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Application Note

Building a Sandwich ELISA for the 2020s: Lot consistency driven by critical reagent characterization

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Introduction

The immunoassay has undergone many re-inventions over the last few decades, moving from single to multiple analyte detection with increasing levels of sensitivity and specificity. Over time there has been an influx of commercially available kits by numerous vendors. The majority of these kits fall within the Research Use Only (RUO) category and are required to be labeled as such by the U.S. Food and Drug Administration (FDA). However, unlike In Vitro Diagnostic (IVD) assays, there are no regulatory requirements for the validation and confirmation of RUO assays. With no requirement for validation, the burden of responsibility falls to the end-user to evaluate and accept the manufacturer's claims. This has led to a range of commentary by both academic and industrial assay users, reflected in the 2015 paper by Khan et al., who are looking to provide a framework for commercial assay validation in the hands of a researcher.[1]

The depth of an assay's validation is dependent on a biomarker's context of use (COU). As stated in the 2019 C-Path "Points to Consider Document" (Piccoli et al.), "the clinical validation of a biomarker requires an extensive analytical validation of the biomarker's assay".[2] This principle builds upon the "fit for purpose" method detailed in papers such as Lee et al. in 2006 which states that "the key component for this approach is the notion that the assay validation should be tailored to meet the intended purpose of the biomarker, with a level of rigor commensurate with the intended use of the data".[3]

A key aspect of an assay's Analytical Validation is the evaluation of lot-to-lot reproducibility, which has often been traced back to the variability within the assay's components. Papers published by King et al. in 2014 and O'Hara et al. in 2012 indicate strategies that may be used to generate and monitor critical reagents used in ligand binding assays, which could be applied to both in-house and commercial vendors.[4,5]

This application note merges these strategies to develop the Conferma™ IL-6 ELISA, which utilized in-house developed reagents and physicochemical characterization to create reproducible kit lots, which were subsequently independently evaluated at the Core Laboratory for Clinical Studies at Washington University in St. Louis.

Reagent Development and Characterization

In-house resources were used to create a capture monoclonal antibody (mAb), a detection mAb, and calibrator material. Three lots of each mAb and two lots of the calibrator material were then sent to our corporate Center of Excellence for Mass Spectrometry in St. Louis MO, who utilized 1D Gel Analysis, Amino Acid Analysis (AAA), Liquid Chromatography–Mass Spectrometry (LC-MS), Reverse Phase – LC-MS (RP-LC-UV-MS), and Surface Plasmon Resonance (SPR, Biacore™ system), where appropriate, to characterize and compare the reagents (**Table 1**). Note that the detection mAb was analyzed pre- and post-biotinylation, as this process can be disruptive to the performance of an antibody.

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Lot	Calibrator	Capture mAb	Detection mAb
1	028M4878V	RB1811028	RB1811029
2	015M4836V	RB1812003	RB1812002
3	NA	RB1912001	RB1912002

Table 1. Lots of materials tested during the reagent characterization process. A third batch of calibrator material was not available for this study, hence the "NA" in Lot 3.

After conducting 1D Gel Electrophoresis, a densitogram was prepared for each reagent to compare the observed bands (**Figure 1**).

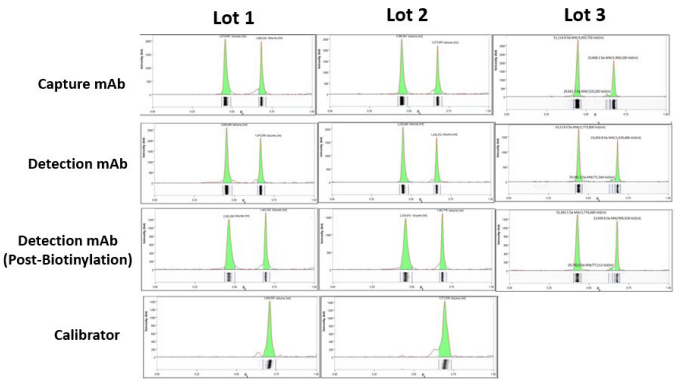

Figure 1. Densitograms for each reagent presented by lot.

The results of this process confirmed that the reagents were of a similar size, but further, the purity of each reagent was greater than 98%. Having confirmed this, three tests were performed on the calibrator material, AAA, peptide mapping, and intact mass analysis. The AAA assay was used to provide an accurate measurement of the material concentrations (**Table 2**). The peptide mapping assay examined each of the two lots for sequence coverage by LC-MS/MS. Sequence coverage of the mature sequence of IL-6 was 81.9% for 028M4878V and 88.5% for 015M4836V (**Figure 2**). The intact mass measurements derived using a second mass spectrometry technique, Reverse-Phased Liqued Cromatography coupled to High Resolution Mass Spectrometry (RP-LC-UV-MS) shown in **Table 3** agree exactly with the mature sequence of IL-6. The peptide mapping and intact mass analyses collectively support that the calibrator material sequence matches the reported sequence for Human IL-6 from the Universal Protein Resource (UniProt) accession # P05231 AA30-212.

