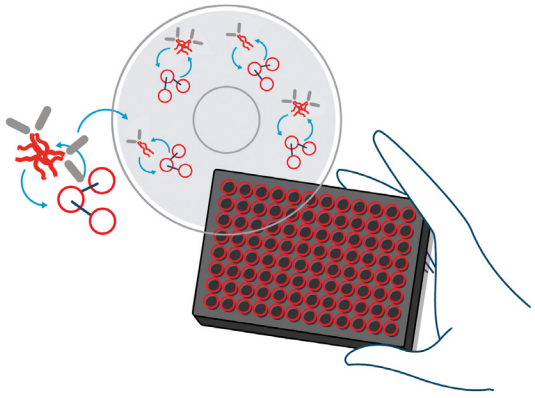
BECKMAN COULTER
Life Sciences

Performance of the Valita Aggregation Pure assay vs HPLC-SEC

Abstract

Aggregation is considered a Critical Quality Attribute in the development and manufacturing of monoclonal antibodies. Current technologies are challenging for scientists for a variety of reasons, including their complexity of execution and analysis, high CAPEX, labor intensity, lack of automation potential, and the need for combinatorial analytics and comprehensive aggregation profiles for clones. The Valita Aggregation Pure assay represents a novel, high-throughput solution for aggregation sample screening that allows users to generate aggregation data for 96 samples in as little as 15 minutes, with minimal sample preparation and a simple, add-mix-read workflow. Here, in collaboration with an industry partner, we showcase the performance of the Valita Aggregation Pure assay versus the current industry standard practice, HPLC Size Exclusion Chromatography (SEC) when used to quantify aggregation in human IgG4 kappa samples. This work highlights the strong correlation between the Valita Aggregation Pure assay and HPLC-SEC and the ease of use of the Valita Aggregation Pure assay.



Introduction

The global biologics market was estimated to have a value of over $500 billion in 2023, with an expected revenue forecast exceeding $1 trillion by 2030 [1]. Currently, monoclonal antibodies (mAbs) account for approximately two thirds of revenue in the biotherapeutics industry, making the continued development of novel mAbs critical for the sustained growth of this market. Within mAb development, there are several critical quality attributes (CQAs) which must be maintained within appropriate specifications to ensure product quality throughout development and manufacturing. One such CQA which has been subject to increased focus in recent years is aggregation. The induction of aggregates during manufacturing has a negative impact on product quality, with the potential to reduce both safety, efficacy, and stability of the final mAb product. As such, aggregation is routinely monitored throughout the bioprocessing pipeline, to ensure levels are minimized.

The formation of aggregates can occur across the development pipeline, with aggregates formed potentially varying in size, structure, charge and solubility. The current state-of-the-art for aggregation screening lacks a "one-size-fits-all" method to accurately measure and quantify aggregation, and scientists typically employ a combinatorial approach to measure and monitor aggregation in their samples. Commonly used methods to measure IgG aggregation include HPLC Size Exclusion Chromatography (SEC) or dynamic light scattering (DLS) technologies. These technologies rely on specialized instrumentation and highly trained analysts. Alongside this, these technologies can require hours of hands-on time per assay, from instrument setup and buffer preparation through to complex data processing and analysis. The manual nature of these steps offers little scope for automation, meaning that these assays are extremely labor intensive, requiring extensive manpower to perform them routinely. Due to these reasons, aggregation testing is often outsourced by Cell Line Development (CLD) and Process Development (PD) labs to analytical and quality control labs. This impacts negatively on the progress of development campaigns, due to increased turnaround time for results and the reduced sample screening throughput, as various labs compete in sending samples for analysis.

