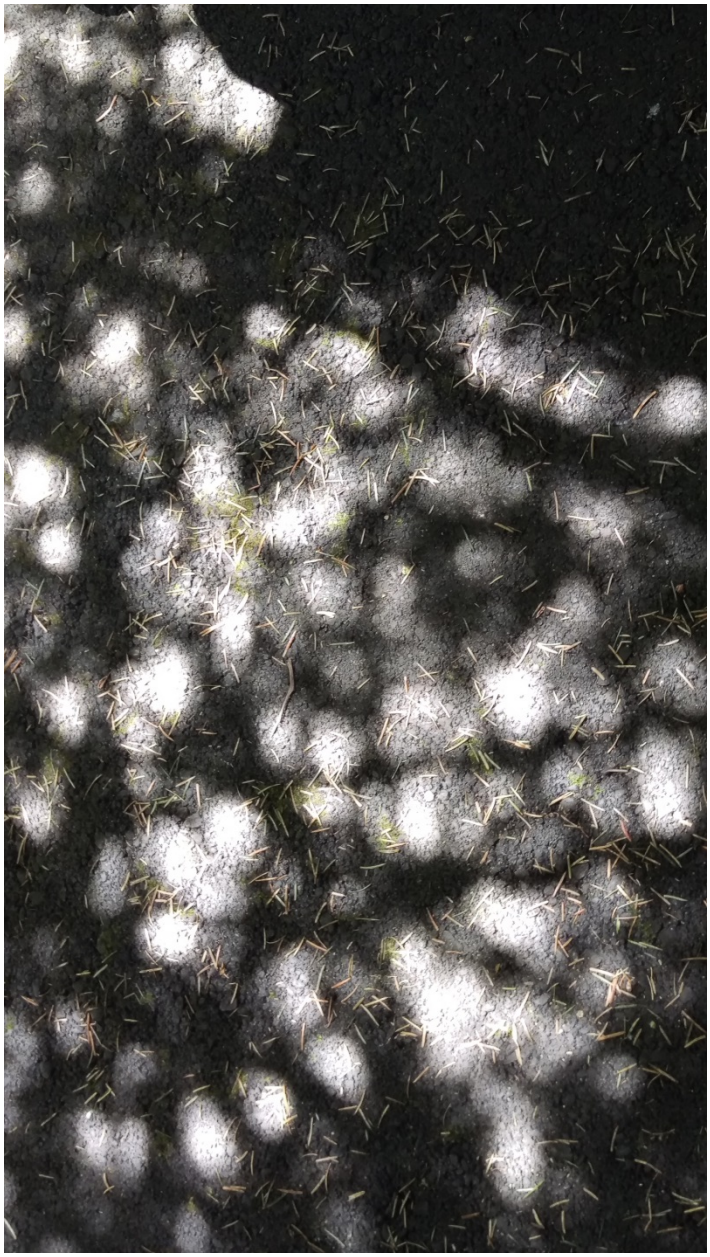

ASSAY ANALYTICS™

IonField Systems Predictive Analytics Software
Reduces Assay Variability When Reusing Cleaned Microplates

Introduction

This article was originally submitted in April of 2018 and published in the August *American Laboratory*. Since then new experiments were run and analyzed using the process described; and there has been feedback, both questions and suggestions. This updated version of the article includes answers to the questions and incorporates many suggestions.

One common suggestion has been to not to use the term "Artificial Intelligence", it is too broad in scope. To address this, we are adopting the term "predictive analytics." Most everyone is aware that organizations like Google, Facebook, Amazon, etc., collect information on people's behavior while visiting their websites and using their applications. They do this to personalize the information presented and match the experience to the individual. The process whereby they accomplish this is predictive analytics. Companies collect everyone's data and use software that goes through a learning process to predict future behavior by comparing each individual's usage patterns to those of thousands to millions of others. Similarly, the software process IonField Systems has developed learns the behavior of every well through a process of running the identical assay in all wells of a microplate and concurrently comparing how each individual well performs relative to all other wells within the microplate and, eventually, to all wells of every microplate the assay has been run in. The data in the original article were based on a limited number of these learning cycles, typically two. A logical next step is to increase the number of learning cycles according to the theory that with more data, the accuracy of prediction increases. We are currently working to see how much improvement is possible.

