

## **Investigating Assay Precision & Validation on Different Multiplex Immunoassay Platforms**

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### **Abstract**

As biomarkers increasingly become incorporated into clinical trials, there is a growing need for a multiplex immunoassay system that can efficiently and reliably deliver high quality data to support drug efficacy and safety. This review compares the performance of four platforms offering multiplex immunoassays for protein biomarker panels: xMAP by Luminex, Discovery by Mesoscale -, Searchlight by Aushon and the AVANTRA<sup>®</sup> by Decision Biomarkers. While each platform incorporates unique detection technologies, they all rely on a traditional sandwich immunoassay format for assay execution. Furthermore, there is considerable overlap in the biomarker panels available for each platform, especially for cytokines and angiogenesis factors. These similarities are fortuitous in that they allow a close comparison of the performance of the platforms. Since each platform is commercially available, third party, peer-reviewed publications were reviewed to collect the data for this comparison. The primary goal of this paper is to shed light on assay processes within each platform. A secondary goal is to identify possible causes for the high degree of variability in performance encountered between the different multiplex assay platforms. Finally, suggestions will be proposed as to how the platforms could attain a higher level of data reliability, with a special focus on automation.

### **1. Introduction, the benefits of multiplex immunoassays**

The merits of multiplex immunoassays have been expressed precisely and succinctly by Ellington and al. (3). *The following three paragraphs are taken from that article in integral form.* The expression uniplex and singleplex are used interchangeably in this document.

Protein immunoassays provide information about quantities and forms of endogenous proteins. Uniplex enzyme immunoassays have been the workhorse for protein measurement for decades, but they can be laborious and expensive and consume relatively large amounts of specimen. Multiplex analysis of protein biomarkers is an attractive strategy for obtaining large numbers of measurements rapidly. Multiplex approaches have already been successfully used in gene expression microarrays. For example, Mammaprint concurrently quantifies expression of 70 genes to evaluate breast cancer prognosis. Thus, there is considerable interest in protein multiplex arrays that enable simultaneous quantification of circulating proteins to improve disease diagnosis and prognosis.

Presently, antibody-based platforms are the core technology for protein multiplex arrays. Assay formats include suspension arrays and planar arrays that use traditional immunometric principles. Several planar multiplex platforms capable of simultaneously measuring up to 16 proteins are commercially available. Multiplexed immunoassays overcome some of the limitations of uniplex assays. For example, measuring several proteins from a single sample conserves specimen, limits sample handling, decreases throughput time, and reduces labor costs. Concerns have been raised, however, about the reliability and consistency of multiplexed protein immunoassays. Analytic challenges include difficulty in optimizing a shared

format that works well for each component protein (e.g., capture/detection system, incubation time, and washing steps) as well as addressing variability in reagent lots and the manufacturing processes. Selection of a single sample dilution factor that enables measurements in physiological ranges for each constituent assay on a panel is often not feasible. Lack of robust multivariate QC algorithms and guidelines for multiplexed protein array data rejection criteria are problematic.

## **2. Multiplexing requirements**

Multiplexing presents additional QC challenges compared with uniplex analyses. The failure of 1 constituent assay to meet QC specifications results in rejection of results for all assays on the panel. Samples failing QC specifications should be retested using the same measurement system, because substitution of a uniplex assay may introduce bias due to differences in assay format. However, the probability of all assays simultaneously meeting QC specifications is much lower than the probability of a uniplex test passing QC. At present, reference guidelines for multiplex QC programs are under development by the Food and Drug Administration (FDA).

Commercial platforms tend to report assay reproducibility from a single plate processing (satisfactory) and omit multiple plate results (not satisfactory). It is the opinion of this reviewer that only multiple plate data has any quantitative validity.

This article explores assay performance and data integrity of multiplex immunoassays in four commercial platforms. It also aims to provide technical insight into the capabilities and limitations of commercial multiplex immunoassay platforms.