Here, we demonstrate the use of the Valita Aggregation Pure assay to quantify aggregation of a human IgG4 molecule rapidly, accurately, and reproducibly in purified solution. Initially, a proof of principle study took place at Beckman Coulter/ValitaCell using 1 mg/mL samples. Following its completion, in-house testing was conducted with an industry partner to establish reproducibility and quantitative capacity of the Valita Aggregation Pure assay. The results of the Valita Aggregation Pure assay are comparable to those obtained by the current gold standard aggregation screening technique HPLC-SEC. The use of the Valita Aggregation Pure assay for high-throughput aggregation screening represents a marked improvement versus standard practice with respect to simplicity, speed of results and potential for automation integration.

Spotlight

The Valita Aggregation Pure assay is a 96-well, plate-based assay that allows users to conduct rapid protein aggregation detection and quantification utilizing fluorescence polarization (FP) technology. FP operates using the principle that, when in solution, a small unbound fluorescent molecule will rotate more rapidly than when this molecule is bound to a larger target. This change in rotational speed can be measured by exciting the fluorescent molecule with polarized light. Unbound molecules will rotate rapidly, depolarizing the light source, while bound molecules will rotate slower, resulting in a retention of polarized light **(figure 1)** that can be measured using a plate reader and correlated with abundance of bound target. In the case of the Valita Aggregation Pure assay, each well of the 96-well plate is coated with a proprietary fluorescent small molecule which can bind to protein aggregates present in solution.

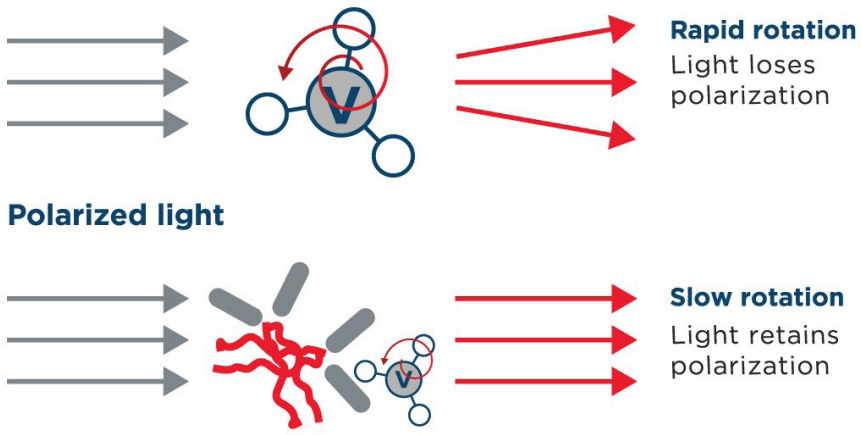


Figure 1. The fluorescence polarization principle used in the Valita Aggregation Pure assay. Small, unbound fluorophores rotate more rapidly in solution, emitting less polarized light **(top)**, while fluorophores bound to their larger targets rotate more slowly, emitting increased polarized light **(bottom)**.

While other technologies to measure aggregation typically have lengthy and complex workflows, the Valita Aggregation Pure assay was developed with speed and simplicity in mind. The Valita Aggregation Pure assay has a simple, 3-step, add-mix-read workflow **(figure 2)**. With no wash steps required and a single 5-minute incubation step, users can generate aggregation data for 96 samples in as little as 15 minutes, representing a significant time-saver versus many other technologies on the market.

