

AN OVERVIEW OF ASSAY QUALITY SYSTEMS AT RULES-BASED MEDICINE®

QA/QC White Paper

By Brian T. Welsh, Ph.D. and

Dr. James Mapes

Abstract

Stringent guidelines of quality assurance and control (QA/QC) serve as the foundation for dependable and reproducible results in any analytical endeavor. To ensure the highest caliber data, Rules-Based Medicine (RBM) has established a rigorous set of criteria that serve as a guide through every stage of sample processing, from the development, validation, and manufacturing of our assays; to the testing, quality controlling and reporting of the data. Here we highlight step-by-step, the measures implemented by RBM that take Luminex xMAP® technology to a new level of reproducibility and ruggedness. The RBM platform combines the sensitivity and dynamic range of microsphere-based immuno-multiplexing with the precision and dependability of automated liquid handling. Together these features set RBM apart from other biomarker testing labs by offering high quality, cost-effective immunoassay measurements.

Introduction

Rules-Based Medicine (RBM) has over twelve years experience developing immunoassays for a vast array of biomarker targets. These assays are quantitative, multiplexed, and highly reproducible. Started as an internal research project at Luminex Corporation in 1998, RBM has grown into one of the world's leading multiplexed biomarker testing laboratories. Drawing on our knowledge and experience of Luminex xMAP® technology, RBM has developed Multi-Analyte Profiles (MAPs) that measure up to hundreds of biomarkers in a single small sample. This provides an efficient approach to rapidly quantify large numbers of biomarkers from a variety of biological samples. Today RBM is testing thousands of samples each month for our customers and generating millions of individual results each year.

In order to successfully manage operations on this scale, we have instituted Standard Operating Procedures (SOPs) that govern the entirety of the testing process. These SOPs provide sample and data handling requirements that begin with the arrival of samples and end with the final report for the customer.

This document presents insight into our quality procedures implemented throughout our operations including validation, assay development, manufacture and sample testing.

Lab Certification

RBM first earned CLIA (Clinical Laboratory Improvement Amendments) accreditation from the Commission on Office Laboratory Accreditation (COLA) in 2005 and has held this level of compliance in an uninterrupted manner. RBM participates in an external proficiency testing program organized by the College of American Pathologists (CAP) as well as internal proficiency testing. In addition, RBM has been audited by many pharmaceutical, biotech, and contract research organizations for compliance with Good Laboratory Practices (GLP) which is required for pre-clinical studies. These audit teams have uniformly found RBM to comply with current GLP standards.

Assay Development

Single-plex

Every immunoassay that we run was first developed as a stand-alone assay using a single Luminex microsphere set. Typically multiple antibody and antigen reagents are sourced from reputable reagent manufacturers so that a variety of options are explored and the highest quality assay can be developed. The goal is to provide the assay sensitivity and dynamic range necessary to measure that 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 which best completes 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 must be prepared that will provide an 8 point calibration curve. If a viable assay is developed that meets the requirements for sensitivity and dynamic range then it is reserved for multiplex development.

Multiplex

Assays are multiplexed according to the concentration of analyte measured in 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 require greater dilution up to 1:200,000. Validation is guided by the principles of immunoassay as defined by the Clinical Laboratory Standards Institute (CLSI formerly NCCLS). The parameters include 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 short term stability of the analyte target using three successive freeze-thaw cycles. All of these data – for each analyte in a multiplex – are compiled in a validation document that is available for every commercial multiplex.

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 lot of reagents through our sample testing process multiple times in order 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 for testing customer samples.

The completed assay kit that we manufacture has five components:

- Microspheres (beads)
- Detection Antibody Cocktail
- Standards
- Standard Diluent Blocker
- Quality controls (3 levels)

Our Sample Testing Process

We have spent years developing multiplexed assays, optimizing the Luminex platform, and automating our sample testing process. It is important to keep in mind that RBM's methodology and biomarker assays are far more advanced than off-the-shelf Luminex bead reagents that are commercially available. This includes a custom-fit, 8-point standard curve, and multilevel controls that are run with each and every assay. In addition we have developed a tightly controlled sample handling and flow process that is customized to our automation processes with quality control checks built into every stage.

Figure 1 is an overview of our sample testing process which is explored in more detail in the following sections.

