WHITE PAPER

Development and Validation of Quantikine® ELISAs and Luminex® High Performance Assays

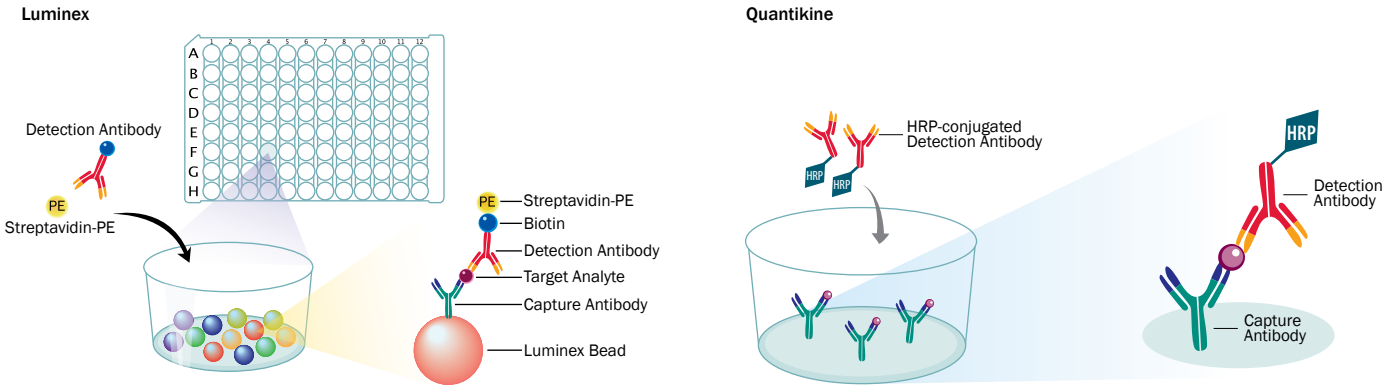
Whether developing a Luminex High Performance Assay or Quantikine ELISA, our assay development process is a rigorous, labor intensive process. We go the extra mile to ensure the correct analyte is being measured, the results are reproducible, and the assay can be consistently manufactured over a long period of time. Our process relies on checkpoint meetings at the end of each development stage. These meetings require the verification of assay development progress before granting approval to begin the next stage of development. We have confidence in every assay we produce, and you should too.

R&D Systems has nearly 30 years of experience designing, testing, and optimizing immunoassay kits to ensure the highest level of performance in analyte quantification. Consistent assay performance is critical for trusting your results, analyzing your data, and forming your conclusions. From experiment to experiment and from project to project, you'll never have to worry about consistent performance with our immunoassay products.

Although sensitivity, sample type, or ease of use may be your primary criterion for selecting an assay, you should expect the assay to work the first time and every time you use it. R&D Systems develops Quantikine ELISAs and Luminex High Performance Assays from materials that we make in-house, giving us unparalleled control over critical kit components. Whether you are breaking new ground or building on the work of others, these assays will give you results you can trust tomorrow, next week, and next year.

Antibody Screening and Titration

High-quality, highly purified antibodies provide the foundation for Quantikine ELISAs and Luminex High Performance Assays. Several different monoclonal and polyclonal antibodies are screened to determine which combination is best suited for analyte detection. Selected antibodies are carefully titered to ensure that the concentrations chosen will give the best possible results for the assay. The capture antibody is coated either on an ELISA microplate or on a Luminex microparticle to determine the optimal coating concentration for analyte binding. The detection antibody is subsequently titered to determine its optimal concentration for pairing with the capture antibody to give the best signal-to-noise ratio.



Assay Principles for Quantikine ELISAs and Luminex High Performance Assays. These assays are based on a two-site sandwich principle in which highly specific antibodies are used to detect the target analyte(s). For Quantikine ELISA Kits, the capture antibody is pre-coated on a 96-well microplate, whereas capture antibodies are pre-coated on microparticles for Luminex High Performance Assays. The sandwiches are completed with a conjugated detection antibody that binds to different epitopes on the target analyte(s). Quantikine ELISA kits utilize HRP-conjugated detection antibodies followed by an HRP substrate solution. Luminex High Performance Assays utilize biotinylated detection antibodies followed by Streptavidin-PE. Each assay format generates a signal that is proportional to the amount of analyte bound.

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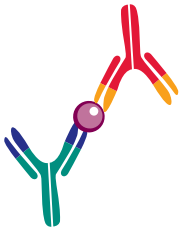
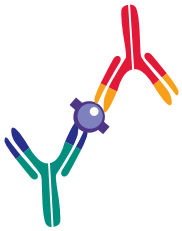
Specificity: Measuring Only the Analyte of Interest

Immunoassay specificity can be compromised by antibody cross-reactivity as well as by interference from materials in the sample. Cross-reactivity occurs when a molecule other than the analyte of interest is bound by both antibodies leading to a false positive result. Interference occurs when substances in the sample matrix (such as serum or plasma) modify the antigen-antibody interaction, preventing an assay from recognizing its designated analyte. These problems can be mitigated by proper development and testing. False positive results due to matrix effects can be identified only by diligent validation and quality control procedures. An immunoassay cannot be evaluated based solely on the detection of a signal. In most cases, confirmation that the signal is due to the analyte of interest can be obtained by testing the linearity of dilution (see below).

