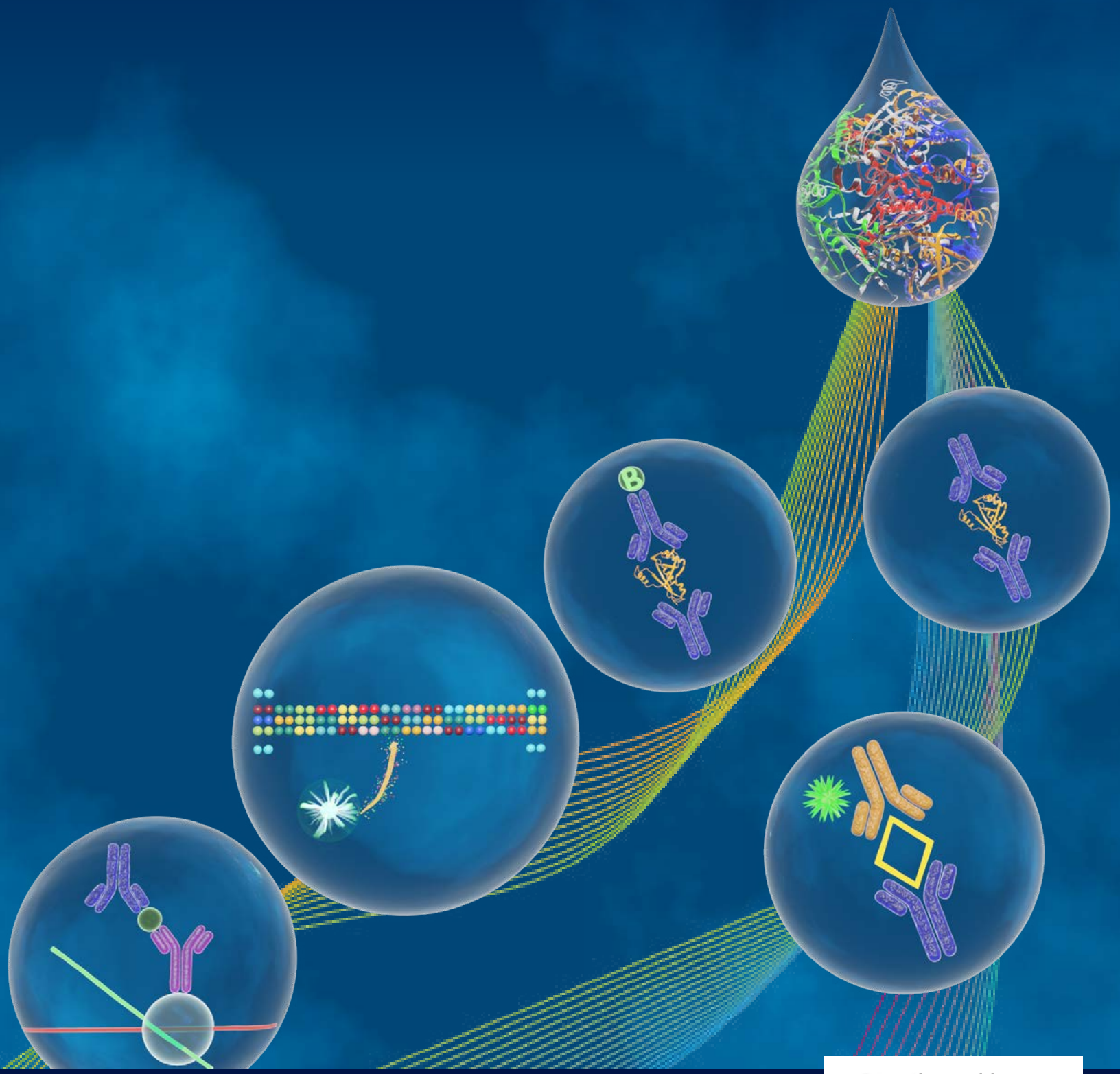
LUMINEX ASSAY USERS GUIDE

A CLEAR AND EASY GUIDE TO LUMINEX MULTIPLEX IMMUNOASSAYS



bio-techne®

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WHAT IS MULTIPLEXING?

To put it simply, multiplexing allows multiple biological target analytes to be simultaneously examined and quantified in a single sample. It's a technique widely used by scientific researchers of all disciplines and provides many benefits.

WHAT ARE THE BENEFITS OF MULTIPLEXING?

The simultaneous analysis of multiple factors provides numerous benefits to you and your research.

• MAXIMIZES LIMITED SAMPLE: data collection from just 25µl or less of undiluted sample.

• MINIMIZES EXPERIMENTAL VARIABILITY: samples are processed only once, so multiple data points are derived from a single manipulation.

• OPTIMIZES PRODUCTIVITY: minimal sample preparation and processing; whilst generating high volumes of data.

• ECONOMICAL: examining multiple analytes in a single sample saves time and resources.

WHAT IS A LUMINEX® MULTIPLEX IMMUNOASSAY?

A Luminex assay is a magnetic microparticle-based immunoassay which utilizes the same sandwich principles as traditional ELISAs. Luminex® multiplex immunoassays allow researchers to quantify up to 100 biomarkers with less sample volume than a traditional plate-based ELISA. Color-coded microspheres, or beads, are internally dyed with different proportions of red and infrared fluorophores that correspond to a distinct spectral signature, or bead region. The quantification of multiple cytokines and other biomarkers in a sample provides critical information about biological processes and diseases.

Antibodies specific to a desired analyte are coupled to a unique bead region and are incubated with sample. After washing away unbound materials, samples are incubated with a mixture of biotinylated detection antibodies and a streptavidin-phycoerythrin (PE) reporter. Using a Luminex instrument, beads are excited by one laser or LED, depending on the instrument, to determine the bead region and corresponding assigned analyte. Another laser or LED determines the magnitude of the PE-derived signal, which is proportional to the amount of analyte bound. Multiple readings are taken at each bead region, ensuring robust detection.

WHY USE A R&D SYSTEMS® LUMINEX® MULTIPLEX IMMUNOASSAY?

Your results matter, so what's inside your Luminex® multiplex immunoassay should too! Our Luminex® assays often utilize the same high-quality antibodies and proteins as our other Bio-Techne immunoassay platforms and are tested for suitability for multiplex immunoassay applications.

Save time and money: Quantify up to 50 analytes with 25-50 ul of sample.

The Highest Flexibility: Customize the R&D Systems® Luminex® Assay to suit your needs.

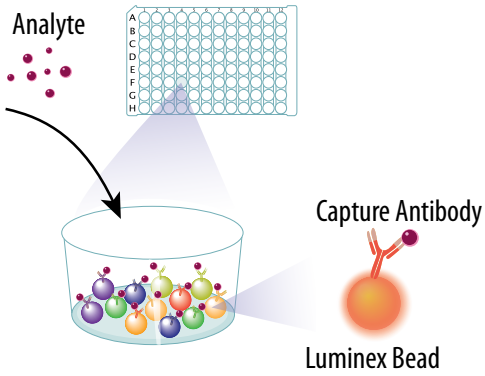
Unparalled Accuracy, Precision and Sensitivity: High Performance assays are validated similar to the gold standard Quantikine® ELISAs.

