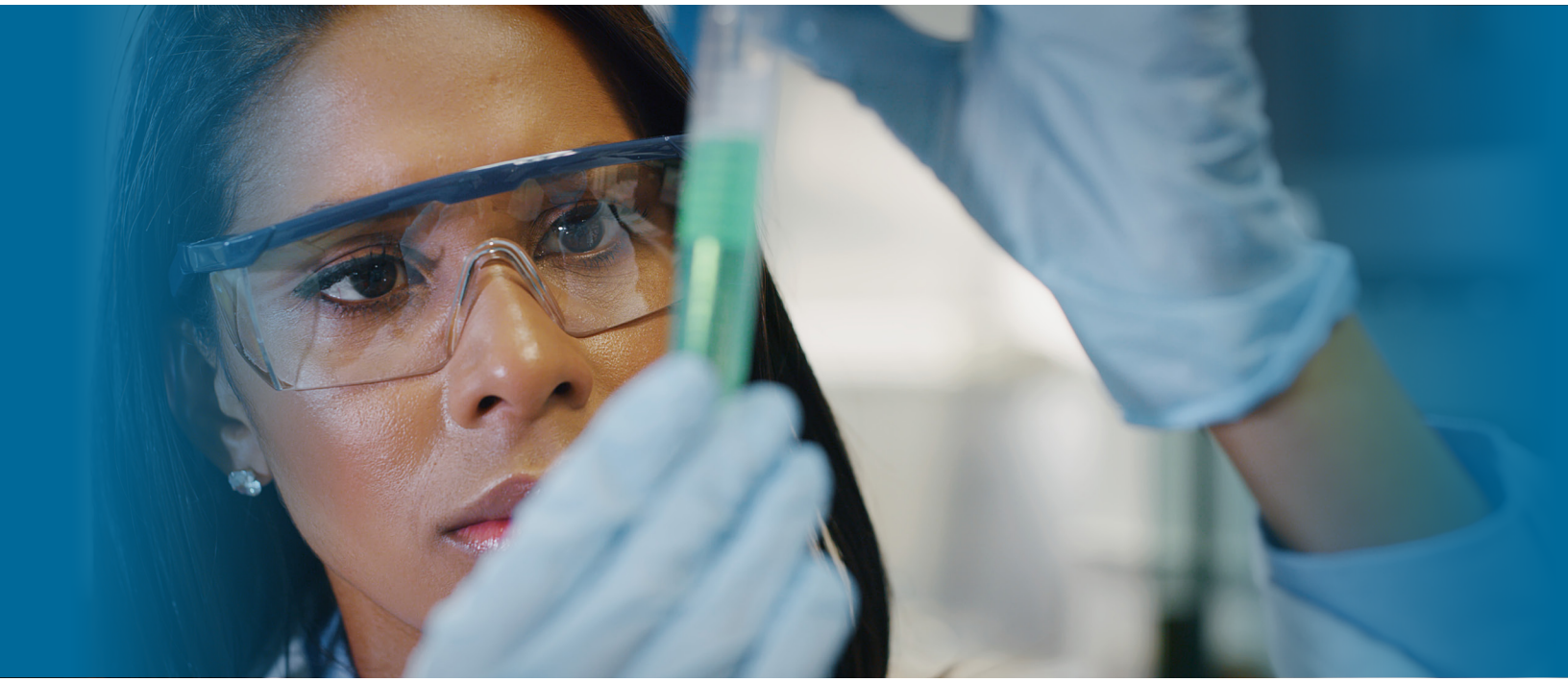
RULES BASED MEDICINE
an IQVIA business



Expertise in assay development, validation and manufacturing with built-in quality controls

Background

With more than 25 years of experience, Rules-Based Medicine (RBM) develops, validates and manufactures its assays with integrity and commitment for the most reliable, reproducible and highest quality assays. RBM was originally established by the founders and innovators of the globally recognized multiplexing technology, xMAP®, from Luminex. Understanding the clinical and research impact of this technology for drug discovery and development, RBM pioneered multiplexing assay development, starting with cytokines and chemokines and expanding to clinical and disease-relevant biomarkers.

Assay validation process

1. **Performance requirements**
   Feasibility to determine if assay will meet specifications.
2. **Singleplex development**
   Combinations of antibodies tested to find the pair with best sensitivity, specificity and dynamic range.
3. **Multiplex development**
   Assays are multiplexed according to the concentration of analyte in the matrix of interest.
4. **Blocking interfering substances**
   Elimination of non-specific interfering signal with proprietary blockers.
5. **Control development**
   Three-level controls that span the range of the standard curve.
6. **Assay validation**
   Validation standards guided by the Clinical Laboratory Standards Institute (CLSI).

