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Life Sciences

# The Valita Aggregation Pure assay: A rapid and accurate alternative for aggregation quantification of purified monoclonal antibodies


## Abstract

Aggregation is considered a Critical Quality Attribute in the development of monoclonal antibodies. Current technologies are challenging for users for a variety of reasons including their complexity of execution and analysis, high CAPEX, labor intensity, lack of automation capabilities and the need for combinatorial analytics to obtain a comprehensive aggregation profile 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, we showcase the precision, accuracy and ease-of-use of the Valita Aggregation Pure assay when used to quantify aggregation in human IgG1 kappa samples. In this work we highlighted how the Valita Aggregation Pure assay is capable of accurately and precisely interpolating test samples with up to 99% accuracy.


## 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, in order to ensure minimal levels.

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 or dynamic light scattering (DLS) technologies. These technologies rely on specialized instrumentation and highly trained analysts. They are typically labor-intensive and time consuming with little to no automation potential to alleviate this burden. Due to the complex nature of these analytics, aggregation testing is often outsourced by Cell Line Development (CLD) and Process Development (PD) labs to analytical and quality control labs, which impacts negatively on the progress of development campaigns due to increased turnaround time for results and reduced sample screening throughput, as various labs compete to send samples for analysis.

Here, we demonstrate the use of the Valita Aggregation Pure assay to rapidly, accurately, and precisely, quantify aggregation of a human IgG1 kappa molecule in purified solution at a total protein concentration of 1000 mg/L. The use of the Valita Aggregation Pure assay for high-throughput aggregation screening represents a marked improvement versus the state-of-the-art with respect to simplicity, speed of results and potential for automation integration.

## Spotlight/Principle

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 uses the principle that a small unbound fluorescent molecule in solution 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 the 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 in solution.

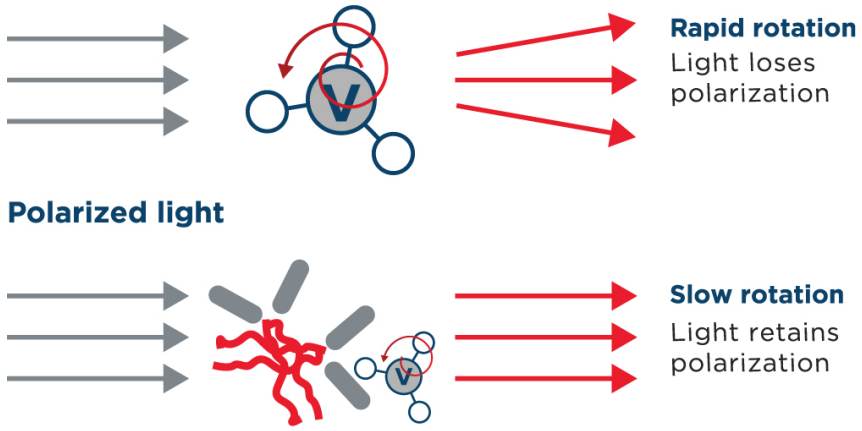


**Figure 1. The fluorescence polarization principle used in the Valita Aggregation Pure assay.** Small, unbound fluorophores rotate more rapidly in solution, emitting less polarized light **(top)**, while fluorophores bound to their larger targets rotate more slowly, emitting increased polarized light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

While other technologies that are used 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.

