPIX® System

- For the MAGPIX® system, choose the “enhanced startup” setting instead of the common startup.
  - This will ensure proper calibration and cleaning prior to running the assay.
  - Working with serum can be “stickier” than other biological fluids and can affect the performance of the instrument unless it is properly cleaned.
  - Luminex® can provide a recommended protocol for maintenance.
- Be sure the needle probe is clean. This may be achieved by sonication and/or alcohol flushes.
- Probe height:
  - When reading an assay on a Luminex® 200™ instrument, adjust the probe height according to the protocols recommended by Luminex® to the kit solid plate using 3 alignment discs.
  - When reading an assay on a FLEXMAP 3D® system, use the probe height adjustment tool (white plate) that has spots for both the 384-well and 96-well plates.
    - The 96-well kit plate well dimensions (3.5 mm) require using location F12 on the white probe height adjustment tool.
  - When reading an assay on a MAGPIX® system, adjust the probe height according to the protocols recommended by Luminex® to the kit solid plate using 2 alignment discs.
- Annotating control wells on the instrument can be tedious, with a lot of manual typing.
  - It is possible to enter the wells as unknowns instead of controls to avoid typing in the annotations for controls, then comparing with your chart later.
- It is important to use the manufacturer’s specific gate settings.

- The Luminex® 200™ system's xPONENT® 3.1 acquisition software has two functions: one for magnetic (MagPlex®) and one for nonmagnetic beads (MicroPlex®).
  - Be sure to select the correct setting in the protocol for your bead type.
  - If the wrong type is selected, the plate does not need to be reread. The batch can be replayed with the corrected protocol setting.
- For information on xPONENT® software and to request software templates go to: MerckMillipore.com/techservice
  or contact Technical Support or your Sales Specialist.
- If a plate cannot be run immediately (within 4 hours) (e.g., it needs to be taken to another site to run the assay), suspend your sample in sheath or drive fluid or assay buffer.
- 10-12 plates can be run with one bottle of drive fluid for the MAGPIX® system.
- To change a standard curve from, for example, a 7-point curve to an 8-point curve, simply make a new protocol and replay the batch.

Running MILLIPLEX® MAP Kits on Other Luminex® Instruments

- A Luminex® 100™ system with IS 2.3 or newer software, Luminex® 200™, FLEXMAP 3D® or MAGPIX® instrument is required to run a MILLIPLEX® MAP assay.
  - If you want to try a kit before purchasing an instrument, ask your Sales Specialist to provide a demonstration using the Luminex® technology and MILLIPLEX® MAP kits.
  - Magnetic bead assays cannot be run on any instruments using Luminex® IS 2.3 or Luminex® 1.7 software.
- Since all Luminex® machines (Luminex® 200™, FLEXMAP 3D® and MAGPIX® instruments) are built by the Luminex® Corporation, MILLIPLEX® MAP kits can be run on any of these machines, regardless of the name given to the machine by a Luminex® business partner.
- If using Luminex® instruments with software other than xPONENT® software (Bio-Plex® Manager™, MasterPlex®, STarStation, LiquiChip, LABScan™ 100), follow instrument instructions for gate settings and additional specifications from the software vendors for reading assays using Luminex® magnetic beads.
- To read a MILLIPLEX® MAP kit on a Bio-Plex® machine, select 5K-25K for magnetic beads, depending on the version of Bio-Plex® Manager™ software.

Overview of Instrument Considerations During a MILLIPLEX® MAP Assay

Starting up and shutting down your system correctly will ensure its longevity. The instructions for the MAPGIX® and Luminex® 200™ systems are located within the systems user manual.* Short-term cleaning will prevent sample induced clogging, while long-term cleaning is important to ensure that sheath or drive fluid does not evaporate and crystallize.

Preparation

- Check probe and insert into reader; set probe height.
- Fill reservoirs (Milli-Q® water, 70% EtOH, 0.1M NaOH).
- Revive instrument: revive from storage, daily start-up.
- Calibrate and verify instrument: system installation.
- Read the entire kit protocol.
- Acquire "Materials Required But Not Supplied."
- Confirm accuracy of pipettes.

Assay

- Follow the kit protocol.
- Set up experimental design on acquisition software.
- Run assay.
- Run "Post Batch Routine."

Shutdown

- Daily shutdown (overnight):
  - Run "Clean Routine."
  - Run "Daily Shutdown Routine."
  - Remove probe and clean in a sonicating water bath.
- Long-term shutdown (longer than one week):
  - Run "Clean Routine" multiple times.
  - Run "Prepare for Storage" part 1.
  - Prime multiple times with Milli-Q® water (use an empty sheath fluid container).
  - Run "Prepare for Storage" part 2.
  - Remove probe and clean in a sonicating water bath.

* Luminex® 200™ User Manual, Section 3, page 17. MAGPIX® User Quick Guide 4.2.

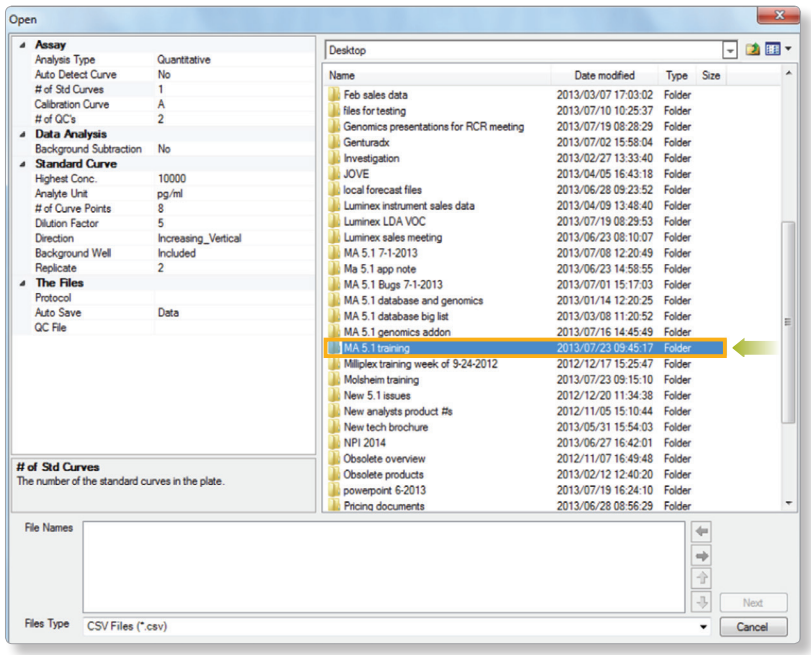
MILLIPLEX® Analyst 5.1 Software

We offer the most powerful combination software package, including powerful multiplex data analysis MILLIPLEX® Analyst 5.1 software coupled with data acquisition using the Luminex® xPONENT® software.
MILLIPLEX® Analyst 5.1 software enables you to manage, track and analyze your multiplex assays rapidly and efficiently, giving you more time to focus on advancing your research.

Data acquisition and analysis integrates seamlessly with all Luminex® instruments, including FLEXMAP 3D®, Luminex® 200™ and MAGPIX® systems. MILLIPLEX® Analyst 5.1 software is available in one- and five-seat licenses, enabling complete flexibility for small, medium and large laboratories.

Step 1:

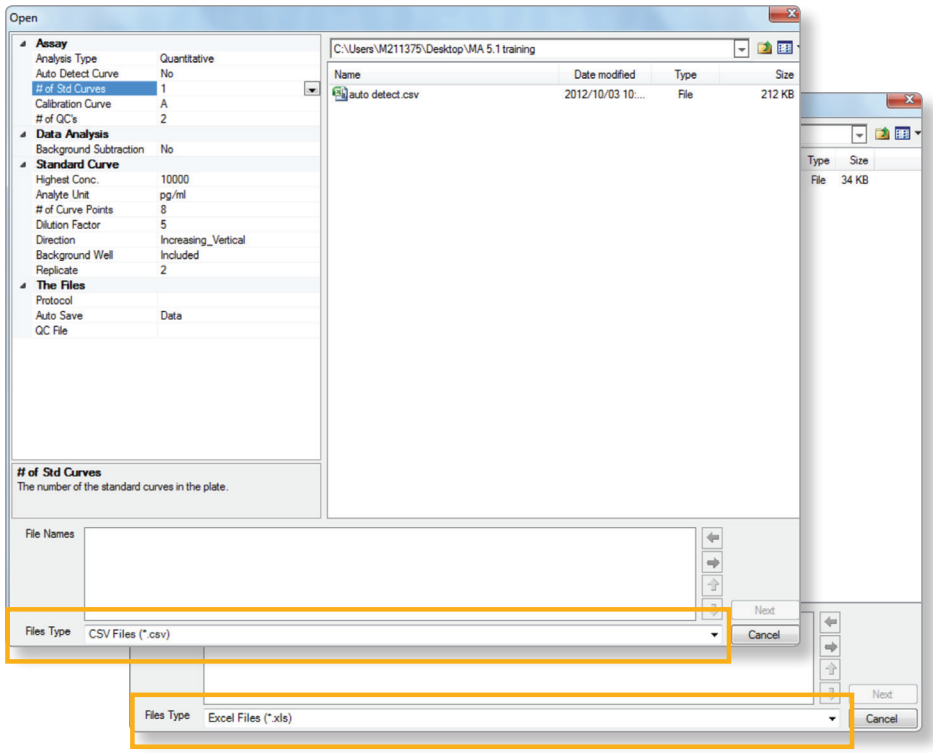
› Choose the folder containing the exported data files.



Step 2:

› Select the appropriate File Type from the pull-down menu:

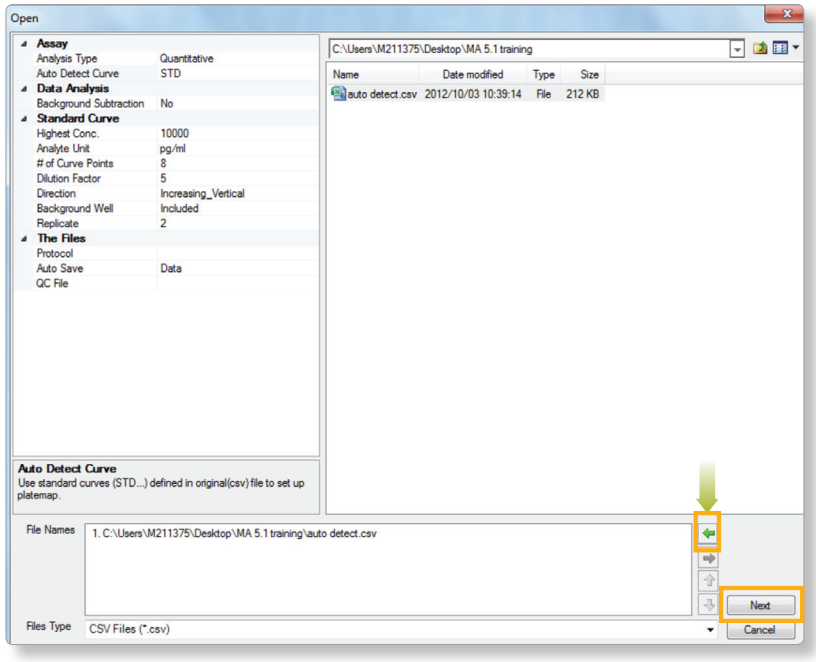
- CSV Files (*.csv) from xPONENT® software.
- Excel® files (*xls) from Bio-Plex® software.



