4 FUNDAMENTALS OF IMMUNOASSAY QUALITY

RECOVERY, LINEARITY, SPECIFICITY & CONSISTENCY

bio-techne®

ARE YOU CONCERNED ABOUT THE QUALITY OF YOUR IMMUNOASSAY, AND REPEATABILITY OF YOUR RESULTS?

HOW DO WE ENSURE YOU GET ROBUST, REPRODUCIBLE, AND RELIABLE RESULTS?

R&D Systems has over 30 years expertise developing and manufacturing ELISA kits that we have leveraged into expanding our immunoassay offerings so you can find the best fit for your workflow. We pride ourselves on the high level of quality we deliver to help you advance research faster. All R&D Systems assays deliver quality at the core; having been built with our own proteins, antibodies, and specialized diluents.
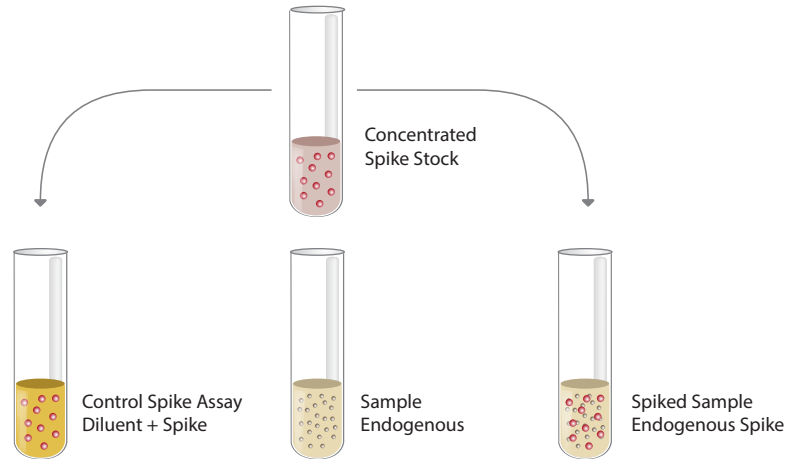
During development, an immunoassay, including components and individual assay steps, goes through many levels of screening to ensure that it accurately and reproducibly measures the target protein of interest, with stringent quality control measures to ensure consistency over time

Before we dig in, lets first cover some basics on complex sample matrices, such as serum and plasma. A sample matrix is an accumulation of anything in your sample that can interfere or cross-react, affecting the ability to accurately quantify the target of interest. Some examples include protein binding partners, rheumatoid factors, anti-animal antibodies, albumins, pH, or salt concentration. Controlling these factors is key to developing accurate immunoassays and we accomplish this through our specialized diluents.

RECOVERY

$$\% \text{ Recovery} = \left(\frac{\text{Concentration}_{\text{CONTROL SPIKE}} - \text{Concentration}_{\text{ENDOGENOUS}}}{\text{Concentration}_{\text{CONTROL SPIKE}}} \right) \times 100$$

Concentrated
Spike Stock

Control Spike Assay
Diluent + Spike

Sample
Endogenous

Spiked Sample
Endogenous Spike

Recovery, or Spike Recovery, is a measurement of accuracy. A spike recovery study provides an estimate of how much of the available analyte is measurable in the assay and if there may be sample components interfering with that measurement. R&D Systems assays are optimized for recovery at three different spike concentrations (low, mid, high) and for every validated sample type, so you can run your assay with ease.

To perform spike recovery, a concentrated recombinant protein, typically the standard, is spiked into a complex sample matrix, such as serum or plasma. A control spike sample is the same amount of recombinant protein spiked into the diluent.

The readout of the spike into the complex sample, minus endogenous, should be equal to the control spike. 100% is ideal. If your sample under-recovers, or falls below 70-80%, depending on the assay, this is indication that something in your sample is causing interference with the assay.

When multiplexing, such as in Luminex assays, achieving acceptable recovery for all analytes is very challenging, but each R&D Systems Luminex panel is optimized to achieve superior performance.