Figure 1 - Sample Testing Process Flow

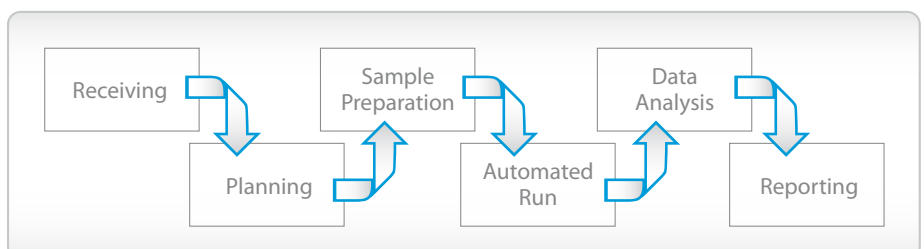


Figure 3A - Schematic of Capture Sandwich

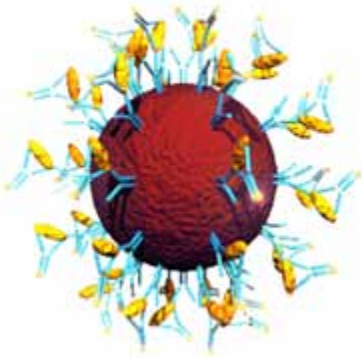
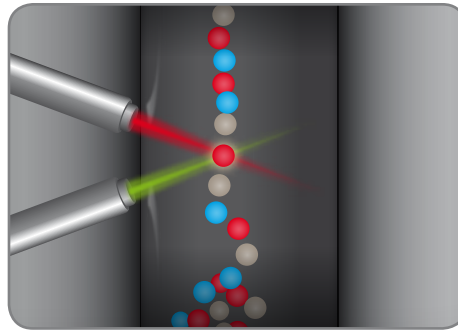


Figure 3B - Detection of Analyte Concentrations



In fact, our data analysis and verification software requires the detection of a minimum of 50 beads per analyte per sample. The median fluorescence intensity (MFI) value of the measured beads is then derived for each protein in the multiplexed assay. Our use of the median reduces the impact that data outliers (i.e. bead measurement errors) may have on the results. These methods allow for more precision than planar array multiplex platforms which look at only one or two “spots” (bead equivalents) per sample and may therefore suffer from spotting reproducibility issues.

Once the beads from each sample are measured and the plate has been completely read, the data are processed, reviewed, and used to create the customer’s report.

Establishing the Standard Curve

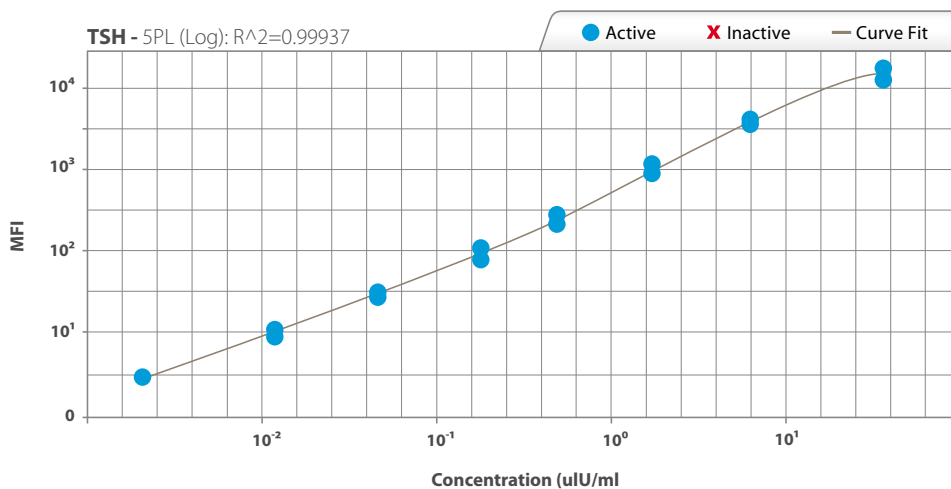
The standard curve serves as the basis for calculating analyte concentrations from customer samples. For each multiplex, calibrator standards are placed in the first and last column of the reaction plate and run alongside the samples. This flanked placement helps control for issues that may arise as the plate is processed because it allows us to detect discrepancies between the duplicate standard values.

During the automated run, the high concentration standards (loaded during the sample plating) are serially diluted to produce two sets of eight-point calibrators that incorporate every analyte in the multiplex. After the plate has been read, this dual set of standard concentration values is fitted using our proprietary curve-fitting routines as seen in **Figure 4**. Our algorithms use four and five parameter equations to produce the best description of the standard values and are specifically tailored to include the “difficult-to-fit” points at the low and high ends of the curve.

Each fit is then visually inspected to ascertain whether or not the data reduction has produced a smooth curve while simultaneously maximizing goodness of fit. We have spent years optimizing our curve-fitting methods and we frequently achieve R-squared values > 0.99. This gives us great confidence that we can accurately quantify the proteins within customer samples.