Step 3:

› Importing data from xPONENT® software using Auto Detect Curve:

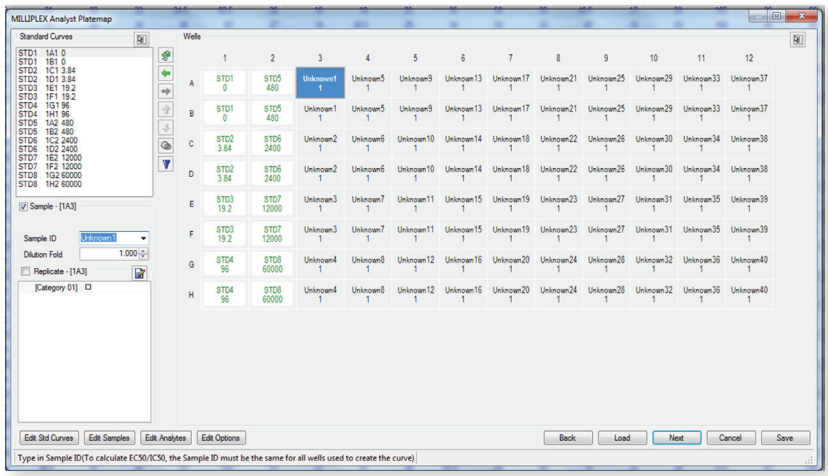
- When analyzing data from xPONENT®, use the name “Standard” for the auto-detect curve function.
- Press the green arrow/icon.
- Loaded files are shown in the File Names window.
- The Standard Curve settings will be ignored and the software will use the Standard Curves as set up in xPONENT® software.
- Click “Next” to continue to the Plate Map screen.



Step 4:

› Importing Data from xPONENT® software using Auto Detect Curve – Plate Map:

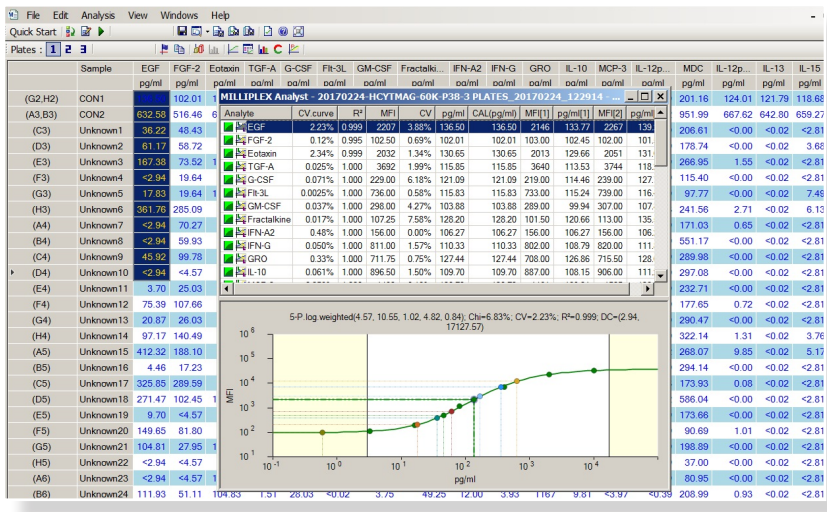
- Assign analytes, dilutions and sample names as needed.
- If the kit and Plate Layout will be used again, save as a “Protocol.”
- When ready for Analysis, click “Next.”



Step 5:

› Data Analysis with MILLIPLEX® Analyst 5.1 software:

- The concentration of each analyte can be calculated independently of the other analytes in any unit (such as pg/mL), using the Standard Curve.
- Best fitting will be the best fitting formula, it may be 5-P log, or it could any of the others.
- Click on different analytes to see where that sample is on the Standard Curve.
- All graphs are updated in real time after hitting the analyze button.
- Detailed Reports can be saved as an Excel® file.



Data Analysis

Bead Counts

- We recommend counting 50 beads.
  - According to Luminex®, a minimum of 35 beads per region need to be counted.
  - Fewer than 35 beads could cause a shift in the MFI (Median Fluorescence Intensity) value of the bead population.
  - However, MFI will not change for bead counts greater than or equal to 35.
  - Therefore, don't worry if there is a 35 bead count on one bead region and 400 for others. MFIs will not be affected.

How to Correct or Prevent Low Bead Counts

- Be sure to specify MagPlex® in the kit protocol for xPONENT® software or use the correct gate setting on Bio-Plex® software.
- Sample preparation: Thaw, vortex and centrifuge samples at a minimum of 10,000 x g. Avoid or remove any fat layers that may develop.
- For samples known to be challenging (e.g., synovial fluid, saliva), one may increase wash steps after incubation with the antibody beads.
- Resuspend beads in wash buffer instead of sheath/drive fluid. However, the plate must be read within four hours.
- Add 1X wash buffer, which contains Tween® 20, to keep the beads from clumping or sticking.
- Store beads only in sheath or drive fluid.
- During the wash steps while using a handheld magnet, decant the liquid, then gently blot the plate.
- When using a plate washer, check the settings to make sure the plate is soaking for 60 seconds and the aspiration is not all the way down in the well.
- Warm the plate to room temperature after an overnight 4 °C capture antibody incubation step. Let the plate shake at room temperature for one hour.
- For MAGPIX® users, cleaning the instrument is critical.
  - Special care should be taken to use the enhanced startup or washing procedures.
  - There is an advanced cleaning method that includes sodium hydroxide (NaOH) and bleach.
  - Washing between wells can also be selected during the plate reading.
  - Cleaning the instrument regularly is important even if the instrument is not being used.

Percent Coefficient of Variation (%CV)

- High %CVs for standards or samples can be due to low bead count.
- For assays that have a standard curve, our target inter-assay %CV is <15% and our target intra-assay %CV is <20%.
- For qualitative assays (no standard curves) the MFI target inter-assay %CV is <20% and the target intra-assay %CV is <15%.

Calculating Lower Limit of Quantification (LLOQ) and Upper Limit of Quantification (ULOQ)

- Defining LLOQ and ULOQ requires a tight standard curve (e.g., a 1:2 or 1:3 serial dilution to the point that you achieve saturation at both ends).
- Choose the lowest and highest standard curve points that have a recovery of +/- 20%.
- Verify that this is the LLOQ and ULOQ by running 5 assays with the LLOQ and ULOQ as samples against a curve using the assay serial dilution factor where the lowest standard is below LLOQ and the highest standard is above ULOQ.
- Inter-assay precision should be within 20% for LLOQ and ULOQ samples.

Curve Performance/Fit

- Standard point %CVs should be <15%.
  - High %CVs here indicate improper technique was used when making standard curve dilutions. Examples of poor technique include:
  - Not vortexing between tubes.
  - Not vortexing while loading the plate.
  - Not pipetting equal amounts into the plate.
    - The lower the concentrations of analytes, the higher the %CVs tend to be. With new users, this improves with time and practice.
    - For any standard points that have high %CVs, samples in that range of the curve should be interpreted with caution.
    - Alternatively, a standard point or one of the replicate wells can be flagged/masked, although it can be difficult to decide which well to flag if only duplicates are run.

Recovery

- Percent recovery should be 100% +/- 30% (industry minimum), although some researchers will have their own acceptance criteria.
- Percent recovery is usually worse at either extreme of the curve, but this also improves with time and practice.
  - For curve statistics, focus on the R^2 value, which approach, but never equal unity. (Note that a R^2 value of "1" is seen with software rounding of 0.9999).

Minimum/Maximum Detectable Concentration (minDC/maxDC)

- For many assays, the minDC/maxDC will be outside the standard points (extrapolated) due to good curve performance and fit.
- To avoid seeing extrapolated data, set the desired range of detection in MILLIPLEX® Analyst 5.1 software.
  - Deciding whether to use the "Best Fit" vs. 5-parameter lot option depends on your comfort level to determine how appropriate it is to "play" with curve fit to find the best one.
  - If samples fall above the dynamic range of the assay, dilute the samples further with the appropriate matrices/media and repeat the assay.

How We Monitor/Avoid Lot-to-lot Drift

- MILLIPLEX® MAP standard points maintain consistent values from lot to lot, unlike other kits that may have values that vary lot-to-lot.
- Lot-to-lot drift is monitored and mitigated using full-curve comparison and comparing the relative potency of each analyte against a reference lot.
- All data are compiled in a single database, and trend charts are maintained in our records.

Comparison of MILLIPLEX® Analyst 5.1, StatLIA and Bio-Plex® Analysis Software

Analyte: IFNγ, Kit: Rat Cytokine/Chemokine Units: (pg/mL)

	MILLIPLEX® Analyst 5.1*	StatLIA®	Bio-Plex®
Standard1	14.7	3.0	
Standard2	57.4	67.0	53.7
Standard3	241.6	245.0	248.6
Standard4	932.1	897.0	908.1
Standard5	3683.0	3858.0	3820.0
Standard6	15184.0	14769.0	14824.5
Standard7	59874.0	61392.0	60975.2
Unknown1	<3.40 ↓	<1.0e-07	OOR <
Unknown2	16.8	8.0	OOR <
Unknown3	51.3	60.0	45.0
Unknown4	197.1	205.0	205.8
Unknown5	844.1	809.0	821.3
Unknown6	3412.0	3564.0	3531.3
Unknown7	14639.0	14296.0	14339.3
Unknown8	70718.0	82002.0	78697.5
Unknown9	<3.40 ↓	<1.0e-07	OOR <
Unknown10	<3.40 ↓	<1.0e-07	OOR <
Unknown11	<3.40 ↓	<1.0e-07	OOR <
Unknown12	3.7	<1.0e-07	OOR <
Unknown13	<3.40 ↓	<1.0e-07	OOR <
Unknown14	<3.40 ↓	<1.0e-07	OOR <
Unknown15	5.0	<1.0e-07	OOR <
Unknown16	16.8	8.0	OOR <
Unknown17	24.0	22.0	OOR <
Unknown18	28.6	29.0	1.1
Unknown19	<3.40 ↓	<1.0e-07	OOR <
Unknown20	<3.40 ↓	<1.0e-07	OOR <
Unknown21	<3.40 ↓	<1.0e-07	OOR <
Unknown22	14.7	3.0	OOR <
Unknown23	<3.40 ↓	<1.0e-07	OOR <
Unknown24	<3.40 ↓	<1.0e-07	OOR <
Unknown25	6.8	<1.0e-07	OOR <
Unknown26	<3.40 ↓	<1.0e-07	OOR <
Unknown27	<3.40 ↓	<1.0e-07	OOR <
Unknown28	5.9	<1.0e-07	OOR <
Unknown29	4.5	<1.0e-07	OOR <
Unknown30	8.2	<1.0e-07	OOR <
Unknown31	<3.40 ↓	<1.0e-07	OOR <
Unknown32	<3.40 ↓	<1.0e-07	OOR <

Magenta: Extrapolated value
Cyan:Extrapolated value
OOR<: Out of Range Below
<3.40↓: Out of Range Below
<1.0e-07: Out of Range Below
*Best Fitting, 5P Log

Table 3. Significantly more IFNγ concentrations could be calculated at the low end of the curve in the Rat Cytokine/Chemokine Magnetic Bead Panel (Cat. No. RECYTMAG-65K) by MILLIPLEX® Analyst 5.1 software compared to the Bio-Plex® and StatLIA® software packages.