1   Percent Recovery of Serum in Luminex Panel

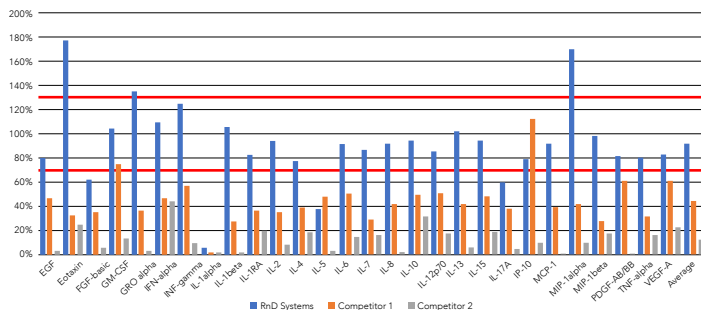### 2   Average Recovery in Quantikine QuicKit ELISA

FIGURE 1. Human Luminex Performance Panel Recovery Competitor Comparison. Human XL Cytokine Luminex Performance Panel (Catalog #FCSTM18) run against similar cytokine panels from two leading competitors. Average percent recovery is shown for all common analytes. Red bars indicate design specifications for High Performance Luminex assays (70%-130%).
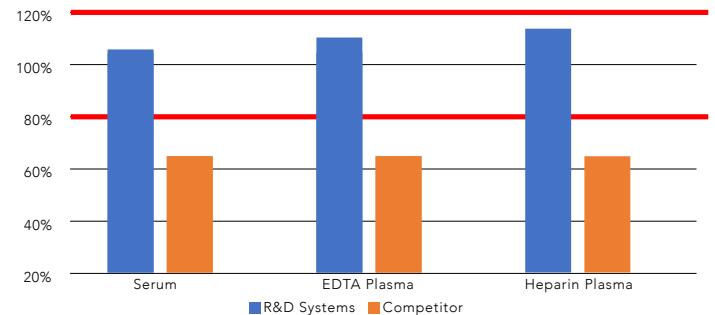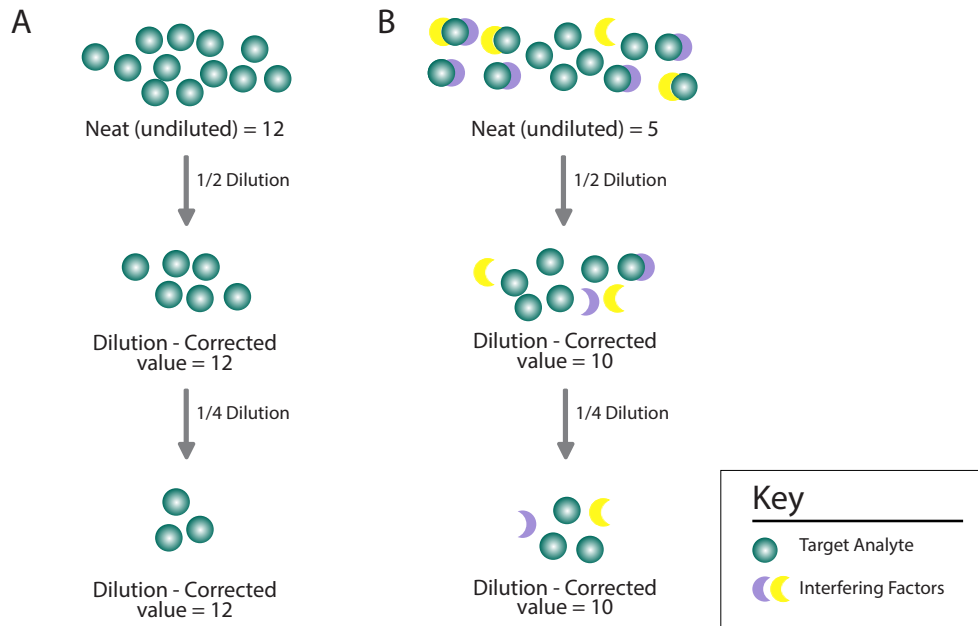
FIGURE 2. Human Quantikine ELISA Recovery Competitor Comparison. Average spike recovery run on R&D Systems Human IL-1 beta Quantikine QuicKit ELISA (Catalog #QK201) and a leading competitor ELISA. Red bars indicate design specifications for single analyte Quantikine ELISAs (80%-120%). Average recovery of R&D Systems QuicKit for serum and plasma samples meet performance specifications, in contrast the competitor assay does not, showing a sample matrix effect.