The next step in assessing the quality of the run is the analysis of the controls. These carefully constructed specimens serve as the best comparison to the samples themselves and by closely monitoring our controls we can continuously track the assay’s performance.

Figure 4: Establishing and Fitting the Standard Curve



WHY YOU SHOULD TRUST RBM WITH YOUR DATA

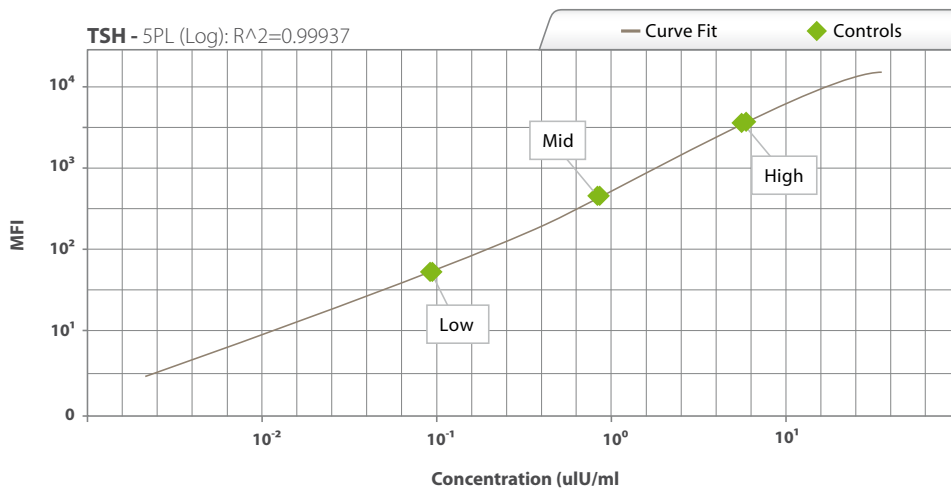
- All data is stored on our local servers which are backed up on-site, daily
- Weekly off-site backups to tape drives gives extra data security
- For GLP studies, we commit to safely archiving your data for up to 10 years

Evaluation of the Controls

All of the tests that we run have unique sets of controls for each of the analytes within the multiplex. These controls are developed in-house to mimic the sample matrix or type, creating a realistic background for our measurements. For the majority of our tests we use native proteins as controls, preferring the use of natural rather than recombinant proteins in our assays. We have found that the benefits of using native controls are two-fold: better assay performance over time and a greater specificity in measuring native proteins within samples.

The three-level controls (low, mid, and high concentrations) can be thought of as supplying known data points across the concentration range that we have already defined by the standards. Measured in duplicate for every analyte, the controls are plotted along the standard curve (**Figure 5**).

Figure 5: Fitting the Three-Level Controls to the Standard Curve



We use the control values to construct a Levey-Jennings chart that follows the assay performance longitudinally. For example, **Figure 6A** shows the average concentration of the low level control for the TSH analyte across 20 runs, with dashed lines indicating the standard deviation (SD) cutoffs around the analyte mean. Charts such as these offer information on lot trends, serve as the basis for the control run's acceptance criteria, and are a standard method of tracking clinical diagnostic immunoassays. Our tests have an exceptionally low intra-assay coefficient of variation (CV), typically less than 10%. The example in **Figure 6B** shows the % CV of all the controls (189 analytes x 3 control levels) for the Human DiscoveryMAP® over 15 runs.

We have adopted a set of modified Westgard rules (<http://www.westgard.com/index.php>) to evaluate the control data in the context of the Levey-Jennings charts. Westgard rules are a set of multirule QC decision criteria that are used to determine whether or not an assay is functioning as expected. These rules alert us to such things as anomalies in individual control value levels, systematic problems among or within the controls, and potentially unwanted trends in the data. We use custom spreadsheets that analyze the control data from every run and automatically alert us to control values that are out of range. The controls must pass all of the Westgard QC criteria to be considered valid and we only report results from assays that meet this standard.

Assuming that the controls have all been verified, the final report is nearly ready to be created. All that remains now is the determination of the sample concentrations and a thorough inspection of the data.