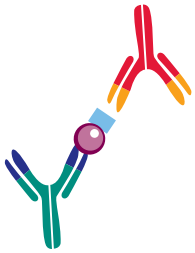
We carefully select antibodies, optimize coating and conjugate buffers, and select assay diluents to eliminate matrix effects. To gauge the specificity of an assay, molecules related to the analyte are tested for cross-reactivity and interference. A list of these molecules and the results of the testing are included in our product datasheets. Natural and recombinant analyte samples may adopt distinct conformations after binding to the capture antibody, and these conformational changes may affect the binding of the detection antibody. Quantikine ELISA Kits and Luminex High Performance Assays are optimized for recognition of both recombinant and natural antigens with equal efficacy.



Non-specific binding occurs in an immunoassay when the antibody pair interacts with assay surfaces and contributes to background. Non-specific binding to microparticles, microplates, or other assay surfaces can be reduced by blocking. Diluents provided with our assays contain optimized blocking reagents to ensure low non-specific binding.



Cross-reactivity, the interaction of the antibody pair with a molecule other than the target analyte, can be caused by either the capture antibody or the detection antibody. It often occurs when proteins in the sample are structurally related to the target analyte. Optimizing antibodies to avoid cross-reactivity with substances in the biological sample is often challenging. We carefully select and exhaustively test our in-house developed antibody pairs against related molecules to ensure analyte specificity.



Immunoassay interference occurs when a substance within the assay prevents the accurate detection of the target analyte. A biological sample is a complex matrix that may contain interfering factors. Sample recovery experiments are used to determine if assays are impacted by interfering factors. Optimal recovery demonstrates that matrix interference does not impact the accuracy of assay results.

Recovery

Complex sample matrices, such as serum and plasma, may contain interfering factors that affect the ability of an assay to accurately quantify the target analyte. Recovery experiments are used to determine if assays are affected by interfering factors. Low, medium, and high concentrations of analyte are spiked into all validated sample types and then analyzed for recovery. The results are expressed as a percentage of analyte recovered and are reported in each product data sheet. If interfering factors are found, we formulate new diluents that minimize their effects.

Human IL-2 Recovery Analysis

Sample	Average % Recovery	Range
Cell Culture Media* (n=4)	96	90–102%
Serum* (n=4)	100	96–105%
EDTA Plasma* (n=4)	104	93–113%
Heparin Plasma* (n=4)	102	88–110%
Saliva (n=4)	108	94–120%

* Samples were diluted prior to the assay as directed in the product data sheet. Preferred recovery range is 70–130%

Human IL-2 Recovery Using the Human XL Cytokine Discovery Panel (Catalog #LUXLM202). Recombinant Human IL-2 (Catalog #202-IL) was spiked at various levels throughout the range of the assay. Sample recovery was assessed for all validated sample types.

Human IL-18 Recovery Analysis

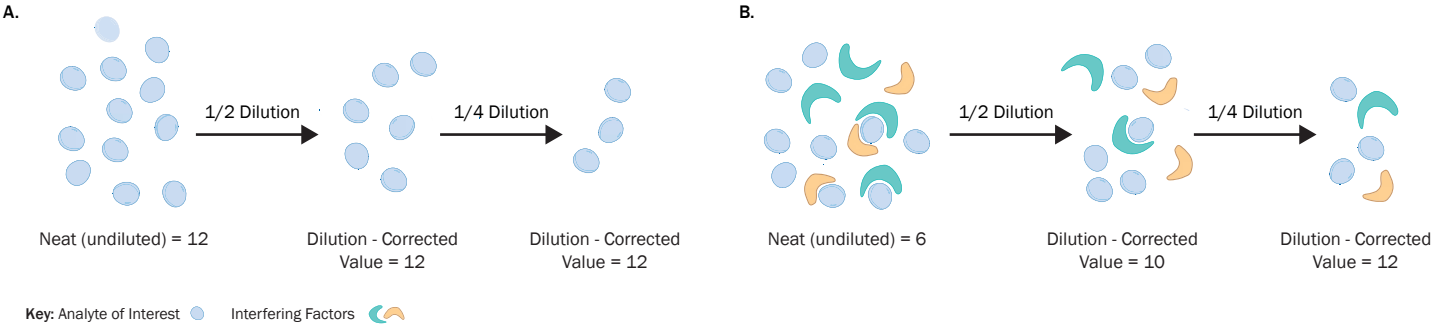
Sample	Average % Recovery	Range
Cell Culture Media* (n=4)	96	90–102%
Serum* (n=4)	100	96–105%
EDTA Plasma* (n=4)	104	93–113%
Heparin Plasma* (n=4)	102	88–110%
Urine (n=4)	91	84–100%
Saliva (n=4)	108	94–120%

* Samples were diluted prior to the assay as directed in the product data sheet.