LINEARITY

A

Neat (undiluted) = 12

↓ 1/2 Dilution

Dilution - Corrected value = 12

↓ 1/4 Dilution

Dilution - Corrected value = 12

B

Neat (undiluted) = 5

↓ 1/2 Dilution

Dilution - Corrected value = 10

↓ 1/4 Dilution

Dilution - Corrected value = 10

Key

● Target Analyte

◐ Interfering Factors

Linearity is another important fundamental of accuracy and specificity. No matter what dilution samples are run at, you should always derive the same concentration once back calculated, giving you consistency in results. If the sample matrix is not properly controlled for, sample values often increase as you perform higher dilutions. This is illustrated in the image above. As you dilute the sample, the interfering factors also dilute out, making the target analyte more accessible, and the concentration of the target analyte increases. When your sample has high endogenous levels, natural linearity can be performed by running a 5-fold serial dilution. If endogenous levels are lower, you can spike recombinant protein into your sample and dilute it out, which is commonly referred to as spiked linearity. In R&D Systems assays, all sample types are optimized for linear dilutions through the standard curve range.

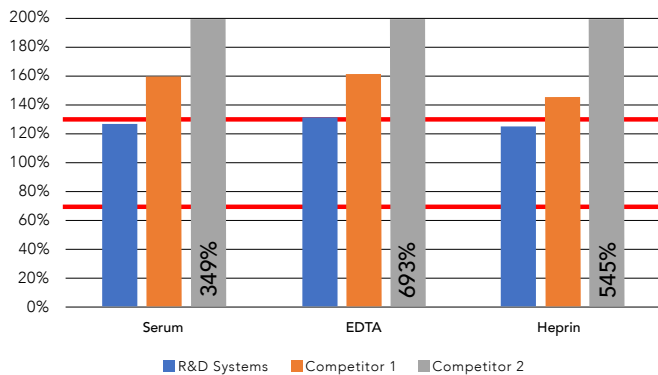**3**   Average Spike Linearity in Luminex Panel