Cell Signaling Assays

Multiplex Assays for Soluble Proteins vs. Cell Signaling Proteins

Soluble Analyte Assay	Cell Signaling Analyte Assay
Quantitative	Qualitative (fold change)
Serum, plasma, tissue culture, urine, CSF, etc.	Cells (must be lysed)
Analytes analytically validated within panel	Fixed kits and individual MAPmates™ are analytically validated
Kit includes standards and QCs	Kits and MAPmate™ assays include positive and negative control cell lysates
Most panels are customizable	Most kits are fixed panels; create custom kits in three ways: 1. Use the Human RTK (Phosphoprotein) configurable kit with pan Tyr analytes 2. Use the 2-plex assays (most can be combined with each other to study multiple total proteins and phosphoproteins in the same well) 3. Combine singleplex cell signaling MAPmate™ assays

Table 4. Comparison of assay characteristics.

Using Cell Signaling MAPmate™ Assays

- All MAPmate™ assays require the Cell Signaling Buffer & Detection Kit (Cat. No. 48-602MAG).
  - This kit contains all necessary reagents except the MAPmate™ assays. Both a filter and flat-bottom plate are included for convenience.

"Plexing" Cell Signaling MAPmate™ Assays

- Up to eight singleplex MAPmate™ assays within the Cell Signaling Buffer and Detection kit can be combined into a custom multiplex kit.
  - Refer to the guidelines provided in the MAPmate™ protocol.
- MAPmate™ assays can also be added to existing intracellular assay kits to enhance the panel or serve as controls.
  - Refer to the guidelines provided in the kit protocol.
- The following MAPmate™ assays should not be plexed together:
  - Phospho-specific and total MAPmate™ pairs.
  - More than 1 phospho-specific MAPmate™ assay for a single target cannot be plexed together.
  - GAPDH and β-Tubulin MAPmates™ can be used for normalization with any of the MAPmates™

Preparation of Cell Lysates for Cell Signaling Assays in 96-well Plates

- For adherent cell lines: seed ~40,000 cells/well and allow growth for 48 hours.
- For suspension cell lines: seed ~250,000 cells/well and collect at desired time.
- For cell lysis: add 30 µL lysis buffer per well and pipet up and down thoroughly without creating too many bubbles. For a more detailed protocol, request info from Technical Support.
  - Unbroken cells/parts can be cleared by either filtration or by centrifugation.
- Lysis buffer can be found in the Cell Signaling Buffer & Detection Kit (Cat. No. 48-602MAG) or it is sold separately (Cat. No. 43-040).
- Add protease inhibitors (such as Aprotinin/Trasylol, Cat. No. 20-201; AEBSF, Cat. No. A8456) and/or phosphatase inhibitors to "home-brew" lysis buffers.
- Other lysis buffer selections:
  - Non-ionic detergents (NP40, Tergitol, IPEGAL) are recommended in lysis buffers for solubilizing cytoplasmic proteins.
  - Partially ionic detergents (Triton® X-100) are recommended in lysis buffers for cytoplasmic or membrane-bound proteins.

- Ionic detergents (sodium dodecyl sulfate, SDS) are recommended in lysis buffers for membrane-bound, nuclear or mitochondrial proteins. If using SDS in the lysis buffer (i.e., Radioimmunoprecipitation assay (RIPA) buffer), then cell lysate must be diluted to less than 0.05% SDS for assays to detect intracellular proteins, such as cell signaling proteins.
  - Note: to solubilize nuclear/mitochondrial proteins, you must use either SDS or another method (such as ultrasonication) to puncture the tough nuclear/mitochondrial membranes.

› Reducing agents, like β-mercaptoethanol or dithiothreitol, are not recommended.

› Perform all dilutions with lysis buffer (not assay buffer or phosphate-buffered saline (PBS)).

› See Table 5 for total protein concentration limits.

Type of detergents	Protein localization	Maximum allowed protein concentration	Luminex® assay compatibility
Non-ionic detergents	Cytoplasm	5 mg/mL	Yes
Partially ionic detergents	Cytoplasm, Membrane-bound	5 mg/mL	Yes
Ionic detergents	Membrane-bound Nucleus, Mitochondria	5 mg/mL	Requires dilution

Table 5. Assay compatible lysis detergents and protein concentrations.

- Do not collect lysates at greater than 5 mg/mL protein concentration.
  - At protein concentrations higher than 5 mg/mL, not all proteins will be solubilized equally by the lysis buffer. Some proteins can be solubilized at a given detergent concentration, while other proteins are not as affected. For example, β-tubulin signal decreases with increasing total protein concentration (signal decrease occurs at 5 to 6 mg/mL for Jurkat cell and PBMC lysates).
- Total protein concentrations should be within a specific range, which is outlined in each protocol.
  - A starting protein amount of 10 µg per well (10 µg protein in the final 25 µL that is loaded into each assay well) is recommended.
  - 10 µg/25 µL = 0.4 µg/µL (mg/mL).
  - All samples must first be brought to a protein concentration of 0.8 µg/µL in lysis buffer.
  - Then dilute the cell/tissue lysates 1:1 in the assay buffer provided in the intracellular kit as recommended.
  - For example, 30 µL of each lysate sample added to 30 µL of assay buffer, diluting the final protein concentration down to 0.4 µg/mL.
  - Then load 25 µL of this diluted sample into each well (duplicate wells are recommended).

Appendix 1: Species Cross-reactivity

Canine

- Four serum or plasma samples were usually run in each kit. Exceptions where more than four samples were run are noted.
- Data below are the number of samples that showed signal above background.
- Untested kit analytes are not listed.
- For more information contact Technical Support.

Cytokine kits

Panel Name	EGF	Eotaxin	FGF-2	Fractalkine	G-CSF	GM-CSF	GRO	IFNα2
Human Cytokine/ Chemokine Panel 1	4	2	1	1	1	1	1	1
	IL-8	IL-9	IL-10	IL-12(p40)	IL-13	IL-15	IL-17A	IP-10
	2	3	3	2	2	2	3	1
Panel Name	SDF-1α+β	EOTAXIN-3	CTACK	IL-23	TPO	TSLP	IL-33	
Human Cytokine/ Chemokine Panel 2	1	1	1	2	1	1	1	
Panel Name	BRAK	CXCL16	HCC-4	IL-34	IL-35	IL-37/IL-1F7	CCL28	HMGB1/HMG1
Human Cytokine Panel 4	4	4	2	3	4	4	2	4
Panel Name	IL-17F	GM-CSF	IL-10	MIP3α	IL-15	IL-17A	IL-22	IL-9
Human Th17 Panel	1	4	3	1	3	1	3	3
Panel Name	GM-CSF	sCD137	IL-10	IL-13	Granzyme B	IL-2	IL-4	MIP-1α
Human CD8+ Panel	1	2	1	1	1	4	1	1
Panel Name	GM-CSF	IFNγ	IL-10	IL-13	IL-17A	IL-1β	IL-2	IL-4
Human High Sensitivity T Cell	2	1	3	1	2	1	1	3
Panel Name	Complement C2	Complement C4b	Complement C4b	Complement C9	Adipsin / Factor D	Mannose-Binding Lectin	Complement Factor 1	
Human Complement Panel 1	4	4	1	2	2	1	2	
Panel Name	Complement C1q	Complement C3	Complement Factor b	Complement Factor H				
Human Complement Panel 2	4	3	2	1				
Panel Name	Eotaxin	G-CSF	GM-CSF	IFNγ	IL-1α	M-CSF	IL-1β	IL-2
Mouse Cytokine/ Chemokine Panel 1	4	1	4	3	2	4	4	2
	IP-10	MIP-2	KC	LIF	LIX	MCP-1	MIP-1α	MIP-1β
	4	2	4	2	4	4	4	1
Panel Name	EPO	Exodus 2	MCP-5	IFNγ	MIP-3β	MIP-3α	IFNβ-1	TARC
Mouse Cytokine/ Chemokine Panel 2	2	4	3	3	1	4	2	4
Panel Name	EPO	Exodus 2	MCP-5	IFNγ	MIP-3β	MIP-3α	IFNβ-1	TARC
Mouse Expanded Cytokine Panel 2	2	4	3	3	1	4	2	4
Panel Name	GM-CSF	IFNβ	MIP-3α	IL-1ß	IL-2	IL-4	IL-5	IL-6
Mouse Th17 Panel	4	3	1	2	3	1	1	1
	IL-17F	IL-33	IL-31	TNFß	TNFα	CD40L		
	4	4	1	4	3	4		

IL-1α	IL-1β	IL-1ra	IL-2	IL-3	IL-4	IL-5	IL-6	IL-7	
3	4	2	1	1	1	2	2	2	
MCP-1	**MCP-3**	**MDC**	**MIP-1α**	**MIP-1β**	**sCD40L**	**TGF-α**	**TNF-β**	**VEGF**	**PDGF-AB/BB**
1	2	4	4	5	4	4	4	4	4

