APPLICATION NOTE

# Rapid Detection and Characterization of Protein Aggregates by Flow Cytometry

**ProteoStat® Protein Aggregation Standards (IgG) (ENZ-51039-KP002)**
**ProteoStat® Protein Aggregation Assay (ENZ-51023-KP002)**

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Therapeutic protein formulations are often plagued by problems with protein aggregation. Contaminants that give rise to protein aggregates can include host cell proteins, nonprotein materials or a damaged form of the protein product itself. Unfortunately, monitoring and identification of these particles has been difficult and is limited to size-exclusion chromatography and native gel electrophoresis. In this study flow cytometry and dyes specific to protein aggregates (ProteoStat®) or oil droplets (Bodipy) are used in combination to identify particles in a therapeutic formulation and assess the size of these particles. The results show that protein aggregates, formed from IgG after heating at low pH, have an average size of less than 2 microns. The aggregates resulting from IgG form a distinct population from silicon oil micro droplets mixed with the protein. This study demonstrates the utility of using flow cytometry combined with the ProteoStat® dye for the analysis of therapeutic protein samples. Using a single simple protocol, one can detect the relative size and number of particles, as well as the nature of the particles (aggregated protein, oil droplet or other solid material).

## Introduction

Proteins, such as monoclonal antibodies, are an increasingly important class of therapeutic drugs. Unfortunately, they often form aggregates during manufacturing, shipping and storage (1-4). In addition to loss of functional protein, aggregates may induce unwanted immune responses once administered to patients (5-7). Because of this, monitoring the type and amount of aggregate in a protein therapeutic has become an important concern for pharmaceutical companies and regulatory agencies over the last few decades (8).

Aggregates from protein formulation cover a wide range of sizes. Recently, subvisible aggregates (0.1-50 µm) have received attention for their potential immunogenicity and the fact that they have been overlooked analytically for many years (3, 8, 9). These small aggregates mimic highly immunogenic viruses and bacteria via size range and closely spaced repetitive epitopes on their surface (8, 10-12).

Therapeutic protein formulations are often contaminated with particles other than protein aggregates, such as silicon oil droplets (13, 14). Several groups are working on a method to detect and differentiate silicon oil droplets, protein aggregates and protein aggregates in silicon oil droplets (13, 15). Filipe *et al.* have used side scatter in flow cytometry, to estimate the size of protein aggregates by comparison to known standards (8).

The following results describe a therapeutic protein formulation analyzed for contaminants and aggregation using ProteoStat® dye with flow cytometry techniques. The size of the particles was estimated by comparison to known size standards. Protein aggregates were identified by binding and fluorescing in the presence of the ProteoStat® protein aggregation dye. Oil droplets were identified using a Bodipy dye which is taken up by the oil droplets.

## Materials and Methods

### Materials

1µm, 2µm, 5µm, 10µm DUKE STANDARDS™ Particle Counter Size Standards were obtained from Thermo Scientific (Waltham, MA). ProteoStat® Protein Aggregation Standards (IgG) (ENZ-51039-KP002) and ProteoStat® Protein Aggregation assay (ENZ-51023-KP002) were sourced from Enzo Life Sciences (Farmingdale, NY). Silicone oil Volatile Alkyl Methyl Siloxane Fluid (FZ-3196) was obtained from Dow Corning® (Midland, MI). Bodipy dye Pyrromethene 546 was obtained from Exciton (Dayton OH).

### Methods

*Making standard beads:*
To disperse the particles, gently invert the bottle several times, then immerse in a low power ultrasonic bath (10 seconds). Dispense 2-3 drops of each size of particles into 4ml PBS tube. Gently vortex tube before running flow cytometry.

*Preparation of 100% IgG aggregates*
Sheep IgG from the ProteoStat® Protein Aggregation standards kit (4mg/ml) was incubated in low pH buffer (1 M Glycine-HCl, pH 2.5) at 80°C for 18 hours to form aggregates.

*Silicone oil emulsion preparation*
1µl silicone oil added into 20ml PBS, vortex and immerse in ultrasonic bath for 10 min. Final silicone concentration will be 0.005%.

*IgG aggregates mixed with silicone oil detected by ProteoStat dye and Bodipy dye*
Sheep IgG aggregate (ProteoStat® Protein Aggregation standards) was diluted with native Sheep IgG to final concentration of 1 mg/ml comprised of 2.5% aggregated IgG. This was mixed with silicone oil emulsion (50µg/ml), ProteoStat® Detection Reagent (final dye dilution 1:2000), and Bodipy dye (final dye concentration 1.5 µM).