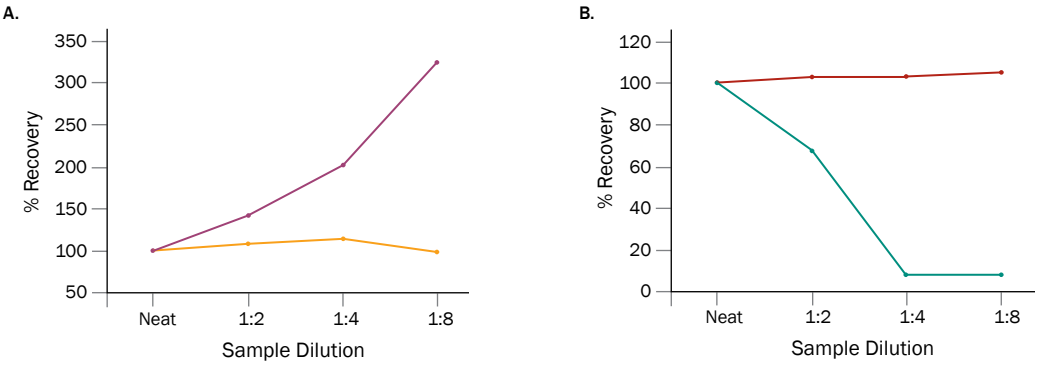
Analysis of the Recovery of IL-18 Using the Human Total IL-18/IL-1F4 Quantikine ELISA Kit (Catalog #DL180). Human Total IL-18 was spiked to various levels throughout the range of the assay. Sample recovery was assessed for all validated sample types.

Linearity of Dilution

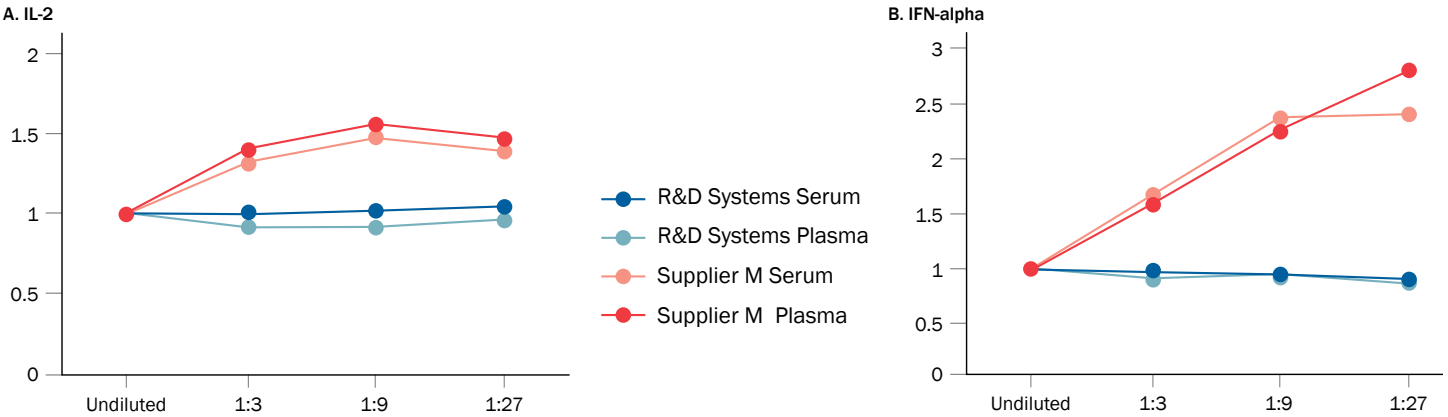
Sample dilutions should always deliver the same calculated analyte concentration for a given sample. This property is known as assay linearity. Interfering factors can compromise assay linearity unless the assay is designed to overcome these effects. We generate a dilution series using kit diluents across the dynamic range of the assay for each validated sample type. The results are expressed as a percentage observed relative to what was expected. Values between 80–120% indicate good assay linearity. Each of our product data sheets documents the percentage mean and range for assay linearity in all validated sample types.



Using Linear Dilution to Assess Matrix Effects. A. Results expected from a linearity of dilution experiment where no interfering factors are present in the sample matrix. **B.** Potential results from the same experiment if interfering factors are present in the matrix. Many factors in complex matrices such as serum or plasma can interfere with the analyte of interest. This effect may be revealed by unexpected linear dilution values.