## **3. Multiplexing Platform description**

The four platforms under consideration incorporate similar epitope/ paratope binding properties of antigen/antibody to measure the total analyte concentration. The four platforms differ in sample and assay processing methodology as demonstrated by the imaging technology chosen to quantify molecular coupling. The publications listed in the reference section are the source of the data used for reliability tabulation. Only Cytokine and Angiogenesis panels are presented for comparison of data reliability. Reliability is determined using the Coefficient of Variation, or CV as an indication of assay reproducibility, and to compare the platforms. The CV's listed in Table 1 have been published and are applicable to each panel on each platform.

### **Similarities**

All four platforms use an optical label to quantify analytes captured by specific antibodies. The choice of optical detection/label is integral to the assay platform.

It is convenient to think of the information content of a single well of a well plate of a uniplex assay as a “packet”. Similarly the information content of a spot or a bead (or a group of identical spots or beads) in a multiplex immunoassay can be considered a “packet” of data. The packet of data is specific to a particular analyte and the data contained in the packet can be used to calculate analyte concentration.

The Luminex platform is a bead system incorporating specific capture antibodies immobilized on a number of almost identical beads. A “packet” is identified via two color optical labeling. The assay result itself is measured via the fluorescence intensity of a label with a third color.

The Mesoscale platform identifies a “packet” from its position in each well of a MULTI-ARRAY plate. Quantification of the assay is obtained as an optical signal produced by the electrochemiluminescence of the label.

The Searchlight-Aushon platform is similar to the Mesoscale platform except that quantification of the assay results is derived from the optical signal level of fluorescence label or a red-light excited fluorescent label.

The AVANTRA-DBI platform also identifies each “packet” of information by its location in a multiplex array as well as the intensity of a fluorescent signal, but in this case via a green-light emission of a fluorescent label.

In an ELISA, a “packet” is identified by its well location on the wellplate, with optical output being either colorimetric or fluorescent.

### Differences

Each of these platform manufacturers has chosen a signal detection method in order to adapt to the demands and limitations of choices made when trying to simulate the ELISA assay process. Such choices are guided by the necessity to preserve the activity of capture antibodies, attached at known locations, or on identifiable beads where the assay takes place.

Proteins are very delicate molecules; best immobilized onto either polystyrene or nitrocellulose surfaces. Unfortunately, both of these plastics are highly fluorescent with short wavelength emission in the UV or Green light range. Each platform circumvents this difficulty of high background in its own way.

The Luminex technology mitigates this shortcoming by attaching the capture proteins to micro-beads of polystyrene where the surface area is large relative to the volume of each bead. The emission (noise) of the bead material is proportional to the bead’s mass, while the specific assay signal label is proportional to the bead’s surface area.

The Mesoscale technology derives its assay signal from an electrically-stimulated chemiluminescent reaction, by-passing any fluorescence excitation of the plastic substrate.

The original Searchlight-Aushon assay used a chemically-induced optical stimulation. A recent technical improvement has offered a red stimulated fluorescent label on a polystyrene plate. The natural fluorescence of both polystyrene and nitrocellulose diminishes substantially at longer wavelengths. Using red light however, requires dyes emitting at longer wavelengths that generally have lower efficiency and more limited stability than their short wavelength emitting counterparts.

The Avantra- DBI platform circumvents the penalties of background fluorescence of the protein support. The AVANTRA<sup>®</sup> uses a sub-micron, thin film nitrocellulose attached to an opaque surface of tantalum oxide adhered to a glass substrate. The thin film nitrocellulose generates substantially less background fluorescence than its thick membrane predecessor, permitting the use of green light stimulated label using a short wavelength dye that is efficient and stable.

### Consequences

The signal detection choices listed above also have assay preparation consequences. The detection technology effectively dictates the number of different manual steps required to satisfy the technical constraints.

All platforms essentially duplicate ELISA methodology. This requires numerous fluid additions, mixing, washing, fluid changes and exchanges and incubation steps. In all cases, reactions are time as well as temperature dependent and all proteins and labels are subject to photodegradation. Exposure to UV light emitted by the sun or fluorescent lighting is extremely damaging to 3-dimensional protein structure.