*Flow cytometry*

Flow cytometry was performed with a BD FACSCalibur flow cytometer (Becton, Dickinson and Company, Franklin lakes, NJ, USA). The data was collected with BD CellQuest software and processed with FlowJo 7.6.3 software (Tree Star, Ashland, OR, USA). The samples were analyzed with high flow rate and PMT voltages were adjusted for the size range of Size Standard from 1 µm to 10 µm. The measurements were stopped after 5000 events were counted. The events were detected by FL1 for Bodipy dye and FL3 for ProteoStat® dye. The size of the detected aggregates was estimated by comparing the amount of side scatter with the amount of side scatter produced by the known size standards.

## Results

Prior publications demonstrated that flow cytometry can be used to determine the size of particles using side-scatter (8). To confirm this finding, beads of known diameter were analyzed using flow cytometry (Fig. 1).

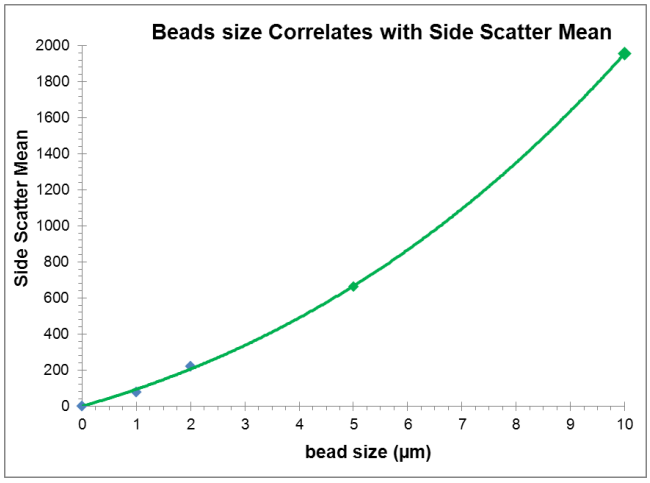



**Figure 1. Bead size correlates with side scatter mean in flow cytometry**.

The ProteoStat® dye delineated protein aggregate particles versus other particles, such as silicone oil, without interfering with size determination. Using ProteoStat® dye detection via flow cytometry, protein aggregates (IgG aggregated standard) were differentiated from oil droplets (Fig. 2).