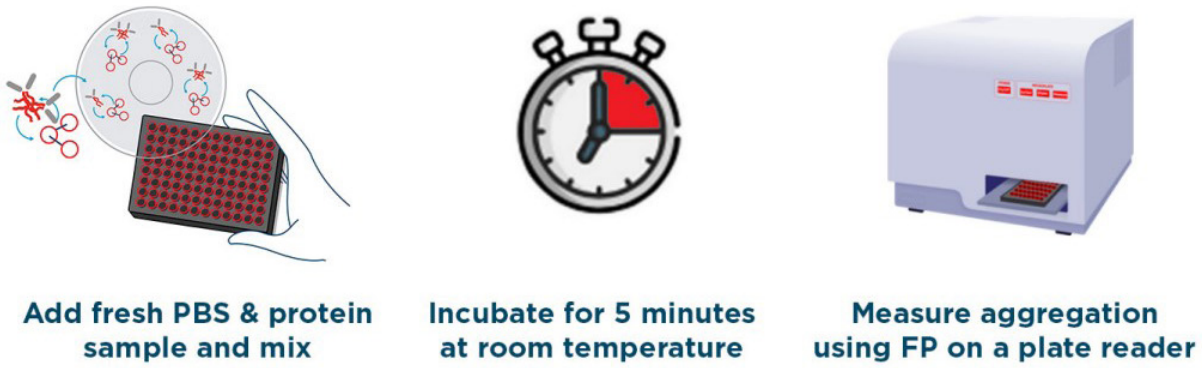


Figure 2. The Valita Aggregation Pure assay workflow. User adds buffer (60 µL) and sample (60 µL) to the assay plate and mixes. Following a 5-minute incubation at room temperature (RT) the plate is read using a fluorescence polarization-enabled plate reader and results are obtained.

Materials and Methods

Preparation of Aggregated material

All standard and sample preparations were made using a human IgG4 kappa molecule (in-house molecule) and PBS (Gibco, P/N 10010031). Highly aggregated IgG4 material was generated via titration to pH 3.5 at 22 °C using 2.0 M acetic acid (Thermo, J/0549/PB17), with stirring to ensure a homogenous solution. Following 72 hours at pH 3.5, the reaction was neutralized and titrated back to the molecule's original pH using 1.0 M Tris (Thermo, J22638.K2). Samples were then filtered using 0.22 µm PES filter and stored at -80 °C until use. This aggregated material was subsequently thawed, diluted to a target concentration (1 mg/mL or 2 mg/mL) using PBS and % aggregation was measured via HPLC-SEC using an Agilent 1260 system. This material was used to prepare standards of known % aggregation via mixing with a monomer IgG4 in fixed ratios, with aggregation percentages confirmed using HPLC-SEC. This process was repeated to generate test samples.

Valita Aggregation Pure Assay

All samples and standards were diluted to a fixed protein concentration (1 mg/mL in study 1, 2 mg/mL in study 2) prior to plating. A 0 mg/L standard (PBS buffer) was prepared as the final point in all standard curves for plate reader optimization. Following the preparation of all samples and standards, 60 µL of PBS was added to the wells of the Valita Aggregation Pure assay plate to allow probe reconstitution. 60 µL of each sample and standard were then added to individual wells in triplicate. Following addition of samples and standards to the Valita Aggregation Pure assay plate, all wells were mixed, and the plate was incubated for 5 min at room temperature, protected from light. Post-incubation the plate was read using a BMG PHERAstar Plus plate reader with settings shown in table 1, or a Biotek Synergy Neo2 plate reader with settings shown in table 2, and raw parallel and perpendicular values were exported for data analysis.

Data Analysis

Parallel and perpendicular fluorescence readings were used to produce raw fluorescence polarization (FP) values for each well. Triplicate wells were averaged for each standard and each individual sample preparation. The coefficient of variation and standard deviations for all triplicates were calculated using Microsoft Excel. Every sample and standard was tested at a concentration of 1 mg/mL. Standard curves were generated using the standard mP shift values and from this, test sample mP shift values were used to interpolate a percentage aggregation. The same was performed for 2 mg/mL samples using the mean raw mP values. All results were interpolated using GraphPad Prism 10. A 2 polynomial (2-poly) fit was applied to the standards and the R-squared was calculated. The aggregation percentage that was derived from Valita Aggregation Pure assay was assessed for accuracy against known HPLC-SEC values.

Parameter	Setting
Measurement Mode	Fluorescence Polarization
Optics Module	FP 560 630 630
G Factor	1.0
Flash number	200
Read Height [mm]	Optimized using most fluorescent well [0 mg/L]
Gain	Optimized using most fluorescent well [0 mg/L]; 70 mP target mP
Settling time	0.5 s

Table 1. Instrument settings used on the BMG PHERAstar Plus plate reader.

