MERCK

Tips & Tricks

MILLIPLEX® Multiplex Assay User Guide

The life science business of Merck operates as MilliporeSigma in the U.S. and Canada.

Millipore®

Preparation, Separation, Filtration & Monitoring Products

Why just multiplex when you can use MILLIPLEX® assays?

For more than 20 years, we have offered the benefits of MILLIPLEX® multiplex assay panels, containing everything you need to detect multiple analytes simultaneously. We're so confident in the benefits of MILLIPLEX® 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® kits, publishing in scientific journals around the world. This guide was created to enhance 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® kits. We cannot guarantee methods presented will work in all cases. These procedures have not been verified.

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Introduction

Luminex® xMAP® Technology

- MILLIPLEX® 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 can perform a variety of bioassays, including immunoassays, on the surface of fluorescent-coded magnetic (MagPlex®) bead microspheres.
- Luminex® technology 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 microspheres are coated with a specific capture antibody/antigen.
- 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 SAPE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere. **Figure 1** illustrates the typical bead-based immunoassay procedure.
- There are multiple Luminex® instruments available to acquire and analyze data. The detection method is illustrated in **Figure 2.**
  - The Luminex® 200™, FLEXMAP 3D®, and INTELLIFLEX® systems are flow cytometry-based instruments that integrate key xMAP® detection components, such as lasers, optics, advanced fluidics, and high-speed digital signal processors.
- 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 experimental analysis capabilities of Belysa® Immunoassay Curve Fitting software, integrating data acquisition and analysis seamlessly on all Luminex® instruments.
- Use of Belysa® Immunoassay Curve Fitting software provides advanced features not available within most data acquisition packages: autocurve fitting functions, data hygiene rules, easy data visualization and standard curve comparison tools, with all data exportable in a variety of file formats.

The capability of adding multiplexed conjugated beads to each sample allows 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.

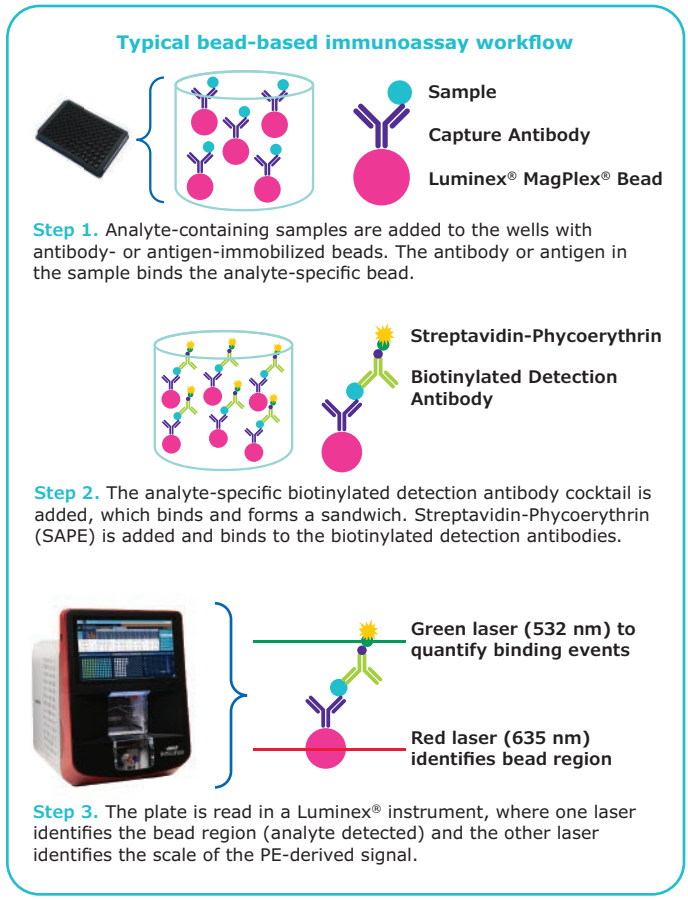


Figure 1. The typical bead-based immunoassay procedure as seen in MILLIPLEX® assays for the Luminex® platform.

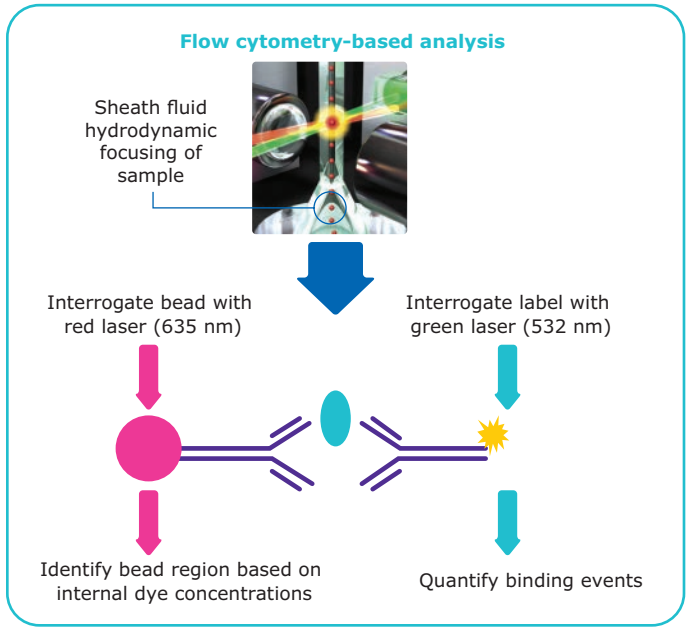


Figure 2. The fluorescence detection method for acquiring and analyzing multiplex assay data.

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

- >130 kits to study circulating soluble proteins
- >500 unique circulating analytes (not counting different species)
- >25 premixed multiplex and singleplex kits to study cell signaling proteins
- >120 cell signaling analytes
- Multiple species
- 96- and 384-well formats

A broad portfolio means you will:

- Find assays for analytes 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 research areas

Let Industry Guidance Lead You to MILLIPLEX® Multiplexing

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 Reproducibility
- Vendor Support

We provide assay performance data in every protocol.

Need more information? Contact:
SigmaAldrich.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.
4. Mathews *et al.* AAPS Open, Vol. 8, No. 2, February 2022; Best practices for the development and fit-for-purpose validation of biomarker methods: a conference report.

Why Choose MILLIPLEX® Assays?

Our quality makes MILLIPLEX® assays stand out. From kit development and verification to manufacturing and quality control, 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 kit development and verification process: cross-reactivity, dilutional linearity, kit stability, and sample behavior (e.g., detectability and stability).

Quality Controls

- We include Quality Controls (QCs) and QC range sheets to qualify assay performance.
  - QC values are based on a minimum of six assays run by at least three different operators.
  - 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 range. This tells the Technical Support Specialist whether or not the kit is performing correctly.
  - Use of high and low QC values serve as an additional checkpoint in case there was user error associated with hydrating or diluting standard.
- We recommend individual labs qualify their own assay performance by including internal controls best suited for their unique experimental samples.
- QCs are important for translational studies that require more validation, ensuring that the data are reproducible across kit lots.
- QCs are also important when comparing data across multiple sites, or assay results from multiple technicians.

Standards

- Each new lot of MILLIPLEX® standards and QCs are compared to previous lots and a “reference” lot to ensure lot-to-lot consistency.
  - All data are compiled in a single database and trend charts are maintained.
  - Full standard curve characteristics and relative potency of analytes are maintained within specifications of the “reference” lot.
  - Since MILLIPLEX® panels stand the test of time, new standard lots are periodically assigned to be the fresh “reference” lot against which subsequent lots are compared.
- Other suppliers compare new standard lots to previous lots, without a "reference" lot.
  - This may make it difficult to compare data from multiple lots since standard curve point values may vary with each new lot and assay drift may occur.

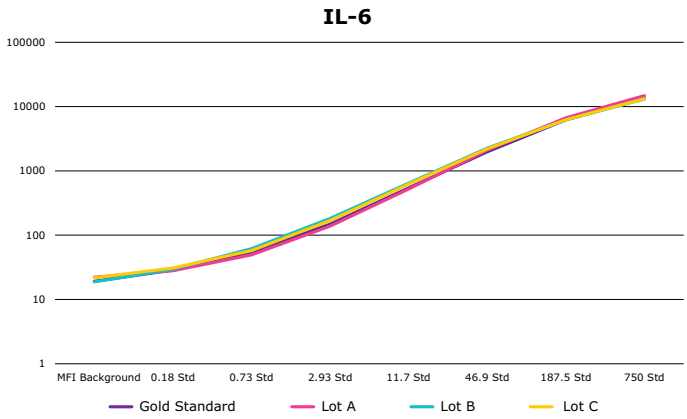


Figure 3. Trend charts are maintained to document lot-to-lot performance. This graph illustrates data generated using the average of all in-house assays for the given lot. The relative potency was 1.12 for **Lot B** and 1.01 for **Lot C** when compared to previous lots, indicating consistent assay performance across multiple lots. This data was generated for IL-6 in the MILLIPLEX® Human High Sensitivity T Cell Panel (Cat. No. **HSTCMAG-28SK**).