One common question is how IonField Systems found something that everyone else has missed. In talking with many scientists, we have come to the conclusion that we have not found something not seen before. The small differences between microplate wells are known to all who run duplicates or triplicates and considered random. Nearly everyone doing assay development knows that different brands of microplates generally give different results for the same assay. In most instances, the selection of microplate is based on which one gives the highest signal. From a practical perspective, our Microplate Cleaning System powered by PlasmaKnife™ for the first time allowed measurement of an individual well's effect on an assay. Such an effect has always been there; however, when it repeats it is no longer random. Statistically, the noise measured as variation in results in a uniform plate-wide assay, is the sum of each well's unique systemic bias together with random errors: accumulation of small differences in pipetting volumes, unevenness of cell suspensions, volume differences due to evaporation and temperature, and other aspects of the processes used that introduce variability. Plate reuse reveals the repeatable differences between wells. The software and testing plans that we have developed allow precise measurement of these important differences.

We originally observed and reported a 30% improvement from correcting the bias due to microplate well differences and received the frequent comment that this seems high. It also did to all of us — at IonField Systems, the National Center for Advancing Translational Science (NCATS) at the National Institutes of Health, and a major pharmaceutical company. It took more than two years of research to gain confidence in our understanding of the underlying material science of microplate surfaces and how the surfaces cause the effects seen in assays.

Understanding Assay Variability

Assay variability has numerous sources, including pipetting variation, non-uniformity of liquids being pipetted, edge effects, and temperature non-uniformity. These sources are well documented in scientific literature [1]. Historically, when using microplates once and disposing, all sources of variability are classified statistically as random. Recently, the technical

challenges of cleaning microplates have been solved. That triggered a new look at random variation and its sources.

Shortly after introduction of the Microplate Cleaning System, IonField Systems observed that when microplates are reused some of the variability was not random and recurred repeatedly with every reuse. Experiments designed to isolate variability by well position over multiple uses showed that individual wells gave the same repeated results with very high precision. Preliminary information on this discovery was presented in three posters at SLAS 2018 [2-4] and an article in *American Laboratory* [5]. This report expands on the previous publications and presents new data and information about reducing variability.

In discovery phase pharmaceutical research, many assays are not developed for inherent accuracy. A series of dilutions of the same compound are used to identify the concentration having the desired modulating effect on a target. Large numbers of compounds are compared and less assay variability results in a greater differentiation between them. Lower variability offers the potential to improve data upon which to base decisions on follow-on testing and subsequent selection of molecules or biologics for moving into later stages of discovery and development.

Assay variability may affect a number of other decisions in the discovery process. During assay development, a common metric is the number of SDs between high and low controls. Too few SDs may render what may be an excellent research method, not suitable for high-throughput screening and many other applications. Another instance of the effects can be on data analysis. As variability is reduced, it allows more of the curvilinear portions of sigmoidal response curves to be used, thereby increasing the potential dynamic range of an assay.

To facilitate the ability to reduce variability, IonField Systems has developed a predictive analytics program that is simple to integrate into data analysis software used in drug discovery. The methodology identifies differences between wells, which are easily seen visually as patterns in microplates. For example, well differences are shown in Figure 1, an image of the results from a cell assay with no added compound using Be(2)-C (a human neuroblastoma cell line).

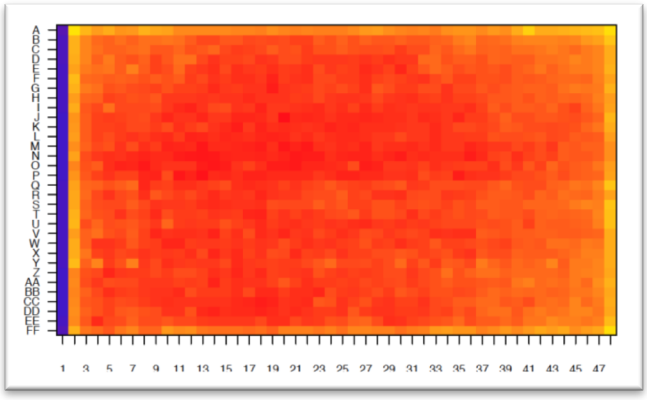

Figure 1

The predictive analytics methodology uses a minimum of two sets of assay results as the learning model, identifying each well's repeating pattern or bias for reporting high or low assay values relative to other wells, and measuring the variability of each well's results. These well-specific results guide the bias prediction for each well upon reuse and can be used to score confidence in that prediction versus results from all other wells.