In all cases, the number of manual steps required (listed in table 1) is derived from the protocol of the platform specified by its respective vendor or as the article indicated from the cited references. The steps are detailed in the Appendix (below). It should be noted that manual steps such as washing, mixing, or agitation, can include a number of sub-steps not described. As an example, insertion of the plate or chip into the reader was not considered a step for any platform.

It is important to realize that many of these steps are different in quality and cannot be readily automated. For instance, consider the case of a triple wash followed by incubation in an agitated environment at 37° C in a dark environment.

It is interesting to note that there is tremendous variation among the protocols, with little similarity in the number of assay steps or concerns.

#### **4. Conclusion**

This paper summarizes available data for the purpose of protein multiplex assay platform comparison and analysis. The major goals were to justify the reliability of assay data and to identify possible causes of any deficiencies in data reliability. The quality of the data is quantified using the Coefficient of Variation (CV).

Table 1 lists the Coefficient of Variation (CV) for the four platforms analyzed in this paper. The data and the protocols are taken either from the reference journals listed below or from the vendor. The emphasis and numbering of steps have been added for clarification.

<b>Platform</b>	<b>Number of Manual Steps</b>	<b>Single plate CV</b>	<b>Multiple plate CV</b>
Avantra-DBI	2	7%-18%	7%-18%
Luminex	39	5% - 15%	5%-85%
Mesoscale-MSD	17	5%- 15%	10%-100%
Searchlight-Aushon	22	7%-67%	11%-67%

**Table 1** – Summary of experiments to investigate assay precision and validation on different multiplex immunoassay platforms. Single plate CV’s are derived from replicates run on the same plate. Multiple plate CV’s are derived from replicates run on different plates. The range of the CV’s listed reflects the CV’s of individual assays within a multiplex panel. For example, on one multi-plate Searchlight panel, the FGF basic assay had a CV of 11% while that of HGF had a CV of 56%.