IFNβ	IL-38	IL-28B/IFN-λ3	BAFF/BLyS	IL-14/α-Taxilin	IL-36β
4	2	4	4	4	4

IL-1β	IL-33	IL-2	IL-4	IL-23	IL-17E	IL-27	IL-31	TNFβ
3	1	3	3	3	1	2	1	3

MIP-1β
1

IL-23	IL-7	IL-8	MIP1α	MIP1β
3	1	3	2	1

IL-3	IL-4	IL-5	IL-7	IL-10	IL-12(P40)	IL-13	IL-15	IL-17A
2	1	3	3	1	1	3	2	2

MIG	RANTES	TNFα	IL-12(P70)	VEGF	IL-9
3	3	4	2	4	3

IL-16	Fractalkine	MDC	TIMP-1	IL-20	IL-11	IL-17A/F
4	3	3	4	3	3	3

IL-16	Fractalkine	MDC	TIMP-1	IL-20	IL-11	IL-17A/F
4	3	3	4	3	3	3

IL-21	IL-22	IL-28B	IL-10	IL-23	IL-12(p70)	IL-27	IL-13	IL-15
4	4	3	2	1	1	0	2	4

Panel Name	GM-CSF	TGFα	G-CSF	IFNγ	IL-2	IL-10	IL-15	CD40L
Non Human Primate Cytokine/Chemokine Panel 1	1	4	3	2	4	0	2	1
	TNFα	IL-12	VEGF	IL-18				
	3	1	4	2				
Panel Name	G-CSF	Eotaxin	GM-CSF	IL-1α	Leptin	MIP-1α	IL-1β	IL-2
Rat Cytokine/ Chemokine Panel	1	1	3	2	4	4	4	4
	IP-10	GRO/KC	VEGF	Fractalkine	LIX	MIP-2	TNFα	RANTES
	4	3	4	4	4	3	2	1
Panel Name	IL1α	IL1β	IL1ra	IL2	IL4	IL6	IL10	IL12
Porcine Cytokine/ Chemokine Panel	1	4	4	4	4	2	4	4

Metabolism/ Endocrinology kits

Panel Name	Apelin	Fractalkine	BDNF	EPO	Osteonectin	LIF	IL-15	Myostatin (MSTN)/GDF8
Human Myokine	1	1	1	2	1	1	1	3

Cardiovascular Disease (CVD) kits

Panel Name	NT proBNP	CK-MB	CXCL6	Endocan-1	FABP4	LIGHT	Oncostatin	Troponin-I
Human CVD1	4	4	1	1	1	1	1	1
Panel Name	ADAMTS13	D-Dimer	FABP5	GDF-15	Myoglobin	sP-Selectin	sVCAM-1	SAA
Human CVD2	4	3	4	4	3	4	3	1
Panel Name	α-2-Macro-globulin	AGP	Fibrinogen	sL-Selectin	Haptoglobin	Platelet Factor 4	von Willebrand Factor	
Human CVD3	4	3	1	4	4	2	4	
Panel Name	Follistatin	dPAPP-A	sPECAM-1	Pentraxin-3	Tissue Factor	Thrombo modulin	Troponin T	
Human CVD4	3	4	2	4	1	1	3	
Panel Name	MMP-9							
Mouse CVD1	1							

Angiogenesis kits: 8 samples run

Panel Name	EGF	ANGPT-2	Leptin	FGF-1	IL-8	HGF	HB-EGF	VEGF-C
Human Angiogenesis/Growth Factor Panel 1	3	8	1	8	1	2	8	2

IL-17A	IL1ra	IL-13	IL-1β	IL-4	IL-5	IL-6	IL-8	MIP-1α
4	3	0	2	2	4	2	1	1

IL-6	EGF	IL-13	IL-10	IL-12(p70)	IFNγ	IL-17	IL-18	MCP-1
2	4	3	4	2	2	1	4	3

IL18
4

FABP3	Irisin	Oncostatin M	FGF21
4	2	1	4

VEGF-D	FGF-2	VEGF-A
6	2	2

Feline

- Four serum or plasma samples were usually run in each kit. Exceptions where more than four samples were run are noted.
- Data below are the number of samples that showed signal above background.
- Untested kit analytes are not listed.
- For more information contact Technical Support.

Cytokine kits

Panel Name	EGF	Eotaxin	FGF-2	FIt-3L	Fractalkine	G-CSF	GRO	IFNα2
Human Cytokine/ Chemokine Panel 1	1	1	1	1	2	2	2	2
	IL-3	IL-5	IL-6	IL-7	IL-8	IL-9	IL-10	IL-12(p40)
	2	1	1	1	1	1	2	1
	MCP-3	MDC	MIP-1α	MIP-1β	sCD40L	TGFα	TNFα	TNFβ
	2	2	2	1	2	2	0	2
Panel Name	SDF-1α+β	I-309	IL-23	TPO	IL-33			
Human Cytokine/ Chemokine Panel 2	3	1	3	2	3			
Panel Name	MPIF-1	BRAK	CXCL16	IL-34	IL-24	APRIL	IL-35	IL-37/IL-1F7
Human Cytokine/ Chemokine Panel 4	4	4	2	4	4	4	4	4
Panel Name	GM-CSF	IL-2	MIP-1α	Perforin				
Human CD8+ Panel	1	2	1	1				
Panel Name	IL-17E	GM-CSF	MIP-3α	IL-1ß	IL-2	IL-4	IL-5	IL-21
Human Th17 Panel	3	3	1	1	1	2	2	3
	IL-27	IL-13	IL-15	IL-17A	IL-17F	IL-33	VEGF	IL-18
	2	1	4	1	3	2	4	3
Panel Name	Complement C2	Completment C4b						
Human Complement Panel 1	4	4						
Panel Name	Exodus 2	MCP-5	MIP-3β	MIP-3α	IFNβ-1	TARC	IL-16	Fractalkine
Mouse Cytokine/ Chemokine Panel 2	4	3	1	3	2	4	4	3
Panel Name	GM-CSF	TGFα	G-CSF	IFNγ	IL-2	IL-15	IL-17	IL1ra
Non-Human Primate Cytokine/Chemokine tPanel 1	1	3	3	2	3	1	2	3
	IL-8	MIP-1α	TNFα	MIP-1β	IL-12	VEGF	Fractalkine	LIX
	3	1	2	0	1	3	4	4
Panel Name	G-CSF	Eotaxin	GM-CSF	IL-1α	Leptin	MIP-1a	IL-4	IL-1β
Rat Cytokine/ Chemokine	2	3	4	3	4	4	3	4
	IL-12(p70)	IFNγ	IL-5	IL-17	IL-18	MCP-1	IP-10	GRO/KC
	3	2	2	2	3	3	4	4
Panel Name	GM-CSF	IFNγ	IL-2	IL-6	IL-7	IL-8	IL-15	IP-10
Canine Cytokine/ Chemokine	4	2	4	4	3	1	3	3
Panel Name	GM-CSF	IL1α						
Porcine Cytokine/ Chemokine	1	1						

Cardiovascular Disease (CVD) kits

Panel Name	FABP3	FABP4	Troponin-I				
Human CVD1	3	1	4				
Panel Name	ADAMTS13	FABP5	GDF-15	Myoglobin	sP-Selectin		
Human CVD2	4	4	4	4	1		
Panel Name	α-2-Macroglobulin	AGP	sL-Selectin	SAP	Haptoglobin	Platelet Factor 4	von Willebrand Factor
Human CVD3	4	1	4	1	3	1	4

Angiogenesis kits: 6 samples run

Panel Name	EGF	G-CSF	ET-1	FGF-1	Follistatin	HB-EGF	VEGF-A
Human Angiogenesis/Growth Factor Panel 1	5	2	1	5	3	5	2

IFNg	IL-1α	IL-1β	IL-1ra	IL-2
1	2	3	2	2
IL-13	**IL-15**	**IL-17**	**IP-10**	**MCP-1**
2	2	1	1	1
VEGF	**PDGF-AB/BB**			
1	3			

IL-19	CCL28	HMGB1/HMG1	IFNβ	IL-38	IL-28B/IFN-λ3	BAFF/BLyS	IL-14/α-Taxilin	IL-36β	IL-32α
4	4	4	4	4	4	4	4	4	4

IL-22	IL-28B	IL-10	IL-23	IL-(12p70)
4	4	3	2	4

MDC	TIMP-1	IL-20	IL-11	IL-17A/F
1	3	3	3	3
IL-13	**IL-1β**	**IL-4**	**IL-5**	**IL-6**
2	3	3	3	2
MIP-2	**TNFα**	**RANTES**		
4	2	2		
IL-2	**IL-6**	**EGF**	**IL-13**	**IL-10**
3	2	4	2	4

KC	IL-10	IL-18	MCP-1	TNFα
3	1	4	4	1

Panel Name	dPAPP-A	Tissue Factor
Human CVD4	2	1
Panel Name	**MMP-9**	**sP-Selectin**
Mouse CVD1	1	4

Guinea Pig

- Four serum or plasma samples were usually run in each kit. Exceptions where more than four samples were run are noted.
- Data below are the number of samples that showed signal above background.
- Untested kit analytes are not listed.
- For more information contact Technical Support.

Cytokine kits

Panel Name	EGF	IL-3	MDC		
Human Cytokine/Chemokine Panel 1	1	2	4		
Panel Name	**SDF-1α+β**	**BCA-1**	**IL-16**		
Human Cytokine/Chemokine Panel 2	4	1	1		
Panel Name	**GM-CSF**	**MIP3α**			
Human Th17 Panel	4	3			
Panel Name	**IL-2**				
Human CD8+ Panel	1				
Panel Name	**Eotaxin**	**IFNγ**	**IL-1β**	**IL-12(P40)**	**IL-13**
Mouse Cytokine/Chemokine Panel 1	2	2	2	4	4
Panel Name	**IL-17E**	**GM-CSF**	**IFNγ**	**IL-2**	**IL-4**
Mouse Th17 Panel	4	3	4	2	1
	IL-15	**IL-17A**	**IL-17F**	**IL-33**	**TNFß**
	2	4	1	4	2
Panel Name	**GM-CSF**	**G-CSF**	**IL-2**	**VEGF**	
Non-Human Primate Cytokine/ Chemokine Panel 1	1	4	4	2	
Panel Name	**Eotaxin**	**GM-CSF**	**IL-1α**	**Leptin**	**IL-4**
Rat Cytokine/Chemokine	1	2	2	4	3
	IL-18	**MCP-1**	**IP-10**	**GRO/KC**	**VEGF**
	1	3	2	2	2
Panel Name	**GM-CSF**	**IL-15**	**IP-10**	**IL-18**	
Canine Cytokine/Chemokine	4	1	4	2	
Panel Name	**GM-CSF**	**IL1β**	**IL1ra**	**IL2**	**IL4**
Porcine Cytokine/Chemokine	3	4	1	2	3

Cardiovascular Disease (CVD) kits

Panel Name	ADAMTS13	D-Dimer	FABP5	GDF-15	Myoglobin
Human CVD2	4	3	4	4	3
Panel Name	**AGP**	**Haptoglobin**	**von Willebrand Factor**		
Human CVD3	2	4	4		
Panel Name	**MMP-9**	**PAI-1 (total)**	**sP-Selectin**		
Mouse CVD1	2	3	3		

Angiogenesis kits: 6 samples run

Panel Name	ANGPT-2	BMP-9	FGF-1	Follistatin	HB-EGF
Human Angiogenesis/Growth Factor Panel 1	5	4	1	5	5

IL-15	**IL-17A**	**IP-10**	**MIP-2**	**LIX**	**RANTES**	**VEGF**
1	1	4	4	4	2	4
IL-5	**IL-22**	**IL-28B**	**IL-10**	**IL-23**	**IL-12(p70)**	**IL-13**
4	4	4	1	4	4	4
CD40L						
3						

IL-1β	**IL-2**	**IL-6**	**IL-13**	**IL-10**	**IL-12(p70)**	**IFNγ**
4	2	1	2	4	1	2
LIX	**MIP-2**	**TNFα**	**RANTES**			
4	4	2	2			

IL6	**IL10**	**IL12**	**IL18**
1	1	2	2

sP-Selectin
1

Hamster

- Four serum or plasma samples were usually run in each kit. Exceptions where more than four samples were run are noted.
- Data below are the number of samples that showed signal above background.
- Untested kit analytes are not listed.
- For more information contact Technical Support.