This method does not predict a result, it predicts the effect on results of the differences between wells. Applying this method using two learning cycles has shown a minimum improvement in precision of 30% in both homogenous and cell assays. A recent run with a new cell line showed a >50% improvement. The new cell line results may indicate greater sensitivity to the well-to-well differences. Testing with more cell lines is planned to determine the frequency and likelihood of higher, and potentially, lower improvement in results.

Addressing Assay Variability

As part of the development of the IonField System Microplate Cleaning System, testing confirmed cleaning effectiveness by comparing the results from a new plate to the same plate used a second time. More testing confirmed the same result over multiple reuses. Of note, results from individual plates in the multiple re-use group indicated more consistency compared to a similar analysis of the identical assay results from new plates.

To better understand these observed and unexpected results, we entered into an informal partnership with the National Center for Advancing Translational Sciences (NCATS), National Institutes of Health, with the goal of understanding the reason(s) for the more consistent results. Our research plan consisted of using best available analytic methods to examine microplate surface properties, including surface "roughness" and chemical composition. Scanning electron micrographs (SEMs) were used to assess microplate surface roughness. Based on the recommendation of EAG Laboratories, a commercial laboratory offering numerous methods of surface chemical testing, we selected Time of Flight Secondary Ion Mass Spectrometry (ToF-SIMS) for the surface chemical assessment. Of interest were the chemicals used in production to initialize and control the polymerization rate, primary and secondary oxidation, and improve polymer characteristics of flow and mold release.

SEM revealed random roughness varying between wells in a microplate, between microplates of the same lot, and between lots. The SEM photo in Figure 2A shows typical surface roughness of a new microplate at extremely high magnification. Figure 2B shows a lower magnification perspective where the randomness of the surface cannot be seen but more macro scale features are. One of our earliest conclusions was that surface roughness per se does not directly affect assay results. Surface area increases significantly with increasing roughness so the net effect of a rougher surface is to expose well contents, i.e., assay fluids, to more surface chemicals. The total surface area of each well is unique.

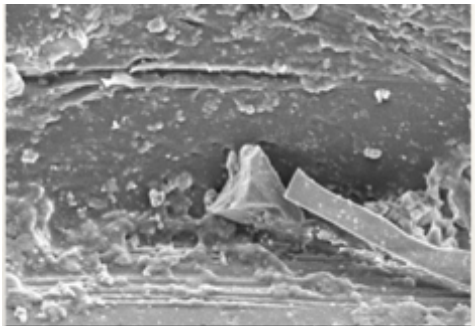

Figure 2A

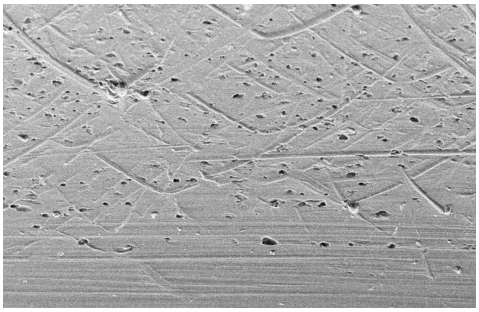

Figure 2B

Shown in Figure 3 is an analysis from performing a well position matrix subtraction of results from two microplates, dividing the absolute value by the SD, and grouping the results by 0.1 SD (X-axis is a well count, Y-axis is 0.1 SD groups). In this analysis, 34.5% of wells differ by <0.2 SD and 97.8% differ by <1.0 SD. Thus, individual wells are extremely precise run to run.

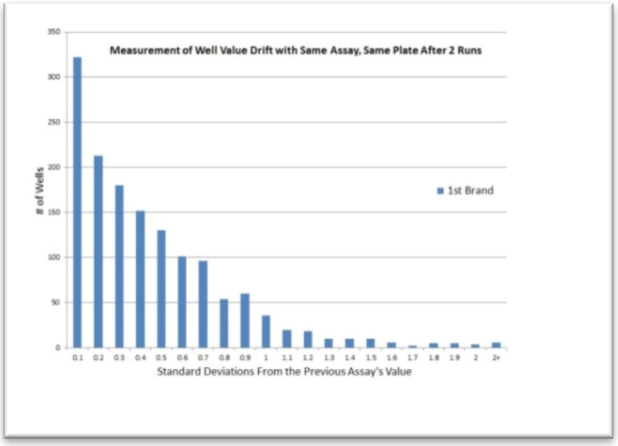

Figure 3

ToF-SIMS, using positive ion analysis, revealed a large number of positive ion species either on or within 1 nm of plate surfaces. Three brands of microplate were tested and all had at least 27 "non-polymer" positive ions. A few ions were unique to a single brand; most were common to all brands, varying in concentration by brand. Na+ varied 498-fold and Ca++ 2860-fold. Trace ions, like Pb++, typically had less than a 10-fold difference.