Consistency: All assays have the most rigorous quality control.

Extensive Analyte portfolio: many unique targets

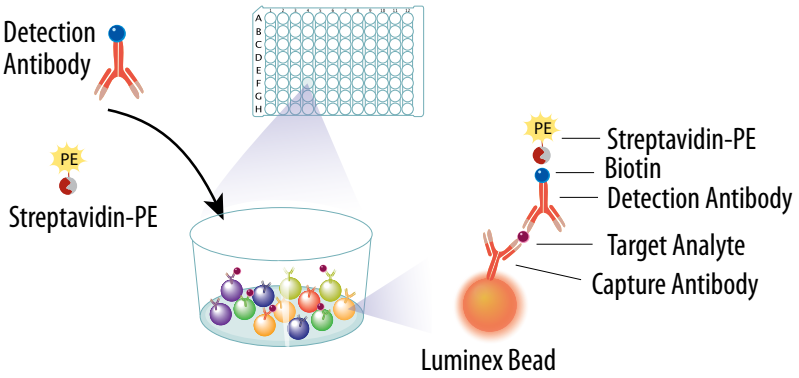
LUMINEX® ASSAY PRINCIPLE

STEP 1



The sample is added to a mixture of color-coded beads, pre-coated with analyte-specific capture antibodies. The antibodies bind to the analytes of interest.

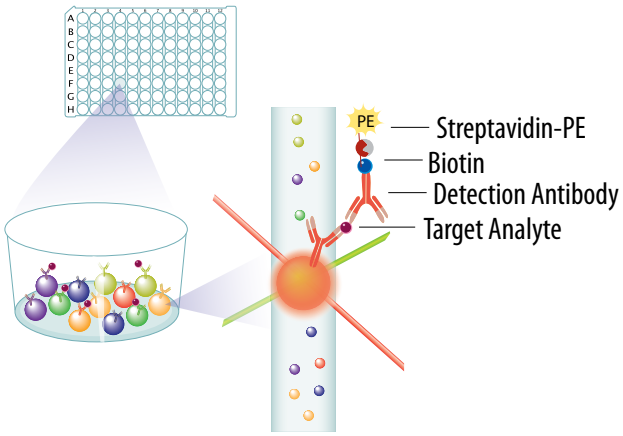
STEP 2



Biotinylated detection antibodies specific to the analytes of interest are added and form an antibody-antigen sandwich. Phycoerythrin (PE)-conjugated streptavidin is added. It binds to the biotinylated detection antibodies.

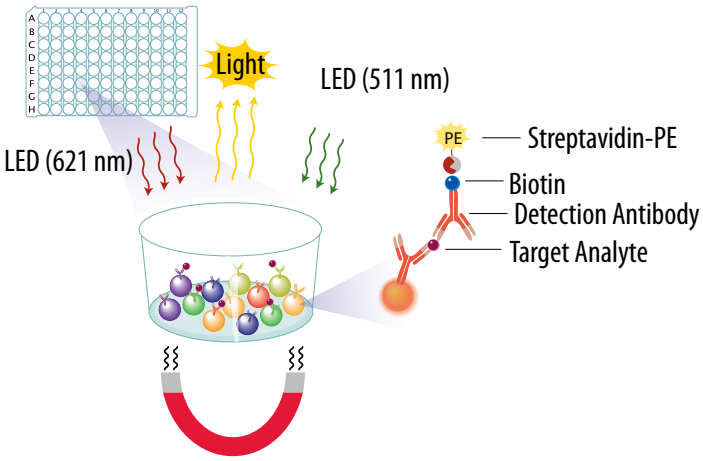
STEP 3

Polystyrene beads:



Polystyrene beads are read on a dual-laser flow-based detection instrument, such as the Luminex 100™, Luminex 200™ or Bio-Rad® Bio-Plex® analyzer. One laser classifies the bead and determines the analyte that is being detected. The second laser determines the magnitude of the PE-derived signal, which is in direct proportion to the amount of analyte bound.

Magnetic beads:



In addition to the Luminex 100, Luminex 200 or Bio-Rad Bio-Plex dual-laser, flow-based analyzers, magnetic beads can be read using the Luminex MAGPIX® Analyzer. A magnet in the MAGPIX analyzer captures and holds the magnetic beads in a monolayer, while two spectrally distinct light-emitting diodes (LEDs) illuminate the beads. One LED identifies the analyte that is being detected and the second LED determines the magnitude of the PE-derived signal. Each well is imaged with a CCD camera.

WHICH R&D SYSTEMS® LUMINEX® MULTIPLEX IMMUNOASSAY IS RIGHT FOR ME?

R&D Systems offers two bead-based multiplex immunoassay options utilizing Luminex® xMAP® microparticle technology. Our immunoassays are intended to help investigators tailor assay selection to their individual research needs. Please note that the sample dilution and the number of standards used can influence plex size and configuration. Design the multiplexing assay you need for your preliminary or discovery investigations with our R&D Systems® Luminex® Discovery assays. These assays are optimized to simultaneously analyze a wide variety and large number of analytes. Next, take your research to the next level by customizing one of our Luminex® High Performance panels to get the most accurate and precise Luminex assays which deliver near single-analyte ELISA performance.

	R&D SYSTEMS® LUMINEX® DISCOVERY ASSAYS	R&D SYSTEMS® HIGH PERFORMANCE LUMINEX® ASSAYS
What	Biomarker Discovery Or Screening	Biomarker Verification
When	Early Stage	Later Stage
Why	Quantitative	Quantitative
	Economical	High Sensitivity
	Maximum Multiplexing Capacity	Greatest accuracy and performance. **Validated similar to R&D Systems gold-standard Quantikine® ELISA**
	Most Flexible Option	Correlated with Quantikine® ELISA pre-defined, configurable panel.

R&D SYSTEMS® HIGH PERFORMANCE LUMINEX ASSAYS COME IN 3 VARIETIES: PREMIXED, USER MIXED AND FIXED.

SEE WHICH ASSAY IS BEST FOR YOUR BIOMARKER VALIDATION NEEDS.