Cytokine kits

Panel Name	EGF	FGF-2	Fracta	IL-1α	IL-3	MIP-1β
Human Cytokine/Chemokine Panel 1	2	1	3	1	4	1
Panel Name	**SDF-1α+β**	**CTACK**	**IL-21**			
Human Cytokine/Chemokine Panel 2	4	1	1			
Panel Name	**GM-CSF**	**MIP3a**	**IL-13**	**TNF-a**		
Human Th17 Panel	4	1	4	4		
Panel Name	**G-CSF**	**GM-CSF**	**IFNγ**	**IL-1α**	**M-CSF**	**IL-7**
Mouse Cytokine/Chemokine Panel 1	1	1	2	1	1	1
	MIG	**RANTES**	**IL-12(p70)**	**VEGF**	**IL-9**	
	2	3	1	4	3	
Panel Name	**IL-17E**	**GM-CSF**	**IFNγ**	**IL-2**	**IL-4**	**IL-5**
Mouse Th17 Panel	2	3	1	2	2	2
	IL-15	**IL-17A**	**IL-33**	**TNFß**	**CD40L**	
	2	1	2	4	1	
Panel Name	**Leptin**	**IL-1ß**	**IL-2**	**IL-13**	**IL-10**	**IL-4**
Rat Cytokine/Chemokine	4	4	1	1	3	2
Panel Name	**GM-CSF**	**IL-1ß**	**IL1ra**	**CD40L**	**IL-17**	**IL12**
Porcine Cytokine/Chemokine	3	3	1	3	2	4
Panel Name	**TGFα**	**G-CSF**	**IFNγ**	**IL-2**	**IP-10**	**IL-13**
Non-Human Primate Cytokine/ Chemokine tPanel 1	1	2	3	4	4	2
Panel Name	**IL-18**					
Canine Cytokine/Chemokine	2					

Cardiovascular Disease (CVD) kits

Panel Name	FABP3	Troponin-I				
Human CVD1	4	4				
Panel Name	**ADAMTS13**	**D-Dimer**	**FABP5**	**GDF-15**	**MPO**	**sP-Selectin**
Human CVD2	4	4	4	4	4	1
Panel Name	**dPAPP-A**	**Tissue Factor**	**Troponin T**			
Human CVD4	3	1	3			
Panel Name	**MMP-9**	**PAI-1 (total)**	**sP-Selectin**			
Mouse CVD1	1	1	4			

Angiogenesis kits: 6 samples run

Panel Name	BMP-9	FGF-1	Follistatin
Human Angiogenesis/Growth Factor Panel 1	3	3	2

PDGF-AA
1

IL-12(p40)	IL-13	IL-15	IL-17	IP-10	LIX	MIP-1α	MIP-1β
1	4	2	1	1	1	1	2

IL-6	IL-21	IL-22	IL-28B	IL-10	IL-23	IL-27	IL-13
2	1	4	4	4	2	1	3

MCP-1	IP-10	GRO/KC	VEGF	Fractalkine	LIX	MIP-2	RANTES
1	4	4	3	2	1	3	2

IL18
1

IL-5	IL-6	IL-8	MIP-1α	MCP-1	TNFα	IL-12	VEGF
4	2	1	1	3	1	1	3

Horse

- Four serum or plasma samples were usually run in each kit. Exceptions where more than four samples were run are noted.
- Data below are the number of samples that showed signal above background.
- Untested kit analytes are not listed.
- For more information contact Technical Support.

Cytokine kits

Panel Name	EGF	Eotaxin	Fractalkine	G-CSF	GM-CSF	GRO
Human Cytokine/Chemokine Panel 1	4	4	1	4	4	4
	IL-4	IL-5	IL-6	IL-7	IL-8	IL-9
	4	4	4	4	4	4
	IP-10	MCP-1	MCP-3	MDC	MIP-1α	MIP-1β
	1	1	4	3	4	1
Panel Name	MCP-4	SDF-1α+β	IL-16	MIP-1δ	6Ckine	CTACK
Human Cytokine/Chemokine Panel 2	1	2	3	2	2	3
Panel Name	MPIF-1	BRAK	CXCL16	HCC-4	MIP-4/PARC	IL-34
Human Cytokine/Chemokine Panel 4	1	4	2	1	1	3
	HMGB1/HMG1	IFNβ	IL-38	IL-28B/IFN-λ3	BAFF/BLyS	IL-14/α-Taxilin
	3	4	4	4	4	4
Panel Name	IL-17F	GM-CSF	IFNγ	IL-10	MIP-3α	IL-13
Human Th17 Panel	2	2	4	4	1	1
	IL-2	IL-21	IL-4	IL-23	IL-5	IL-6
	4	1	1	2	1	1
Panel Name	GM-CSF	sCD137	IFNγ	IL-10	Granzyme A	IL-13
Human CD8+ Panel	2	1	4	1	2	3
Panel Name	Eotaxin	G-CSF	GM-CSF	IFNγ	IL-1α	M-CSF
Mouse Cytokine/Chemokine Panel 1	4	4	4	4	4	4
	IL-7	IL-10	IL-12(p40)	IL-13	IL-15	IL-17
	4	4	4	4	4	4
	MIP-1α	MIP-1β	MIG	RANTES	TNFα	IL-12(p70)
	4	4	4	4	4	4
Panel Name	Exodus-2	MCP-5	IFNβ-1	TARC	IL-16	Fractalkine
Mouse Cytokine/Chemokine Panel 2	1	1	2	2	2	1
Panel Name	GM-CSF	TGFα	G-CSF	IFNγ	IL-2	IL-15
Non-Human Primate Cytokine/ Chemokine Panel 1	1	4	3	2	4	4
	IL-5	IL-6	IL-8	MIP-1α	TNFα	MIP-1ß
	1	4	4	4	2	1
Panel Name	G-CSF	Eotaxin	GM-CSF	IL-1α	Leptin	MIP-1α
Rat Cytokine/Chemokine	1	1	1	2	2	2
	IL-10	IL-12(p70)	IFNγ	IL-5	IL-17A	IL-18
	2	2	2	1	1	2
Panel Name	GM-CSF	IFNγ	IL-2	IL-6	IL-7	IL-8
Canine Cytokine/Chemokine	3	4	4	4	4	2

Cardiovascular Disease (CVD) kits

Panel Name	CK-MB	FABP3	Troponin-I			
Human CVD1	4	1	1			
Panel Name	α-2-Macroglobulin	Fetuin A	AGP	Fibrinogen	sL-Selectin	SAP
Human CVD3	4	1	4	3	4	2
Panel Name	sE-Selectin	dPAPP-A	sPECAM-1	Pentraxin-3	Tissue Factor	Troponin T
Human CVD4	1	4	1	4	4	4

Angiogenesis kits: 6 samples run

Panel Name	EGF	ANGPT-2	ET-1	FGF-1	Follistatin	IL-8
Human Angiogenesis/Growth Factor Panel 1	1	3	3	3	3	3

IFNα2	IFNg	IL-1α	IL-1β	IL-1ra	IL-3			
4	2	4	4	4	4			
IL-10	IL-12(p40)	IL-12(p70)	IL-13	IL-15	IL-17			
4	4	1	4	4	1			
sCD40L	TGFα	TNFα	VEGF	PDGF-AB/BB				
4	4	0	1	4				
IL-23	LIF	TPO	TSLP	IL-28A	IL-33			
3	1	3	2	2	2			
IL-24	APRIL	IL-35	IL-37/IL-1F7	IL-19	CCL28			
1	1	4	4	2	3			
IL-36β	IL-32α	YKL40/CHI3L1						
4	1	1						
IL-15	IL-17A	IL-22	IL-9	IL-1ß	IL-33			
2	4	1	3	4	3			
IL-17E	IL-27	IL-31	TNFα	TNFß	IL-28A			
1	2	1	1	4	1			
Granzyme B	IL-2	IL-4	IL-5	IL-6	MIP-1α	MIP-1β	TNFα	Perforin
1	4	1	1	2	3	1	1	1
IL-1β	IL-2	IL-3	IL-4	IL-5	IL-6			
4	4	4	4	4	4			
IP-10	MIP-2	KC	LIF	LIX	MCP-1			
4	4	4	4	4	4			
VEGF	IL-9							
4	4							
MDC	TIMP-1	IL-20	IL-11	IL-17A/F				
1	1	2	2	2				
CD40L	IL-17	IL1ra	IL-13	IL-1ß	IL-4			
1	4	4	1	4	4			
IL-12	VEGF	IL-18						
1	3	4						
IL-4	IL-1ß	IL-2	IL-6	EGF	IL-13			
2	2	2	2	2	2			
MCP-1	IP-10	GRO/KC	VEGF	Fractalkine	LIX	MIP-2	TNFα	RANTES
2	2	2	2	2	2	2	2	2
IL-15	IP-10	KC	IL-10	IL-18	MCP-1	TNFα		
4	4	3	2	4	4	2		

Haptoglobin	Platelet Factor 4	von Willebrand Factor
3	4	3

HB-EGF	VEGF-D	VEGF-A
3	2	3

Porcine

- Four serum or plasma samples were usually run in each kit. Exceptions where more than four samples were run are noted.
- Data below are the number of samples that showed signal above background.
- Untested kit analytes are not listed.
- For more information contact Technical Support.