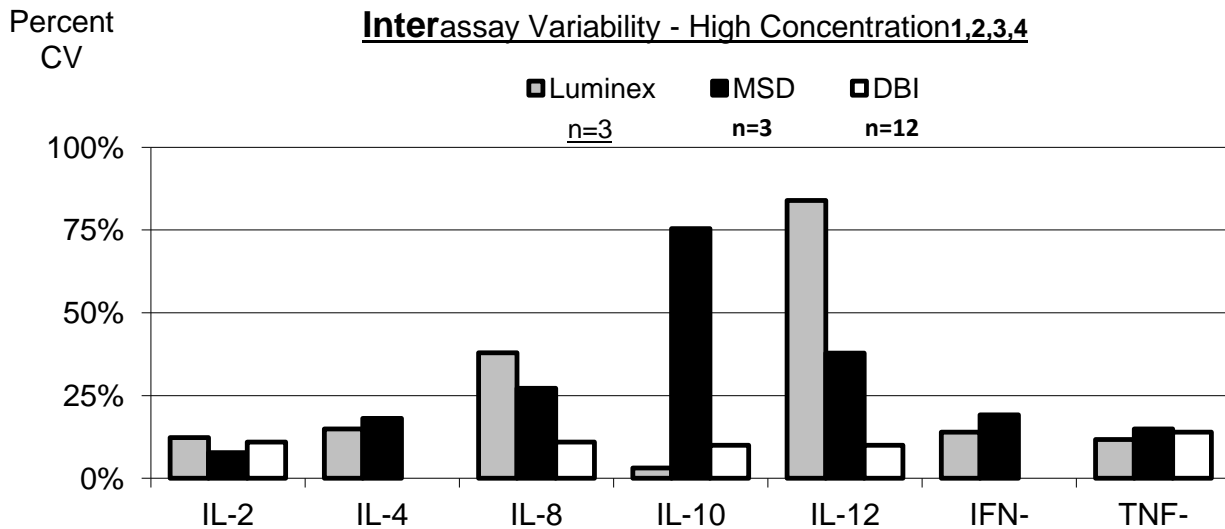


Figure 1 Interassay variable at high concentration of analyte

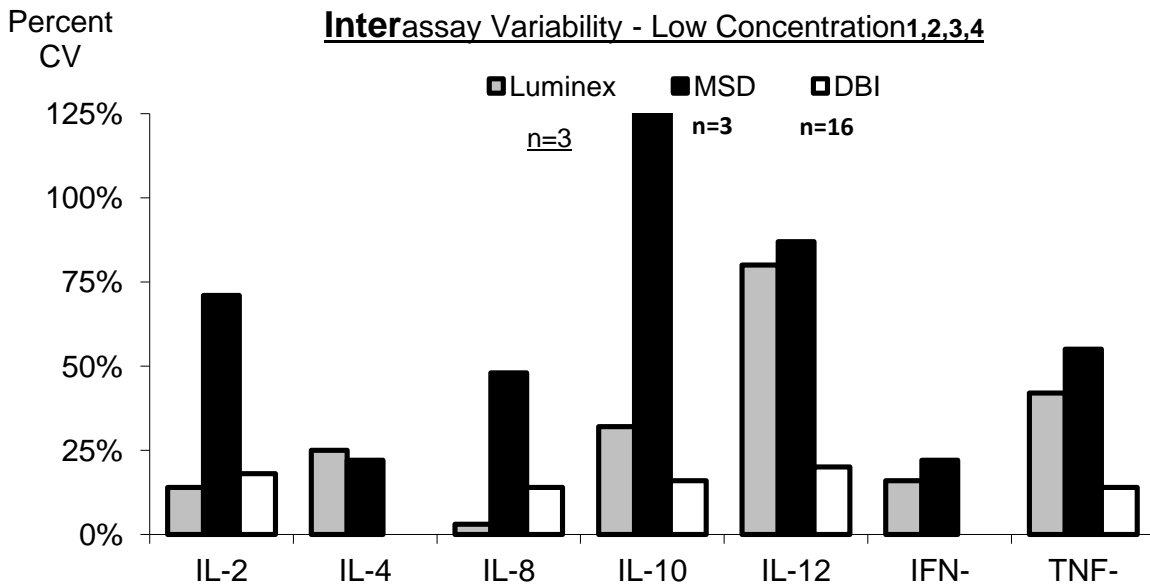


Figure 2: Inter-assay variability at low concentration of analyte

Table 1 is a general listing as this presentation aims to identify possible causes of weakness in processes rather than to recommend a specific platform for a specific assay.

A more specific comparison is presented below. The publications listed give data for a number of common assays. Figures 1 and 3 are constructed from those data.

These figures compare variability of specific assays from data listed in references 1, 2, 3 and 4. We can see that:

1. AVANTRA's inter-assay CV remains well below 25% at both high and low concentrations

2. AVANTRA's CV remains below 15% at high concentrations; CV  $\leq$ 20% at low concentrations
3. Luminex and MSD have higher inter-assay variability:
  - At high concentrations, 2 out of 7 analytes exceed 25% CV for both Luminex and MSD
  - Using MSD at low concentrations, 4 out of 7 analytes exceed 50% CV
  - Using Luminex at low concentrations, 3 out of 7 analytes exceed 25% CV

In addition, Table 1 and Figures 1 & 2 illuminate a number of trends:

- A strong correlation exists between the number of manual steps and the resulting CV's. The higher the number of manual steps, the higher the CV's.
- It is quite notable that both the Luminex and MSD platforms offer very good within plate CV's but quite poor plate to plate CV's. This casts doubt on the validity of the CV's derived from a single plate analysis.
- The CV's on the Searchlight platform are equivalent for within plate and plate to plate analysis.
- The Avantra platform does not incorporate a plate-based technology but a fully automated single assay process that employs a cartridge containing all assay reagents and simply requires addition of sample. The CV's presented were generated from data from multiple runs at multiple sites and from different instruments at different times. The Avantra platform employs a computer-controlled process with no operator interaction after the sample has been introduced.

