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Belysa™ Immunoassay Curve Fitting Software

Monitoring Immunoassay Variability by Comparing Calibration Curves

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Belysa™ Immunoassay Curve Fitting Software (Cat. No. 40-122) was designed and built in-house, stemming from the need for researchers to be able to quickly and easily evaluate the performance of an immunoassay. Firstly, the individual plate, and then more broadly in comparison to other plates within a study. To address this latter point, this technical note looks at the application of the mathematical Parallelism tool contained within the software and demonstrates its functionality in evaluating multiplate data.

The Parallelism tool is found within the software's Relative Potency experimental feature. It measures the mathematical similarity of two or more curves, one of the founding calculations for relative potency (which is used to compare the performance of two lots of reagents within the same assay). In the case of an immunoassay, these curves are normally created by the company or laboratory that has designed the assay, from recombinant calibrator material (aka the standard curve). Assessing the curves' statistical similarity allows the scientist to have confidence that their method was correctly executed and reproducible as the relationship between the signal from the assay and the slope of the curve has remained consistent throughout the experiment.

When using this function, the first curve added to the tool is considered the reference curve with each subsequent curve added to the function being contrasted against it to generate a Slope Ratio with an ideal value of 1. Acceptable deviation from this number may be positioned within a laboratory's own acceptance criteria, however our internal acceptance criteria is a ratio change of +/- 0.1 when undertaking a comparison.

Our method adopts the parallel line model as, described by Bortolotto et al, essentially comparing the linear portions of each curve against that of the curve defined as the reference. The automatic method used by the software for this calculation is outlined in the user manual (page 94). As the end user builds their curves into the tool, the Belysa™ software will plot them on the same graph as a visual aid under which the Slope Ratio will also be displayed. **Figure 1** shows this function utilzed for a multiplexed Luminex® assay, however it will also both work for individual assays (e.g. ELISA or SMCxPRO™ instruments).
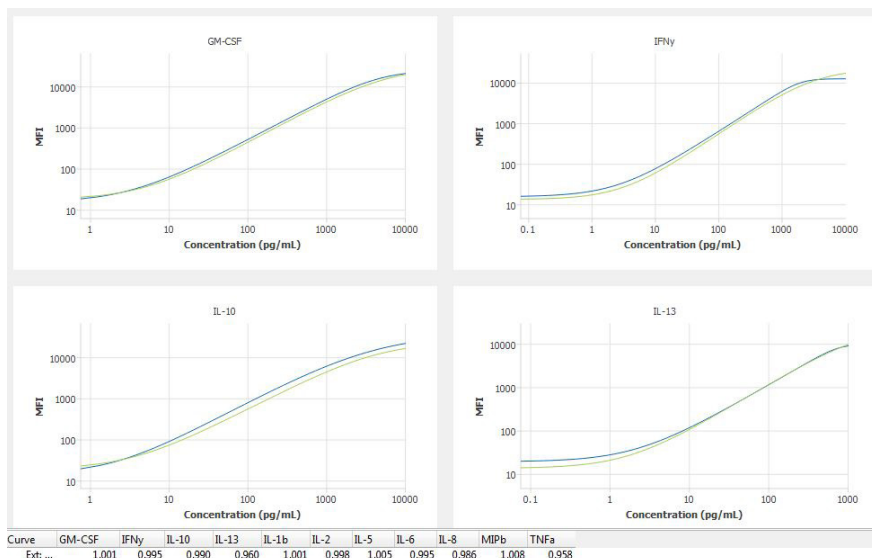

Figure 1. A screenshot of Belysa™ software demonstrating a MILLIPLEX® multiplexed cytokine assay with two sets of calibrators compared and the ratios displayed at the bottom of the curve.

Curve	GM-CSF	IFNy	IL-10	IL-13	IL-1b	IL-2	IL-5	IL-6	IL-8	MIPb	TNFa
Ext: …	1.001	0.995	0.990	0.960	1.001	0.998	1.005	0.995	0.986	1.008	0.958

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Some examples to demonstrate the utility of this function are described below.