	LUMINEX® DISCOVERY ASSAYS	LUMINEX® "PREMIXED" HIGH PERFORMANCE ASSAYS	LUMINEX® "USER MIXED" HIGH PERFORMANCE ASSAYS	LUMINEX® FIXED HIGH PERFORMANCE ASSAYS
	ORDER NOW!	ORDER NOW!	ORDER NOW!	ORDER NOW!
Maximum Analyte Multiplex	50	45	45	45
Sample Volume (µL)	25-50	*25-50	*25-50	*25-50
Microparticles Premixed	Yes	Yes	No	Yes
Biotin Antibodies Premixed	Yes	Yes	No	Yes
Controls Available	No	Yes	Yes	Yes
Premix QC Prior to Shipping	Yes, full QC	Yes, Confirmational QC	Yes, Confirmational QC	Yes, Confirmational QC
# Analytes Available	>450	>100	>100	45
# Species Available	3	2	1	1
Validated sample types	Cell culture supernatants, serum, EDTA plasma, Heparin plasma. For mouse analytes only, tissue lysates.	Cell culture supernatants, serum, EDTA plasma, Heparin plasma; For select panels**: saliva, urine, milk.	Cell culture supernatants, serum, EDTA plasma, Heparin plasma; for select panels**: saliva, urine, milk.	Same as "Premixed"
Analyte Selection	User defined*	Predetermined but customizable.	Predetermined but customizable.	Predetermined not customizable.
Assay Format	Bead sets include base kits.	Bead sets include base kits.	Order the bead sets and base kits separately.	Bead sets include base kits.
Delivery Time	U.S. orders ship in 7 business days.	U.S. orders ship in 5 business days.	U.S. orders ship in 2 days.	U.S. orders usually ship in 1 day.
# Panels Available	Custom	10 customizable panels, including 2 high sensitivity panels.	13 customizable panels, including 2 high sensitivity panels.	9 fixed panels.

ENSURING LUMINEX® PERFORMANCE & CONSISTENCY

R&D Systems® Luminex assays outperform competitor assays in a number of dimensions, including natural sample linearity, lot-to-lot consistency, precision and reproducibility, sensitivity, and minimization of false positive signals.

ACCURATE DETECTION OF NATURAL PROTEINS

Antibody pairs recognize both the supplied recombinant standard and the natural proteins in biological samples. Natural sample linearity (sometimes referred to as parallelism) is a hallmark of the R&D Systems® Luminex® High Performance assay and confirms that the kit can accurately measure the relative mass values of the natural analyte. R&D Systems also determines the ideal standard curve range for each assay, ensuring optimal sensitivity and reproducibility of results.

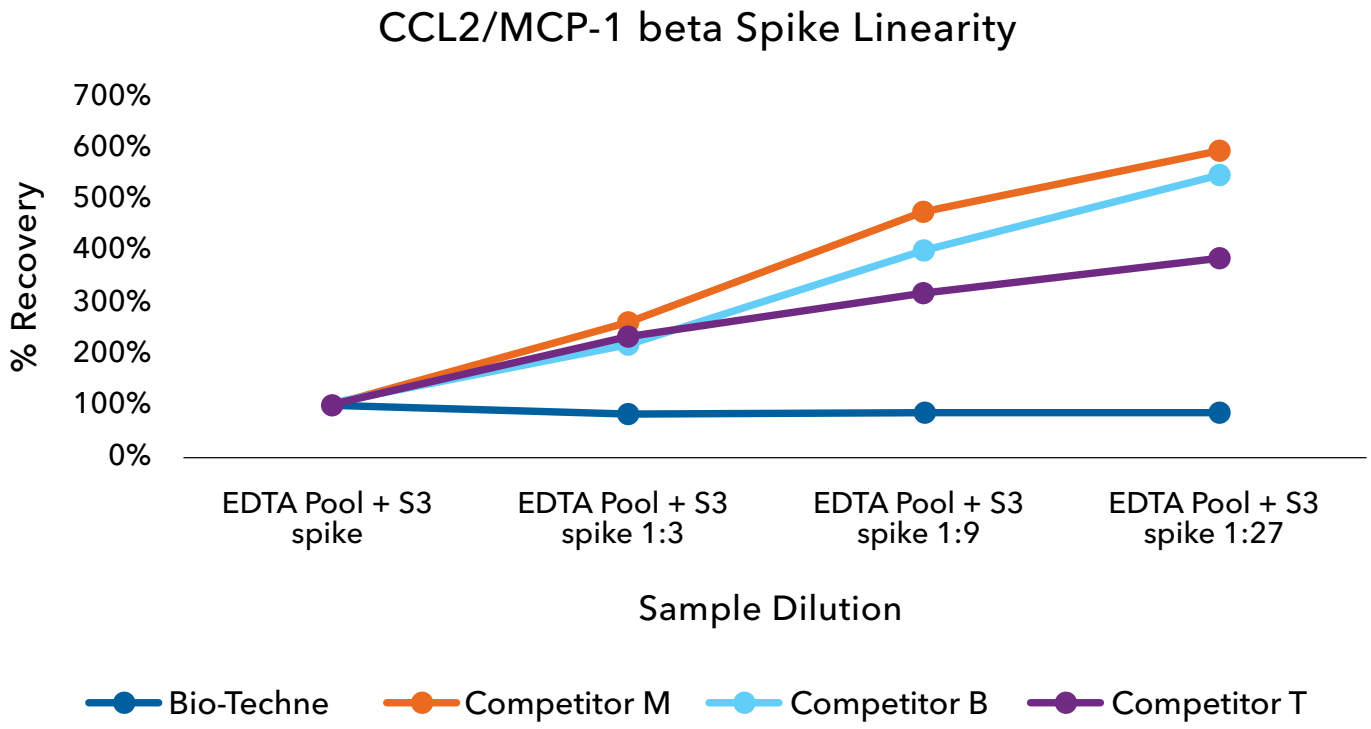


Figure 1. MCP-1 (Catalog # LUXLM279) natural sample linearity is maintained with the R&D Systems® recombinant standard but not with competitor B.

CONFIRMED LOT-TO-LOT CONSISTENCY

Another hallmark of the R&D Systems® Luminex® High Performance products is that all lots are tested to ensure low background, consistent standard curves and dynamic ranges. Each standard is anchored to the same master calibrator to ensure that sample data is comparable over time.