Cytokine kits

Panel Name	EGF	Eotaxin	FGF-2	Fractalkine	GM-CSF	GRO	IFNα2	IFNg
Human Cytokine/ Chemokine Panel 1	1	2	4	3	3	3	3	2
	IL-9	IL-10	IL-12(p40)	IL-12(p70)	IL-13	IL-15	IL-17A	MCP-3
	3	4	4	0	3	3	2	3
Panel Name	SDF-1α+β	IL-16	IL-23	TPO	IL-20	IL-28A	IL-33	
Human Cytokine/ Chemokine Panel 2	4	1	2	1	1	1	1	
Panel Name	BRAK	HCC-4	IL-34	IL-35	IL-37/IL-1F7	IL-19	CCL28	HMGB1/HMG1
Human Cytokine/ Chemokine Panel 4	3	3	3	4	4	1	4	4
Panel Name	IL-17F	GM-CSF	IL-10	IL-15	IL-22	IL-9	IL-1ß	IL-33
Human Th17 Panel	3	3	1	3	4	3	3	3
Panel Name	GM-CSF	sCD137	IFNγ	IL-10	Granzyme A	IL-13	Granzyme B	IL-2
Human CD8+ Panel	2	2	2	1	2	3	2	2
Panel Name	GM-CSF	IFNγ	IL-10	MIP3α	IL-13	IL-17A	IL-1β	IL-2
Human High Sensitivity T Cell	1	1	2	1	2	3	2	2
Panel Name	Exodus 2	MCP-5	MIP-3α	IFNβ-1	TARC	IL-16	Fractalkine	MDC
Mouse Cytokine/ Chemokine Panel 2	4	1	2	2	3	4	3	4
Panel Name	IL-17E	GM-CSF	IFNγ	MIP-3α	IL-1ß	IL-2	IL-4	IL-5
Mouse Th17 Panel	2	3	2	2	2	3	2	3
	IL-13	IL-15	IL-17A	IL-17F	IL-33	IL-31	TNFß	TNFα
	2	4	2	4	4	2	4	2
Panel Name	GM-CSF	TGFα	G-CSF	IFNγ	IL-2	IL-10	IL-15	CD40L
Non-Human Primate Cytokine/Chemokine Panel 1	0	4	2	3	4	0	4	1
	MCP-1	TNFα	MIP-1ß	IL-12	VEGF	IL-18		
	1	1	2	1	2	3		
Panel Name	Eotaxin	GM-CSF	IL-1α	Leptin	MIP-1α	IL-4	IL-1ß	IL-2
Rat Cytokine/ Chemokine	2	2	2	4	4	3	3	3
	GRO/KC	VEGF	Fractalkine	LIX	MIP-2	TNFα	RANTES	
	3	4	4	3	4	2	2	

Metabolism/ Endocrinology kits

Panel Name	Apelin	Fractalkine	BDNF	EPO	Osteonectin	LIF	IL-15	Myostatin (MSTN)/GDF8
Human Myokine	2	2	1	2	1	1	3	4

Cardiovascular Disease (CVD) kits: 8 samples run

Panel Name	NT proBNP	CK-MB	CXCL6	Endocan-1	Oncostatin	Troponin-I		
Human CVD1	1	8	2	2	2	5		
Panel Name	ADAMTS13	D-Dimer	FABP5	GDF-15	Myoglobin	sP-Selectin	Lipocalin-2	SAA
Human CVD2	8	3	3	8	7	8	1	1
Panel Name	α-2-Macroglobulin	CRP	Fetuin A	AGP	Fibrinogen	sL-Selectin	Haptoglobin	Platelet Factor 4
Human CVD3	4	1	1	4	2	4	4	1
Panel Name	Tissue Factor	Troponin T						
Human CVD4	5	7						
Panel Name	sP-Selectin							
Mouse CVD1	1							

Angiogenesis kits: 6 samples run

Panel Name	EGF	ANGPT-2	ET-1	FGF-1	IL-8	HB-EGF	PLGF	VEGF-C
Human Angiogenesis/ Growth Factor Panel 1	1	5	5	5	1	5	1	1

IL-1α	IL-1β	IL-1ra	IL-4	IL-5	IL-6	IL-7	IL-8		
3	4	4	3	2	2	3	3		
MDC	**MIP-1α**	**MIP-1β**	**sCD40L**	**sIL-2Rα**	**TGFα**	**TNFβ**	**VEGF**	**RANTES**	**PDGF-AB/BB**
3	4	3	4	0	4	4	1	1	4

IFNβ	IL-38	IL-28B/IFN-λ3	BAFF/BLyS	IL-14/α-Taxilin	IL-36β
4	4	4	4	4	1

IL-2	IL-4	IL-23	IL-17E	IL-27	IL-31	TNFß	IL-28A
4	1	3	2	3	3	2	1
IL-4	**IL-5**	**IL-6**	**sFasL**	**MIP-1α**	**MIP-1β**	**TNFα**	**Perforin**
2	2	2	2	2	2	2	2
IL-4	**IL-23**	**IL-5**	**IL-6**	**IL-8**	**MIP1α**	**MIP1β**	
2	3	1	1	4	1	1	

TIMP-1	IL-20	IL-11	IL-17A/F
2	1	3	3

IL-6	IL-21	IL-22	IL-28B	IL-10	IL-23	IL-12(p70)	IL-27
2	4	4	3	3	3	3	2
CD40L							
4							
IL-17A	**IL-1ra**	**IL-13**	**IL-1ß**	**IL-4**	**IL-5**	**IL-6**	**IL-8**
4	4	0	4	3	2	3	4

IL-6	EGF	IL-13	IL-10	IL-12(p70)	IL-18	MCP-1	IP-10
1	2	2	3	3	3	2	4

FABP3	Irisin	FSTL-1	Oncostatin M	IL-6	FGF21	Osteocrin/ Musclin
4	3	3	3	3	3	1

von Willebrand Factor
4

VEGF-D	FGF-2	VEGF-A
4	5	1

Rabbit

- Four serum or plasma samples were usually run in each kit. Exceptions where more than four samples were run are noted.
- Data below are the number of samples that showed signal above background. A "+" indicates a positive result in cross-reactivity where the number of samples tested is not indicated.
- Untested kit analytes are not listed.
- For more information contact Technical Support.

Cytokine kits

Panel Name	EGF	Eotaxin	FGF-2	G-CSF	GRO	IFNg	IL-1α	IL-1β	IL-1ra	IL-3
Human Cytokine/ Chemokine Panel 1	4	4	4	2	4	4	3	4	2	1
	IL-17A	IP-10	MCP-1	MCP-3	MDC	MIP-1α	MIP-1β	sCD40L	TGF-α	TNF-β
	3	1	1	2	4	4	2	4	4	4
Panel Name	MCP-2	MCP-4	ENA-78	SDF-1α+β	I-309	TARC	6Ckine	EOTAXIN-3	CTACK	IL-23
Human Cytokine/ Chemokine Panel 2	0	0	0	3	2	1	3	3	1	3
Panel Name	MPIF-1	BRAK	CXCL16	HCC-4	IL-34	IL-35	IL-37/IL-1F7	IL-19	CCL28	HMGB1/HMG1
Human Cytokine/ Chemokine Panel 4	4	4	4	0	4	4	4	4	4	4
Panel Name	GM-CSF	IL-17A								
Human Th17 Panel	4	4								
Panel Name	GM-CSF	sCD137	IFNγ	IL-10	IL-6					
Human CD8+ Panel	2	4	4	2	4					
Panel Name	IFNγ	IL-1α	M-CSF	IL-1β	IL-2	IL-3	IL-4	IL-5	IL-12(p40)	IL-15
Mouse Cytokine/ Chemokine Panel 1	**1**	**2**	**2**	**1**	**1**	**1**	**1**	**1**	**4**	**1**
	MCP-5	MIP-3α	IFNβ-1	IL-16	Fractalkine	TIMP-1	IL-20	IL-11	IL-17A/F	
Mouse Cytokine/ Chemokine Panel 2	**1**	**1**	**4**	**4**	**4**	**4**	**4**	**4**	**4**	
Panel Name	IL-17E	GM-CSF	IFNγ	MIP-3α	IL-2	IL-4	IL-5	IL-6	IL-22	IL-28B
Mouse Th17 Panel	2	4	3	1	3	1	1	4	4	4
Panel Name	GM-CSF	TGFα	G-CSF	IL-2	IL-17	IL-13	IL-5	IL-8	MIP-1α	VEGF
Non-Human Primate Cytokine/ Chemokine	0	4	2	4	4	1	1	4	3	4
Panel Name	G-CSF	GM-CSF	IL-1α	Leptin	MIP-1a	IL-4	IL-1ß	IL-2	IL-6	EGF
Rat Cytokine/ Chemokine	1	3	3	4	3	3	3	3	2	2
Panel Name	IFNγ	IL-18	TNFα							
Canine Cytokine/ Chemokine	4	3	1							
Panel Name	IL-4	IL-18								
Porcine Cytokine/ Chemokine	4	1								

Metabolism/ Endocrinology kits

Panel Name	Apelin	BDNF	EPO	Osteonectin	LIF	Myostatin (MSTN)/GDF8	FABP3	FSTL-1
Human Myokine	4	4	1	1	4	1	4	4

IL-4	IL-5	IL-6	IL-7	IL-8	IL-9	IL-10	IL-12(p40)	IL-12(p70)	IL-13	IL-15
1	2	2	2	2	3	3	2	0	2	2

VEGF	PDGF-AA
3	4

LIF	TSLP	IL-33
3	3	3

IFNβ	IL-38	IL-28B/ IFN-λ3	BAFF/ BLyS	IL-14/α-Taxilin	IL-36β	IL-36α
0	4	4	4	4	4	4

IL-17	IP-10	MIP-2	LIF	LIX	MIP-1α	MIP-1β	RANTES	TNFα	VEGF	IL-9
1	**2**	**3**	**1**	**3**	**1**	**2**	**4**	**1**	**4**	**2**

IL-10	IL-23	IL-12(p70)	IL-27	IL-13	IL-15	IL-17A	IL-17F	IL-33	IL-31	TNFß	CD40L
3	2	2	3	1	1	3	1	2	3	2	4

IL-13	IL-10	IL-12(p70)	IFNg	IL-17	IL-18	MCP-1	IP-10	GRO/KC	VEGF	Fractalkine	LIX	MIP-2	RANTES
3	4	2	2	1	4	2	4	4	3	3	3	4	1