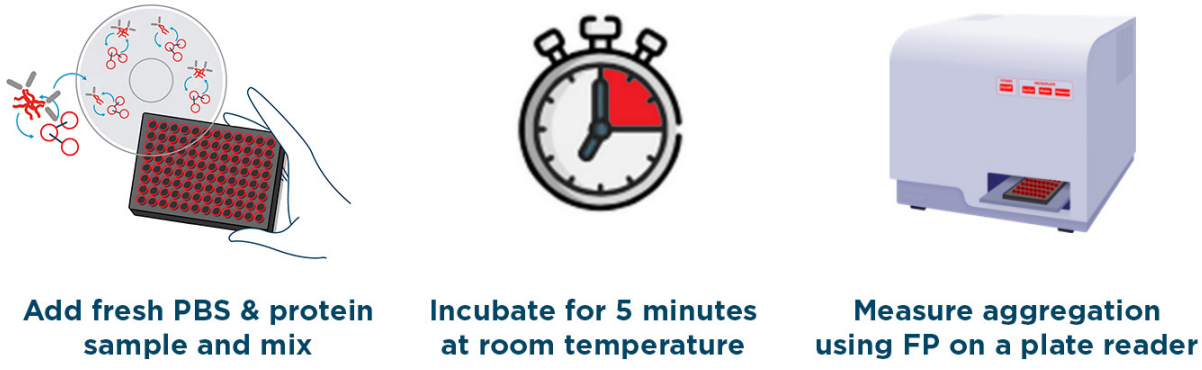


**Figure 2. The Valita Aggregation Pure assay workflow.** User adds buffer (60 µL) and sample (60 µL) to the assay plate and mixes. Following a 5-minute incubation at room temperature (RT) protected from light, the plate is read using a fluorescence polarization-enabled plate reader and results are obtained.

## Methods

### Preparation of High Molecular Weight Species (HMWS) material

All standard and sample preparations were made with Infliximab (Human IgG1 kappa) stock solution (10,000 mg/L) along with 0.1 M Glycine pH 2.5 (Sigma-Aldrich, 100327731) and 1 M Tris pH 8.0 (Thermo, J22638-AE) for the generation of HMWS material. PBS (Cytiva, SH30256.01) was used for all sample dilutions.

In all assessments, a standard curve was generated as demonstrated in Table 1 with a total protein concentration of 1000 mg/L. The standard curves also included a blank well containing 0 mg/L protein prepared using 60 µL PBS/well. This 0 mg/L well is used to optimise plate reader settings prior to reading the samples and standards to ensure optimal data quality.

To prepare the HMWS for the standard curve preparation, Infliximab underwent a 15-minute low pH hold using 0.1 M glycine, pH 2.5 to induce aggregation. The resulting material was considered 100% aggregated. Following neutralization of this reaction using 1 M Tris, the HMWS was mixed in fixed ratios with its associated monomer at the same concentration to prepare standards of a known % aggregation with a total protein concentration of 1000 mg/L.

| Tube | % Aggregation | Final volume [µL] | Volume of HMWS [µL] | Volume of monomer [µL] |
|---|---|---|---|---|
| 1 | 10% | 1000 | 100 | 900 |
| 2 | 7.5% | 1000 | 75 | 925 |
| 3 | 5.0% | 1000 | 50 | 950 |
| 4 | 2.5% | 1000 | 25 | 975 |
| 5 | 1.25% | 1000 | 12.5 | 987.5 |
| 6 | 0.50% | 1000 | 5 | 995 |
| 7 | 0% | 1000 | 0 | 1000 |

**Table 1.** Valita Aggregation Pure assay Standard Curve Preparation.

## Experimental design

In this study, precision was tested using the International Conference for Harmonisation (ICH) Q2 parameters for repeatability and intermediate precision (IP) [2]. This testing assesses the intra-assay (repeatability) (IP1) and inter-assay (intermediate precision) (IP1-3) variability that can be expected with the Valita Aggregation Pure assay. Accuracy was also assessed during this study, by comparing the interpolated value for % aggregation of test samples vs the known value. Two methods of calculating inter-assay precision and interpolating test samples were utilized in this report: once where all test samples were interpolated using the corresponding standard curve run on their test plate (method 1); and once where all test samples were interpolated using the standard curve generated in IP1 (method 2). The purpose of this was to assess whether sufficient accuracy and precision is retained when a standard curve is not included on the assay plate, as users have expressed an interest in using "banked" standard curves for the interpolation of test samples to increase ease of use of the assay.

### Valita Aggregation Pure Assay

All assay components were brought to room temperature prior to commencing the assay protocol. A standard curve was prepared as detailed in table 1. Test samples of 0.5%, 2.5% and 7.5% aggregation were also prepared following the same protocol for IP1. Three individual sample preparations for each test sample were included. On assay occasions IP2 and IP3, 2.5% aggregation test samples were prepared and assessed (prepared as in IP1). A 0 mg/L standard (sample buffer alone - no protein content) was also prepared as the 8$^{th}$ point in the standard curves. 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 sample or standards was then added to individual wells in triplicate. Following addition of samples and standards, 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 the instrument settings specified 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 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 Excel. Standard curves were generated using the standard raw FP values and from this, test sample raw FP values were used to interpolate a degree of aggregation in %. All results were interpolated using GraphPad Prism 10. A 5-parameter logistic (5-PL) fit was applied to the standards and the R-squared for all standard curves was calculated. For inter-assay precision, sample interpolation was calculated twice, once where all test samples were interpolated using the corresponding standard curve run on their test plate, and once where all test samples were interpolated using the standard curve generated in IP1. The interpolated % aggregation values were further analyzed in Excel to calculate accuracy of interpolation.

| 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 2.** Instrument settings used on the BMG PHERAstar Plus plate reader.

