Challenges of Multiplex Assay Validation

The word Multiplex originated from Latin and means “having many folds’. In the present era, especially in the field of science, multiplexing has become a trend. Multiplex assays are being widely used to identify large number of biomolecules in a single biological sample. Some of these “multiplex assays” include techniques like ELISA, PCR, Luminex, Quantigene and Meso Scale Diagnostic (MSD). Two White papers that have been published previously by Cambridge Biomedical (Authored by Dr. Sonal Gupta), describe in detail about one of these multiplex assays: Luminex. In this white paper some of the challenges that we face while validating Luminex Assays using guidance provided by FDA will be addressed.

Obtaining Positive Samples

The most important and foremost thing one needs prior to beginning (Luminex) assay validation is, obtaining single samples that are positive for all biomarkers that need to be analyzed in the relevant Luminex assay panel. This may involve screening hundreds of donors before one can obtain a few samples that may be positive for a few biomarkers, if not all. If the aim is to validate inflammatory cytokines then there are published methods that may be used for procuring positive samples[1] for some or most cytokines in a single sample. In spite of following the published methods, one may still need to optimize conditions to obtain quantifiable levels of the cytokines one is interested in. Repositories that possess and are capable of storing positive samples may be the answer to this challenge.

FDA Requirements

The FDA guidance for Bioanalytical Method Validation[2] provides “General” recommendations that need to be addressed during validation of an assay:

> **Accuracy** - accuracy is determined by replicate analysis of samples containing *known amounts of the analyte*. For determining accuracy, a known concentration of analyte is spiked into the sample (plasma/serum) and if the recovery is between 70-130% the result is considered accurate.

Samples used for Luminex assays usually consist of Plasma and Serum. Both plasma and serum is complex in nature and consist of components other than the analyte of interest. These components often cause what is called the “Matrix Effect” which hinders the results quantitatively thus affecting the accuracy of the analyte. It is a general practice to dilute the samples in order to minimize matrix effect and obtain accurate results.

While validating Luminex assays we have observed that a single dilution will not necessarily work with all biomarkers or cytokines in the assay panel. As an example, if we are testing 15 cytokines in a single sample then 6 cytokines may show accurate results at a dilution of 1:2 while the rest may give accurate results at a dilution of 1:5. Thus necessitate running of the same sample at two or more dilutions in

order to obtain accurate results for all the cytokines being analyzed. In such cases, to arrive at a scientifically productive data one may need to either: **a)** validate the **same** sample at **multiple dilutions** and obtain accurate results (which conflicts with the idea of multiplexing) or **b)** run the sample undiluted along with the baseline (pre- treatment) sample in order to evaluate the differences between the pre- and post- treatment.

> **Precision –** the precision of an analytical method describes the *closeness* of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. It is recommended by the FDA that the precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV.

While validating Luminex assays we have noticed that most cytokines fall within the recommended precision of 15% CV. However, there are certain cytokines that consistently fall outside the recommended precision range but show precision at higher acceptable range of 25% CV except at LLOQ where the precision is closer to 30% CV (see Table 1). This suggests that that either we utilize a higher acceptance range for precision (25% except at LLOQ where it is 30%) for all cytokines/biomarkers being evaluated by Luminex, than what has been recommended by the FDA for general bioanalytical methods, or have different acceptance criteria for individual cytokines. The worst case scenario would be avoiding analysis of those cytokines that are outside the FDA recommended 15% CV threshold.

Table 1: Inter Assay Precision of Cytokine 1 and 2

Experiment #	Cytokine 1 (pg/mL)	Cytokine 2 (pg/mL)
Experiment 1	3967.51	4.66
Experiment 2	5200.00	6.12
Experiment 3	3528.52	5.00
Experiment 4	4969.46	5.68
Experiment 5	3141.00	6.88
Experiment 6	4925.21	5.16
%CV	20.05	14.66

In order to avoid using multiple acceptance criteria for different cytokines in the same sample and the same run, we often have to modify the acceptance criteria for Luminex assays to 25% CV except at LLOQ where the precision is 30% CV.