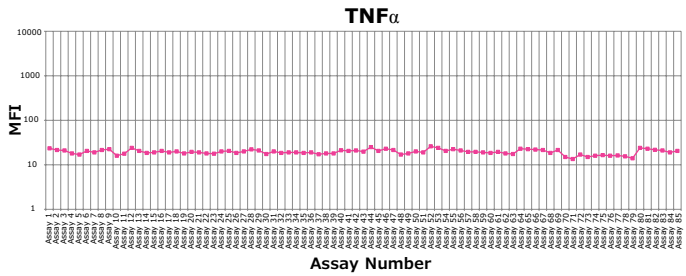


Figure 4. Trend chart standard curve point-to-point analysis shows consistent MFI values for TNFα across 85 assays and 4 standard lots. This data was generated using MILLIPLEX® Human Cytokine/Chemokine/Growth Factor Panel A (Cat. No. **HCYTA-60K**). Data for one standard point is shown.

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 non-specific 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 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 analyte measurement.
  - It is very difficult to predict the effect of mixing serum matrix with samples from a randomly sampled population.

Optimized Serum Matrix

- 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 non-specifically bind to the capture and detection antibodies simultaneously, thus generating a false positive signal.
  - Our bead diluents contain a cocktail of proprietary reagents that significantly reduce this false signal without reducing the true analyte measurement.
  - Our bead diluents may also contain factors for detection. For instance, we add 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® 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.

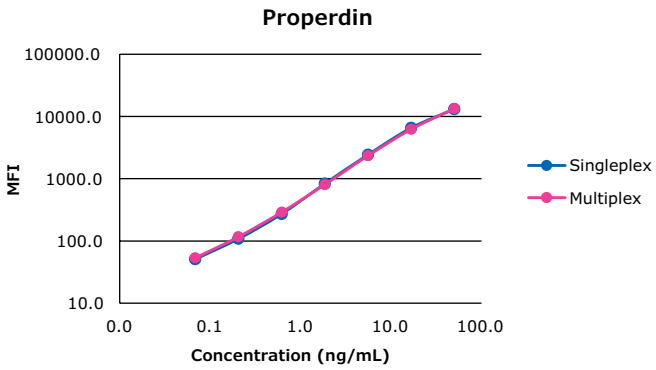


Figure 5. Consistent analyte profiles are seen when comparing multiplex and singleplex assays from the same MILLIPLEX® panel, (Cat. No. **HCMPEX1-19K**) as in this example of the analyte Properdin.

Selecting the Best MILLIPLEX® Assay for Your Research

- Our assays for the same analyte often (not always) use the same antibody pairs and conditions.
  - In method comparison tests, while the absolute values may not be exactly the same, the results correlate. Hence, when switching from one assay platform to another, a correlation factor may 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 **SigmaAldrich.com** using the catalog number.
  - Click on the link to go to the kit page and then scroll to the documentation section.
  - There are three easy methods to find your analytes of interest:
  - The MILLIPLEX® Analyte Kit Finder located at **SigmaAldrich.com/milliplex-order.**
  - Search the latest edition of the Analyte Quarterly, available for download at **SigmaAldrich.com/milliplex**.
  - Contact Technical Support, your Sales Specialist, or Field Application Scientist.
- To find publications citing a specific panel or analyte, contact Technical Support or your Sales Specialist. You may also find featured publications on the product detail pages on our website, or at **SigmaAldrich.com/milliplex-learn**.
- To determine cross-reactivity for other species for a panel or analyte:
  - For a subset of kits, see the Species Cross-Reactivity information at **SigmaAldrich.com/milliplex-species**.
  - Contact Technical Support.
  - For cell signaling kits, we analytically verify 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.
- How to design a configurable kit:
  - Human Cytokine/Chemokine/Growth Factor Panel A (Cat. No. **HCYTA-60K**).
  - Choose 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, for example **HCYTA-60K-05,** and list the specific analytes.

Luminex® Technical Support:
Phone: +1-512-381-4397
Toll-free: +1-877-785-2323
Contact: **int.diasorin.com/contact-us**

Agilent® Technical Support:
Phone: +1-800-766-7000
Email: **customer.service@agilent.com**

Please visit **SigmaAldrich.com/techservice** to find your regional Technical Support Team or contact your Sales Specialist.

Materials Required But Not Provided

- Adjustable pipettes with tips capable of delivering 5 µL to 1,000 µL

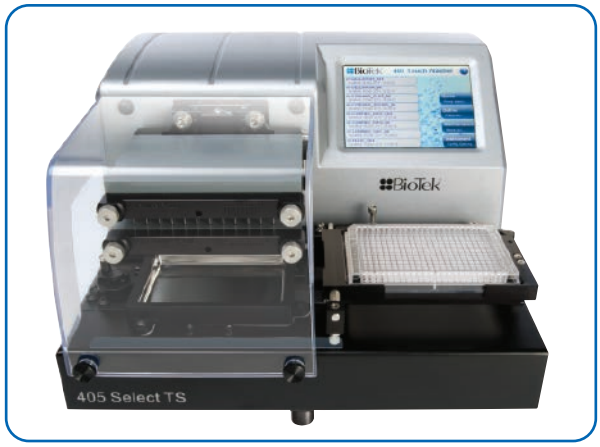
- Multichannel pipettes capable of delivering 5 µL to 50 µL or 25 µL to 200 µL

- Laboratory vortex mixer

- Ultrasonic waterbath (Branson Ultrasonic Cleaner Model #B200 or equivalent)
  - Sonicator probes should not be used

- Orbital titer plate shaker

- Automatic Plate Washer for magnetic beads or Handheld Magnetic Separation Block (BioTek® 405 Plate Washer is shown). See next page for additional plate washer information.



Materials Required But Not Provided

- Luminex® 200™, MAGPIX®, FLEXMAP 3D®, or xMAP® INTELLIFLEX® RUO instruments, with analysis software.
- Sheath fluid (Luminex® 200™, FLEXMAP 3D®, or INTELLIFLEX® RUO systems) or drive fluid (MAGPIX® instrument).
  - Sheath Fluid PLUS or Drive Fluid PLUS can be reordered directly from us:
    - Sheath Fluid PLUS, 20L, RUO (Cat. No. **40-50021**)
    - MAGPIX® Drive Fluid PLUS 4PK, RUO (Cat. No. **40-50030**)
    - Sheath Concentrate PLUS, RUO (Cat. No. **40-50023**)
- Maintenance kits for Luminex® instruments are available directly from us:
  - Luminex® 200™ Instrument (xPONENT® Software)
    - Calibration Kit (Cat. No. **LX2R-CAL-K25**)
    - Performance Verification Kit (Cat. No. **LX2R-PVER-K25**)
  - MAGPIX® Instrument
    - Calibration Kit (Cat. No. **MPX-CAL-K25**)
    - Performance Verification Kit (Cat. No. **MPX-PVER-K25**)
  - FLEXMAP 3D® Instrument
    - Calibration Kit (Cat. No. **F3D-CAL-K25**)
    - Performance Verification Kit (Cat. No. **F3D-PVER-K25**)
  - xMAP® INTELLIFLEX® RUO Instrument
    - Calibration Kit (Cat. No. **IFX-CAL-K20**)
    - Performance Verification Kit (Cat. No. **IFX-PVER-K20**)
- Bead washer (either automated or manual):
  - Automated magnetic bead plate washers:
    - BioTek® 405 TS Magnetic 96-Well Washer Complete with Touch Screen and Ultrasonic Cleaning (Cat. No. **40-092AB**)
    - BioTek® MultiFlo™ FX Automated Reagent Dispenser, optimized for both 384- and 96-well plates (Cat. No. **40-999**)
  - Handheld Magnetic Separator Block for 96-Well Flat Bottom or Conical Well Plates (Cat. No. **40-285**)

BioTek® MultiFlo™ FX Automated Reagent Dispenser

Sample Collection and Preparation

General Sample Information

- 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 more precise delivery of contents during pipetting, reverse pipetting is recommended. Once you have set your pipette to the required volume, press the plunger down completely, past the first stop. Aspirate liquid and dispense to the first stop. A small volume of liquid will remain in the pipette, which can be discarded. Reverse pipetting helps ensure accurate delivery of small volumes of liquid to achieve best results by eliminating bubbles or air from liquid dispensing steps.
- 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.
- 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.
  - Still unsure? Contact Technical Support.

Species Cross-Reactivity

As part of the MILLIPLEX® immunoassay verification process, we test each kit with a selection of samples from multiple species to determine species cross-reactivity. This compiled information is helpful when studying serum/plasma samples from various species where a verified kit may not be available.

Visit **SigmaAldrich.com/milliplex-species** for more information on a subset of our kits.