Appendix 2: Sample Preparation

MILLIPLEX® MAP Kits Requiring Special Sample Preparation

Kit Name	Cat. No.	Sample Type	Sample Trt Inhibitors	Inhibitor Source
Canine Gut Hormone Magnetic Bead Panel	CGTMAG-98K	SER / PLA / CCS	DPP-IV, Aprotinin, AEBSF, Protease Cocktail	See Notes 1, 2, 3, 4
Human IGF Magnetic Bead Panel	HIGFMAG-52K	SER / PLA / CCS	Extraction	NONE
Human IGF Binding Protein Magnetic Bead Panel	HIGFBMAG-53K	SER / PLA / CCS	Protease Inhibitor Cocktail	See Note 4
Human Metabolic Hormone Magnetic Bead Panel	HMHEMAG-34K	SER / PLA / CCS	DPP-IV, Aprotinin, AEBSF, Protease Cocktail	See Notes 1, 2, 3, 4
Human Neuropeptide Magnetic Bead Panel	HNPMAG-35K	SER / PLA / CCS	Extraction	NONE
Mouse Gut Hormone Magnetic Bead Panel	MGTMAG-78K	SER / PLA / CCS	DPP-IV	See Note 1
Mouse Metabolic Hormone Magnetic Bead Panel	MMHMAG-44K	SER / PLA / CCS	DPP-IV, Aprotinin, AEBSF, Protease Cocktail	See Notes 1,2, 3, 4
Non-Human Primate Metabolic Magnetic Bead Panel	NHPMHMAG-45K	SER / PLA / CCS	DPP-IV, Aprotinin, AEBSF, Protease Cocktail	See Notes 1,2, 3, 4
Multi-Species Hormone Magnetic Bead Panel	MSHMAG-21K	SER / PLA / CCS	Extraction	NONE
Rat Metabolic Hormone Magnetic Bead Panel	RMHMAG-84K	SER / PLA / CCS	DPP-IV, Aprotinin, AEBSF, Protease Cocktail	See Notes 1,2, 3, 4
Rat/Mouse Neuropeptide Magnetic Bead Panel	RMNPMAG-83K	SER / PLA / CCS / CSF	Extraction	NONE
Human Skin Magnetic Bead Panel	SKINMAG-50K	SER / PLA / CCS	Validated for skin lysates	NONE
Steroid/Thyroid Hormone Magnetic Bead Panel	STTHMAG-21K	SER / PLA / CCS	Extraction	NONE
Multi-Species Hormone Magnetic Bead Panel	MSHMAG-21K	SER / PLA / CCS	Extraction	None
TGFβ1 Singleplex Magnetic Bead Panel	TGFBMAG-64K-01	SER / PLA / CCS	Acidification	NONE
TGFβ1, -2, -3 Panel Magnetic Bead Panel	TGFBMAG-64K-03	SER / PLA / CCS	Acidification	NONE

Notes:

1. DPP-IV (Cat. No. DPP4-010) is used at 10 µL per mL of blood.
2. Pefabloc or AEBSF (Cat. No. 101500) is used at 1 mg/mL in blood.
3. Protease Inhibitor Cocktail I (Cat. No. 20-201).
4. Protease Inhibitor Cocktail (Sigma-Aldrich Cat. No. P2714).
5. Active and Total cannot be run together in the same assay.

Appendix 3: Other Sample Types

Protocols Using Other Sample Types

Sample Type	Species/Kit Run	Procedure	Reference (if available)
Adipose Tissue Homogenates	Human	Approximately 4 g of adipose tissue from each subject was homogenized in 16 mL of ice-cold deoxygenated homogenization buffer containing 10% glycerol, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 25 mM benzamidine, 10 µM leupeptin, 2.5 µmol/L pepstatin A, and 50 U/mL aprotinin in 10 mM Tris-HCl (pH 7.0), with four up/down strokes at Setting No. 3 using a Polytron (Brinkmann Instruments, Inc., Westbury NY). The crude homogenate was centrifuged at 3,000 x g for 15 minutes, and the fat cake was discarded. The infranate was made up to 1% (vol/vol). Triton® X-100 was used to solubilize PTPase enzymes from the particulate compartment into the tissue homogenate. The supernatant resulting from centrifugation at 15,000 x g for 20 minutes at 4 °C was stored in aliquots at -80C	J Clin Endocrinol Metab. 2001 Dec;86(12):5973-80. PMID:11739472
Adipose Tissue Extract	Human/ Apolipoprotein Panel	Adipose biopsies (50–75 mg) were homogenized on ice in 1 mL of the kit assay buffer. (10 mmol/L PBS, 0.08% (wt./vol.) sodium azide, 1% (wt./vol.) BSA, pH 7.4). The homogenate was further diluted 25-fold in assay buffer to minimize assay interferences. 10 µL of dilute homogenate was incubated in a 96-well plate with 25 µL of capture antibody-conjugated beads and 65 µL assay buffer for 1 hour ambient. Beads were washed (10 mmol/L PBS, 0.05% (vol./vol.) Proclin, 0.05% (vol./vol.) Tween-20, pH 7.4) and 50 µL biotinylated detection antibody cocktail added for 30 minutes ambient, followed by further washing and incubation with 50 µL streptavidin–phycoerythrin for 30 minutes at ambient temperature. After final washing, beads were resuspended in 150 µL Luminex® sheath fluid for analysis.	Diabetologia. 2008 Nov;51(11):2041-8. doi: 10.1007/s00125-008-1126-5. Epub 2008 Aug 19 PMID:18712345
Adipose Tissue Extract	Human/CVD Panel	Approximately 100-200 mg adipose tissue (SAT and VAT) from each subject was homogenized in 250 µL of ice-cold homogenization buffer. The homogenate was centrifuged at 3,000 x g for 15 minutes at 4 °C, the fat cake was discarded and the homogenate was centrifuged again at 14,000 x g for 20 minutes at 4 °C. The supernatant was stored in aliquots at -70 °C.	Physiol Res. 2010;59(1):79-88. Epub 2009 Feb 27. PMID:19249917
Aorta Tissue Extract	Guinea Pig/ Human Cytokine/ Chemokine Panel 1	The vessel was dissected and all the surrounding tissues removed. The vessel was mixed and homogenized in a rotor-stator with 1 mL of lysis buffer (0.1 g of bovine serum albumin, 5 µL of Triton® X-100, 100 mg of gentamycin sulfate, 100 µL of HEPES buffer-1M, 23 µL of aprotinin, 18.391 mg of sodium orthovanadate and PBS to complete 1 mL). After this, 2 mL of the lysis buffer was added to the content and was homogenized in a Potter-Elvehjem tissue grinder. This was centrifuged at 400 × g for 10 minutes at 4 °C. The supernatant was analyzed.	BMC Cardiovasc Disord. 2009 Feb 17;9:7. doi: 10.1186/1471-2261-9-7. PMID:1922285
Brain Tissue Extract	Rat/Rat Cytokine	Plasma and brain tissue from injured (hyperintense tissue on DW-MRI during occlusion) and anatomically matching tissue from the contralateral hemisphere were collected from control and minocycline- or PBS-treated rat pups following 24 hours of reperfusion. The flash-frozen brain tissue was homogenized in a buffer containing 20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L PMSF, 0.05% Tween® 20, and a cocktail of protease inhibitors (Roche), and protein concentration was measured in each sample.	J Cereb Blood Flow Metab. 2005 Sep;25(9):1138-49 PMID: 15874975
Bronchoalveolar Lavage (BAL) Samples		For lavage samples, use 50 µL sample + 25 µL beads in sample wells. Set up standards using one additional lower point and dropping the highest concentration standard point. Use a buffer matrix or medium used to collect the lavage sample as the matrix, i.e., 25 µL standard/control/blank + 25 µL assay buffer /medium + 25 µL beads. The first incubation with standard/ sample should be overnight, 4 °C. Final results should be divided by 2.	
Dried Blood Spot Samples		Two 3.2-mm (1/8-inch) diameter disks were punched from dried blood-spot calibrators or controls and eluted in 100 µL of 0.075 mol/L sodium barbital buffer (pH 8.6) containing 0.5 g/L anilinonaphthalenesulfonic acid and 0.5 g/L sodium azide by sonication at room temperature for 30 minutes. The volume of blood per 3.2-mm disk was 3 µL. The eluate was filtered in a 0.45 µm centrifugal filter unit.	Clin Chem. 2000 Sep;46(9):1422-4. PMID: 10973880
Dried Blood Spot Samples		Whole blood samples were “spotted” onto Whatman 3 mm filter paper, air-dried, and stored at 4 °C prior to extraction and testing. Areas equivalent to a 25-µL drop were punched from the filter paper and eluted in 25 µL of 0.01 M phosphate buffer, pH 7.4 prior to analysis. The protein content of each eluate was measured spectrophotometrically at 260/280 nm and the samples normalized to a standard protein content of 1 µg/mL.	J Chromatogr B Biomed Sci Appl. 1998 Sep 11;715(1):55-63. PMID: 9792497

Sample Type	Species/Kit Run	Procedure	Reference (if available)
Cervical Secretions, Vaginal Secretions, and Saliva Secretions		Saliva, cervical and vaginal secretions were collected using ophthalmalic sponges (Wek-Cel, Xomed Treace, Orlando, FL) after exposure of the cervical os with the speculum. The secretions were collected by placing the ophthalmalic sponge directly into the cervical os and allowing it to absorb secretions for approximately 1 minute. Vaginal secretions were collected by placing the ophthalmalic sponge against the vaginal wall and allowing the sponge to collect secretions. In a similar fashion, saliva was collected by placing the ophthalmalic sponge over the parotid duct and allowing the sponge to absorb saliva. All sponges were immediately placed on ice and then frozen at -20 °C. The secretions were extracted from the sponges just prior to analysis. Each individual sponge was weighed to determine the volume of secretions absorbed into the sponges. The sponges were then equilibrated in 300 µL phosphate-buffered saline (PBS) + 0.25 M NaCl with 10% fetal calf serum for 30 minutes at 4 °C. The secretions were separated using a spin-x centrifuge filter unit (Costar, Cambridge, MA), centrifuged at 12,000 x g rpm for 20 minutes. A dilution factor for the final extract was determined based on the following formula: dilution factor = [(x-0.0625 mL) + 0.3 mL buffer]/x-0.0625 mL)], where x equals the volume of material collected and 0.06 equals the weight of the dry spear (mg=mL). (Note: The weight of the dry sponge is dependent on the lot number. Each lot must be weighed.) This dilution factor was used to calculate the final units of specific antibody and total immunoglobulin measured.	J Clin Immunol. 1997 Sep;17(5):370-9. PMID: 9327336
Colorectal Tissue Extracts	Human/Human Cytokine Panel 1	Normal and cancer tissue specimen weights were determined before protein extraction with Tissue Protein Extraction Reagent (T-PER; Pierce, Rockford, USA) as recommended by the manufacturer. Briefly, 20 mL of P-TER was added to 1 g of tissue and homogenized. Samples were centrifuged at 10,000 x g for 5 minutes and the supernatant (protein extract) was stored at -80 °C until cytokine/chemokine profiling.	Gut. 2009 Apr;58(4):520-9. doi: 10.1136/gut.2008.158824. Epub 2008 Nov 20. PMID: 19022917
Ear Lysates	Mouse/Mouse Cytokine Panel 1	Skin ear biopsies were pooled from four test animals. Biopsies were minced and then repeatedly homogenized with beads in phosphate buffered saline (PBS) plus Complete Protease Inhibitor Cocktail (Roche Applied Science, Indianapolis, IN) at 4 °C. Supernatant was collected and analyzed for the presence of cytokines.	J Invest Dermatol. 2010 Apr;130(4):1023-33. doi: 10.1038/jid.2009.358. Epub 2009 Nov 12. PMID: 19907432
Ear Lysates	Mouse/Mouse Cytokine Panel 1	Ear tissue from mice treated with vehicle or R348 (120 mg/kg) were harvested after 6 weeks of treatment and snap-frozen in liquid nitrogen. Ears were homogenized under liquid nitrogen with radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitors, centrifuged at 13,000 rpm, and the supernatant was collected to generate ear lysates. Ear lysates were normalized for equal protein concentration.	J Immunol. 2009; 183(3):2183-92. PMID: 19596999
Infectious Samples		For infectious samples: If washing with an automatic plate washer, add 30% bleach to the waste bottle before washing/aspirating the plate. If washing with a handheld magnetic bead separator, add 30% bleach to a container capable of catching the wash solution decanted from the plate. Then at the end of the assay, resuspend the beads in 0.1ml of 4% formaldehyde made in 10mM PBS (prepared fresh daily) instead of sheath fluid, before running the plate in the Luminex® machine. Prolonged incubation in this solution may cause bead aggregation. Consequently, after agitating the plate for 5 minutes on an orbital plate shaker, read the plate immediately.	
Jejunal Extracts	Human/Human Cytokines	Jejunal biopsy specimens were fixed with formalin or embedded in optimal-cutting-temperature (OCT) compound and snap frozen in liquid nitrogen. Protein extracts were prepared from jejunal biopsies embedded in OCT compound by washing them twice with a phosphate-buffered saline lysis buffer containing 0.05% sodium azide, 0.5% Triton® X-100, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors (Complete Mini protease inhibitor cocktail; Roche Diagnostics, Indianapolis, IN). After OCT compound removal, the tissues were minced in 1 mL of lysis buffer with a sterile disposable homogenizer on ice for 5 minutes. The homogenates from the tissues were then sonicated for 1 minute on ice. After centrifugation at 10,000 x g for 15 minutes, the supernatant was collected and stored at -80 °C or immediately assayed to determine the protein concentration with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL).	Infect Immun. 2007 Jan;75(1):481-7. Epub 2006 Oct 16. PMID: 17043107
Lipemic Samples		For lipemic and plasma samples, the blood needs to be collected on ice, centrifuged in a refrigerated centrifuge, aliquoted and frozen at -20 °C for short term (<2 months) and -70 °C for long term. Prior to assay setup, thaw samples and centrifuge at 10,000 rpm for 5 minutes. Spool off the lipid layer from the surface using a cotton swab and use the supernatant below lipid layer for the assay.	