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Lower Limit of Quantitation- According to FDA, the lowest standard on the calibration curve should be accepted as the LLOQ if the lowest standard is *at least 5 times* the response as compared to the blank response.

One of the advertised advantages of Luminex technology is that it has a wider dynamic range than for example ELISA where the ULOQ can be as high as 10000 pg/mL (or even higher) and the LLOQ can be as low as 2-3 pg/mL (or in certain cases even lower) with the manufacturer guaranteed dynamic range of ≥4.5 logs. While validating Luminex assays we have noticed that the FDA recommended "LLOQ be at least 5 times as compared to the blank" **does not always apply to Luminex assays, where the LLOQ is very often only *1.5 to 3 times the blank value (or LOD)***.

Stability- According to FDA, stability testing should evaluate the stability of the analytes after short-term (bench top, room temperature) storage, long-term (frozen at the intended storage temperature), and after going through freeze and thaw cycles.

An analyte is considered stable if the difference between the baseline sample and the stored sample (Relative Error) is within ±30%. We have observed two scenarios:

1. Scenario1 –where cytokines show inter assay precision of 25-30% CV (higher than FDA recommended precision of 15-20% CV).
2. Scenario2- where cytokines have values closer to LLOQ but show inter assay precision at 15-20% (within FDA recommended precision).

In both scenarios, certain cytokines "wobble" from being stable to unstable to stable state. As shown in Table 2, cytokine 1 (*Scenario1*) and cytokine 2 (*Scenario2*) go from being unstable (%RE >30%) to a stable state (%RE <30%) when analyzed at 1 month and 3 months respectively.

Table 2: Stability of Cytokine 1 and Cytokine 2 at -80°C

Cytokine	Time Point	Concentration (pg/mL)	%RE
Cytokine1	Baseline	3967.51	N/A
	1 Month	5200.00	31.06
	3 Month	3828.52	-3.50
Cytokine2	Baseline	4.66	N/A
	1 Month	6.12	31.33
	3 Month	5.00	7.30

Interestingly, when inter assay precision is analyzed between the baseline, 1 month, and 3 month time points, both cytokine 1 (*Scenario1*) and cytoine2 (*Scenario2*) fall within *their* respective acceptance criteria (see Table 3).

Table 3: Inter Assay Precision of Cytokine 1 and Cytokine 2

Cytokine	Time Point	Concentration (pg/mL)	%CV
Cytokine1	Baseline	3967.51	17.43
	1 Month	5200.00	
	3 Month	3828.52	
Cytokine2	Baseline	4.66	14.52
	1 Month	6.12	
	3 Month	5.00	

Therefore, unless trending of the data is observed **we often use both %RE and %CV for making a decision for stability studies, as the perceived failure of stability (1 Month) is in actuality a product of acceptable inter-assay variability.**

Conclusion: Multiplex technologies have become very popular in the present era because of their advantages over the single plex assays. Advantages of multiplexing include high throughput, lower cost, and outmost important, use of smaller sample volume. Due to these advantages, multiplex technologies including Luminex are being widely used in basic research[3] as well as for screening and for correlative assays in clinical trials[4]. Our observations have shown that in spite of their advantages over single plex assays we still need to address challenges that will help us use these new technologies in a regulated environment.

References:

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Archana Kapoor, PhD

Dr Kapoor is a Research Scientist III at Cambridge Biomedical Inc. She is a Molecular and Developmental Biologist and specializes in the field of Host Pathogen Interactions, Enteroendocrine Cell Development and Diabetes. She has extensive experience in Molecular Biology, Immunohistochemistry, Western Blotting, Cell Culture, ELISA, Luminex, Transfections (both stable and transient), PCR and RT-PCR. She worked in these fields at Massachusetts General Hospital, Umass Medical School and Joslin Diabetes Center.

Dr Kapoor holds a PhD in Zoology from University of Delhi, India and Post Doctoral Fellowships at Harvard Medical School and Umass Medical School.