Assay development

Singleplex

Every immunoassay developed at RBM begins as a standalone singleplex assay. First, multiple antibodies and antigens are sourced from reputable manufacturers, and after screening, the optimal reagents are selected. The goal is to provide the assay sensitivity and dynamic range necessary to measure the analyte in a biological fluid such as serum or plasma. For immunometric assays, a combination of two antibodies is selected by identifying the pair of capture (covalently attached to the microsphere) and reporter antibodies that best complete the sandwich-capture assay. For competitive-inhibition immunoassays, a single antibody and antigen are required. Once the antigen and antibodies are chosen, a standard solution is prepared that provides an eight-point calibration curve. If a viable assay that meets sensitivity and dynamic range requirements is developed, then it is reserved for multiplex development.

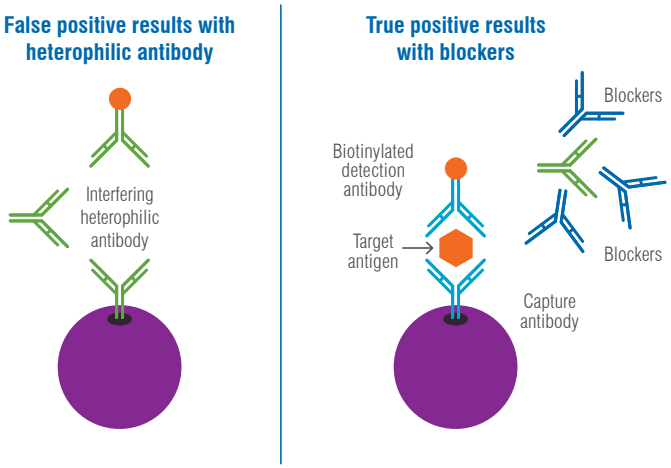
Multiplex

Assays are multiplexed according to the concentration of analyte measured in the matrix. This will drive the multiplexing of analytes with similar concentrations into specific multiplexes. The minimum dilution in serum/plasma is 1:5 and is commonly used for low concentration analytes such as cytokines and growth factors. Some analytes at higher concentrations may require greater dilution up to 1:200,000.

Blocking buffer

Each assay includes a blocking buffer developed for the analytes in the multiplex and optimized to prevent nonspecific binding and matrix effects from rheumatoid factor, human anti-mouse antibodies (HAMA) and other interfering substances. Without appropriate blocking buffers, assay results are subject to inaccurate measurements, both false positives and false negatives (Figure 1). RBM's proprietary blocking buffers mitigate false positives and false negatives and are critical for accurate results.

Figure 1
Blockers mitigate complex matrix interference issues associated with immunoassays



Standards

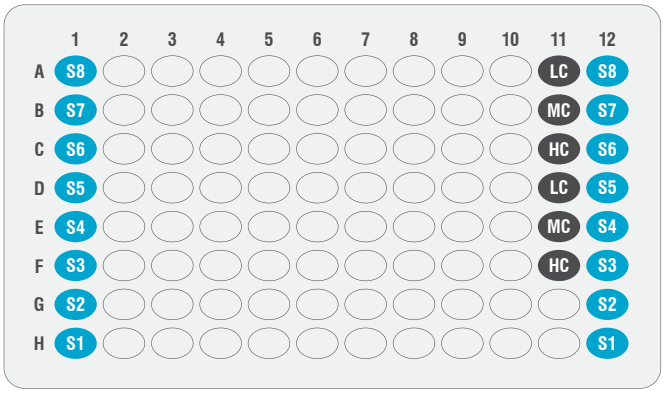
A series of standards at a range of concentrations are used to determine the concentration of the target analytes. The standards are added to a surrogate matrix that closely resembles human serum or plasma. RBM standard curves are generated using eight standards analyzed in duplicate and included with each sample plate (Figure 2).

Controls

Controls are used to monitor the performance of the assay and are included with each plate of samples. Three levels of controls, which fall on the low, mid and high parts of the standard curve, are formulated for each kit. The target proteins are added to human serum to mimic the matrix of the samples being tested. Each control level contains all the multiplexed analytes in the kit.

Figure 2: Sample plate layout

Standards placed in the first and last column of the reaction are processed alongside samples, enabling rapid detection of any inconsistencies.



Manufacturing

The final stage of kit manufacture is highly controlled, guided by a batching template and two individuals who verify the part numbers, lot numbers and volumes of each component as the kit is assembled. Completed kits undergo correlation assays that bridge the performance of the new lot with previous lots, ensuring that our assays produce the same results across different kit lots. The operating parameters of the new multiplex kit are defined by running the new lot of reagents through our sample testing process multiple times to establish the Lower Limit of Quantification (LLOQ), Least Detectable Dose (LDD) and assay precision of the new lot. Once the kit passes the entire set of acceptance parameters, it is ready to test customer samples.