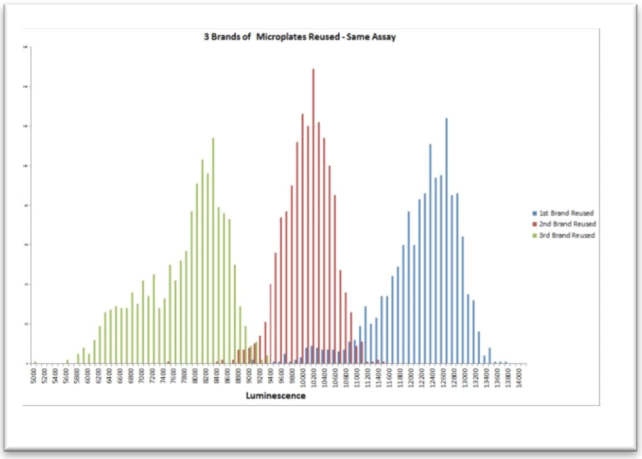

Figure 4

Figure 4 compares the assay results of three microplate brands run with Be(2)-C cells without added compounds. CellTiter-Glo® was used to measure the number of cells (Y-axis) and luminescence was measured on a ViewLux® (X-axis).

The variation within brand is due to well-to-well differences, while the variation between brands shows the effect of chemical differences between brands. Cell growth results are clustered by brand around a central value. All brands showed a skewing toward low values, primarily due to parallax from the ViewLux® reader. The three plate groupings confirm the effect of well surface chemical differences between brands on assay results. The plates were processed as a single batch to normalize any other protocol related biases. Plates were run in duplicate.

The data for the three curves in Figure 4 were analyzed statistically to determine whether the curve shapes were due to exposure to plate chemicals, very small differences in well dimension/shape/volume, or other non-random causes. The approach used was to compare the data from each well position to data from the corresponding well in the paired plate. The curve shapes for each pair were identical in this comparison, with the well positions on the graph remaining stationary. Subsequently, wells were mapped by the SD groups they fell into (in Figure 3). Their distribution over the microplate was even.

Using the statistical technique of matrix subtraction previously described, not only was cell growth rate found to be altered by brand, but assay variability differed by brand. For the Blue brand, the plate average was 11,860, and 1 SD was 700; 74.1% of well results were within 0.5 SD, and 97.8% were within 1.0 SD. Results for the other brands showed progressively more variability, indicating less uniformity between plate pairs. For the Red brand (#2), 51.5% of results were within 0.5 SD, and 79.1% were within 1.0 SD; 37.7% of results from the Green brand (#3) were within 0.5 SD, and 56.9% were within 1.0 SD.

This statistical analysis demonstrates the potential of using predictive analytics in methods development to identify optimally matched microplates for an assay. The output of the Assay Analytics™ software consists of two matrices, one with a bias adjustment factor derived from the median value for each well pair, and the other scoring the variance of each well. The scoring ranks wells based on the difference between results used in the learning process. This scoring has application to statistical methods such as Least Squares curve fitting that analyze data without regard to the measured precision of the data.

The predictive analytics method uses the first software matrix to make adjustment for well position bias. In the example below in

Figure 5, this statistical analysis process yielded a reduction in SD of >30% for the Red brand of plate. This brand was used because its raw data had the least skew with a distribution more typical of results for most assays.

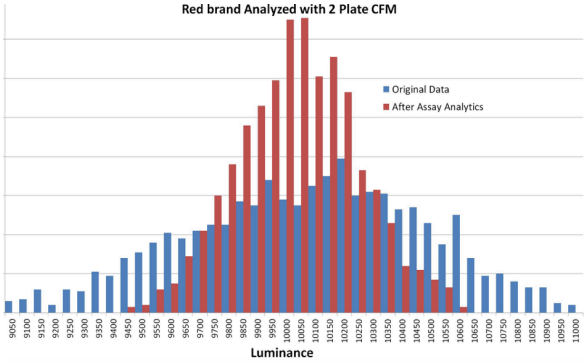

Figure 5

The ToF-SIMS data, discussed above, show differences in microplate surface chemicals that are intriguing. Once matched with assay data, this information establishes a link between surface chemicals for each brand and effects on assay results. For example, in the Be(2)-C assay in Figures 1, 3, 4, and 5, the lower signals from plate brands 2 and 3 are directly correlated to a reduced number of live cells detected by CellTiter-Glo®. The surface chemical data in Figure 6 indicate that the Blue plate brand (#1 in Figure 6) had low concentrations of all surface chemicals examined except Ca++. The Red brand (#2) had high concentrations of Na+, K+, Ca++, and Al+++. The Green brand (#3) had high concentrations of Al+++ and Zn++ and was only brand with ethylene bis stearamide (EBS).