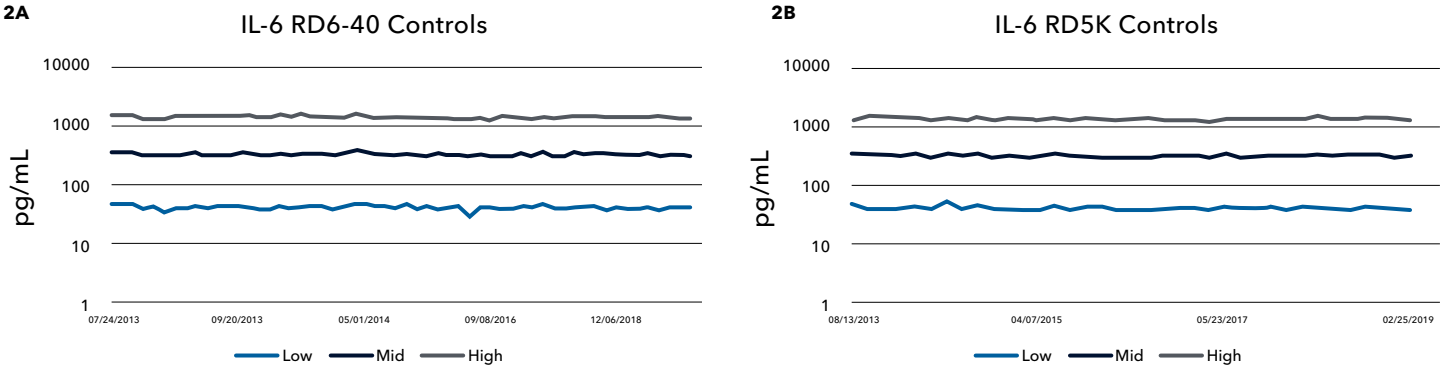


Figure 2. Levey-Jennings control plots show IL-6 (Catalog # LUXLM206) lot-to-lot consistency in RD5K diluent (**2A**) and RD6-40 diulent (**2B**) over the course of 6 years.

PRECISION AND REPRODUCIBILITY: PROVIDING CONFIDENCE IN YOUR RESULTS

Immunoassay precision is defined as the reproducibility of results within and between assays. This characteristic of an immunoassay is extremely important in order to: 1) provide assurance that the results obtained throughout a study are accurate and reproducible from one experiment to the next and 2) determine if two results are the same or different. Precision is measured as a coefficient of variation (CV) from the mean value. Two types of precision should be considered, intra-assay precision and inter-assay precision. Intra-assay precision is the reproducibility between wells within an assay. This allows the researcher to run multiple replicates of the same sample on one plate and obtain similar results. Inter-assay precision is the reproducibility between assays. Inter-assay precision guarantees that the results obtained will be reproducible using multiple kits over time. R&D Systems® Luminex® Assays typically have CV values less than 30% across the standard curve for both intra- and inter-assay precision, while our High Performance Assays typically have CV values less than 17%. These low CV values allow the researcher to perform repeated assays and be confident that the results are consistent throughout the study.

ANALYTE	INTRA-ASSAY (%CV)	INTER-ASSAY (%CV)
BDNF	8.16	13.3
CCL2/MCP-1	3.02	10.5
CCL5/RANTES	3.72	17.0
CCL11/Eotaxin	7.98	15.4
CCL20/MIP-3a	8.41	17.3
CD40 Ligand	9.30	15.0
CXCL2/GRO□	7.76	13.1
CXCL10/IP-10	2.95	12.2
CXCL11/I-TAC	6.23	13.7
CXCL13/BLC	5.79	12.5
FGF basic	5.60	13.1
G-CSF	5.55	14.2
GM-CSF	7.11	14.1
Granzyme B	9.75	18.6
INF-□	5.17	12.4
INF-□	10.9	15.2
INF-γ	6.36	13.0
IL-1□	2.55	12.7

ANALYTE	INTRA-ASSAY (%CV)	INTER-ASSAY (%CV)
IL-10	8.55	14.1
IL-12 p70	4.92	17.1
IL-13	7.97	17.5
IL-15	4.99	18.2
IL-17A	4.38	19.0
IL-2	5.32	18.1
IL-21	5.84	19.0
IL-4	4.92	17.5
IL-5	4.14	16.5
IL-6	6.80	17.8
IL-7	6.57	17.7
IL-8/CXCL8	6.87	17.5
PDGF-AA	6.52	25.0
PDGF-BB	3.37	16.9
PD-L1	8.36	19.4
TGF-□	5.43	18.7
TNF-□	3.68	17.2
VEGF	4.79	18.4

Figure 3. Data from the Non-Human Primate XL Cytokine panel (Catalog # FCSTM21) indicate that all analytes have an intra assay CV below 11% from 40 reportable results and an Inter-assay CV below 26% across 31 assays.

SENSITIVITY

The minimum detectable dose is the lowest measurable value that is statistically different from zero. It is calculated by adding two standard deviations to the mean optical density value of several zero standard replicates and determining the corresponding analyte concentration from the standard curve.

The better the sensitivity of an assay, the lower the useful working range (standard curve range) will be. R&D Systems® Luminex® Assays and High Performance Assays are optimized to ensure high signal, low background, and the best sensitivity possible.

ANALYTE	MEAN (PG/ML)	RANGE (PG/ML)
ICAM-1	130	64-303
E-Selection	2.1	0.9-7.4

ANALYTE	MEAN (PG/ML)	RANGE (PG/ML)
P-Selection	6.4	3.0-12.2
VCAM-1	252	122-529

Figure 4. Examples of minimal detectable dose (MDD) from the Luminex Human Adhesion Molecule Luminex assay (Catalog # LKTM007). Twenty-nine assays were run and the MDD was determined by adding 2 standard deviations to the MFI of twenty zero standard replicates and calculating the corresponding concentration.

LINEARITY EXPERIMENTS IDENTIFY FALSE POSITIVE SIGNALS

A false positive result incorrectly detects an analyte not detectable in a given sample. Linearity of dilution is commonly used to identify false positives. Sample values should remain consistent when running multiple dilutions and back-calculating concentration. If sample values increase with increasing dilutions, this indicates a specificity issue. R&D Systems® Luminex® Assays and High Performance Assays are built, split if necessary, to prevent false positives.