## Results

### Intra-assay precision/ repeatability (IP1)

Standard curves were automatically generated using a 5-PL fit using GraphPad Prism 10. For IP1, the triplicates produced a maximum % relative standard deviation (RSD) of 1.3% **(table 3)**. The standard curve generated had an $R^2$ of 0.9981 **(figure 3)**.

| % Aggregation | Raw FP | | | | | RSD (%) |
|---|---|---|---|---|---|---|
| | R1 | R2 | R3 | Average | Std Dev | |
| 10 | 185.69 | 182.94 | 187.68 | 185.43 | 2.38 | 1.29 |
| 7.5 | 158.31 | 155.78 | 155.76 | 156.62 | 1.47 | 0.94 |
| 5 | 135.05 | 135.34 | 133.24 | 134.54 | 1.14 | 0.84 |
| 2.5 | 108.79 | 109.66 | 109.37 | 109.27 | 0.44 | 0.40 |
| 1.25 | 96.13 | 94.79 | 96.38 | 95.77 | 0.86 | 0.89 |
| 0.5 | 87.14 | 86.71 | 86.43 | 86.76 | 0.36 | 0.41 |
| 0 | 80.08 | 80.55 | 81.61 | 80.75 | 0.79 | 0.97 |
| 0 mg/L | 70.49 | 68.70 | 69.83 | 69.67 | 0.90 | 1.30 |

**Table 3.** Raw FP values and associated variability between triplicates for the IP1 standard curve.

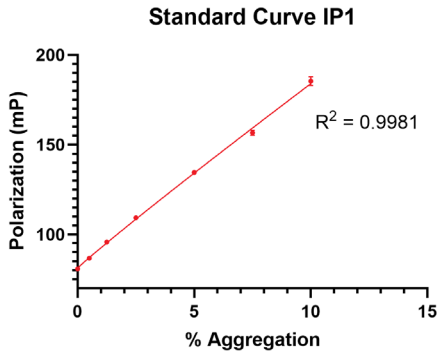


**Figure 3.** Standard Curve for assay IP1. The x-axis displays each standard percentage aggregation. The y-axis displays the mean raw FP ± SD for each data point.

Using this standard curve, the aggregated percent of the 0.5%, 2.5% and 7.5% aggregate test samples were derived and averaged for three individual preparations. The %RSD was calculated from the three individual preparations for both raw FP measurements and aggregated percentage of the samples. Variation in raw FP between replicates was low for all sample subsets with triplicates reporting an overall %RSD of < 2.5%. The general average % aggregation of these sample sets was interpolated with > 95% accuracy versus their starting concentration with less than 5% RSD **(table 4)**. The exception here is the 0.5% sample subset, whose average % aggregation was recovered with 93% accuracy versus its starting concentration but displayed a higher RSD value (13%) than the other sample subsets. The 0.5% sample subset likely displays this higher level of variation as previous results have demonstrated that the theoretical limit of detection of the assay is approximately 0.5% aggregation in 1000 mg/L samples. The RSD of 13% in the 0.5% test sample subset translate to a real difference of approximately ± 0.03% aggregation. When examining the standard deviations for all samples this appears to be fixed at approximately 0.08 % aggregation, or 0.8 mg/L aggregated protein. This apparent fixed error of the assay will result in higher RSD values for samples at the lower end of the curve.

| Sample type | Average resulting concentration | Std Dev | RSD (%) | Accuracy | Difference [%Agg] |
|---|---|---|---|---|---|
| | % Aggregation | | | % | |
| 0.5 | 0.53 | 0.07 | 13.2 | 93.87 | 0.03 |
| 2.5 | 2.48 | 0.09 | 3.50 | 99.09 | -0.02 |
| 7.5 | 7.13 | 0.08 | 1.2 | 95.02 | -0.37 |