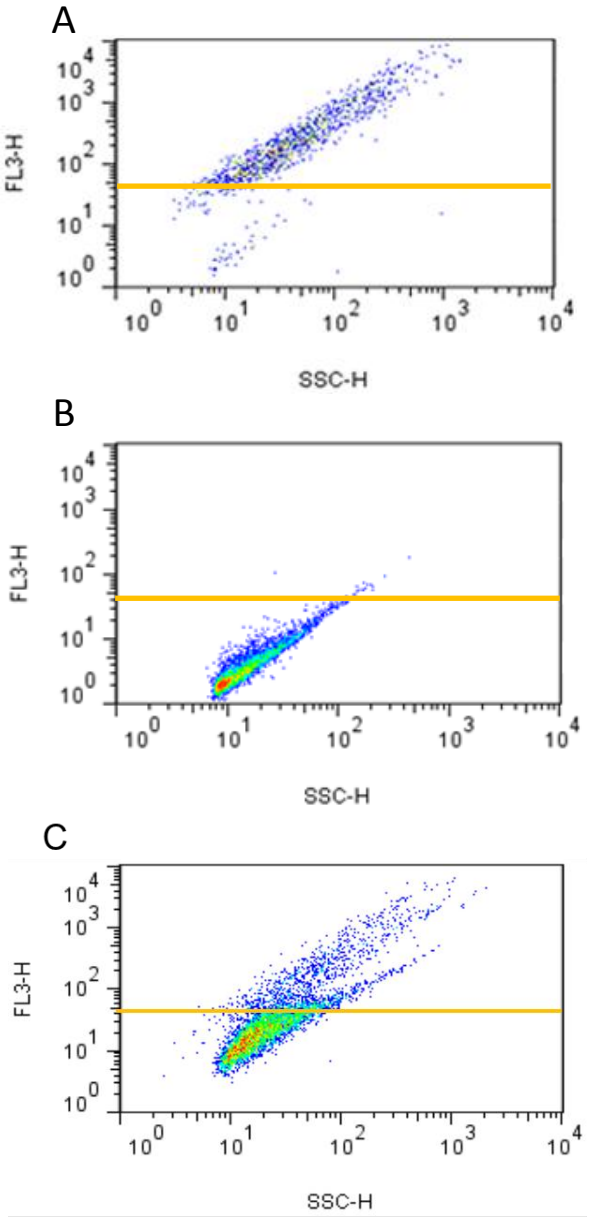


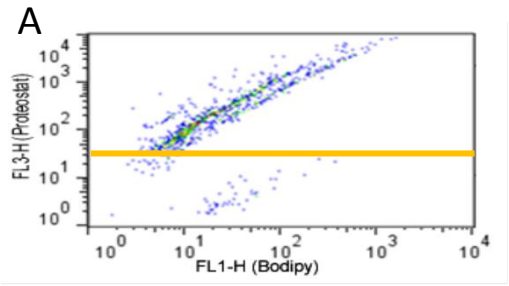

**Figure 2. Side Scatter vs ProteoStat® fluorescence of IgG aggregates (A), silicon oil droplets (B) and a mixture of silicon oil droplets and IgG aggregates (C).**

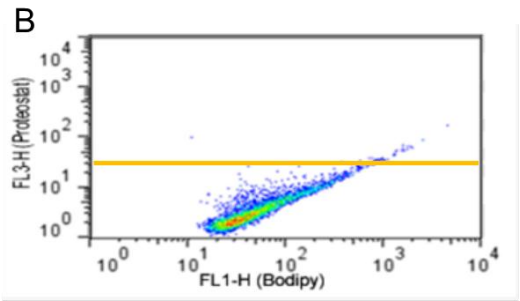
Based on this analysis, >97% aggregates from the ProteoStat® protein aggregation standards were less than 2 µm in size (Table 1).

| | 0-2µm (%) | 2-5µm (%) | 5-10µm (%) | >10µm (%) |
|---|---|---|---|---|
| Aggregate from 2.5% standard alone | 97.90% | 1.92% | 0.22% | 0.16% |
| Aggregate from 2.5% standard with ProteoStat dye | 97.50% | 2.31% | 0.20% | 0.02% |

**Table 1. Size range of the IgG aggregation standards (ENZ-51039-KP002)**

When treated with ProteoStat® dye, non-protein aggregate particles did not fluoresce. Yet, aggregates bound to silicone drops were still detected. ProteoStat® dye can be mixed with other dyes, such as Bodipy dye (Pyrromethene 546), and was used to differentiate oil droplets from true protein aggregates. Figure 3 shows the combined fluorescence of ProteoStat® (FL-3) and Bodipy (FL-1). Particles that did not stain with either ProteoStat® or Bodipy were likely debris (plastic or glass from the container). Note, some of the aggregated protein was detected on the oil drops (Fig. 3C).



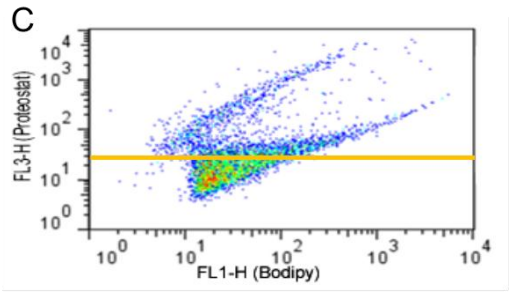




**Figure 3. Bodipy vs Proteostat® fluorescence of IgG aggregates (A), Silicon oil droplets (B) and a mixture of Silicon oil droplets and IgG aggregates (C). The threshold value (orange line) was determined as in Figure 2.**

## Discussion

The aim of this work was to develop a method for monitoring and detecting aggregation of protein drugs during biomanufacturing. By providing a high-throughput screening tool, bioprocessing facilities will be better equipped to devise strategies to limit or prevent protein aggregation, and, ultimately, safer and more effective protein drugs.

Flow cytometric analysis of protein formulations has many advantages. It is rapid, requires little sample volume and provides quantitative results of the nature and size of particles in solution. The addition of the red fluorescing ProteoStat® dye differentiates protein aggregates from other particles in the solution. A second dye in the green channel, such as Bodipy, can be used to detect oil droplets that often occur in bio-formulations. The method described here allows for accelerated analysis of parameters impacting monoclonal antibody and recombinant protein stability (pH, ionic strength, cryoprotectants, and excipients). This high-throughput screening capability allows for the optimization of different protein drug formulations at one time, ensuring production of the most stable and particle free formulation.

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