- Freeze/thaw limits:
  - Multiple freeze/thaw cycles may reduce the stability of some analytes; however, this is analyte dependent. When aliquoting samples to freeze, carefully determine what volume to aliquot. If in doubt, freeze single-use aliquots.
  - Most MILLIPLEX® protocols suggest avoiding more than 2 freeze/thaw cycles.
- Vortexing samples:
  - Vortexing is recommended for 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 metabolic biomarkers require an addition of protease and/or phosphatase inhibitors to samples. Others may require a sample extraction or acidification.
  - Consult the kit protocol.
  - Review the Sample Preparation information in the kit protocol, required serum matrix (if needed), dilutions, and sample type in Appendix 1.

What If I Have Other Sample Types?

If you want to run a MILLIPLEX® kit using samples other than what we have tested (reference the protocol), we have protocols available for the following sample types: tissue lysates, urine, blood spots, gingival fluid, nasal lavage fluid, tears, cerebrospinal fluid (CSF), bronchoalveolar fluid, saliva, cervical/vaginal secretions, and many more. We also have protocols that are modified for use with small volume samples.

Please refer to Appendix 2 or contact Technical Support.

Preparation of Serum/Plasma Samples

› Serum Preparation

- Serum separator tubes (SST) are recommended for higher quality separation.
- Allow the blood to clot for at least 30 minutes before centrifugation for 10 minutes at 1,000 x g to separate cells.
- Remove serum and run your assay immediately or aliquot and store samples at < -20°C.
- 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 likely interfere with assay performance.
  - If you must use serum with gross hemolysis or lipemia, avoid debris, lipids, and cells.
- Hemoglobin (at >10 mg/mL) is known to interfere with antigen/antibody interactions.
- Dilute samples according to the specific MILLIPLEX® kit protocol.

› Plasma Preparation

- Plasma collection using EDTA as an anticoagulant is recommended.
- Care must be taken when using heparin as an anticoagulant since an excess of heparin will provide falsely high values.
  - Use no more than 10 IU heparin per mL of blood collected.
- Other anticoagulants have not been extensively tested and are not recommended.
- Centrifuge for 10 minutes at 1,000 x g within 30 minutes of blood collection.
- Remove plasma and assay immediately or aliquot and store samples at < -20°C.
- Dilute samples according to the specific MILLIPLEX® kit protocol.

› General Dilution Guidelines

- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing, and centrifuge prior to use in the assay to remove particulates.
- For serum or plasma samples that require a dilution beyond “neat,” use the serum matrix provided in the kit as the diluent. Refer to the kit protocol for sample preparation details.
- For serum or plasma samples that require high dilution, use the assay buffer provided in the kit as the diluent. Refer to the kit protocol for sample preparation details.

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.

Cell Lysates

› For more information about the compatibility of buffers with MILLIPLEX® cell signaling kits, contact Technical Support.

› A selection of pre-made lysis buffers and protease/phosphatase inhibitors are available at SigmaAldrich.com.

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

› For more information on preparation of cell lysate samples and protein concentration requirements for MILLIPLEX® assays, refer to the Non-Traditional Format (Cell Signaling) Assays section in this guide.

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. Do not exceed total protein concentrations of 5-6 mg/mL. A drop in signal has been observed for several analytes using PBMC samples at >6 mg/mL total protein (not enough lysis buffer was added to solubilize proteins).
- Because strong detergents are used in the lysis buffer, lysate samples require enough dilution in assay buffer to dilute strong detergents. Avoid lysate total protein concentrations below 2 mg/mL. If lysate protein concentration is below 2 mg/mL, then too much lysis buffer, with strong detergents, will be present in the assay and will result in decreased signal. If protein concentration below 2 mg/mL is unavoidable, we recommended running less sample, thus minimizing the volume of lysis buffer present in the assay.
- The optimal total protein concentration is 2-6 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 which does not contain strong detergents.

Short Protocol for PBMCs:

- If PBMCs are from frozen stock, it is recommended to allow cells to recover 24 hours in complete media. (Less than 24 hours recovery leads to decrease in signal.)
- After 24 hours of recovery, count cells using an appropriate cell counter.
- Pellet the PBMCs at 1,000 x g using a table top centrifuge for 5 minutes at room temperature.
- Remove supernatant and wash cells with PBS.
- Pellet the PBMCs at 1,000 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 MILLIPLEX® cell signaling kits, 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 detergent), 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.

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 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 Detergent	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. Assay compatible lysis detergents and protein concentrations.

Non-Traditional Format Assays

Cell Signaling Assays

What's in a typical MILLIPLEX® Cell Signaling kit?

- Assay Buffer
- 96-Well Plate
- Plate Sealers
- Cell Lysates
- Lysis Buffer
- Streptavidin-Phycoerythrin
- Mixing Bottles
- Amplification Buffer
- Detection Antibodies
- Magnetic Beads

Using Cell Signaling MAPmate™ (Singleplex) Assays

- Plex loading control MAPmate™ assays into exisiting MILLIPLEX® Cell Signaling panels to enhance the panel within guidelines provided in the protocols.
- Our Cell Signaling Buffer & Detection Kit (Cat. No. **48-602MAG**) may be purchased if additional buffers are required.
  - A flat-bottom plate is included for convenience.

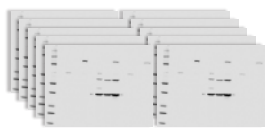
"Plexing" Cell Signaling MAPmate™ Assays

- MAPmate™ assays can be added to existing cell signaling kits to serve as controls.
  - Refer to the guidelines provided in the kit protocol.
- GAPDH and β-Tubulin MAPmate™ assays can be used for normalization with any of the MAPmate™ assays.

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 pipette up and down thoroughly without creating too many bubbles. Request a more detailed protocol from Technical Support.
  - Unbroken cells/parts can be cleared by either filtration or 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 Protease Inhibitor Cocktail I, Cat. No. **20-201**; AEBSF, Cat. No. **A8456**) and/or phosphatase inhibitors to "home-brew" lysis buffers.
- Perform all dilutions with lysis buffer (not assay buffer or phosphate-buffered saline (PBS)).

Our Cell Signaling multiplex kits give you the gift of time and sample.

10 Proteins 12 Samples	MILLIPLEX® Assays	Western Blots
Number of 96-well plates or acrylamide gels		
Number of samples per plate or gel	12 (>40 samples can be measured in duplicate)	12
Total data points per plate or gel	120 (>400 data points can be measured per plate)	12
Total time to result	2.5 hours + overnight incubation (~18 hr)	Days/weeks
Amount of protein required (µg)	1-25 µg of total protein per well (all 10 proteins measured in same sample)	10-50 µg total protein per lane (100-500 µg total protein for 10 gels)

Antibody Detection Assays

What's in a typical MILLIPLEX® Antibody Detection kit?

- Mixing Bottle
- 96-Well Plate
- Plate Sealers
- Assay Buffer
- Phycoerythrin-Ig Conjugate
- Antigen-Immobilized Magnetic Beads
- Wash Buffer

- Antibody detection assays are qualitative (no standard included), with relative levels of detection presented as MFIs.
- Each kit includes four assay control beads, three with a different amount of immunoglobulin, providing varying levels of MFIs. One is a negative assay control bead that produces background-level MFIs.
- The assay control beads are not intended to be used for sample determination, only as assay controls for plate-to-plate consistency.
- It is recommended that experiment-specific positive and negative control samples are run to determine the appropriate assay cut-off MFI.
- Anti-human immunoglobulin conjugated to phycoerythrin is used in all antibody detection assays in place of detection antibody and SAPE steps.
- Each assay detects one immunoglobulin type: IgM, IgG, or IgA.