1. The Perfect Curves

Figure 2 demonstrates more or less the perfect outcome of this calculation. In this example, the end user, a contract research organization (CRO), purchased a set of assays twice in the course of a multi-year study and looked to compare the two. This is significant as over time the components between lots had been remade. An important consideration was that the analyst had stayed the same. Visually it is clear that the ratio between the curves is going to be tight ,which is reflected in the slope ratio of 1.001. This allowed the scientist to confirm that despite changes between lots the method had remained consistent between the two plates for this analyte.
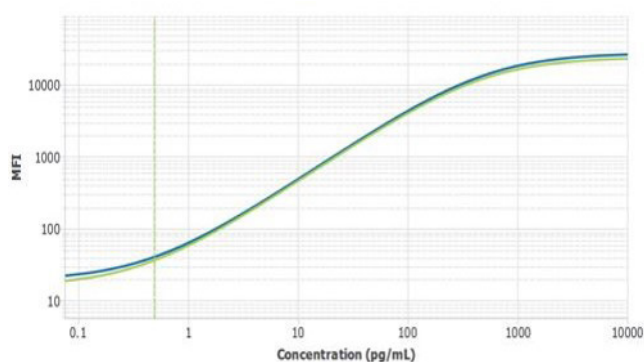

Figure 2. Two IL-1β standard curves from independently manufactured lots of a MILLIPLEX® Multiplex Immunoassay (Cat. No. HSTCMAG-28SK) run by the same operator at a CRO (Slope Ratio = 1.001).

2. The Complex Experiment

Small scale experiments can often be contained within a couple of plates under the care of the same analyst. However, as the sample numbers grow and/or the experiment becomes elongated over time, it is likely new variables will be introduced such as multiple lots of kits with two or more analysts performing the work. While some laboratories will compare the assays individually against a validation run in the manner of the above **Figure 1**, others will collate the data and then examine it in a single batch. By looking at all the curves together in the Belysa™ software and comparing them against a reference curve, the user can see if all the plates ran similarly or if there was a specific deviation. In **Figure 3**, three lots of the same type of ELISA were run by two end users. When the curves were compared with each other it was clear that Plate 2 (lime green line) had run differently to the reference curve delivering a Slope Ratio of 0.866. All of the others fell within the +/- 0.1 acceptance criteria. By performing this analysis, the end user is able to quickly determine that one of the assays has performed differently and if necessary, exclude it or repeat the run to ensure data consistency.
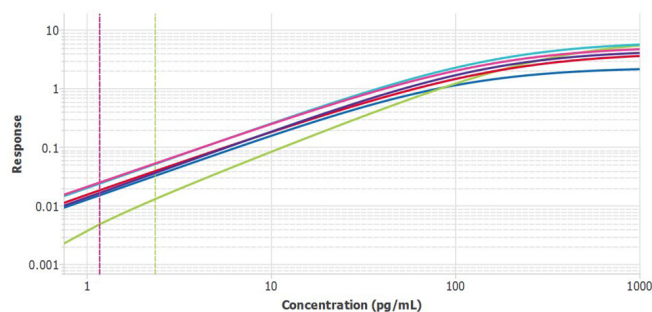

Figure 3. Six ELISAs from three lots run by two users. When compared to the reference curve (dark blue line), the Slope Ratio of 0.866 for Plate 2 (lime green line) indicates an out of conformance assay.

3. Containing the Error

Identifying the source of the error and correcting it can be important both within the context of the experiment but also for the technical capabilities of the lab. The Belysa™ software was able to show that in **Figure 3** the fault was isolated to one user error, allowing the lab to rule out other technical or reagent errors in their work. Firstly, the results between the two users within the same lot were examined.

Figure 4 indicates the deviance between two plates run by two different users A & B. To confirm this was an isolated case, the three lots run by user A **(Figure 5)** were compared to confirm that in their hands each lot of the assay performed consistently.