**Table 4.** Valita Aggregation Pure assay intra-assay precision and accuracy results.

### Inter-assay precision/ Intermediate precision method 1

Using a 5-PL fit, the concentrations of 2.5% aggregation test samples were derived using their corresponding standard curve and average results were interpolated for each individual preparation across the 3 assays. Similarly, the %RSD was calculated for the individual preparations in terms of raw FP and this variation was < 2.5% for all sample subsets. The results from the 3 individual assay occasions were used to assess the RSD of the interpolated % aggregation. The RSD for these 3 assay occasions was calculated at 3.16%, with a standard deviation of 0.08% aggregation, or 0.08 mg/L aggregated protein **(table 5)**. The averages of all these test sample sets were interpolated with > 96% accuracy versus their starting concentration.

| Samples at 2.5% Aggregation | Result | Average | Std Dev | RSD (%) | Accuracy | Difference [%Agg] |
|---|---|---|---|---|---|---|
| | % Aggregation | | | % | | |
| Assay 1 | 2.48 | 2.42 | 0.08 | 3.16 | 96.66 | -0.02 |
| Assay 2 | 2.44 | | | | | -0.06 |
| Assay 3 | 2.33 | | | | | -0.17 |

**Table 5.** Valita Aggregation Pure assay Inter-assay precision and accuracy results method 1.

### Inter-assay/precision/Intermediate precision method 2

While the Valita Aggregation Pure assay was designed to contain a standard curve on every plate, and users should run a standard curve on every plate to maximize accuracy of results, we also wished to assess the ability of the assay to interpolate test samples run across multiple plates, while using a single standard curve generated during IP1 using a 5-PL fit. As described for the inter-assay precision 1, the results from the 3 individual assay occasions were used to assess the RSD. The RSD for these 3 assay occasions was calculated at 7.02%, with a standard deviation of 0.16% aggregation, or 0.16 mg/L aggregated protein **(table 6)**. The averages of all these sample sets were recovered within 9% of their starting concentration.

| Samples at 2.5% Aggregation | Result | Average | Std Dev | RSD (%) | Accuracy | Difference [%Agg] |
|---|---|---|---|---|---|---|
| | % Aggregation | | | % | | |
| Assay 1 | 2.48 | 2.29 | 0.16 | 7.02 | 91.68 | -0.02 |
| Assay 2 | 2.19 | | | | | -0.31 |
| Assay 3 | 2.21 | | | | | -0.29 |

**Table 6.** Valita Aggregation Pure assay Inter-assay precision and accuracy results method 2.

### Accuracy

Each aggregated test sample measured for intra-assay precision was assessed against their expected % aggregation (for the purposes of this experimentation the aggregation percent is considered known, as the samples come from the same lot as the aggregated standard from which all results are derived). All results were within 7% of their predicted values with a mean difference vs known % aggregation of ± 0.14% aggregation. Accuracy was improved to within 5% in samples with > 0.5% aggregation **(Table 4)**.

The 2.5% aggregation samples run across the inter-assay precision experiments gave an average of 2.42% aggregation providing a 96.66% accuracy with a mean real difference vs known aggregated material of ± 0.08% aggregated protein **(Table 5)**.

When the standard curve generated during IP1 was used to interpolate all test samples measured during IP1-3, an average interpolated value of 2.29% aggregation was obtained, giving 91.68% accuracy with a mean difference vs known % aggregated material of ± 0.21% aggregated protein **(Table 6)**.

## Discussion

The assays performed in this work have demonstrated that analysts can produce rapid, highly accurate aggregation data using the Valita Aggregation Pure assay. When users interpolate samples run on the same plate as their standard curve at a total protein concentration of 1000 mg/L (and > 0.5% aggregation in their samples), intra- and inter-assay precision of RSD ≤ 3.5% can be obtained. These results were obtained using plates tested by different analysts and were all performed manually, with no automated contributions. The consistency of the Valita Aggregation Pure assay was demonstrated across the standard curve, when samples are > 0.5% aggregated in a 1000 mg/L sample, interpolation can be performed with > 95% accuracy. For test samples interpolated using a standard curve run on a different plate, a mean accuracy of > 91% accuracy was observed.

As the Valita Aggregation probe may exhibit different binding profiles with different molecules, and as the resolving power of the assay is impacted by the total protein concentration of the sample, these results should be considered representative of the assay's performance and not absolute for all potential experimental conditions. As such, the increased variation observed for the 0.5% sample (RSD 13%) could likely be improved by increasing the total protein concentration of the samples and standard curves to enhance the accuracy and resolution of the high-sensitivity region of the curve.

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 clone selection and development, ultimately increasing the quality of goods produced while also potentially limiting late-stage project failures and decreasing the time from discovery to clinic.

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