Typical Antibody Detection Protocol

- Prewet 96-well plate with 200 µL wash buffer and decant
- + 25 µL assay buffer
- + 25 µL sample (diluted serum or plasma); 25 µL assay buffer to background wells
- + 25 µL bead mixture
- Shake for 2 hours at RT or overnight at 4°C
- Wash beads x3 with wash buffer
- + 50 µL PE-Ig Conjugate
- Shake 1.5 hours at RT
- Wash beads x3 with wash buffer
- + 150 µL sheath fluid and read on Luminex® instrument

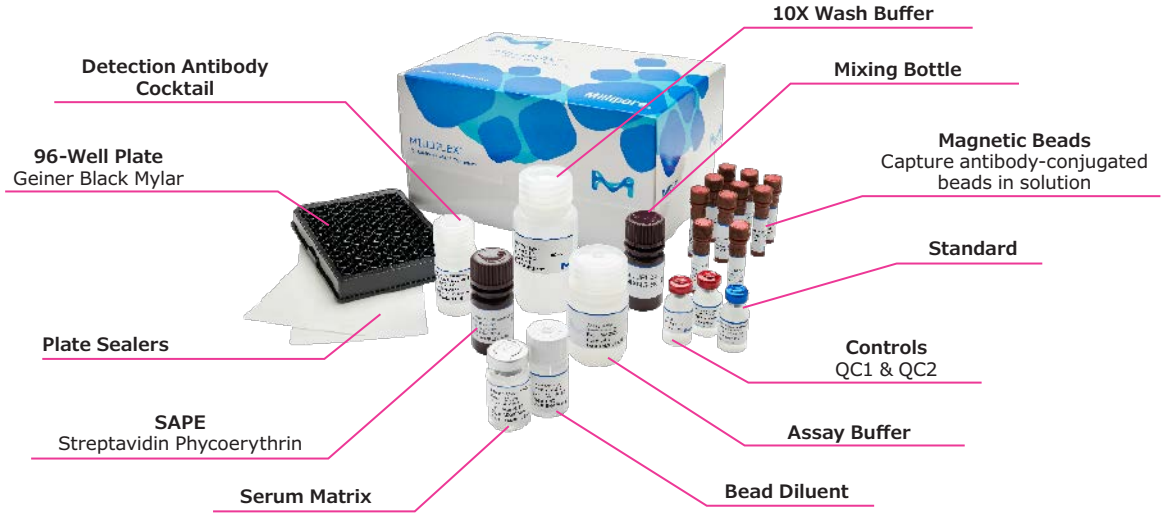
Multiplex Assays for Soluble Proteins vs. Antibodies vs. Cell Signaling Proteins

Soluble Analyte Assay	Antibody Detection Assay	Cell Signaling Analyte Assay
Quantitative	Qualitative (reported as MFIs)	Qualitative (fold change)
Serum, plasma, tissue culture, urine, CSF, etc.	Serum, plasma	Cells (must be lysed)
Analytes analytically tested and verified within panel	Analytes analytically tested and verified within panel	Fixed kits and individual MAPmate™ assays are analytically tested and verified
Kits include standards and QCs	Kits include 4 assay control beads	Kits and MAPmate™ assays often include positive and negative control cell lysates
Most panels are configurable	Panels are configurable	Most kits are fixed panels; create custom kits in three ways: 1. Use the Human RTK (Phosphoprotein) configurable kit with pan Tyr analytes (Cat. No. **HPRTKMAG-01K**) 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 3. Comparison of assay characteristics.

Preparation of Reagents

What's in a typical MILLIPLEX® soluble protein kit?



General Information

- 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.
- 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.
- Deliver 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 being used.

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 QC 1 and QC 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.
  - Refer to lot-specific QC range sheet for appropriate QC concentration ranges. QC range sheets are included in each MILLIPLEX® kit box or can be found on MILLIPLEX® product detail pages.

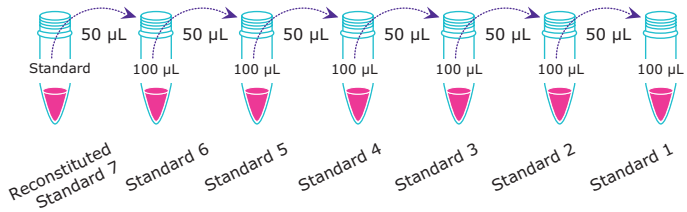
Standards/Calibrators

- 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.
- Hydrate the bottle containing lyophilized serum matrix. Refer to specific kit protocol for detailed instructions on Serum Matrix preparation.
- 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.

Example of Standards Preparation



- 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.

Why Use Serum Matrix?

Blood is a complex matrix, containing proteins that may interfere with the accurate measurement of your specific analytes, so using an optimized serum matrix in the standard curve when measuring analytes in serum/plasma samples more accurately simulates the conditions of the native analytes, significantly improving the accuracy of measurement.

Antibody-Immobilized Beads

- For individual vials of beads, sonicate each antibody-bead vial in an ultrasonic waterbath for 30 seconds; vortex for 1 minute.
  - Sonicator probes should not be used.
- 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 sales representative about receiving premixed beads for select kits. Premixed beads are background tested and assessed for the appropriate bead regions.

Keep Up To Date!

Don’t miss out on updates by signing up for our newsletter:
SigmaAldrich.com/analyteupdate

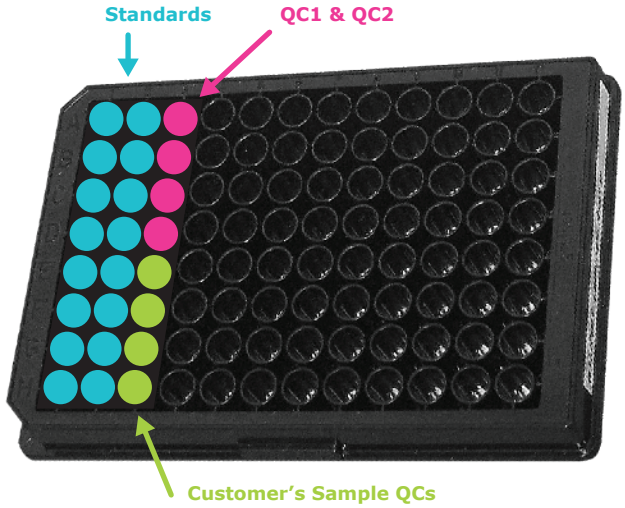
Immunoassay Procedure

General Information

- 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.
- Procedure information is for 96-well plate formats unless otherwise indicated.

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 debris, lipids, and cells that may be present.
- 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 may need to 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.
- 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.



96-Well Plate Map. Sample plate showing placement of Standards, QCs, and samples. In this format, 38 samples can be analyzed in duplicate. 384-well plate set-up is similar, but with 182 samples in duplicate.

- In a typical MILLIPLEX® soluble protein assay, when running samples in duplicate, a maximum of 38 samples can be run per 96-well kit. If using a MILLIPLEX® 384-well kit, a maximum of 182 samples may be run in duplicate. Cell Signaling and Antibody Detection assays allow for more samples to be tested per plate.
- To pre-wet the plate, use 200 µL wash buffer or assay buffer. Refer to kit protocol for appropriate buffer to use.
- 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 to remove particulates.

- Be precise when adding samples, standards, and QCs to the plate.
  - Pipette onto the sides of the wells.
  - Be sure the appropriate amount of fluid is expelled from the pipette tips. Use fresh tips for each addition.
  - For more precise fluid additions, use reverse pipetting technique.
- 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 designed to hold a 96-well plate firmly, and it should reach at least 500 rpm. Do not use 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.

How to Correct or Prevent Low Bead Counts

- Be sure to specify MagPlex® microspheres 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 debris, lipids, and cells layers that may be present.
- For samples known to be challenging (e.g., synovial fluid, saliva), you 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 reagent, 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.

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 visit **SigmaAldrich.com/customassay**

For protocols that do not include a wash step between detection and SAPE incubations:

- 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 using the following options:
  - Add appropriate volume as indicated in the protocol of detection antibody and continue to follow the protocol.
  - If additional detection antibody cocktail is not available, replace with assay buffer, add SAPE and continue to follow the protocol. Alternatively, you may add SAPE and continue to follow the protocol, keeping in mind that with both options the signal may be lower.
- Use the sheath fluid, drive fluid (if using the MAGPIX® instrument), 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 leftover reagents at appropriate conditions quickly after the first use (e.g., stock standard at -20°C or lower).
  - 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. See specific kit protocol for directions on storage and use of leftover components.
  - 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**).

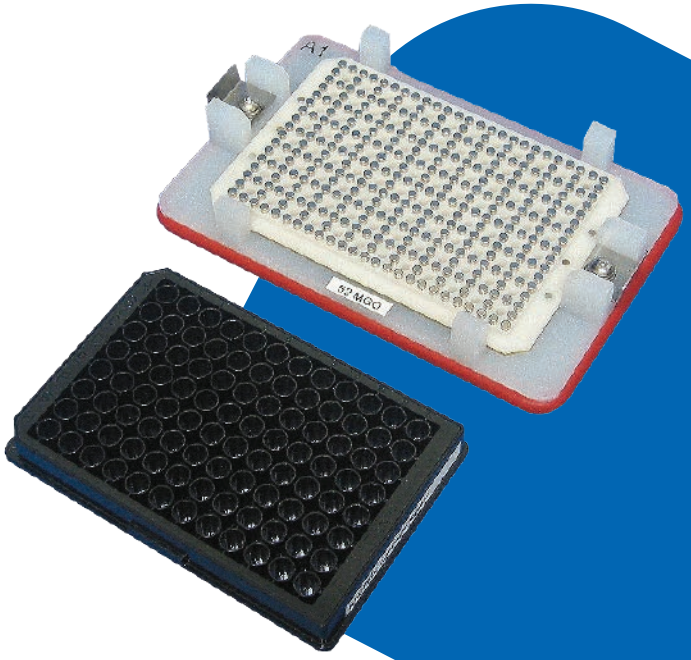
What If I Have Sticky Samples?