FIGURE 3. Human Luminex Performance Panel Linearity Competitor Comparison. Serial dilutions of serum and plasma samples in the Human XL Cytokine Luminex Performance Panel (Catalog #FCSTM18) and two competitor panels shown with average percent linearity for all dilutions. R&D Systems meets the acceptable range (70%-130%) for Luminex Performance assays. In contrast, the competitor panels did not, showing matrix effects.
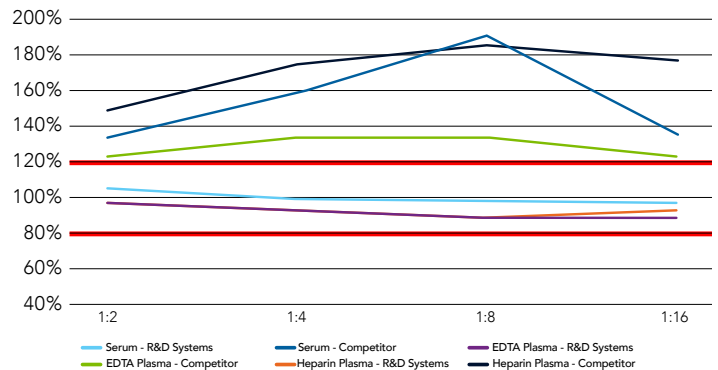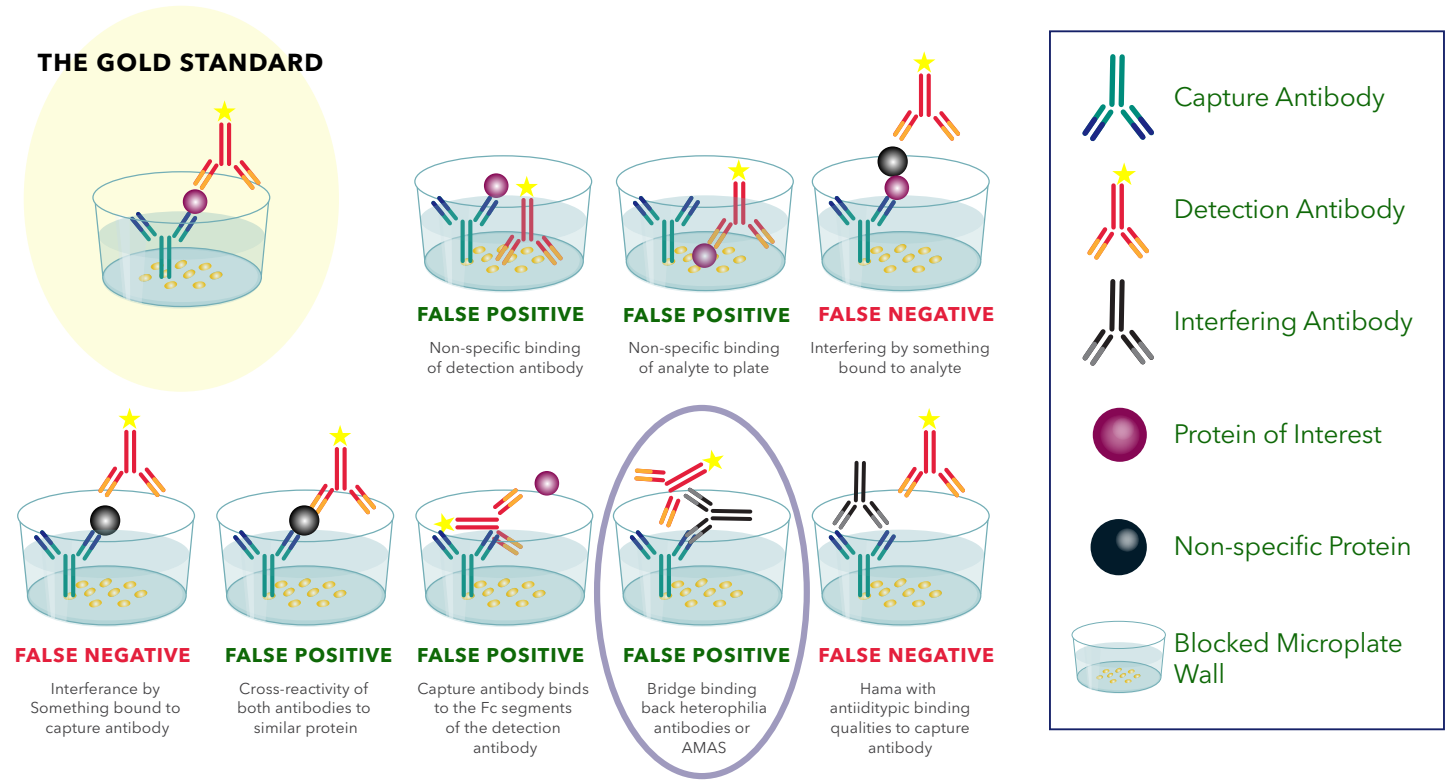
4   Spiked Linearity in Quantikine QuicKit ELISA

FIGURE 4. Human TNF-alpha QuicKit Spiked Linearity Competitor Comparison. TNF-alpha is spiked at high concentration in various sample matrices, then diluted with the appropriate calibrator diluent and run on the Human TNF-alpha Quantikine QuicKit ELISA (Catalog #QK210). R&D Systems ELISA meets acceptable range (80%-120%). The competitors do not, showing matrix effects.