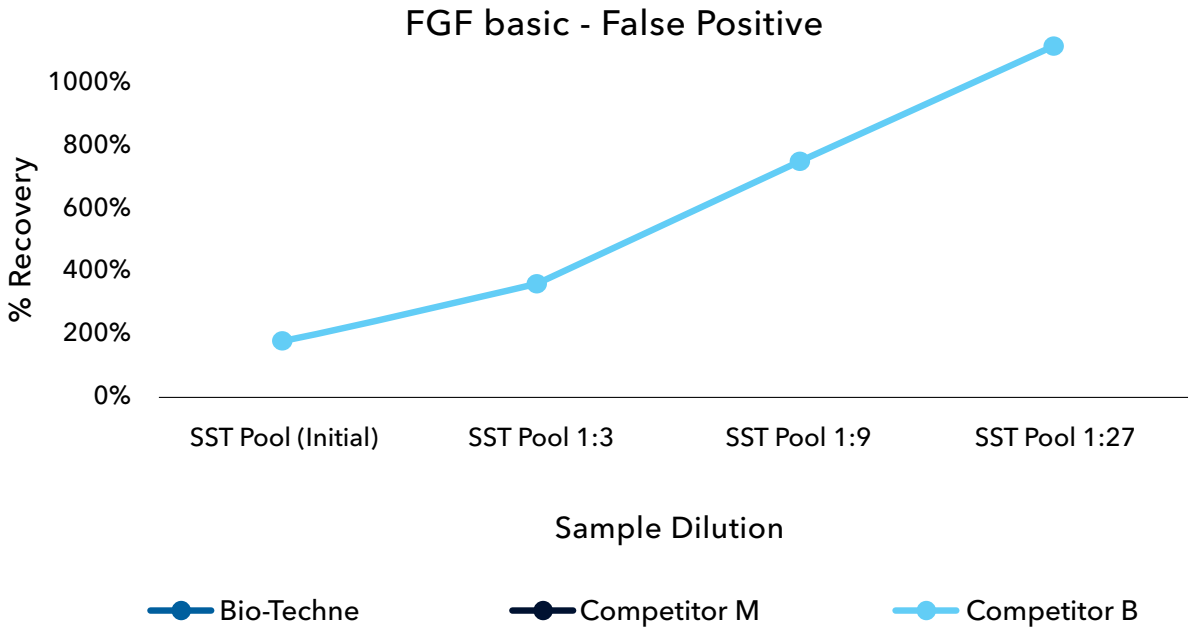


Figure 5. Linearity of dilution is used to identify false positive signals in Competitor B's Luminex Assay. Serum samples were not detected in assays from Bio-Techne (Catalog #LUXLM233) and Competitor M, which is consistent with Bio-Techne's FGF basic Quantikine ELISA Kit (Catalog #DFB50) where serum levels of FGF basic are below the detectable range.

SAMPLE PREPARATIONS

The sample collection and storage conditions listed below are intended as general guidelines. Samples require a minimum 2-fold dilution. High abundance biomarkers may require additional dilution for samples to meet performance criteria such as being within the dynamic range of the assay. Sample stability has not been evaluated. Hemolyzed, icteric and lipemic samples are generally not suitable for Luminex® assays. Note that each of the following sample types have not been validated for all Luminex assays. Check the kit insert to determine validated sample types.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Tissue Lysates – Rinse tissue with PBS and cut into 1-2 mm pieces. Homogenize with a tissue homogenizer in PBS. Add an equal volume of Cell Lysis Buffer 2 (R&D Systems®, Catalog # 895347) and lyse at room temperature for 30 minutes with gentle agitation. Remove debris by centrifugation. Assay immediately, or aliquot and store at ≤ -70 °C. Avoid repeated freeze-thaw cycles.

Platelet-poor Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. An additional centrifugation step of the plasma at 10,000 x g for 10 minutes at 2-8 °C is recommended for complete platelet removal. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

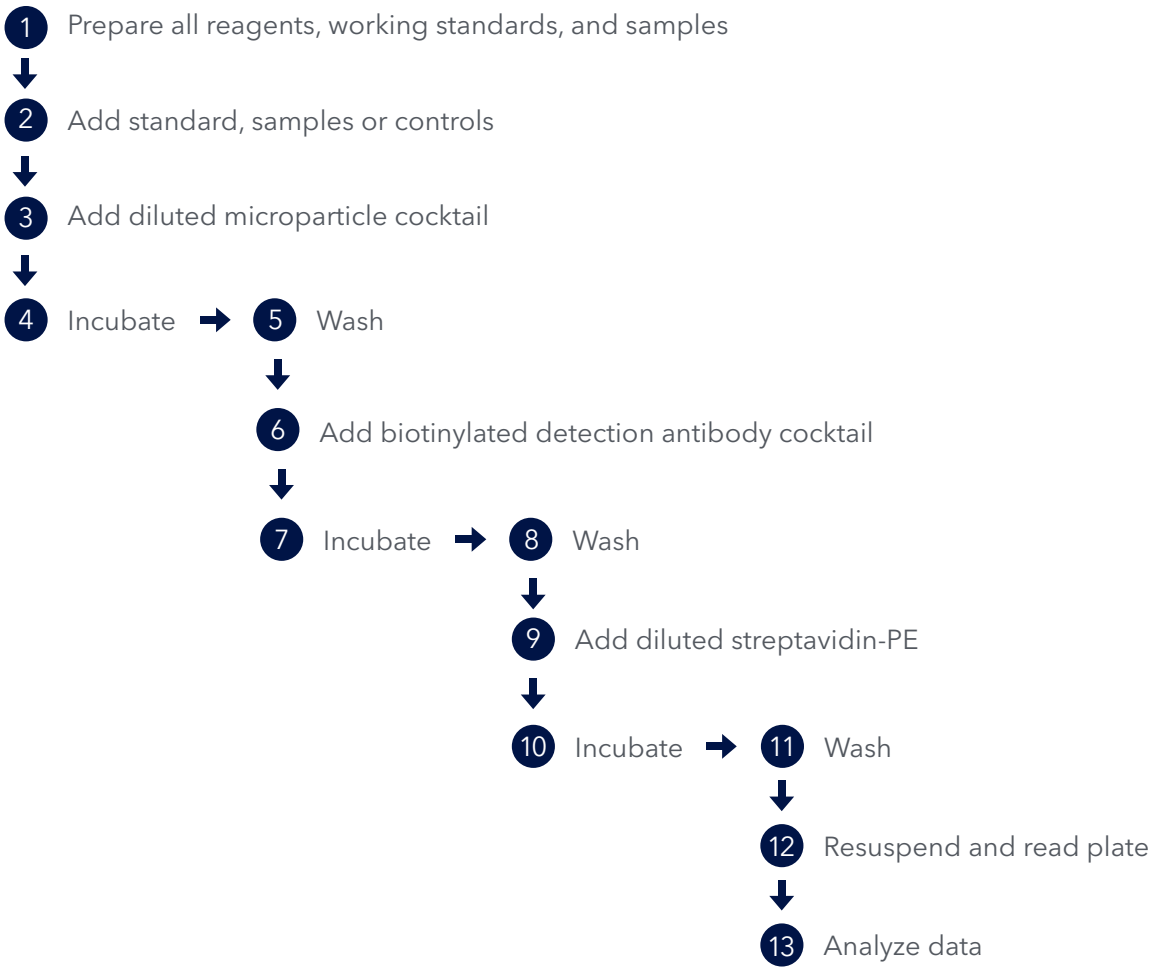
Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Saliva - Collect saliva in a tube and centrifuge for 5 minutes at 10,000 x g. Collect the aqueous layer, assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge at 16,000 x g for 4 minutes to remove particulate matter. Assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Human Milk - Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

STANDARD PROCEDURE



DATA ANALYSIS: CALCULATION OF RESULTS

CALCULATING CONCENTRATION OF TARGET PROTEIN IN THE SAMPLE

The values of the unknown samples are assigned in relation to the standard curve. Since samples are diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Always run samples in duplicate or triplicate, to provide enough data for statistical validation of the results. Average the duplicate or triplicate readings for each standard, control, and sample and subtract the average zero standard (blank) median fluorescence intensity (MFI). The coefficient of variation (CV) of duplicates should be ≤ 20%.