		Vial Content (µg/mL)					
No.	Calibrator	Replicate 1	Replicate 2	Replicate 3	Mean	SD	% RSD
1	Lot 028M4878V	140.751	134.053	140.384	138.40	3.77	2.7
2	Lot 015M4836V	154.477	176.465	183.233	171.39	15.03	8.8

*SD = standard deviation; RSD = relative standard deviation

Table 2. Two lots of calibrator, tested using AAA with a National Institute of Standards and Technology (NIST) calibrator (standard reference material (SRM) 2389) and a NIST bovine serum albumin (BSA) standard (SRM 927) for control purposes.

Sequence Coverage for Calibrator Lot 028M4878V
VPPGEDSKDVAAPHRQPLTSSERIDKQIRYILDGISALRKETCNKSNMCESS
KEALAENNLNLPKMAEKDGCFQSGFNEETCLVKIITGLLEFEVYLEYLQNRFE
SSEEQARAVQMSTKVLIQFLQKKAKNLDAITTPDPTTNASLLTKLQAQNQWL
QDMTTHLILRSFKEFLQSSLRALRQM

Sequence Coverage for Calibrator Lot 015M4836V
VPPGEDSKDVAAPHRQPLTSSERIDKQIRYILDGISALRKETCNKSNMCESS
KEALAENNLNLPKMAEKDGCFQSGFNEETCLVKIITGLLEFEVYLEYLQNRFE
SSEEQARAVQMSTKVLIQFLQKKAKNLDAITTPDPTTNASLLTKLQAQNQWL
QDMTTHLILRSFKEFLQSSLRALRQM

Figure 2. Sequence coverage of two lots of IL-6 calibrator material based on LC-MS analysis. The blue highlight shows the identified sequences of IL-6.

With the strong sequence coverage, the material was deemed fit for use as the calibrator material for the assay's standard curve and quality controls. Turning our attention to the mAbs, the RP-LC-UV-MS technique was used to compare the similarity of each lot of mAb by its size in Daltons (Da), accounting for instrument error. Secondly the technique also allows for the calculation of the average number of moles of biotin that have been conjugated to the detection mAb (**Table 3**).

Sample	Measured masses (Da)	Moles of Biotin/moles of antibody
Lot 028M4878V	20977	NA
Lot 015M4836V	20977	NA
IL6.2F2 (Lot RB1811028)	148728	NA
IL6.2F2 (Lot RB1812003)	148735	NA
IL6.2F2 (Lot RB1912001)	148728	NA
IL6.2E3 (Lot RB1811029)	148937	NA
IL6.2E3 (Lot RB1812002)	148934	NA
IL6.2E3 (Lot RB1912002)	148933	NA
IL6.2E3 Bt (Lot RB1811029)	Distribution with Bt=4 at 150744	3
IL6.2E3 Bt (Lot RB1812002)	Distribution with Bt=4 at 150744	4
IL6.2E3 Bt (Lot RB1912002)	Distribution with Bt=4 at 150748	8

*Bt = biotin; Da = Dalton; MS = mass spectrometry

Table 3. RP-LC-UV-MS results per reagent lot compared by mass (Da) and distribution of moles of biotin-conjugated detection antibody.

This data indicates that each of the reagent lots was identical or nearly identical at the Da level, while the biotin comparisons indicate that the third batch of detection mAb had a higher number of incorporated biotin molecules than the prior two. It could be hypothesized that the increased number of biotins in a batch could lead to a curve with a higher optical density (OD), although other parts of the assay can affect this outcome.

The final technique utilized was SPR using the Biacore™ T200 platform. This allows for the understanding of the affinity and activity of each mAb to the calibrator material and ensures that the biotinylation process could be achieved consistently without affecting the binding of the detection mAb. **Table 4** demonstrates

the relationships between each lot of mAb (pre- and post-biotinylation for the detection mAb) and the calibrator material. Each of the mAbs maintained a high affinity against the calibrator with an equilibrium dissociation constant (Kd) <0.5 nM. The activity of each lot of mAb was also consistent. While there was a drop in activity of the detection mAb upon biotinylation, it was minor.