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

- Recommended Oribital Titer Plate Shaker (Microplate Shaker Cat. No. **CLS67804** 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 is 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.



Handheld Magnetic Separation Block

Equipment Settings and Maintenance

xMAP® INTELLIFLEX® System

Probe Height Adjustment

- Navigate to “Probe Height” from the Maintenance Tab
- Select New or Saved Plates from menu
- If new, select 96-well or 384-well format
- Add off-plate reagent block and plate to the plate holder
- Select “Auto-Calibrate”

xMAP® INTELLIFLEX® Maintenance Schedule	
Daily	Laser warm-up, daily start-up, verify system, check fluid levels, daily shutdown
Weekly	Visual inspection, calibrate system, clean sample probe, run clog removal, run weekly maintenance
Monthly	Clean exterior surfaces
Semi-Annual	Replace HEPA filter, replace syringes, replace sheath-in tubing

FLEXMAP 3D® System

Daily Maintenance

› The software provides three options for system initialization:

- Warmup, fluidics
- Warmup, fluidics, verification
- Warmup, fluidics, verification, and calibration

› Select the option you want. Warmup, fluidics, and verification should be part of the daily maintenance of the instrument; calibration can be limited to weekly maintenance unless otherwise specified by assay instructions.

Probe Maintenance (once per month)

1. Turn off instrument and open access door
2. Unscrew cap above sample probe, remove probe by pushing it up through the controller arm
3. Submerge probe in sonication bath for 30-60 seconds, pat dry with KimWipe
4. Drop probe back through the controller arm, screw cap on until finger tight
5. Reset Probe Height after replacing the probe

Warm-up takes 30 minutes

- Note: The lasers will shut down after 4 hours of inactivity

Daily Startup from the Maintenance Tab → Cmds & Routines → Daily Instrument Startup

- Fill reservoirs with appropriate reagents (deionized water, 70% ethanol)
- Routine takes 10 minutes

Run Calibration/Verification if needed; This takes approximately 20 minutes

› Probe Height Adjustment

- Use the Probe Height Adjustment Tool; If not available, use 96-well plate
- Select “Probe & Heater” from the Maintenance Tab
- 96-well plate: Use well F12 on the Probe Height Adjustment Tool or 1 disc in a 96-well flat bottom plate
- 384-well plate: Use well B7 or C7 on the Probe Height Adjustment Tool

Shutdown System from Maintenance Tab → Auto Maint → Shutdown

- Fill reservoir block with appropriate reagents and run Shutdown
- Empty reservoir block and place back in instrument
- Exit software and shutdown
- Routine takes 5 minutes

Luminex® 200™ System

Probe Height Adjustment

- Select “Probe & Heater”
- Drop 3 discs in well D6 of a clean/dry MILLIPLEX® plate
- Place the plate on the tray; Retract tray
- Loosen thumb screw on the front of the probe arm
- Click Lower Probe Arm, CONFIRM probe is resting on discs by gently pushing the thumb screw down
- Manually move probe up (~1 mm), re-tighten thumb screw
- Eject the plate and return the discs to their storage tube

The Luminex® 200™ system’s xPONENT® acquisition software has two functions: one for magnetic (MagPlex®) beads and one for nonmagnetic (MicroPlex®) beads. 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. If the system is powered on, but idle for more than four hours, click the Maintenance tab. Click Warmup. Wait 30 minutes for the analyzer and the optics system to warm up.

Daily Startup from the Maintenance Tab → Cmds & Routines → Daily Instrument Startup

- Fill reservoirs with appropriate reagents (deionized water, 70% ethanol)
- Routine takes 10 minutes

Run **Calibration/Verification** if needed; This takes approximately 20 minutes

- When running assays, Calibration should be performed once per week, and Verification within one day of assay set-up

Shutdown System from Maintenance Tab → Auto Maint → Shutdown

- Fill reservoir block with appropriate reagents and run Shutdown
- Empty reservoir block and place back in instrument
- Exit software and shutdown
- Routine takes 5 minutes

Sanitization Procedure After Running Samples

1. Sanitize with a 10-20% household bleach solution
2. Run two Wash cycles with distilled water
3. Soak with distilled water; Wait until the soak completes
4. If desired, turn off the instrument

MAGPIX® System

Choose **Enhanced Startup** setting instead of the common startup. This will ensure proper calibration and cleaning prior to running the assay.

Adjust the probe height according to the protocols recommended by Luminex® experts to the kit solid plate using 2 alignment discs.

10-12 plates can be run with one bottle of drive fluid.

Stringent Soak Routine

- Used to clear the chamber of beads and debris; After a calibration and/or verification failure, generate the Calibration & Verification report
- Look for failures in Percent Total Misclassification (> 2.0% is a failure) and Percent Classification Efficiency (any region failure); These failures indicate a dirty chamber
- The Stringent Soak Routine can be performed for 20-30 minutes or overnight
  - Best to use with the Enhanced Startup routine
  - Make sure the probe is clean and the correct height has been set
  - Make sure the side door is open as you will need to watch the syringe pump for proper shutdown

Instructions

1. Go to Maintenance > Cmds & Routines > Clean command; Add 0.1 N NaOH to the designated reservoir well
2. Click Run and watch the syringe pump; When the syringe pump takes a stroke downwards, the reagent is being acquired and sent to the sample line
3. When the syringe pump takes a stroke upwards, the reagent is pushed onto the chamber; Turn the instrument off from the back power switch to allow the reagent to soak the chamber
4. With the instrument turned off, close out of the xPONENT® software, and shut the PC down; Do not use Restart
5. When soaking is complete, turn the instrument on first, then the PC, and allow connection to the software*
6. Run the Daily Fluidics Prep by going to Maintenance > Cmds & Routines > Daily Fluidics Prep routine
7. Run System Initialization for calibration and verification

*Note: If you experience a step-loss error after soaking, cycle the power the system by shutting down the instrument, software, and PC. Let set for 30 seconds and restart the system by turning on the instrument first, then the PC and software.

General Instrument Tips

- For full instrument details, reference the Luminex® instrument manuals
- Be sure the needle probe is clean; This may be achieved by sonication and/or alcohol flushes
- Working with serum can be “stickier” than other biological fluids and can affect the performance of the instrument unless it is properly cleaned
- 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
- For information on xPONENT® software and to request software templates 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
- 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® Kits on Other Luminex® Instruments

- Luminex® 200™, FLEXMAP 3D®, and MAGPIX® systems with xPONENT® software and xMAP® INTELLIFLEX® systems with the INTELLIFLEX® Software are required to run a MILLIPLEX® assay
- Magnetic bead assays cannot be run on any instruments using Luminex® IS 2.3 or Luminex® 1.7 software
- Since all Luminex® machines are built by Luminex® Corporation, MILLIPLEX® 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 other software (e.g. Bio-Plex® Manager™, MasterPlex®, STarStation, LiquiChip, LABScan™ 100 software), follow instrument instructions for gate settings and additional specifications from the software vendors for reading assays using Luminex® magnetic beads
- To read a MILLIPLEX® kit on a Bio-Plex® instrument, select 5K-25K for magnetic beads, depending on the version of Bio-Plex® Manager™ software

Belysa® Immunoassay Curve Fitting Software

(Cat. No. **40-122**)

We offer a powerful combination for analyzing immunoassay data, including multiplex experimental analysis with Belysa® Immunoassay Curve Fitting software coupled with data acquisition using the Luminex® xPONENT® software. Belysa® 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® xMAP® instruments.

Step 1:

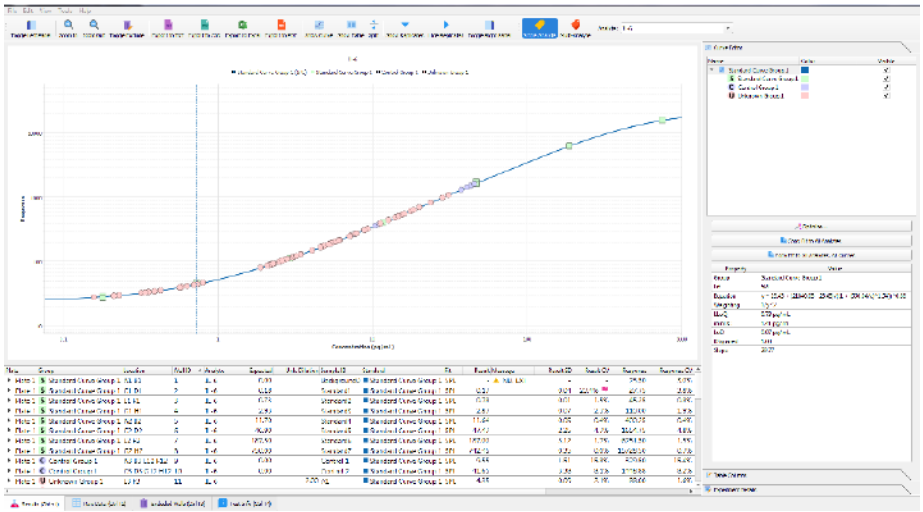
Drag and drop your .csv file obtained from the xPONENT® acquisition software

- Belysa® software will accept acquisition files from all RUO Luminex® instruments. Files can be dragged into the open browser to be opened in the Belysa® platform.