Parameter	Setting
Measurement Mode	Fluorescence Polarization
PMT	Dual PMT
Filter Cube	Dual FP Red Cube (#62 EX 530/25, EM 590/35, DM570)
G Factor	1.0
Measurements per data point	200
Read Height [mm]	Optimized using most fluorescent well [0 mg/L]
Gain	Automatic gain adjustment, scale to high wells, adjustment using most fluorescent well [0 mg/L]
Read speed	Normal
Lamp energy	High
Delay after plate movement	0 msec
Dynamic Range	Standard

Table 2. Instrument settings used on the Biotek Synergy Neo2 plate reader.

Results

Study 1: Proof of principle

Initially, the Valita Aggregation Pure assay was assessed using IgG4 with a total protein concentration of 1 mg/mL. This work utilized a BMG PHERAstar Plus plate reader at Valitacell/ Beckman Coulter Life Sciences to assess performance and establish proof of principle. Standard curves were automatically generated using a poly-2 fit using GraphPad Prism 10. For the standard curve, variation between replicates was minimal, with all triplicates reported an RSD of < 2.5%. The standard curve generated had an R^2 of = 0.995 **(figure 3)**.

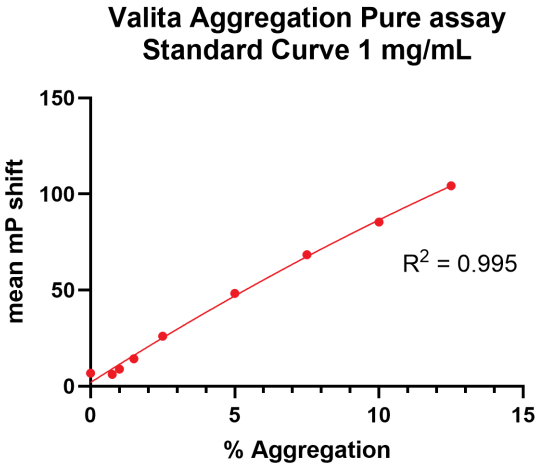


Figure 3. Standard curve at 1 mg/mL. The x-axis displays each standard's percentage aggregation. The y-axis displays the mean mP shift. Error bars display ± SD for each data point.

Using this standard curve, the aggregate percentages of the test samples were derived. Variation in mP between sample replicates was low, with all triplicates reporting an RSD of < 4 %. The correlation between the interpolated Valita Aggregation Pure assay results vs the % aggregation as measured by HPLC-SEC was calculated and an R^2 of = 0.995 was achieved **(figure 4)**.