No.	Ligand (Antibody)	IL-6 Calibrator	KD (nM)	% Activity
1	17M4830V-control	028M4878V	0.04	85
2	IL6.2F2 Capture RB1811028	015M4836V	0.13	86
3	IL6.2F2 Capture RB1811028	028M4878V	0.05	84
4	IL6.2F2 Capture RB1812003	015M4836V	0.14	88
5	IL6.2F2 Capture RB1812003	028M4878V	0.06	88
6	IL6.2F2 Capture RB1912001	028M4878V	0.05	93
7	IL6.2E3 Detection RB1811029	015M4836V	0.13	67
8	IL6.2E3 Biotinylated Detection RB1811029	015M4836V	0.11	63
9	IL6.2E3 Detection RB1811029	028M4878V	0.08	70
10	IL6.2E3 Biotinylated Detection RB1811029	028M4878V	0.06	68
11	IL6.2E3 Detection RB1812002	015M4836V	0.11	66
12	IL6.2E3 Biotinylated Detection RB1812002	015M4836V	0.12	64
13	IL6.2E3 Detection RB1812002	028M4878V	0.1	72
14	IL6.2E3 Biotinylated Detection RB1812002	028M4878V	0.1	68
15	IL6.2E3 Detection RB1912002	028M4878V	0.06	83
16	IL6.2E3 Biotinylated Detection RB1912002	028M4878V	0.06	75

Table 4. The Biacore™ T200 SPR comparison of lot-specific affinity (Kd) and activity of each mAb lot against its calibrator lot. Note that the 3rd lot of capture mAb was only tested against two batch of calibrator material.

The overall impression from the SPR comparison was that each generation of mAb was produced with consistently high affinity against the different lots of calibrator material indicating stability in the process. Further, the biotinylation of the detection mAb lots did not seriously impact their relationship either.

Final Kit Testing at the Core Laboratory for Clinical Studies (CLCS), Washington University St. Louis

The R&D group at MilliporeSigma created multiple lots of the IL-6 assays components, performed feasibility tests, and created a protocol. Subsequently, three lots of assays were manufactured from the evaluated lots of components (**Table 5**). The first two lots were made by the R&D group and the third lot was from the manufacturing group at MilliporeSigma in St. Louis, MO. Kits from each lot were then sent to the CLCS at Washington University St. Louis (Wash U).

Material	Lot 1	Lot 2	Lot 3
Capture mAb	RB1811028	RB1812003	RB1912001
Detection mAb	RB1811029	RB1812002	RB1912002
Calibrator Material	028M4878V	028M4878V	028M4878V

Table 5. Materials used in the creation of the three initial lots of the IL-6 Conferma™ ELISA.

The study used samples provided by the CLCS and Professor Clay Semenkovich at Wash U. The same patient serum samples, standards, and quality controls (QCs) (constructed from the same lot of the recombinant calibrator material) were run in duplicate or triplicate on multiple plates by two analysts. This testing examined the reproducibility of the assay with both the endogenous IL-6 and calibrator material. Data was calculated and displayed using the Belysa™ Immunoassay Curve Fitting software (Cat. No. 40-012). The serum samples kindly provided by Prof. Semenkovich were from patients demonstrating at least three indicators of metabolic syndrome, a pathology with well-characterized low-grade systemic inflammation. **Figure 3** demonstrates the distribution of the samples, standards, and QCs (low, middle, and high) on a representative plate. Further, the Belysa™ software displays the Lower Limit of Quantitation (LLOQ) of the standard curve (the lowest point at which % CV and % Recovery is 20% or less, where all points above it also conform). In the case of this study, it was calculated to be the lowest point on the standard curve, 1.17 pg/mL.

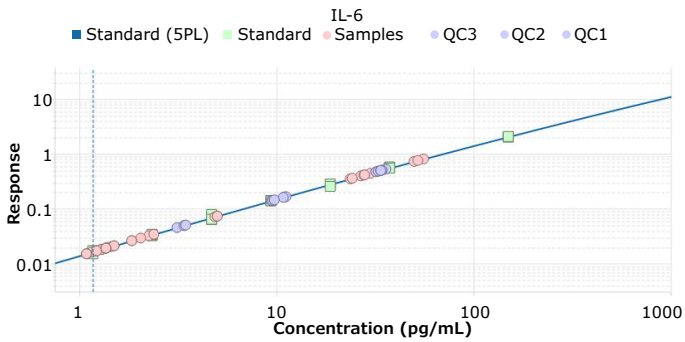

Figure 3. Plate 1, demonstrating the distribution of samples (pink circles), QCs (purple circles), and calibrator points (green boxes). The Response is in O.D. LLOQ of the standard curve is displayed as a dotted line at 1.17 pg/mL.