Step 2:

Examine and automatically optimize the curve fit for your data

- Belysa® tools will parse out the raw data and present it to the user, annotating the curve with Standard, Control, and Sample points. Through a simple wizard function, the software will provide the best fit for the acquired data. A manual curve fit is also an available option.



Step 3:

Scan data to ensure replicate hygiene

- Belysa® software notifies the user in two ways:
  - Belysa® tools alert to data that is at the low end of the assay (e.g. below the limit of quantitation or non-detectable).
  - Belysa® tools also alert to technical errors within an assay, whether at the raw data level (such as bead count), or in calculated parameters (for instance recovery or %CV).

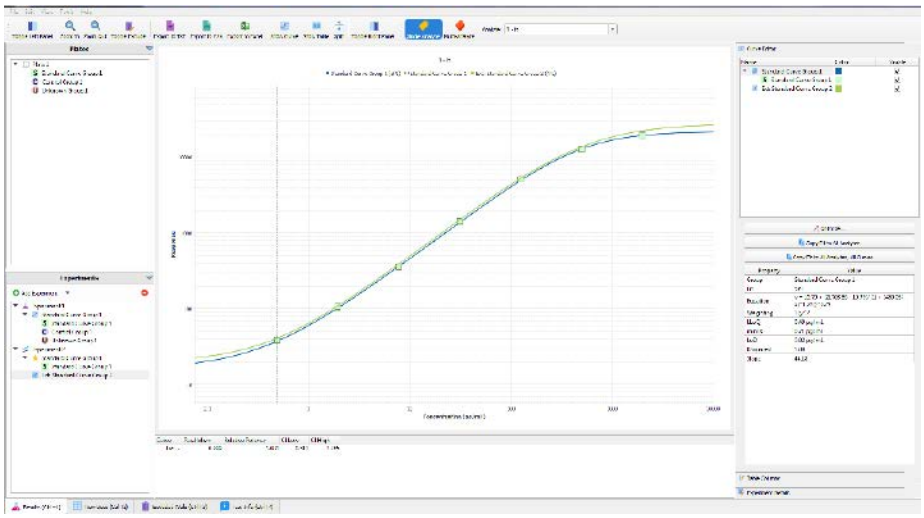
    These are user-defined and present as a pink flags in the data table.

Message	Result SD	Result CV	Response	Response CV
EXT, ND	-	-	15.75	2.2%
BLOQ	0.03	3.6%	35.50	2.0%
BLOQ	0.08	10.4%	32.75	5.4%
BLOQ	0.13	16.4%	33.00	8.6%
BLOQ	0.03	6.0%	27.50	2.6%
BLOQ	0.03	3.8%	34.50	2.0%
BLOQ	0.03	4.2%	32.50	2.2%
BLOQ	0.03	3.4%	36.50	1.9%
BLOQ	0.18	25.9%	30.75	12.6%
BLOQ	0.00	0.0%	23.00	0.0%
BLOQ	0.05	13.3%	23.75	4.5%
BLOQ	0.06	27.1%	21.00	6.7%
BLOQ	0.03	10.5%	22.50	3.1%
BLOQ	0.10	10.7%	35.50	6.0%

Step 4:

Compare standard curves against a previous experiment to confirm mathematical similarity

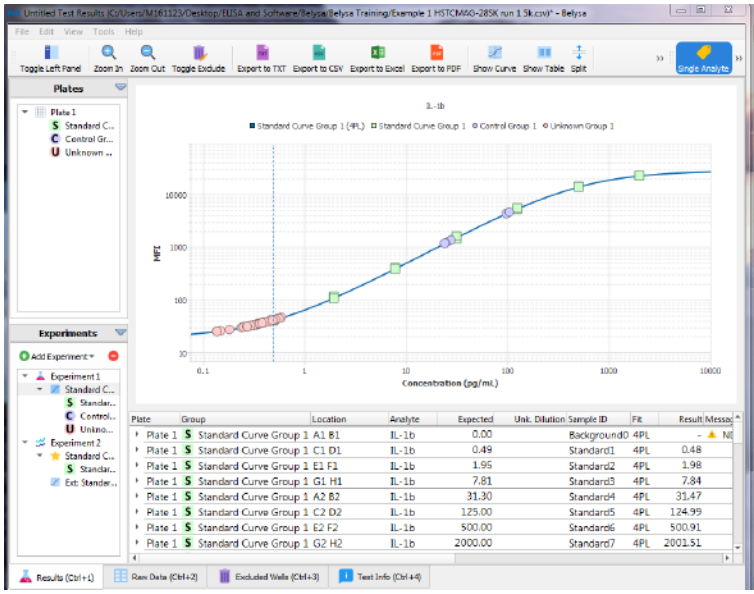
- Comparing standard curves' slopes between batches or runs ensures that different kits perform equally, either within a run or through the course of a longitudinal study. The first curve is established as a reference curve to which subsequent assay curves are compared. If the calculated slope ratio is equal to 1, then the curves are statistically similar.



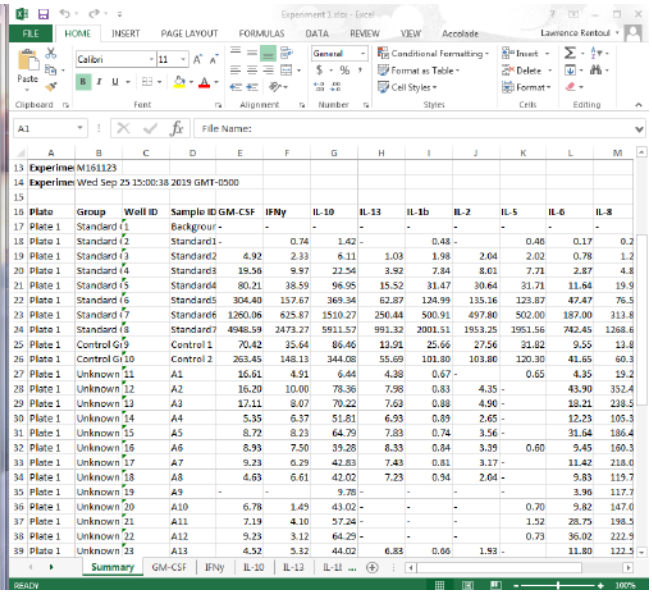
Step 5:

Export your data

- Each experimental mode in the software offers a slightly different report structure, single analyte and multi analyte. These are available in .csv, .txt, Excel, and PDF for future analysis or record keeping.



PDF export



Excel report

Learn more about Belysa® Software at **SigmaAldrich.com/belysa**

Data Analysis

Bead Counts

- We recommend counting 50 beads.
  - According to Luminex® experts, a minimum of 35 beads per region need to be counted.
  - Fewer than 35 beads could cause a shift in the MFI value of the bead population.
  - MFI will not change for bead counts ≥35.
  - Don't worry if there is a 35 bead count on one bead region and 400 for others. MFIs will not be affected.

Percent Coefficient of Variation (%CV)

- High %CVs for standards or samples can be due to low bead count.
- Our target intra-assay %CV is <15% and our target inter-assay %CV is <20%.

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 standard), 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 approaches, but never equals 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 the 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® standard points maintain consistent values from 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.

Appendix 1: Sample Preparation

MILLIPLEX® Kits Requiring Special Sample Preparation

Kit Name	Cat. No.	Sample Type Requiring Preparation	Sample Trt Inhibitors	Inhibitor Source
Human Circadian Stress Panel	**HNCSMAG-35K**	SER / PLA	Acetonitrile Extraction	NONE
Human IGF Binding Protein Panel	**HIGFBMAG-53K**	SER / PLA	Protease Inhibitor Cocktail	See Note 4
Human IGF Panel	**HIGFMAG-52K**	SER / PLA	Extraction	NONE
Human Metabolic Hormone V3 Panel	**HMH3-34K**	SER / PLA	DPP-IV, Aprotinin, AEBSF, Protease Inhibitor Cocktail	See Notes 1, 2, 3, 4, 5
Human Neuropeptide Panel	**HNPMAG-35K**	SER / PLA	Acetonitrile Extraction	NONE
Mouse Metabolic Hormone Expanded Panel	**MMHE-44K**	SER / PLA	DPP-IV, Aprotinin, AEBSF, Protease Inhibitor Cocktail	See Notes 1, 2, 3, 4, 5
Rat/Mouse Neuropeptide Panel	**RMNPMAG-83K**	SER / PLA	Extraction	NONE
Rat Metabolic Hormone Panel	**RMHMAG-84K**	SER / PLA	DPP-IV, Aprotinin, AEBSF, Protease Inhibitor Cocktail	See Notes 1, 2, 3, 4, 5
Non-Human Primate Metabolic Panel	**NHPMHMAG-45K**	SER / PLA	DPP-IV, Aprotinin, AEBSF, Protease Inhibitor Cocktail	See Notes 1, 2, 3, 4
Canine Gut Hormone Panel	**CGTMAG-98K**	SER / PLA	DPP-IV, Aprotinin, AEBSF, Protease Inhibitor Cocktail	See Notes 1, 2, 3, 4
Multi-Species Hormone Panel	**MSHMAG-21K**	SER / PLA	Acetonitrile Extraction	NONE
Multi-Species TGFβ1 Singleplex Panel	**TGFBMAG-64K-01**	SER / PLA / CCS	Acidification	NONE
Multi-Species TGFβ1, -2, -3 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 (Cat. No. **P2714**).
5. Active and Total cannot be run together in the same assay.