Create a standard curve by using computer software capable of plotting the mean MFI (y axis) against the protein concentration (x axis) and generating a five-parameter logistic (5-PL) curve-fit.

CALCULATING THE COEFFICIENT OF VARIATION

The coefficient of variation (CV) is the ratio of the standard deviation to the mean, which is usually expressed as a percentage.

BEST PRACTICES

Make sure all reagents are brought to room temperature before using (unless instructed to keep them cold).

Standards and controls are single-use and should not be aliquoted or frozen after reconstitution.

Multichannel pipettes speed the ability to plate your standard and samples and lead to more consistent results. When pipetting, dispense liquid with the pipette tips held at an angle and avoid touching the bottom of the well.

While it is not necessary to change your pipette tips between each replicate, it is recommended that you change them between different samples or standards to prevent contamination.

It is highly recommended that automatic plate washer is used to achieve the most consistent assay results.

When washing plates, either manually or with a plate washer, be sure to give the wash buffer time to work by adding a 30 second soak time in between washes.

Pay close attention to the incubation times. As a general guide the incubation time should not vary by more than +/- 5 minutes per hour of incubation time.

On the day of the assay, all fresh and previously frozen serum and plasma samples require centrifugation at 16,000 x g for 4 minutes immediately prior to use or dilution.

LUMINEX® ASSAY AND HIGH PERFORMANCE FAQS

WHAT IS INCLUDED IN AN R&D SYSTEMS® LUMINEX® IMMUNOASSAY?

Pre-mixed Luminex® immunoassays are a complete kit consisting of precoated microparticle cocktails, biotinylated detection antibody cocktails, standards, diluents, calibrator diluent, assay diluent, streptavidin-PE concentrate, wash buffer, plate and plate sealers. Assay instructions and product inserts are also included to help guide users through the assay.

User mixed Luminex® High Performance Assays are supplied as a base kit containing all of the reagents necessary to run the assay with separate microparticle and biotinylated detection antibody concentrates. Note: Newer user mixed High Performance Assay kits, such as XL panels, will have a lyophilized biotinylated antibody cocktail supplied with the base kit and will not contain a "matched set" of microparticles and detection antibody.

HOW MANY SAMPLES CAN BE ASSAYED IN A LUMINEX® KIT?

Typically, 40 user supplied samples can be assayed in duplicate. This will depend on the number of points being evaluated for the standard curve and the inclusion of any controls. The R&D Systems® Luminex® Assay is typically run with a six-point standard curve and High Performance Assays are run with a seven-point standard curve. Please refer to the datasheet for details that may be specific to your kit.

WHAT SAMPLES CAN BE TESTED IN THE KIT?

Typically, the R&D Systems® Luminex® kits are validated for sera, two types of plasma (collected in EDTA or Heparin), and cell culture supernate. However, the samples validated can vary from product to product. The product datasheet and product-specific web page states all sample types that have been validated for use with the specific kit. These are the only samples for which we can support the claims. References may exist for other sample types. See the "Citations" tab on the product-specific webpage for any published references citing the use of the kit with an alternate sample type. Unvalidated sample types should be validated by the customer.

HAS THIS KIT EVER BEEN TESTED WITH MY SAMPLE TYPE?

R&D Systems has not routinely tested all sample types such as tissue homogenates or bronchoalveolar lavage for Luminex® kits. This does not mean that the Luminex® kit is not suitable for other sample types. Each investigator will need to perform a spike and recovery study to determine if an unvalidated sample type will work with the kit. To perform a spike and recovery experiment, investigators should divide a sample into two aliquots. In one of the aliquots, the user should spike in a known amount of the kit standard. A dilution series is performed comparing the spiked versus the unspiked sample. This method may be used to validate any sample type that has not been evaluated by R&D Systems. For a more detailed spike and recovery protocol, please contact Technical Service. Note: Acceptable ranges should be determined individually by each laboratory. Please see the Citations tab for peer-reviewed papers utilizing a wide range of sample types.

WHY CAN'T I DETECT ANY OF MY SAMPLES?

You will be able to quantify samples down to the lowest point on the standard curve. In some cases, the standard curve does go down low enough to detect normal samples. You can check the sample values section of the analyte specific datasheet to find what kind of sample values we obtained from apparently healthy individual donors. You may also want to review the literature to find out if there is an established normal range for your target. It is important to recognize that assay platforms and manufacturers differ in their calibrations for their unique assay products and reported measurements may not directly correlate.

CAN I EXTEND THE STANDARD CURVE (IN EITHER DIRECTION)?

R&D Systems cannot support kit results outside the stated range under any circumstances. A specific range was chosen because of confidence in the reproducibility of the assay.

WHAT IS ASSAY SENSITIVITY?

Sensitivity is the lowest measurable value that is statistically not equal to zero. It is calculated based on the signal of the background and the inherent variability of the assay. It is commonly determined by taking the mean fluorescence intensity (MFI) plus two standard deviations from 20 zero replicates. This value is converted into analyte concentration from the standard curve. The low standard is the lowest possible point at which R&D Systems feels confident that the value is in the linear portion of the standard curve and, therefore, quantifiable. Values which are greater than the sensitivity can be distinguished as separate from the background or the noise of the assay, however the confidence level for reporting these values is lower than if the sample values fall within the standard curve range.

WHY IS A SAMPLE DILUTION NECESSARY?

There are primarily two reasons for dilutions. In some assays most samples read above the standard curve, thus requiring a dilution for analyte levels to fall within the range of the assay. A second reason for dilution is to limit interference due to factors in complex matrices.

WON'T ADDITION OF AN ASSAY DILUENT CAUSE FURTHER DILUTION OF THE SAMPLE?

Since the assay diluent is added to all wells, standards and specimens are treated equally. Therefore, sample concentration can be read from the standard curve without adjusting for this dilution.

continued on next page

IS THERE ENOUGH CALIBRATOR DILUENT FOR ALL MY SAMPLE PREPARATIONS?

The kits are designed with enough calibrator diluent to ensure that the vast majority of samples fall within the indicated range of the assay. Should you find that there is not enough diluent provided in the kit to dilute your samples, you have at least two options. Option 1: Samples can be diluted in two steps. The initial dilution in culture medium and a final dilution, of at least 1:10, into the Calibrator Diluent provided in the kit. Option 2: You can purchase additional diluent provided the same lot included in the kit is still available. Contact Technical Service for more information.

MY DILUENTS APPEAR TO CONTAIN PRECIPITATE, IS THIS OKAY?

Due to saturating amounts of some buffer components, some of the RD1 Assay Diluents contain a light to heavy precipitate. This should not affect the assay. In these instances, it will be noted in the specific protocol booklet. If it is not noted in the protocol booklet, please contact Technical Service.

THE ASSAY PROTOCOL SPECIFIES THE USE OF A SHAKER AT 800 RPM. THIS IS TOO FAST FOR MY SHAKER. IS THIS CORRECT?