The completed assay kit that we manufacture has five components:

1. Microspheres (beads)
2. Detection Antibody Cocktail
3. Standards
4. Standard Diluent Blocker
5. Quality controls (three levels)

Control of lot-to-lot variation

It is essential for biomarker testing laboratories to control for and minimize lot-to-lot variation. This is particularly important for long-term studies that will span several months or years, requiring testing with different lots of reagents. RBM has optimized a method for controlling lot-to-lot variation using reference samples. These samples are frozen at -80°C in numerous small aliquots. They are analyzed with each new lot of reagents, and the results are compared to those of the previous lot. Accordingly, the standards or calibrators for each assay are then adjusted to bring the concentration of each analyte within these reference samples to match that of the previous lot. This is necessary as many of the antigens used in our testing as calibrator material are derived from recombinant proteins. These recombinant molecules often quantitate differently in the sample matrix when compared to the native protein. Thus, standardization based on recombinant protein standards alone can often lead to significant lot-to-lot variability. The use of reference samples in the appropriate matrix is the preferred choice for controlling the variability of multiplexed immunoassays.

Quality processes in testing your samples

Phase 1 Instrument QC → **Phase 2** Standard curve QC → **Phase 3** Assay QC → **Phase 4** Sample QC

Robust curve fitting

We have developed our own curve-fitting routine that uses weighted and unweighted four- and five-parameter equations. This routine is based on a proprietary algorithm specifically designed to accommodate the difficult-to-fit points at the low end and high end of the curve, providing a more accurate readout of the data.

Figure 3: Standard curve fitting
Our curve-fitting methods are tailored to include the "difficult-to-fit" points at the low and high ends of the curve.

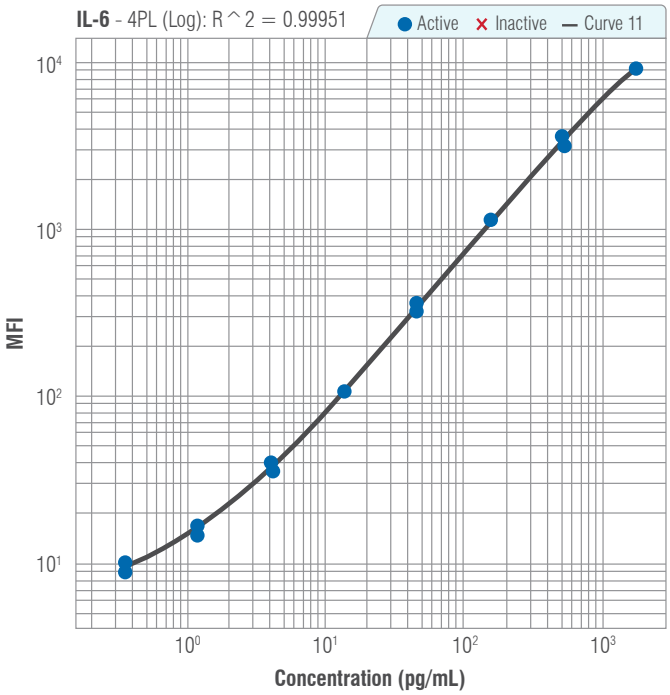
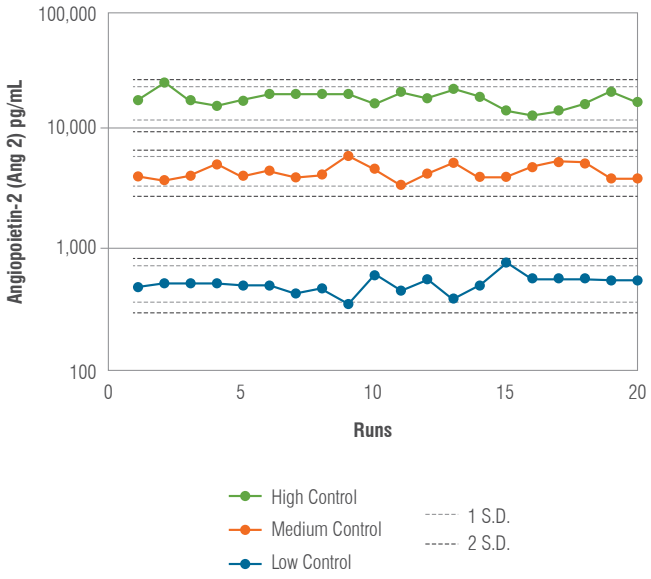


Figure 4: Sample Levey-Jennings chart
Our assay QC uses 3 levels of controls with known values. A Levey-Jennings chart is constructed for easy assay to evaluate assay performance over time.



Validation

Validation is guided by immunoassay principles as defined by the Clinical Laboratory Standards Institute (CLSI, formerly NCCLS) in a CLIA-accredited laboratory. The parameters include the determination of each assay's sensitivity, dynamic range, linearity, cross-reactivity, and precision. Additionally, we determine if there are any matrix effects in the measurement and investigate the stability of the analyte target over three successive freeze-thaw cycles. All the data — for each analyte in a multiplex — are compiled in a validation document that is available for every multiplex assay.

Conclusion

RBM is committed to the highest level of quality in our products and services. We've structured our assay development, validation, and manufacturing workflow and processes to comply with the highest industry standards for reliable and reproducible data. These standards are essential for ensuring that laboratory practices meet the highest-quality industry standards, which ultimately lead to data you can trust.

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