Although this experiment was not designed to identify the biological processes resulting in lower cell growth rates, it demonstrated that low concentrations of chemicals on microplates surfaces mix with assay liquids and affect cell growth rates and assay results — and could be considered as potential "contaminants" to be minimized when selecting the microplate for each assay. Preliminary results from homogeneous assays using the same predictive analytics process show similar improvements in reducing variability and need for optimum microplate selection.

We are collaborating with clients to conduct additional experiments and expect results later in 2019.

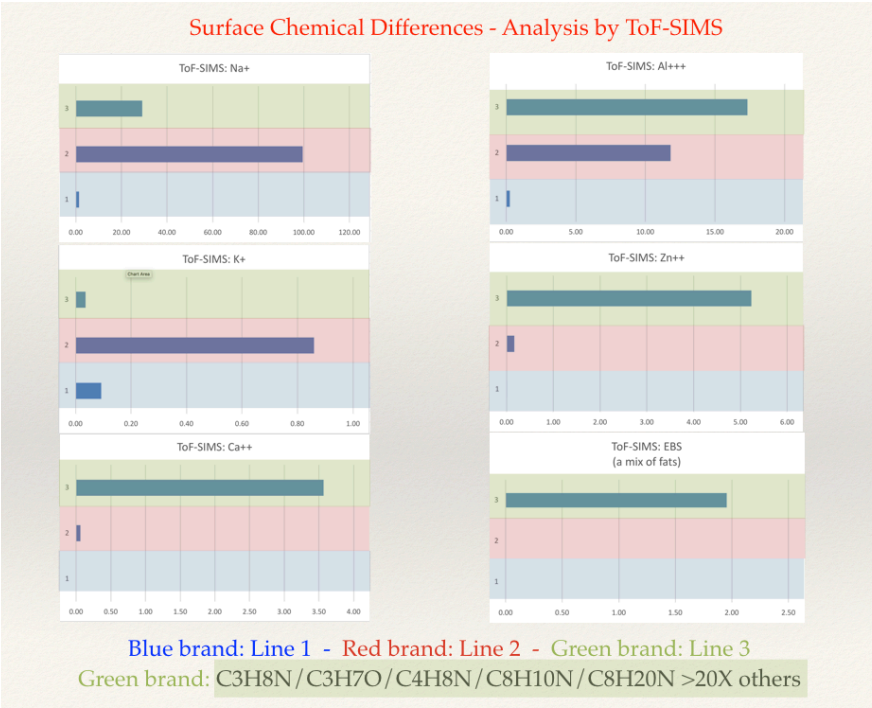

Figure 6

Key Takeaways

- Assay results from individual microplates after cleaning and multiple reuse show better consistency compared with results from new plates.
- Surface chemicals are detectable in varying concentrations across all brands of new microplates and, together with well surface "roughness" that increases exposure of well contents to chemicals, can substantially affect assay results.
- Optimal microplate selection using predictive analytics is a critical first step to assay success.
- Predictive analysis of result patterns from individual plate wells can substantially reduce assay variability.
- The IonField Systems predictive analysis process, Assay Analytics™, adjusts assay results using multiple actual measurements from each well to yield a more precise correction than methods using estimated variance or correction factors.
- This novel predictive process yields improvements in assay precision, routinely achieving a 30% reduction in SD.
- The method described will likely be effective for identifying and correcting other sources of repeating assay variability.
- Because it does not require any modification to assay reagents or methods, measurement modality, or instrumentation, the method is simple to implement.
- Repeated use of cleaned plates eliminates the risks associated with assay results shifting when changing lot numbers of microplates and helps insure optimum long-term assay stability.

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Author Information

Paul Hensley is the CEO of IonField Systems, and was the founder and CEO of Cerionx, which first brought atmospheric pressure plasma cleaning products for the life science laboratory to market. Earlier in his career, Mr. Hensley held management positions at Beckman Instruments and the Zymark Corporation.