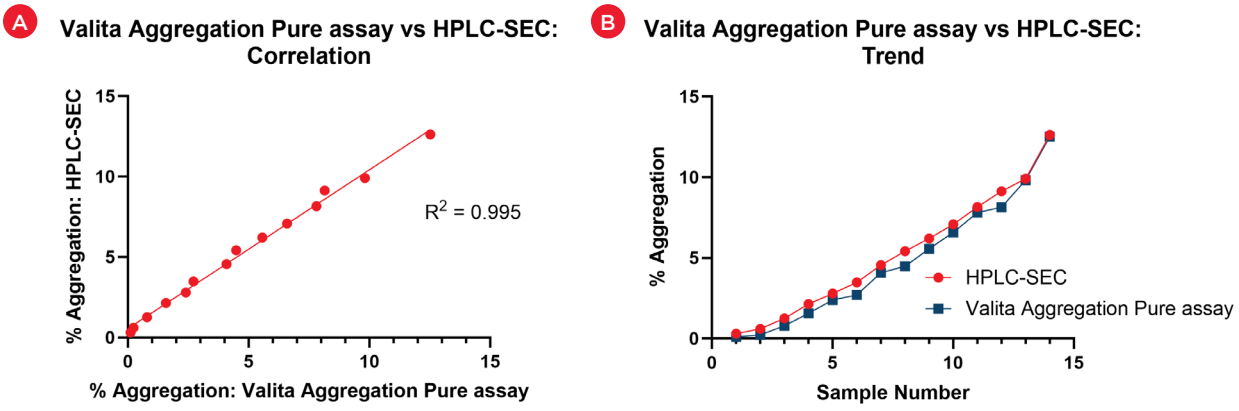


Figure 4. Valita Aggregation Pure assay vs HPLC-SEC. (A) Correlation between the % aggregation calculated using Valita Aggregation Pure assay vs % aggregation measured by HPLC-SEC. The x-axis displays the mean aggregation percentage derived using Valita Aggregation Pure assay while the y-axis displays the % aggregation as determined by HPLC-SEC. **(B)** The trend in percentage aggregation as measured by Valita Aggregation Pure assay vs HPLC-SEC. The x-axis displays the sample number while the y-axis displays the mean % aggregation calculated for each sample.

Study 2: in-house testing at an industry partner

Following the completion of this proof of principle at Beckman Coulter Life Sciences, in-house testing of the Valita Aggregation Pure assay was performed at our collaborator's site. Here, the Valita Aggregation Pure assay was assessed using IgG4 samples and standards at 2 mg/mL, previously characterized using HPLC-SEC. The Valita Aggregation Pure assay signal was measured using a Biotek Synergy Neo2 plate reader. Prior to commencing assay validation, the Synergy Neo2 plate reader was qualified for use with the Valita Aggregation Pure assay. IgG4 material was prepared and characterized using HPLC-SEC at our collaborator's site. This material was shipped to ValitaCell/Beckman Coulter Life Sciences for testing with the BMG PHERAstar Plus plate reader, while alternate IgG4 material was tested at the industry partner's site using a Biotek Synergy Neo2 plate reader. Testing revealed that both curves performed comparably despite differences in plate reader and analyst, with the RSD for all replicates across both assay occasions < 2%. Standard curves were generated automatically by GraphPad Prism 10 using a poly-2 fit. Both standard curves generated had an R^2 of > .0.99 **(figure 5)**.

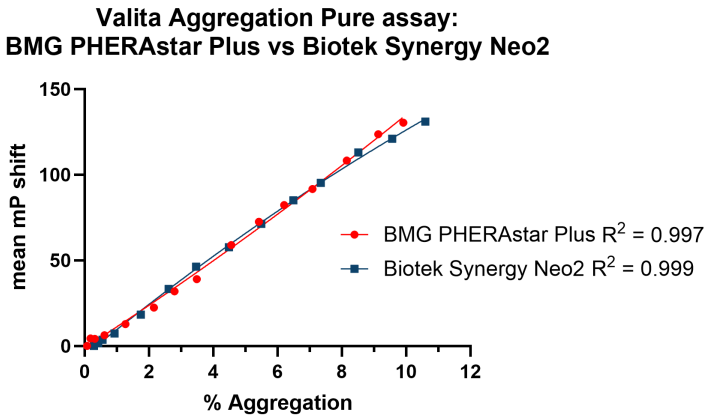


Figure 5. Valita Aggregation Pure assay performance using BMG PHERAstar Plus and Biotek Synergy Neo2. The x-axis displays each standard's percentage aggregation. The y-axis displays the mean mP shift ± SD for each data point.

Following plate reader qualification, the reproducibility, repeatability, and correlation of the Valita Aggregation Pure assay versus HPLC-SEC was assessed. Standards and samples were prepared at 2 mg/mL. A 16-point standard curve was prepared on plate 1. The standard curve was automatically generated using a poly-2 fit on GraphPad Prism 10. For the standard curve, minimal variation between replicates was observed, with all triplicates reporting an RSD of < 2.5 %. The standard curve generated had an R^2 of 0.999 **(figure 6)**.