SPECIFICITY

THE GOLD STANDARD

FALSE POSITIVE	**FALSE POSITIVE**	**FALSE NEGATIVE**
Non-specific binding of detection antibody	Non-specific binding of analyte to plate	Interfering by something bound to analyte

FALSE NEGATIVE	**FALSE POSITIVE**	**FALSE POSITIVE**	**FALSE POSITIVE**	**FALSE NEGATIVE**
Interference by Something bound to capture antibody	Cross-reactivity of both antibodies to similar protein	Capture antibody binds to the Fc segments of the detection antibody	Bridge binding back heterophilia antibodies or AMAS	Hama with antiiditypic binding qualities to capture antibody

Legend:
- Capture Antibody
- Detection Antibody
- Interfering Antibody
- Protein of Interest
- Non-specific Protein
- Blocked Microplate Wall

One of the most important features of an immunoassay is that you are measuring the correct intended analyte, so you can have confidence in results and move your research forward. Immunoassays are, by nature, subject to several potential interferences from components in complex samples like serum or plasma. The nature of these interferences can be both positive and negative as shown in this graphic, but all result in an inaccurate values. The burden is on the assay manufacturer to recognize these and correct them, but not all manufacturers are equal. Removing false positives and false negatives are key to obtaining the correct specificity. R&D Systems verifies specificity through multiple measures, but here we will demonstrate the lower right panel: false positives due to bridging from endogenous antibodies.