Assay Linearity Measurement of Quantikine ELISA Kits. Spiked heparin plasma samples were serially diluted and assayed for human Thrombomodulin (panel A) or human TFPI (panel B). A. Samples measured with the Human Thrombomodulin/BDCA-3 Quantikine ELISA Kit (Catalog # DTHBD0) showed acceptable assay linearity with recovery values between 90–110% of the neat sample (gold line). In contrast, the percent recovery of diluted samples measured with a competitor's kit had a range of 141–325% (burgundy line), suggesting that interfering factors were preventing accurate measurement of the target analyte. **B.** Samples measured with the Human TFPI Quantikine ELISA Kit (Catalog # DTFP10) showed acceptable assay linearity with recovery values between 90–110% of the neat sample (red line), while the same samples showed poor assay linearity of a competitor's kit with values between 80–7% of the neat sample (green line).



Assay Linearity Measurement of Luminex High Performance Assays. Spiked heparin plasma and serum samples were serially diluted and assayed for human IL-2 (panel A) or IFN-alpha (panel B) using the Human XL Cytokine Discovery Panel (Catalog #LUXLM000) and a competitor's kits. **A.** Samples measured with the Human XL Cytokine Discovery Panel for IL-2 (Catalog # LUXLM202) displayed acceptable assay linearity with recovery values between 90–103% of the neat sample (blue lines). In contrast, the percent recovery of diluted samples measured with the competitor's kit had a range of 133–155% (red lines), suggesting that interfering factors were preventing accurate measurement of the target analyte. **B.** Samples measured with the Human XL Cytokine Discovery Panel for IFN-alpha (Catalog # LUXLM9345) showed acceptable recovery values (88–95%) of the neat sample (blue lines), while those measured with the competitor's kit had a range of 162–283% of the neat samples (red lines).

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: 1) to provide assurance that the results obtained throughout a study are accurate and reproducible from one experiment to the next, and 2) to determine if two independent assay results are the same or different. Precision is measured as a coefficient of variation (CV) relative to the mean value. Intra-assay precision refers to 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 refers to the reproducibility between assays. Inter-assay precision ensures that the results will be reproducible using multiple kits run at different times. Low CV values allow a researcher to perform repeated assays and be confident that their results are consistent throughout the duration of the study.

n=27	C1-Low	C2-High	Intra-assay Precision	Inter-assay Precision
	Mean (pg/mL)			
IFN-gamma	27.20	1065.00	14.8%	14.5%
IL-8/CXCL8	8.83	325.00	13.4%	13.7%

Intra-assay and Inter-assay Precision for Human IFN-gamma and Human IL-8/CXCL8 Using the Human XL Cytokine Discovery Panel (Catalog # LUXLM000). Intra-assay precision was determined by running 27 replicates of a low and high control within a single assay. Inter-assay precision was determined by running low and high controls over multiple independent assays.

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	155	304	606	155	298	592
Standard deviation	4.08	6.62	18.3	11.3	21.8	49.9
CV (%)	2.6	2.2	3.0	7.3	7.3	8.4

Intra-assay and Inter-assay Precision for Human TNF-alpha Using the Human TNF-alpha Quantikine ELISA Kit (Catalog #DTA00D). Intra-assay precision was determined by running 20 replicates of three known samples in a single assay. Inter-assay precision was determined by running 20 replicates over multiple independent assays.

Calibration: Ensuring Consistency from One Lot to the Next

Each Quantikine ELISA Kit and Luminex High Performance Assay includes an assay standard that is calibrated against highly purified material. R&D Systems assigns a mass value to a standard with reference to a master calibrator. Master calibrators are manufactured during the development of a Quantikine ELISA or Luminex High Performance Assay and are used to maintain the consistency of kit standards. All future lots of standards are compared to the master calibrator to ensure that no drift in sample values occurs. Due to differences in how manufacturers assign mass values for standards, sample values produced using one manufacturer's kit may not be directly comparable to those obtained with another manufacturer's kit. R&D Systems supplies the correlation of its standards to WHO international reference materials, when available. This calibration allows a researcher to compare values obtained with a Quantikine ELISA Kit or a Luminex High Performance Assay with values obtained with other assays (if the other assay's manufacturer likewise provides their conversion factor).

Summary

R&D Systems has developed immunoassay kits for nearly 30 years, and we leverage our expertise to manufacture the highest quality products available. Our immunoassay developers are held to stringent assay performance requirements – particularly specificity, sensitivity, precision, accuracy, and reproducibility. Our attention to these assay parameters allows investigators to focus on generating high-quality data rather than spending their time and resources on rigorous assay development. The development of robust immunoassays is a complex process – let us do what we do best so you can do what you do best!

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