This is 800 rpm with a 0.12 orbit. If the plate shaker has a larger orbit, then 800 rpm will be too fast. R&D Systems® recommends the Thermo Fisher® Model # 4625 microtiter plater shaker. R&D Systems® Luminex® immunoassays have been optimized for performance with these shaker specifications only.

ARE CONTROLS AVAILABLE FOR KITS?

R&D Systems® Luminex® Assays do not have kit controls available for purchase. However, R&D Systems® Luminex® High Performance Assays either include kit controls or are available as supplemental products. Please inquire for specific ordering information.

WHAT IS THE STABILITY OF SUPPLEMENTAL CONTROLS?

Controls are assigned an expiration date of 6 months from date of receipt. They are to be used once and discarded. If the lyophilized controls are stored properly, it is possible that they will remain stable for an extended period of time, although we have not conducted extended stability testing. The controls have not been tested for stability after reconstitution.

I USED YOUR RECOMBINANT PROTEIN AS A CONTROL IN THE CORRESPONDING R&D SYSTEMS® LUMINEX® IMMUNOASSAY KIT. WHY AM I SEEING DISCREPANCY IN MASS VALUES?

First, a large dilution is required to place the recombinant protein on the standard curve range. Typically, this is a dilution from µg/mL to pg/mL. Any dilution step can introduce inaccuracy and the larger the dilution step the greater the potential for error. Any pipetting error or mis-calibrated pipet can result in apparent over- or under-recovery. Second, R&D Systems® Luminex® immunoassays have been developed to measure a level of protein captured by one antibody and detected by a second antibody. This measurement is calibrated to standards established when the kit was initially developed. The protein determination of these initial standards became the Master Calibrators to which all new standards are formulated. This provides R&D Systems® Luminex® immunoassay kits with consistency between manufacturing lots. In general, we would expect +/- 30% recovery of the amount stated on the vial when using the Luminex® Assay to determine a protein concentration. There may be slight differences in the immunologically recognizable mass between lots of protein, so the apparent concentration provided on the vial may vary from lot-to-lot when measured in the immunoassay. If you are using proteins to make controls, it is better to value assign the mass based on measurement in the immunoassay and not use the mass on the vial when setting control levels.

WHY MUST I USE POLYPROPYLENE TUBES FOR STANDARD CURVE DILUTIONS?

Certain proteins or analytes will bind to glass and polystyrene, but do not readily bind to the polypropylene tubes.

CAN I RUN A PARTIAL PLATE?

While it is possible to run less than a full plate and retain the microparticle or biotinylated detection antibody cocktails, this only applies to the concentrates. A fresh working stock of material (microparticles, biotinylated detection antibody, standards and controls) should be generated at the time of assay. Lyophilized standards and controls are single use only and should be freshly prepared at the time of assay.

CAN I ADJUST THE INCUBATION TIMES OR TEMPERATURES FROM THE INSTRUCTIONS IN THE KIT INSERT?

R&D Systems® has optimized the assays for both incubation times and temperatures. Each kit has been validated for the protocol described in the kit datasheet. We cannot guarantee the performance of our kits when the protocol has been altered in any way.

CAN REAGENTS FROM DIFFERENT KITS BE INTERCHANGED?

Assay Diluent(s), Calibrator Diluent(s), and other kit components may only be interchanged if they have the same part number AND lot number. R&D Systems® does "whole kit QC" which means that we cannot support the use of reagents from other lots or sources being substituted into an assay.

WHY AM I SEEING HIGH VARIABILITY BETWEEN SAMPLE DUPLICATES?

High intra-assay variability can be caused by poor pipetting and/or poor washing technique.

OBSERVATION	POSSIBLE SOURCE	SUGGESTION
Acquisition Problems and Error Messages	Incompatible instrument	Use the instrument that is compatible to the microparticle type. R&D Systems® Luminex® assays are compatible with all Luminex® instruments. • Luminex® 100/200™ • FLEXMAP 3D™ • Luminex® MAGPIX®
	Instrument is out of calibration	To obtain accurate measurements, regular calibration of the instrument is required. Best practice is to run assays within one week of calibration. Luminex® recommends running verification the day of the assay to confirm the instrument is functioning properly and is within current calibration settings. Perform instrument calibration and verification per the instrument user's manual.
	Incorrect probe height	Adjust the sample probe vertical height and align to the plate per the instrument user's manual.
	Sample probe is clogged	Clean the sample probe per the instrument user's manual. Replace the sample probe if necessary. Remember to readjust the vertical height each time the probe has been removed.
	Microparticle spectral address is not assigned correctly	Ensure microparticle regions are assigned correctly per the kit insert or the Certificate of Analysis. Microparticle maps are custom created depending upon the analytes selected. In the event of omission or an incorrect assignment of a microparticle region, data will be missing in the CSV file, try the "Replay" function to retrieve the data following selection of the appropriate microparticle regions.
	Incorrect instrument settings	Follow the insert instructions on instrument settings.
Low Microparticle Count	Instrument is out of calibration	Perform instrument calibration and verification. To obtain accurate measurements regular calibration of the instrument is required. Best practice is to run assays within one week of calibration. Luminex® recommend running verification the day of the assay to confirm the instrument is functioning properly with current calibration settings.
	Wrong event or microparticle setting	Verify that the events/bead is set at 50. A 50-count per analyte is sufficient to produce a statistically accurate result. A bead count of about 25 may be acceptable if the samples are run in duplicates and other parameters of measuring the performance of the assay are fine.
	The system is timed-out (Luminex® 100/200™ and FLEXMAP3D™)	If your instrument times out when using flow cytometry-based instruments such as the Luminex® 100/200™, stop the plate run. Check the probe height and confirm the magnetic microparticle type is selected. Then re-run the plate. As each microparticle has a different rate for acquisition and the instrument is set to collect 50 microparticles in a designated time, a "time-out" may result in insufficient microparticle counts for one or more analytes. The MagPIX® instrument has a fixed read time for each well and does not have time-out functionality.
	Sample contains debris which affects acquisition	Centrifuge samples on the day of the assay at approximately 16,000 x g for 4 minutes immediately before use. In rare cases, an extended centrifugation may be necessary.
	Miscalculation of microparticle dilution/ lower number of microparticles added per well	Confirm microparticles were diluted according to the kit insert.
	Microparticles are clumped or aggregated	Centrifuge the microparticle cocktail concentrate (for 30 seconds at 1,000xg) and gently vortex the concentrated before preparing the 1X diluted microparticle cocktail.
	Microparticles not in suspension during acquisition	Immediately before placing the plate on the reader, shake the plate for one additional minute in 1X Wash Buffer to resuspend the microparticles.
	Shaker with incorrect settings	Use a horizontal orbital microplate shaker with a 0.12" orbit. Ensure the shaker speed is set per recommendations from the kit insert.
	Magnetic microparticles not collected at the bottom of plate during wash steps	Use an appropriate magnetic device designed to accommodate a microplate. Wash by applying the magnet to the bottom of the microplate, allow 1 minute before decanting wash buffer. Do not blot dry as this may cause a loss of microparticles.
	Sample was run undiluted	Samples require at least a 2-fold dilution with the appropriate Calibrator Diluent. Mix thoroughly. Samples may require higher than 2-fold dilutions. Review the Product Insert, Certificate of Analysis or R&D Systems® Luminex® Assay Customization Tool for the suggested starting dilution for each sample type.
	Blockage of sample probe	See above under acquisition and error messages.
Low Fluorescence Intensity (FI) signal or Poor Sensitivity	Non-optimal preparation of the standard curve	Confirm the standard reconstitution volume from the standard value card or the Certificate of Analysis. Incorrect reconstitution of the standard will result in inaccurate sample value calculations. Be sure to follow reconstitution instructions for all lyophilized reagents outlined in the kit insert.
	Non-optimal dilution of the detection antibodies or streptavidin-PE concentrates	Confirm reagent dilutions were performed according to the kit insert.
	Photo-bleaching of the PE signal	Streptavidin-PE is light sensitive. Protect from light.
	Incorrect shaker settings	See above.
	Incorrect instrument settings	See above.