## **5 – Recommendations and guidelines**

This analysis and review of the protocols for each of these platforms listed in the appendix suggests a number of follow-up studies. It would be of interest to perform a multiple plate study where all steps were controlled in order to identify sources of variability. The following should be considered:

1. Verify that all plates and analytes are treated in an identical manner.
2. Maintain records of the duration of each step, e.g. wash, agitation and specially incubation. This is important as incubation does not proceed at similar kinetics for all proteins. In some conditions, assay kinetics is a linear function of time.
3. Verify that all experiments are performed at the same temperature, preferably 37° C. This is important because the coefficient of diffusion in water increases by about 1.5% per degree C at room temperature range.
4. Ascertain that agitation is always performed at the same speed, as proteins have a non-Newtonian diffusion behavior. An orbital rocker is preferred to an angular one.
5. Ascertain that all plates and processing are shielded from light, especially UV and fluorescent lighting.

These variables are usually controlled in all central lab processing equipment running singleplex protein immunoassays. They are also fully controlled in the Avantra platform.

Central Lab assays are all singleplex (single assays) and exhibit within plate CV's typically around 5% and plate to plate CV's in the 10-15% range. The Avantra platform processes simultaneously a panel of 8 or 10 assays and generates CV's equivalent to ELISA. The 3 other multiplex platforms studied here exhibit CV's ranging from 10% to as high as 100% and consequently deliver data with an uncertainty 10 or 20 times higher than those obtained from a Central Lab..

The fact that the CV's from the Avantra platform are higher than that of central lab systems may be due to the imperfect specificity of protein to protein interaction which only affect multiplex assays. A judicious dilution and Ph choice as well as virtual dilution minimizes these effects in the Avantra platform.

## **6 - Discussion**

The high variability of molecular concentrations derived from any assay run on the Luminex, the MSD or the Searchlight platform brings into question the reliability of those results.

It is important to consider that analyte concentrations encountered between a normal individual and an individual with solid tumors may vary by greater than 100% and frequently many times more than that (Reference 1, page 111). These platforms therefore, may offer a practical triage function.

Most platforms may not be applicable during treatment however, where accurate quantification of analyte concentrations is crucial. The exception is the AVANTRA, which may be the only platform with diagnostic value, as it meets required accuracy and timeline restrictions.

## **7 - References**

1. A.C. Backen et al. "Fit-for-purpose" validation of Searchlight multiplex ELISA of angiogenesis for clinical trial use. – Journal of Immunological Methods Vol. 342 (2009) pp.106-114.
2. F. Chowdhury et al. "Validation and comparison of two multiplex technologies, Luminex and Mesoscale Discovery, for human cytokine profiling" - – Journal of Immunological Methods Vol. 340 (2009) pp. 55-64.
3. A. Ellington et al. "Measurement and Quality Control Issues in Mutiplex Protein Assays: A Case Study" – Clinical Chemistry Vol. 55:6 (2009) pp. 1092-1099.
4. DBI website and data presented from MD Anderson private communications.

## **Appendix**

**SEARCHLIGHT Protocol:** Text from reference 1 pp. 107-108 with numbering and underlines added .See also Reference 3.

“The multiplex ELISAs were performed essentially according to the manufacturer's instructions. Briefly, [1] 50 µl calibration standards or samples were added in [2, 3] duplicate to the ELISA plate which was [4] covered with an adhesive lid and [5] incubated for one hour at room temperature [6] shaking on a horizontal orbital micro-plate shaker at 200 rpm. At the end of the incubation, the plate was [6, 7, 8] washed three times with kit wash buffer, using a wash bottle. Then, 50 µl biotinylated antibody reagent was [9] added to each well of the plate which was 10] covered with an adhesive lid and [11] incubated for 30 min at room temperature [12] shaking at 200 rpm. The plate was [13, 14, 15]washed three more times as before. Streptavidin-HRP reagent (50 µl) was [16] added to each well of the plate which was again [15] covered with an adhesive lid and [17] incubated for 30 min at room temperature [18] shaking at 200 rpm. The plate was [19, 20, 21] washed three times as before. Prepared SuperSignal substrate (50 µl) was [22] added to each well of the plate which was then imaged.”