Sample Type	Species/Kit Run	Procedure	Reference (if available)
Lymph Node Homogenates	Mouse/Mouse Cytokines	Footpad popliteal lymph nodes from mouse subjects were harvested, combined and placed in 200 µL of PBS containing 1 × protease inhibitors (Roche). The lymph nodes were mechanically homogenized with a pestle, followed by centrifugation at 4 °C. Supernatant was transferred to another tube and frozen on dry ice.	Vaccine. 2010 Apr 19;28(18):3238-46. PMID: 20184975
Saliva	Human	Add protease inhibitor cocktail at 1:500 to saliva. Centrifuge at 10,000 rpm 10 minutes and dilute supernatant 1:2 with assay buffer prior to assay setup. This method significantly improves recovery and reduces bead aggregation. Run assay with assay buffer as matrix in standard curve. Use an overnight option if available.	
Skin Extracts	Human/Human Cytokine Panel 1	D-Squame® tape strip samples of human scalp skin were extracted with PBS containing 0.2% SDS and 0.5% propylene glycol (PG) for 30 minutes with sonication on ice. The extracts were then centrifuged for 5 minutes at 2,100 × g to remove skin solids that might interfere in the assay. Subsequently, the extracts of D-Squame® tape samples were transferred into 96-well polypropylene deep-well plates and frozen at -80 °C.	Int J Dermatol. 2011 Jan;50(1):102-13. doi: 10.1111/j.1365-4632.2010.04629.x. PMID: 21182510
Tears	Human/Human Cytokines	Polyurethane minisponges were obtained commercially (PeleTim; VOCO GmbH, Cuxhaven, Germany). A single polyurethane minisponge was laid on the outer third of the lower eyelid margin. After 5 minutes of tear collection, the sponge was recovered and placed in the narrow end of a truncated Gilson micropipette tip adapted to a 1.5 mL tube (Eppendorf, Fremont, CA) and centrifuged at 6,000 rpm for 5 minutes. Tear samples from both eyes were pooled and immediately stored at -80°C until they were used for the immunoassay.	Invest Ophthalmol Vis Sci. 2012 Aug 13;53(9):5443-50. PMID: 22789923
Tears	Human/Human Cytokines	Tear collection was performed before any other test and with a minimum of 10 minutes after the patient answered the two symptom questionnaires. Unstimulated tear samples were collected non-traumatically from the external canthus of open eyes, avoiding additional tear reflex as much as possible. Glass capillary micropipettes (Drummond, Broomall, PA) were used to collect 1 µL of tears. Each sample was then diluted 1:10 in a sterile collection tube containing ice-cold Cytokine Assay Buffer. Tubes with tear samples were kept cold (4 °C) during collection, and stored at -80 °C until assayed.	Mol Vis. 2010 May 19;16:862-73. PMID: 20508732
Cell or Tissue Extraction		Protocol varies depending on tissue types and/or analytes of interest. Generally, most protocols that are used in ELISAs can be used, but here are some guidelines in selecting a method. 1) Homogenize cells or tissues mechanically (eg. ultrasonication) in a PBS-based buffer containing protease inhibitors (like aprotinin or an inhibitor cocktail) and low (< 0.2%) non-ionic detergent concentration. 2) Extraction medium should not contain any organic solvents like DMSO, etc. 3) Centrifuge the extract and freeze supernatant at <-20 °C. 4) Use the extraction medium as matrix in blank, standard curve and QCs.	
Tumor Homogenates	Mouse/Mouse Cytokines	Tumors were treated with DMXAA. After harvesting at 6, 24, and 48 hours, the tumors were sonicated for 30 seconds in 1 mL of complete buffer (50 mL PBS containing one tablet of antiprotease cocktail, Roche, Indianapolis, IN). Tissues were then spun at 3,000 rpm for 10 minutes and filtered through a 1.2 µm syringe filter unit. Total protein in each sample was determined.	Cancer Res. 2005 Dec 15;65(24):11752-61. PMID: 16357188
Urine		Typically, measurement of analytes in urine requires either a 24 hours urine collection or second morning void collection. For the second morning void urine, the analyte value is normalized against creatinine, i.e., the analyte is expressed as units/mg of creatinine. Mix urine samples 1:1 with assay buffer and incubate on the plate approximately 20 minutes on a shaker prior to addition of the beads. Use assay buffer as matrix for standard curve, controls and blank. The assumption is that this helps neutralize the sample, thereby improving recovery.	

Glossary*

Accuracy

Data representing mean percent recovery of spiked standards ranging from low, medium and high concentrations in serum matrices for a defined number of samples (see Spike Recovery).

Analyte

A chemical substance that is the subject of chemical analysis.

Configurable/customizable kit

A type of MILLIPLEX® MAP kit that enables you to choose the analytes within a specific panel that best meet your research needs.

Drive fluid

Luminex® Drive Fluid is the delivery medium to transport the sample to the optic components of the MAGPIX® system.

Fixed kit

A type of MILLIPLEX® MAP kit that is not configurable. All of the analytes are sold together with the capture beads already premixed.

FLEXMAP 3D® system

A Luminex® instrument that combines differentially dyed fluorescent microsphere sets with an innovative instrument design to enable precise, rapid multiplexing of up to 500 unique assays within a single sample. Other features include an automated probe height adjustment, simplified routine maintenance operations and an intuitive software interface.

Inter-assay precision (%CV)

Precision generated across two different concentrations of analytes across a defined number of different assays.

Intra-assay precision (%CV)

Precision generated across two different concentrations of analytes in a single assay.

Linearity

The ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

Luminex® 200™ system

A Luminex® instrument that provides a complete mid- to high-range solution for rapid, accurate biomarker quantification. The Luminex® XY Platform™ (Luminex® XYP™) complements this instrument system by automating the sequential positioning of each well of a microtiter plate.

Magnetic beads (MagPlex®)

Similar to MicroPlex® microspheres, MagPlex® microspheres are carboxylated polystyrene micro-particles, or "beads," that have been dyed into spectrally distinct sets, or "regions"; allowing them to be individually identified by a Luminex® instrument. These uniquely coded beads provide a user an addressable substrate on which to perform multiple bio-analytical reactions in a single well.

MAGPIX® system

A Luminex® instrument based on CCD imaging technology, which allows for a compact, more robust system. Streamlined startup and shutdown protocols and minimal maintenance requirements make the system easy to operate and maintain.

MFI

Median fluorescence intensity.

MILLIPLEX® 5.1 Analyst software

Our analysis software package that is able to automatically import data from Luminex® instruments, providing better data from the low and high ends of standard curves, comprehensive detailed reports and enhanced visualization.

MILLIPLEX® MAP

A broad portfolio of multiplex immunoassays that includes immunology, metabolism, cardiovascular disease, cancer, neuroscience, toxicity and cell signaling pathways for a variety of species.

MinDC (minimum detectable concentration)

The lowest concentration at which an analyte can be reliably detected.

Precision

Measure of statistical variability generated from the mean of the %CVs from a defined number of reportable results.

Premixed kit

A MILLIPLEX® MAP kit in which the capture beads have been mixed together. There is often an option of choosing either a premixed kit or a configurable kit.

Quality controls (QCs)

Medium-low and medium-high standard points used to qualify assay performance.

SAPE

Streptavidin-phycoerythrin, in Luminex® assays, it is excited by the green light source in the instrument.

Serum matrix

An appropriately selected matrix found only in MILLIPLEX® MAP kits that is added to the standard wells to mimic the environment in which native analytes are present in serum/plasma. The selected matrix most often consists of a serum/plasma pool with all endogenous and cross-reacting proteins extracted.

Sheath fluid

Luminex® Sheath fluid is intended for use as the delivery medium of the sample to the optics component of the Luminex® 100/200™ system and the FLEXMAP 3D® systems.

Spike recovery

Data representing mean percent recovery of spiked standards ranging from low, medium and high concentrations in serum matrices for a defined number of samples (see Accuracy).

Stability

Resistance or the degree of resistance to chemical or thermal change or disintegration.

Standard curve/Calibration curve

A graphic plot of median fluorescence intensity versus the known concentration of test substances in a set of standards usually prepared by serial dilution or incremental addition.

xMAP® (Multi-Analyte Profiling) technology

Flexible, open-architecture design that can be configured to perform a wide variety of bioassays developed by the Luminex® Corporation.

xPONENT® software

Luminex® acquisition software with analysis capabilities.

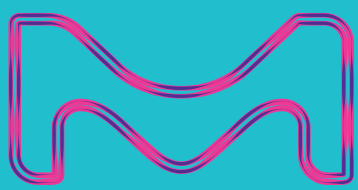
* Sources include: thefreedictionary.com; merriam-webster.com; luminexcorp.com; scientistsolutions.com; regulatory.com; dictionary.com; and MILLIPLEX® systems protocols.

Notes:

Notes:

Merck KGaA
Frankfurter Strasse 250,
64293 Darmstadt, Germany

Merckmillipore.com



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