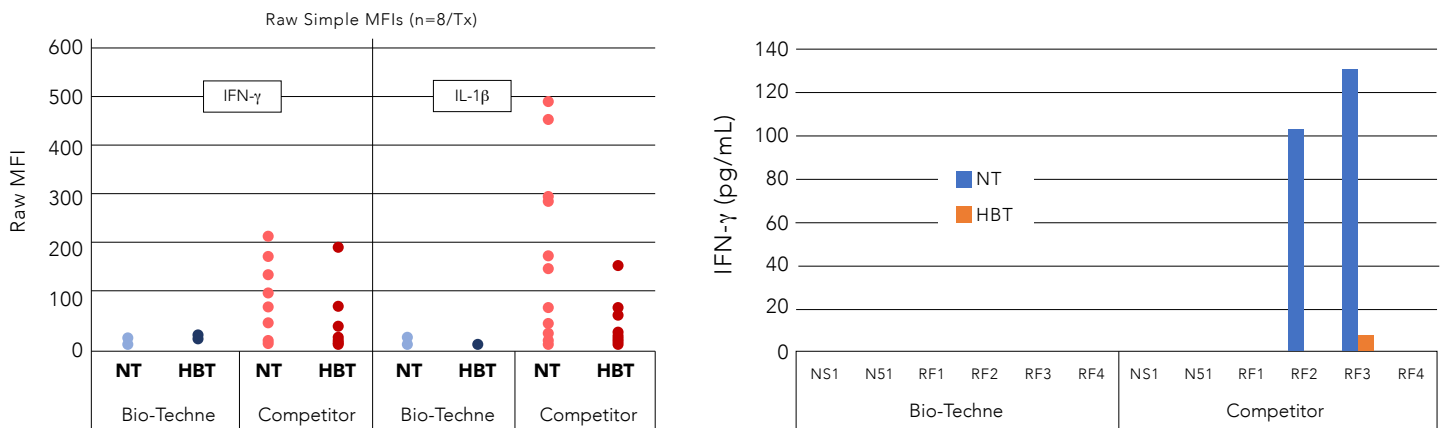5

Removal of False Positives in Luminex Panel

FIGURE 5. Removal of False Positives. To demonstrate the false positive phenomenon, we acquired samples from patients with known values of rheumatoid factor (RF) or HAMA (human anti-mouse antibodies) both of which are known to be potential interferants in immunoassays. One portion of each sample was treated with a commercial quenching reagent (HBT) designed to block those interferences and the other portion was left untreated (NT). All samples were run in both R&D Systems and in a competitors Luminex assay according to the manufacturers protocol and the data collected. The chart on the above left is a composite graph showing all samples for IFNg and IL-1b. This shows that the competitor's assay is prone to false positive interference, but the R&D Systems assay is not. The chart on the right is a dramatic example of the effect it can have on data where the blue bars represent false positive values in the competitor's assay, but not in the R&D Systems assay.

Quantikine Serum Values

Human GDNF Concentration (pg/mL)

Legend: ■ Quantikine Diluent   ■ Quantikine Diluent w/o Blockers

Competitor A Serum Values

Human GDNF Concentration (pg/mL)

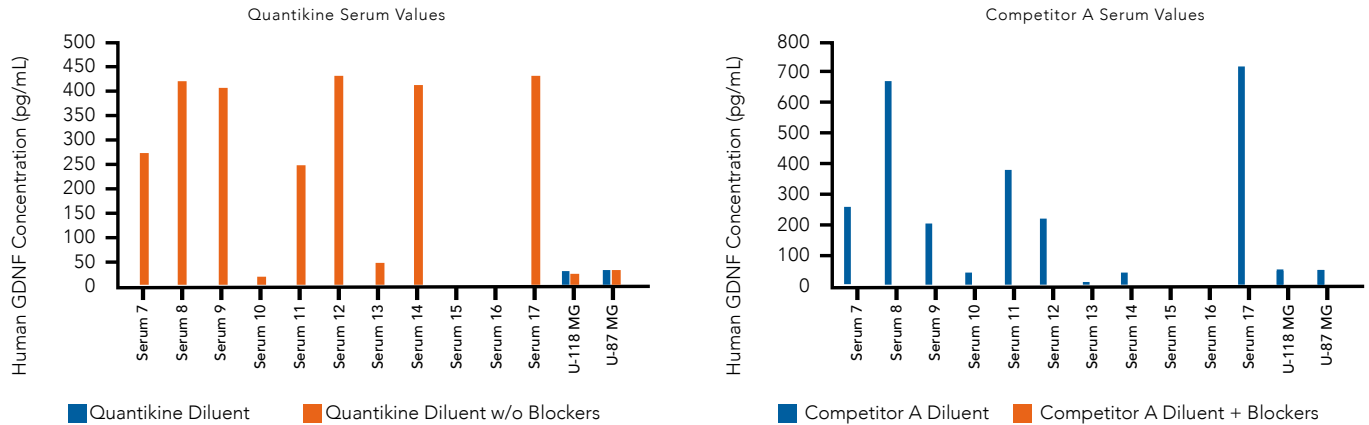Legend: ■ Competitor A Diluent   ■ Competitor A Diluent + Blockers

FIGURE 6. The importance of blocking reagents when detecting human GDNF. Blocking reagents are commonly used in ELISA kits to reduce interference from proteins in samples which may produce false positive results. In this experiment, we ran our human GDNF Quantikine ELISA (Catalog #DGD00) with our Quantikine diluent which contains blockers and with the Quantikine diluent with blockers removed. The absence of our proprietary blocking reagents resulted in false positives in 9 of 11 samples using the Quantikine ELISA (8A). Conversely, the same samples were tested on a competitor Human GDNF ELISA with their provided diluent and with their diluent plus our proprietary blocking reagents. There were 9 of 11 false positives using the Competitor A ELISA as provided, there were no false positive results in Competitor A's ELISA when our blocking reagents were added to the diluent (8B). Note that Competitor A lacked the sensitivity needed to detect GDNF in positive control supernates from U-118MG and U-87MG cell cultures.

CONSISTENCY

R&D Systems assays ensure lot-to-lot consistency, enabling long-term research studies. Extensive quality control testing is done on each kit component and each complete kit to make sure standard curves, background, and sample values remain consistent.