Figure 6A - Example Levey-Jennings Chart for TSH Analyte Over 20 Runs

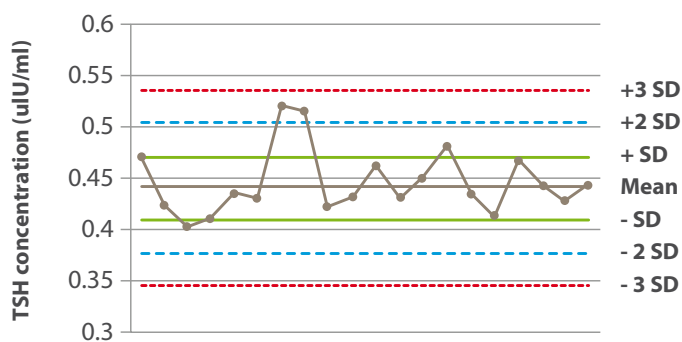
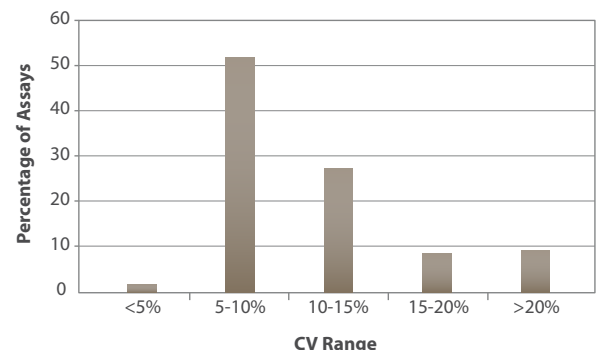


Figure 6B - Coefficient of Variation for Controls from DiscoveryMAP (n=568)



Determining the Sample Concentrations

The final step in the quality process is the calculating and reporting of the sample data. We begin by plotting the MFI for each sample along the standard curve to derive the protein concentration (Figure 7). In this example, each individual green mark on the graph represents the MFI measured from TSH-specific beads from a single sample in a run. The corresponding concentration determined for that sample can then be adjusted by the appropriate dilution factor to calculate the ultimate protein concentration.

Once plotted, all of the data is manually reviewed on a sample-by-sample basis for possible issues. Due to our considerable knowledge and experience with the expected results for each multiplex, we often identify problems before they would be reported to the customer.

One of the benefits of multiplexing is that since we measure many analytes within the same sample, we can use proteins that are likely to be present in a particular sample type as a point of reference. Thus the absence of a robust signal from a given marker may indicate reagent or liquid handling issues and prompt us to rerun some of the samples to check the data. Analytes that appear to have atypical results, e.g., the analyte concentrations that fall outside the range of the standard curve, may also be verified through repeat testing. Throughout this entire review process the data file is electronically tracked, recording information about who has viewed, edited or analyzed the file and logging the changes that have been made. This plate/data managing system is a feature that provides a degree of data traceability that is not available in any other Luminex software package. After we are satisfied that the entire run is up to our standards and has passed all of our quality control measures, we produce a final report to deliver the results to the customer.

Conclusion

Our long history of developing and validating assays allows us to provide customers with unmatched expertise in the field of biomarker testing. We have quality control measures in place for each and every step of the process (Figure 8), all in accordance with our strict standard operating procedures. Furthermore, all of the data that we produce is inspected both automatically and manually to validate the standard curves, verify three-level controls, and double-check the sample results themselves. These rigorous measures give us the utmost confidence in the accuracy of the final results that we report to our customers.

Figure 7: Measuring the Unknown Samples along the Standard Curve

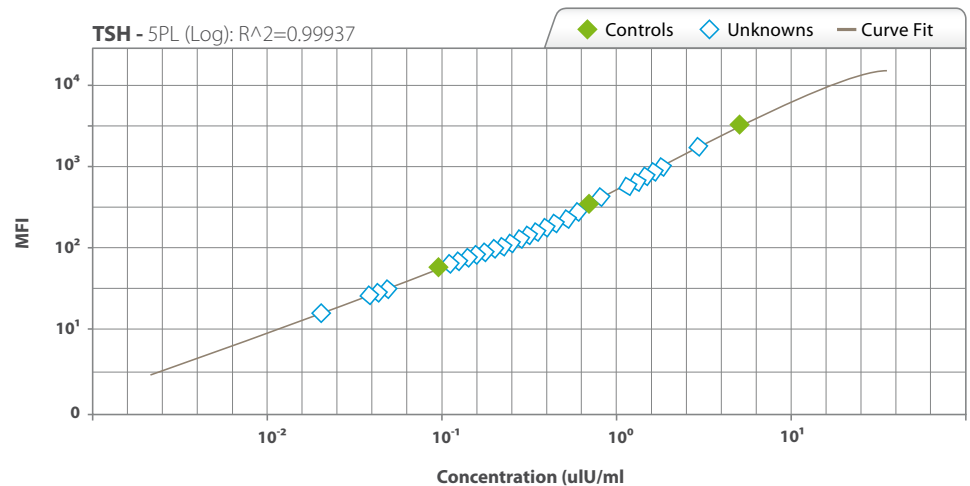


Figure 8: Summary of RBM's Testing Process