Having confirmed a that each lot ran consistently in the hands of user A, plates from a both users from a second lot were then compared **(Figure 6)**. The mathematical parallelism tool confirmed the similarity between the assays run indicating that the user error was limited to user B's lot 1 plate.
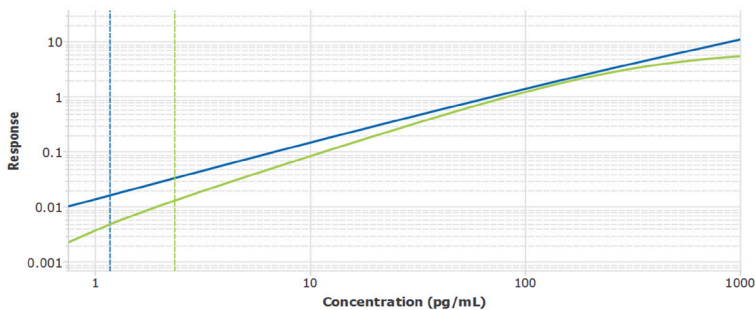

Figure 4. Two ELISAs from the same lot run by users A & B with poor parallelism (Slope Ratio = 0.866). The blue line (user A) was the reference curve to which user B's plate (green line) was compared.

Figure 5 Three individual lots of the ELISA run by a User A with ratios of 1.001 (green line) and 1.035 (red line) when compared against the reference curve (blue line). Having confirmed that the error was unlikely to be at the kit level via the multi-lot reproducibility demonstrated by one of the users, it was important to confirm that this error was a single occurrence by comparing performance in a second lot **(Figure 6)**.
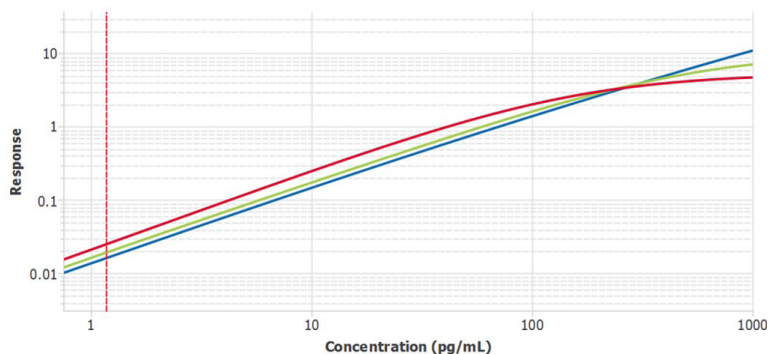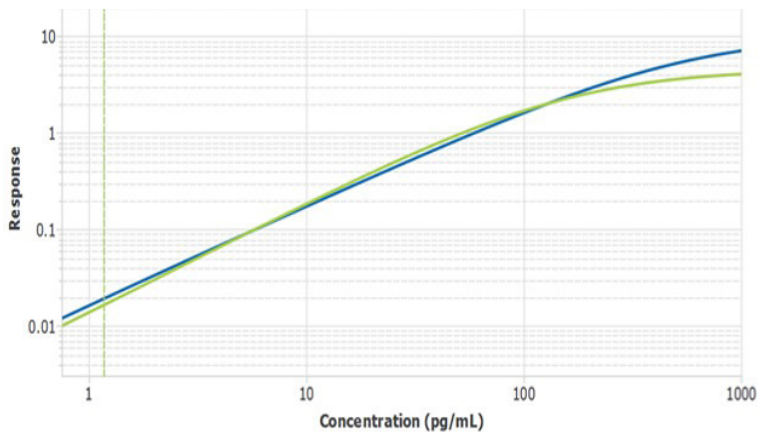

Figure 6. A second batch of ELISAs with two users compared by using one curve as a reference (blue line) for testing the other. The two users achieved a Slope Ratio of 1.004.

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Summary

The mathematical Parallelism tool within the Belysa™ Immunoassay Curve Fitting Software **(Cat. 40-122)** allows the end user to quickly assess multiple plates of an immunoassay to confirm that the standard curves of each performed similarly. Similarity is determined statistically using a Parallel Line model to provide a Slope Ratio of each curve against an initial reference curve. A score of 1 is considered to demonstrate that slopes are statistically similar. This function allows the end user to demonstrate that their method was executed reproducibly either within the same batch of assay or across multiple lots.

References

Bortolotto, Erica, Rousseau Rejane, Teodorescu Bianca, Wielant Annemie, and Debauve Gael. 2015. "Assessing Similarity With Parallel-Line and Parallel-Curve Models: Implementing the USP Development/Validation Approach to a Relative Potency Assay." BioProcess International 13(6).

Acknowledgments

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