In particular, master calibration is used to prevent variability over time due to differences in protein immunoreactivity. Each new standard lot is calibrated against a master calibrator which is manufactured during assay development. This, combined with our stringent manufacturing and quality control measures, ensures reliable and reproducible results between kit lots and prevent drift in sample values.

7   Consistent Control Values in Quantikine® ELISAs

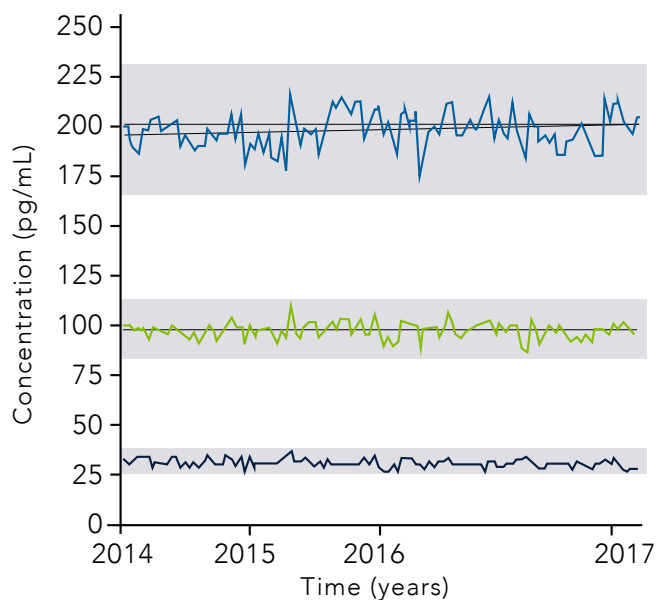**8   Consistent Control Values in Luminex Panel**

FIGURE 7 Consistent Control Values in Quantikine® ELISAs. High (blue line), medium (green line), and low (dark blue line) controls are assayed with every manufactured lot of the Human IL-6 Quantikine® ELISA Kit (Catalog #D6050). Controls for the Human IL-6 Quantikine® ELISA Kit fall within acceptable ranges (gray bars) and remain consistent from lot to lot.
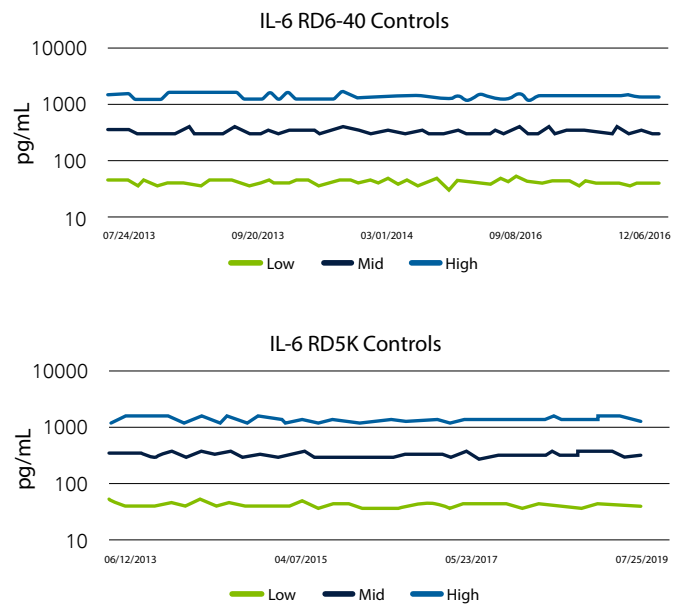
FIGURE 8 Consistent Control Values in Luminex Panel. High (blue line), medium (dark blue line), and low (green line) controls are assayed with every manufactured lot of the Human IL-6 Performance Luminex Assay (Catalog# LUXLM206). Controls for the Human IL-6 Luminex Assay remain consistent from lot to lot over the course of 6 years.

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