**LUMINEX Protocol** Text from reference 2 pp.56-57 with numbering and underlines added.

“The assays were performed in 96- well filter bottom plates according to the protocol provided. Antibody conjugated beads are used at a concentration of 5000 beads per marker, per well[1] requiring a 30 s vortex [2] followed by a 30 s sonication to prevent bead aggregation. The volume of concentrated beads required for each assay was determined using the protocol chart and [3] diluted to the correct volume using wash solution. The beads were protected from light throughout the course of the assay. The lyophilized standard was [4] reconstituted in 1 ml of assay diluent and 1/3 serial dilutions undertaken to [5]generate a 7 standard concentration set. Diluent alone was used as a blank. The wells to be used were [6] pre-wet with 200 µl of wash solution for 30 s, and then [7] aspirated using a vacuum manifold with the bottom of the plate blotted on paper towels to [8] remove excess fluid. The bead solution was [9] vortexed and [10] sonicated immediately prior to [11] adding 25 µl into each well. The plate was [12] washed [13] twice by adding 200 µl of wash solution, [14] soaking for 30 s and then [15] aspirating using a vacuum manifold with the bottom of the plate once again blotted to remove excess fluid. Incubation buffer (50 µl) was [16] added to each well

and each standard was [17] added in duplicate (100 µl per well). A further 50 µl of assay diluent was [18] added to each sample well [19] followed by the addition of the sample (50 µl). The plate was [20] covered in foil and [21] incubated for 2 h at room temperature on an orbital plate shaker (600 rpm). At the end of this incubation, the fluid from the wells was [22] removed using a vacuum manifold, and then the plate was [23] washed [24] twice as before. Biotinylated detection antibody (100 µl) was [25] added to each well, [26] covered and [27] incubated on the orbital shaker for a further 1 h at room temperature. The plate was then [28] washed [29] twice prior to [30] addition of Streptavidin-RPE (100 µl). The plate was [31] covered and [32] incubated for a further 30 min at room temperature on an orbital shaker. Finally the plate was [33] washed [34] & [35] three times as before. Wash solution (100 µl) was [36] added to each well, and the plate [37] covered and [38] placed on an orbital shaker for 2–3 min at room temperature prior to analysis. The plate was [39] uncovered.”

**MESOSCALE Protocol** Text from reference 2 pp.57 with numbering and underlines added.

“The standards were [1] reconstituted in the assay diluent provided. Assay diluent (25 µl) was added to all wells and the plate [2] sealed and [3] incubated for 30 s at room temperature on an orbital shaker (600 rpm). Samples, standards and controls were [4&5] added at 25 µl per well. The plate was [6] sealed and [7] incubated for 2 h at room temperature on an orbital shaker (600 rpm). At the end of the incubation the wells were [8, 9, 10] washed three times using 200 µl PBS+0.05%Tween 20, soaking for 30 s and then discarding. Detection antibody was [11] added at 25 µl per well, and the plate [12] sealed and [13] incubated for 1 h at room temperature on an orbital shaker (600 rpm). At the end of the incubation the plate was [14, 15, 16] washed three times as before. 150 µl of the MSD Read Buffer was [17] added to each well and the MSD plates were measured on the MSD Sector Imager”

**DBI Protocol** from reference 4 and Company website. Underlines and numbering added.

Select sample and [1] reconstituted in the assay diluent provided\_ as per specification. Inject [2] in entry port of cassette, close trap door and press start button. Please see Figure below.



**STEP 1:**  
**Open the door and insert the biochip.**  
You don't have to worry about handling or adding reagents.



**STEP 2:**  
**Inject the sample.**  
Serum or plasma are the routine types but we can handle others. See "Sample Injection Instructions" on previous page for more details.



**STEP 3:**  
**Close the door.**  
Our simple process reduces waste and maintenance failures and eliminates contamination issues.



**STEP 4:**  
**Push start. Results are ready in approximately one (1) hour.**  
Remember our system runs the assay without user intervention. Test results are displayed on the screen and stored in the database.