Appendix 2: 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 surfactant 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 -80°C.	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 reagent, 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/ Chemokines	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 surfactant, 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 reagent, 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
Breast Milk	Human	Milk samples were thawed, centrifuged at 800 g for 15 min at room temperature, fat was removed, and supernatant transferred to a new tube. Centrifugation was repeated 2x to ensure removal of all cells and fat. Skimmed acellular milk was aliquoted and frozen at -80°C until testing.	Fox et al., iScience 23, 101735 November 20, 2020 ª 2020. doi:10.1016/j. isci.2020.101735
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	Human	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

Appendix 2: Other Sample Types

Sample Type	Species/Kit Run	Procedure	Reference (if available)
Dried Blood Spot Samples	Human	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
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 Cytokines	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
Exosomes/ Soluble Extracellular Vesicles (sEV)	Human/Human Cytokines	For RNAseq and Luminex® analysis, sEVs were isolated using the patented ExoRelease™ technology (Clara Biotech; Lawrence, KS). Briefly, 1.0 mL of plasma was incubated with magnetic immunoaffinity beads, which bind to the sEV markers CD63, CD9, and CD81 according to the manufacturer's protocol. sEV were released by ultraviolet light 5 exposure and stored in 200 µL of PBS at -80°C.	Molecular Carcinogenesis. 2023;62:1428-1443
Gingival Crevicular Fluid (GCF)	Human/Human Cytokines	GCF samples were collected from each site with paper filter strips (PerioPaper) gently inserted into the sites 1 to 2 mm for ~10 s. Volume of GCF was calculated with a micro-moisture meter (Periotron 8000) and through calibration curves. Samples were frozen at −80 °C until analyses of soluble biomarkers were performed.	JDR Clin Trans Res. 2017 Jul;2(3):258-268. doi: 10.1177/2380084417701898. Epub 2017 Apr 14. PMID: 28879248; PMCID: PMC5576058.
Fecal Samples	Mouse/Mouse Immunoglobulin Isotyping	Fecal contents from small and large intestines were collected when mice were euthanized and were frozen at −80 °C. Samples were thawed out, weighed, and resuspended in PBS 5% gelatin supplemented with complete protease inhibitor cocktail (Roche), with the help of vigorous vortexing. Samples were prepared as concentrated as possible to facilitate detection of low abounding cytokines. Insoluble fraction was separated by centrifugation at 10,000 ×g for 10 min at 4 °C, in an Eppendorf® 5417R centrifuge. Supernatants were either test immediately or frozen at −80 °C.	Toxicology and Applied Pharmacology, Volume 333, 2017, Pages 84-91, ISSN 0041-008X, doi: 10.1016/j.taap.2017.0
Follicular Fluid	Human/Human Soluble Cytokine Receptors	During oocyte retrieval, the monofollicular fluid from the leading follicle was aspirated and collected. No flushing procedures were performed during oocyte retrieval. The follicular fluid samples were centrifuged for 10 min at 800 x g to separate red blood cells, leukocytes, granulosa cells, and debris. The supernatants were frozen without preservatives and stored at -80°C until assayed.	Reproductive BioMedicine Online, Volume 26, Issue 1, 2013, Pages 62-67, ISSN 1472-6483, doi: 10.1016/j.rbmo.2012.10.001.

Sample Type	Species/Kit Run	Procedure	Reference (if available)
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 surfactant, 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.	
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
Peritoneal Fluid	Mouse/Mouse Cytokines	After animals were euthanized, 1 mL of sterile PBS was injected into the peritoneal cavity, the abdominal area was gently massaged, and the fluid collected. The collected fluid was centrifuged at 1390 RPM for 5 min at 4 °C and the resulting supernatant was then stored at −80 °C.	Cell Death and Disease (2016) 7, e2059; doi: 10.1038/cddis.2015.361
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.	
Seminal Fluid	Human/Human Cytokines	An aliquot of each semen sample from normal fertile donors and infertile men were collected by centrifugation at 400 x g for 5 minutes to obtain spermatozoa-free seminal fluid as previous reported and then frozen at -80°C until analysis was made. Absence of spermatozoa was verified by H&E staining of seminal fluid.	PLoS ONE 8(7): e70470. doi:10.1371/journal.pone.0070470
Seminal Fluid	Non-Human Primate/Non-Human Primate Cytokines	Seminal plasma was isolated from total semen immediately after collection, by centrifugation for 15 minutes at 7756 x g. The seminal cell pellet was resuspended in 14 mL of complete medium, consisting of RPMI-1640 medium enriched in glutamine (Invitrogen, Carlsbad, USA) supplemented with a mixture of penicillin, streptomycin, and neomycin (Invitrogen) and 10% FCS (Lonza, Allendale, USA), and kept at room temperature for no more than one hour. Cells were then centrifuged for 10 min at 1,5006 x g, filtered through a sieve with 70 mM pores, and washed with 5 mL of PBS supplemented with 10% FCS.	PLoS Pathog 9(12): e1003810. doi:10.1371/journal.ppat.1003810
Sputum	Human/Human Cytokines	Fresh, spontaneously expectorated sputum samples were frozen and stored at −80 °C until preparation. Samples were ultra-centrifuged for 4 h at 38,000 RPM. All samples were stored at −80 °C prior to analysis.	Journal of Cystic Fibrosis 9 (2010) 51–58 doi:10.1016/j.jcf.2009.10.005
Synovial Fluid (SF)	Human and Mouse/Human and Mouse Cytokines	Add an additional standard point compared to standard protocol in a buffer matrix. Use 100 µl buffer + 25 µL std/sample. Agitate for 10 mins at room temperature. Add 25 µL beads and incubate with agitation overnight at 4°C. On the next day, before reading, resuspend the beads in 200 µL of 1X wash buffer prior to loading onto the instrument. Volume read is 100 µL on the instrument.	

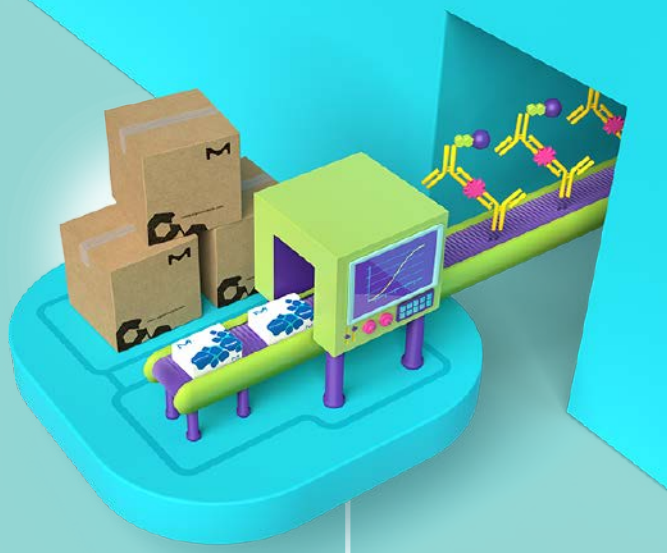
Appendix 2: Other Sample Types

Sample Type	Species/Kit Run	Procedure	Reference (if available)
Synovial Fluid (SF)	Human/Human Adipokine/ Adipocyte	Fluid samples were kept out of the light and stored in sterile containers at -80 °C until analyzed to minimize inter-assay variation. Samples were then centrifuged at 3,000 × g for 30 min. Protease inhibitors were added immediately, and all samples were analyzed not more than 3 weeks after collection.	Obes Facts 2013;6:536-541. doi: 10.1159/000357230
Synovial Fluid (SF)	Rat/Rat Cytokines	SF was collected by impregnation of standardized small pieces (4 mm^2) of filter paper (Schleicher & Schuell GmbH, Dassel, Germany). To prevent any proteolytic cleavage of cytokines in arthritic fluids, these paper pieces were left for 12 hours at 4°C in 150 µL of PBS containing a cocktail of protease inhibitors (cOmplete™ Mini reagent; Roche, Basel, Switzerland). After initial and final agitations for 30 seconds on a mechanic stirrer, the 'joint-derived' eluates were frozen at -80°C until processing.	Arthritis Res Ther 14, R60 (2012). doi: 10.1186/ar3774
Synovial Fluid (SF)	Equine/Equine Cytokines	Synovial fluid (2 mL) was aseptically collected and aliquoted (EDTA and Protein LoBind microfuge tubes, Eppendorf® Labware, Westbury, CT). Anticoagulant-free synovial fluid was immediately centrifuged at 12,000 × g for 10 min at 4°C and the supernatant stored at −20°C. Thawed samples (200 µL) were hyaluronidase-digested (10 µL of 100 IU hyaluronidase/ mL acetate buffer; Worthington Biochemical Corporation, Lakewood, NJ) for 30 min at 37°C, centrifuged at 12,000 × g for 10 min at 4°C, and the supernatant recovered.	Front. Vet. Sci., 7:568756. 26 November 2020. doi: 10.3389/ fvets.2020.568756
Skin Extracts	Human/Human Cytokines	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® Labware, 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.	