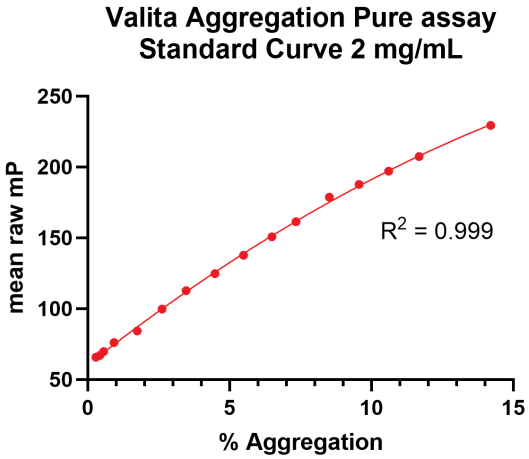


Figure 6. Standard curve at 2 mg/mL. The x-axis displays each standard's percentage aggregation. The y-axis displays the mean raw mP ± SD for each data point.

Using this standard curve, three preparations of 15 test samples were measured in triplicate across three different Valita Aggregation Pure assay plates (plates 2-4). Variation for all preparations was low with all triplicates across the three preparations reporting an RSD of < 3.5 %. The correlation between the interpolated Valita Aggregation Pure assay results vs the known % aggregation as measured by HPLC-SEC was calculated and an R^2 of > 0.99 was achieved across the three plates of test samples, indicating reproducibility and repeatability when using the Valita Aggregation Pure assay to assess % aggregation of test samples **(figure 7)**.