The standard curve performance was evaluated across all three kit lots using the Belysa™ Immunoassay Curve Fitting software. The software's mathematical Parallelism tool was used to calculate the slope ratio of the standard curves in each kit lot relative to Lot 1. This is demonstrated in **Figure 4** where the slope ratios of Lots 2 and 3 relative to Lot 1 were 1.035 and 1.001, respectively, and met the acceptance criteria of 1.0 ± 0.1. The excellent slope ratio indicated that while there were slight differences in the raw data output (Response on graph), the relationships between the points in the curves remained consistent. A second observation was that the curve from Lot 3 shifted to the left with a higher Response compared to Lots 1 and 2. Lot 3 included a detection mAb with a higher affinity and activity due to a higher rate of biotin incorporation. This will be closely monitored in future reagent lots.

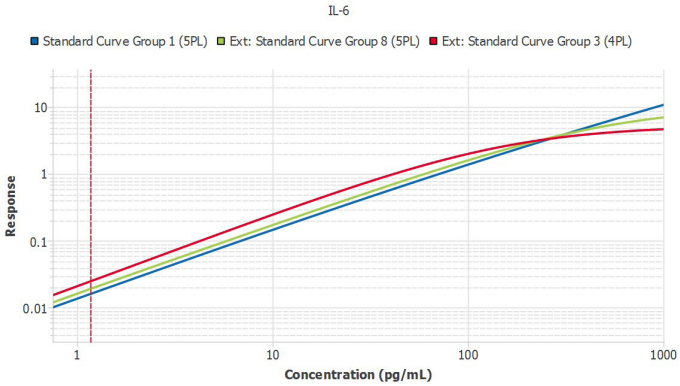

Figure 4. Mathematical parallelism of contrasted standard curves from individual plates of three lots of IL-6 ELISAs run by the same analyst. Lot 1 (blue line) was used as the reference curve for Lot 2 (green line) and Lot 3 (red line).

Inter- and intra-assay precision were evaluated using the quality controls provided with the kit. Two kits from each lot were evaluated by two analysts. The coefficient of variance (% CV) for intra- and inter-assay precision were ≤6.9% and ≤9.1%, respectively. In addition, the pair of analysts gave a good indication of precision and reproducibility across the three lots (**Table 6**).

QC	Inter-Assay % CV	Intra-Assay % CV
Low	11.8	3.7
Mid	7.6	1.4
High	6.8	1.8

Table 6. Inter- and intra-assay % CV of QCs. Each QC point n=32 replicates (2 Assays per Lot, 3 Lots, 2 Analysts, 6 kits in total were run).

Similarly, performance was evaluated at the sample level. Having been run in duplicate or triplicate, each sample had a replicate number of n=17. **Figure 3** demonstrated that the samples were distributed across the range of the curve, so the %CVs were calculated above and below 2 pg/mL, around the second point in the standard curve (**Table 7**). One sample was excluded as it was below the limit of detection (LOD) of 0.6 pg/mL.

Value	Inter-Assay % CV	Intra-Assay % CV
<2 pg/mL (n=5 samples)	29.1	11.5
>2 pg/mL (n=6 samples)	10.5	4.7

Table 7. Inter- and intra-assay % CV of endogenous markers in patient serum samples. Each sample had n=17 replicates (2 Assays, 3 Lots, 2 Analysts).

The samples that were near the lowest non-zero standard in the curve demonstrated a % CV of 29.1% inter-assay and 11.5% intra-assay precision. This was calculated by including samples above the assay's LOD but below the lowest non-zero standard of the curve. Samples at or above the second non-zero standard (2 pg/mL) in the curve behaved more like the QCs provided with the assay. Overall, both the samples and the controls performed well in the hands of the two analysts over the three lots of kits. This is reflective of the reproducibility, precision, and robustness of the assay across multiple analysts.

Observations and Conclusions

Creating these reagents and submitting them to the characterization process provided excellent insight into the various batches. From research and development to their utilization in the creation of multiple lots, these pieces of information added value at each stage. Although we have not included details of reagent batch failure in this application note, on one particular occasion, we were able to reject a batch of mAbs based on the LC-MS data, which convinced us of the value of that technique. However, the real evidence was provided when the assays were in the hands of the CLCS at Washington University in St. Louis. Their two analysts were able to achieve excellent reproducibility for both QCs and samples across three lots of kits.

This application note deals with one assay and will need to be repeated multiple times with other analytes to see the true effectiveness of these techniques. We have established a new brand called Conferma™ ELISA, to which we will be adding assays that are aligned to this process. The IL-6 ELISA under Cat. No. EZIL6-98K will be the first in the family.

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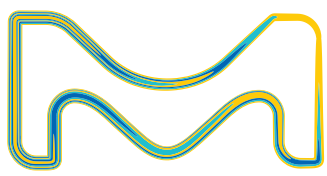

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