continued on next page

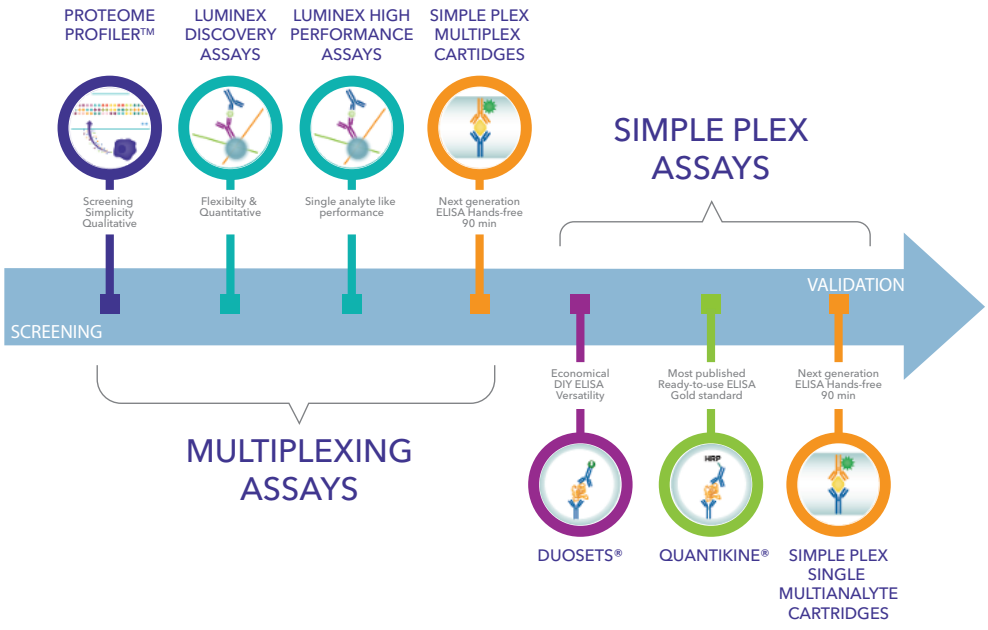
TROUBLESHOOTING GUIDE TROUBLESHOOTING GUIDE CONT.

OBSERVATION	POSSIBLE SOURCE	SUGGESTION
Sample Readings are Out of Range (OOR Error Message)	Samples are below assay range (<OOR Error Messages) and contain no analyte, or the analyte level is below the level of detection, or the sample may be too diluted.	Analyte of interest may be undetectable in the assay range due to low abundance of natural protein. Check kit instructions or the R&D Systems® Luminex® Assay Customization Tool for suggested sample dilution. Suggested dilution factors are based on samples from healthy volunteers. Depending on the unique nature of an individual sample, a different dilution factor may be needed to bring the reading within the dynamic range of the assay.
	When readings are above assay range (>OOR Error Messages)	Review the Product Insert, Certificate of Analysis or Check kit instructions or the R&D Systems® Luminex® Assay Customization Tool for the suggested initial dilution. Suggested dilution factors are based on samples from healthy volunteers. Depending on the nature of the sample, it may require a further dilution to bring the reading within the assay range.
		Note - Samples may require dilution and re-analysis if a specific analyte is out of range.
Poor Precision with sample measurements	Presence of interfering components in samples, especially samples with complex matrices such as plasma and serum	Check for the presence of interfering components, additives, or if gel separators were introduced into the sample by performing a Spike/Recovery and Linearity test. Contact R&D Systems® Technical Service if you require assistance with this test.
	Sample type not validated for the assay	Check the kit insert to confirm if the sample type has been validated for the assay.
	Samples with hemolyzed and hyperlipidemic matrices	Avoid the use of samples with hemolyzed or hyperlipidemic matrices. Such samples may disrupt antibody binding or clog the probe. See discussion above on how to clean the sample probe.
	Integrity of the sample is compromised while in storage	Follow the kit insert on Sample Collection & Storage. Observe best practices for processing and storing the samples after collection. Avoid repeated freeze-thaw cycles.
	Non-optimal pipetting technique	Ensure a consistent and accurate pipetting method. Dispense microparticles, diluents and samples accurately. Change pipette tips between samples and dilutions. Pre-wet tips for sample replicates. Ensure that your pipettes are calibrated regularly.
	Assay reagents not equilibrated to room temperature prior to use	All assay components should be equilibrated to room temperature prior to use.
High Background Signal	Incorrect buffer used for the dilution of standards and/or samples	Ensure the use of the recommended Calibrator Diluent for the dilution of standards/samples per kit insert.
	Blank wells accidently spiked with standard or samples	Do not add standard or samples to wells designated as blank. Add Calibrator Diluent only.
	Extended incubation with detection Abs or streptavidin-PE	Follow the kit instructions for incubation times and follow precisely.
Microparticle Aggregation	Samples with hemolyzed and hyperlipidemic matrices	See above
	Microparticles not thoroughly mixed	Follow the kit instructions on the preparation of the diluted microparticle cocktail. Use a plate shaker with appropriate settings for the assay. Shake plate for one additional minute in 1X Wash Buffer immediately before analysing in an appropriate instrument.
	Doublet Discriminator gates setting is incorrect	Check the kit insert for the Doublet Discriminator gate settings and adjust settings as needed.

FURTHER YOUR IMMUNOASSAY CAPABILITES

FROM SCREENING TO VALIDATION

We have your total worflow solution in mind at Bio-Techne, we have a solution to assis you at every stage of your research.



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