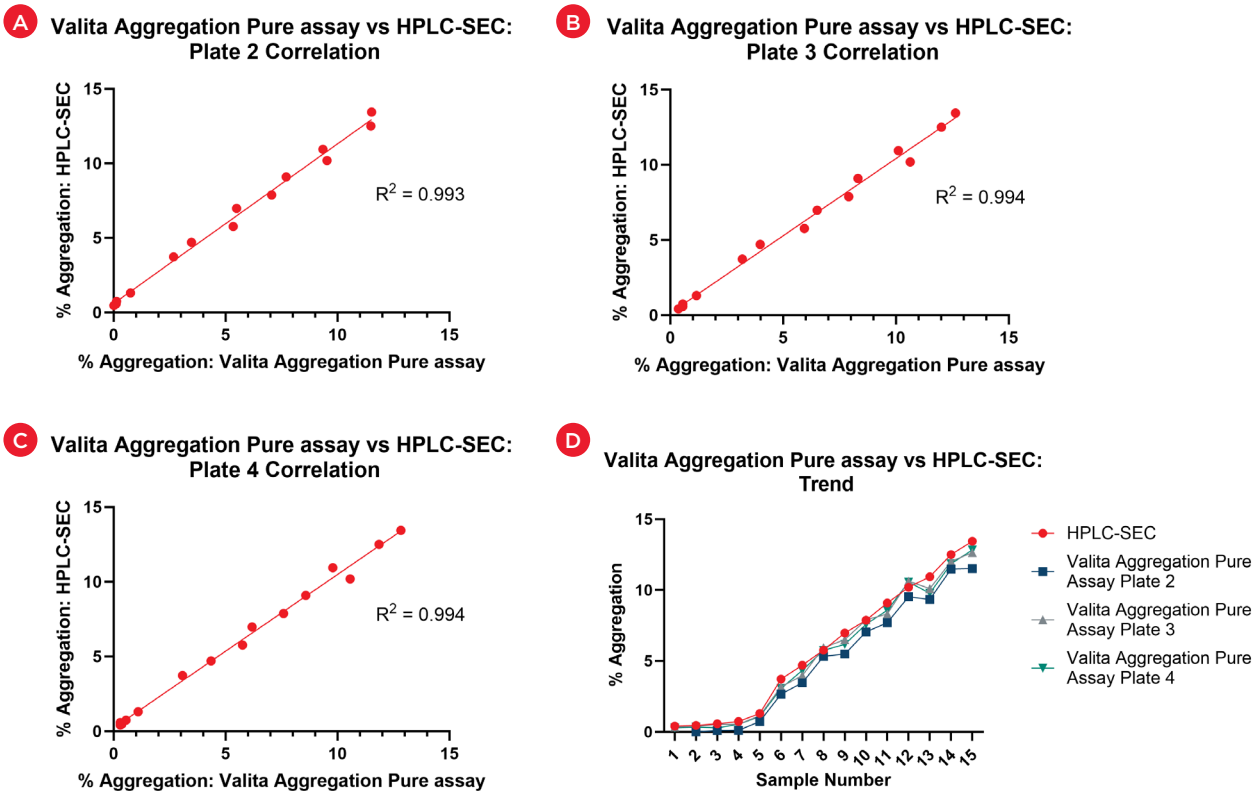


Figure 7. Valita Aggregation Pure assay vs HPLC-SEC. (A-C) Data from 3 separate Valita Aggregation Pure assay plates highlighting the correlation between the % aggregation calculated using Valita Aggregation Pure assay vs % aggregation measured by HPLC-SEC. The x-axis displays the mean aggregation percentage interpolated by Valita Aggregation Pure assay while the y-axis displays the % aggregation as determined by HPLC-SEC. **(D)** The trend in percentage aggregation as measured Valita Aggregation Pure assay vs HPLC-SEC. The x-axis displays the sample number while the y-axis displays the mean % aggregation calculated for each sample by either Valita Aggregation Pure assay or HPLC-SEC.

Discussion

The assays performed in this work demonstrate that analysts can produce rapid, reproducible, and repeatable aggregation data using the Valita Aggregation Pure assay that is comparable to that obtained using HPLC-SEC, the current gold standard technique for aggregation assessment in the Biologics industry. In this work, both the BMG PHERAstar Plus plate reader and Biotek Synergy Neo2 plate reader are shown to be suitable for the generation of high-quality, reproducible aggregation data **(figure 5)**, highlighting the versatility of the Valita Aggregation Pure assay and its ability to be integrated with a wide range of high-performance plate readers with fluorescence polarization capabilities.

Here, we tested IgG4 samples and standards at both 1 mg/mL and 2 mg/mL total sample concentration and demonstrated excellent intra- and inter-plate reproducibility, with excellent interpolation of test samples whether the interpolating standard curve is measured on the same plate as the test samples (1 mg/mL samples, figure 4) or on a different plate (2 mg/mL, figure 7).

We further demonstrated the reproducibility of the interpolation ability of the assay via incubation of test samples across 3 test plates (plates 2-4), with the standard curve used for interpolation generated on a separate plate (plate 1) **(figure 7)**.For both sample sets, excellent correlation of the interpolated % aggregation by Valita Aggregation Pure assay versus % aggregation measured by HPLC-SEC was observed, with an $R^2 \geq 0.993$ for all sample sets in study 1 and study 2.

Both sample sets contained 15 test samples. Due to the rapid, 3-step protocol of the Valita Aggregation Pure assay **(figure 2)**, aggregation data for these test samples was generated in approximately 15 minutes and could easily be scaled to measure up to 96 samples in this time. The corresponding HPLC-SEC data, with a run time of approximately 10 minutes per sample, would take 2.5 hours for 15 samples, increasing to > 8 hours for a 96-sample batch.

The Valita Aggregation Pure assay allows analysts to rapidly obtain aggregation data without laborious experimental workflows and complex data analysis. The ability to conduct rapid, simple aggregation screening in a high-throughput manner will enable scientists to generate aggregation data with greater ease and frequency, providing valuable extra insight into product quality and stability profiles throughout development. This in turn will facilitate better decision making throughout development, ultimately increasing the quality of mAbs produced while also potentially limiting late-stage project failures and decreasing the time from discovery to clinic.

References

1. https://www.grandviewresearch.com/industry-analysis/biologics-market

For Beckman Coulter's worldwide office locations and phone numbers, please visit Contact Us at beckman.com
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