Notes:

Beyond Biomarkers

We are committed to delivering the best possible immunoassays for your research needs. Whether you are using our industry-leading MILLIPLEX® multiplex panels to broadly survey multiple analytes, performing femtogram/mL biomarker analysis using the SMC® ultrasensitive immunoassay platform, or examining single proteins with our tried-and-true ELISAs and RIAs, you can confidently expect reliable measurements, a simplified user experience, and knowledgeable scientific partners to help drive your biomarker project from hypothesis to publication.



Designed RelyAbility

Our Conferma® ELISAs are engineered for endogenous detection and lot-to-lot consistency of routine-use biomarkers to keep your data reproducible, run after run.

Genuine PlexAbility

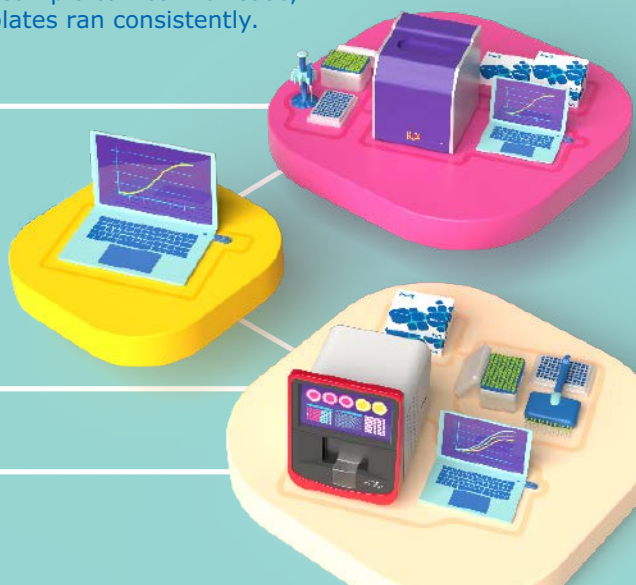
Our industry-leading MILLIPLEX® multiplex assays for the Luminex® platform give you the FlexAbility to select your combination of biomarkers to simultaneously analyze with ease.

Next-Level DetectAbility

Our ultrasensitive SMC® kits for the SMCxPRO® platform take your research to the next level by detecting biomarkers down to fg/mL levels.

Complete CompareAbility

Our Belysa® Immunoassay Curve Fitting Software helps you quickly compare your standard and sample curves with ease, confirming that your methods and plates ran consistently.



Expert CustomAbility

Our Custom Assay Development & Innovation (CADI) team of experienced scientists partner with you to develop custom immunoassays for your biomarker(s) of interest, accelerating your projects from discovery to clinical trials.

keep your focus on the next stage

Rigorous Assay Development

Specificity

- Antibody specificity is tested to ensure negligible cross-reactivity in the tested sample types
- Assay specificity is ensured by confirming consistent performance in singleplex and multiplex formats

Method Comparisons

- We test our kits against in-house kits and kits from other vendors when available, ensuring the highest performing kits

Selectivity

- Assay buffers and bead diluents are optimized to enhance antibody binding to only those analytes of interest in samples

Detection and Sensitivity

- The assay range and minimum detectible concentrations (minDCs or LLOQ) for each analyte is published in our protocols, and is based on actual samples

Performance in a Sample Matrix

- Assays are verified for the sample type noted in the protocol; for serum/plasma sample kits, we provide an optimized serum matrix to mimic the environment of the sample, normalizing assay performance

Reproducible Results You Can Trust

Stability

- All kits are rigorously tested for shipping stability to determine impact on each analyte
- Sample stability is examined at a range of temperatures, and by freeze/thaw

Precision and Accuracy

- Precision of control standards are typically within 15% CV for intra-assay and 20% CV for inter-assay
- Spike recovery in sample is performed for each analyte at three concentrations; accuracy is normally within ± 30%

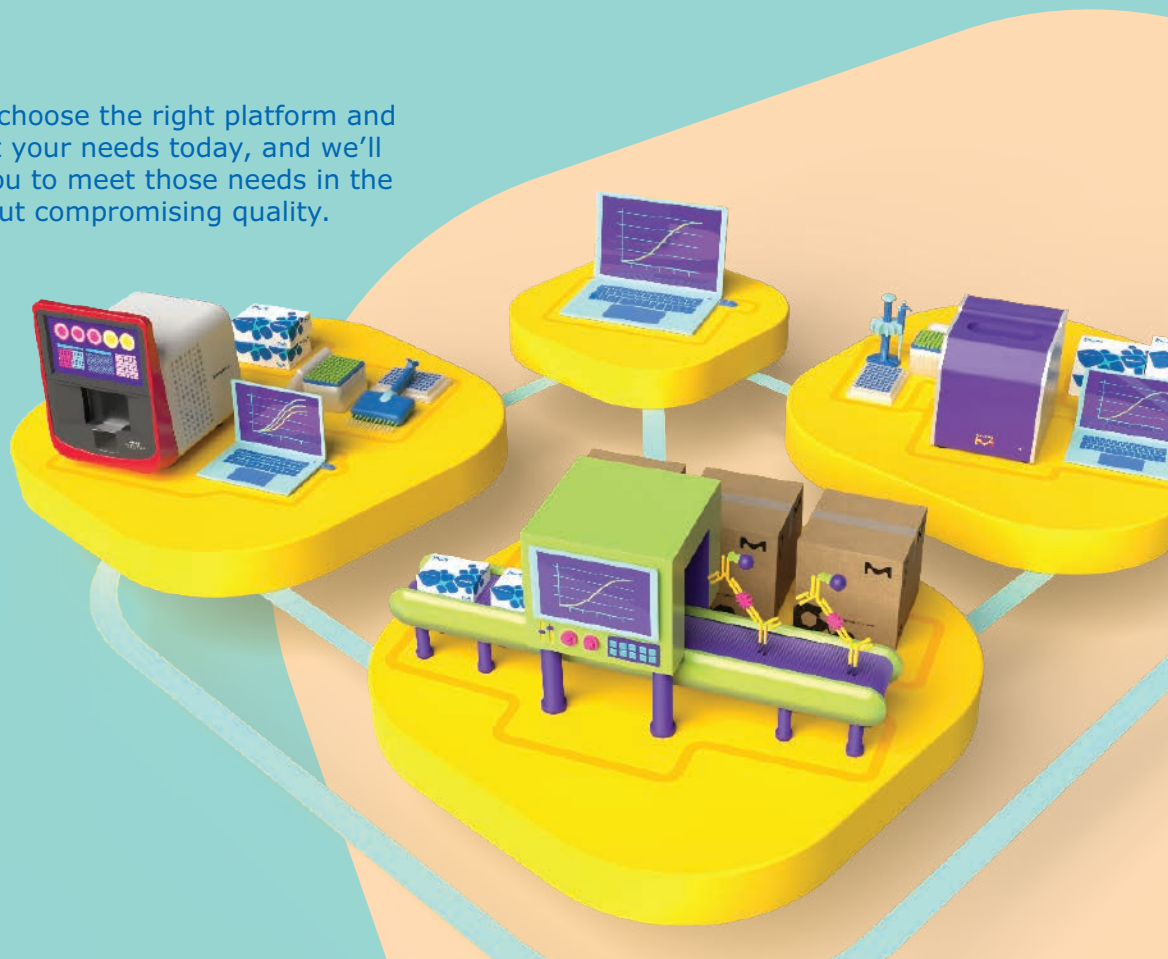
Linearity of Dilution

- Diluted sample results must be directly proportional to the concentration of analyte in the sample, typically within ± 30%

Lot-to-Lot Consistency

- A “gold standard” calibrator is defined during assay development and used as a reference for all future production lots

We’ll help you choose the right platform and assays to meet your needs today, and we’ll partner with you to meet those needs in the future — without compromising quality.



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