



## **PROTEOSTAT<sup>®</sup> Protein Aggregation Assay**

**For microplates or flow cytometry**

### **Instruction Manual**

#### **ENZ-51023-KP002**

2 x 96-well tests or 70 flow cytometry tests

#### **ENZ-51023-KP050**

50 tests in a 96 well plate or 16 flow cytometry tests



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Please read entire booklet before proceeding with the assay.

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Carefully note the handling and storage conditions of each kit component.



Please contact Enzo Life Sciences Technical Support if necessary.

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## I. INTRODUCTION

Biochemical and biophysical assays for monitoring protein aggregation are often cumbersome, relying upon ultracentrifugation, size-exclusion chromatography, gel electrophoresis, dynamic light scattering, or turbidity measurements.<sup>1,4</sup> None of the above mentioned techniques works well for every protein, nor are the assays ideal for tackling the wide range of aggregation problems that can arise during the manufacture of protein pharmaceuticals.

Enzo Life Sciences' PROTEOSTAT<sup>®</sup> Protein Aggregation Assay provides a simple, homogenous assay format for monitoring peptide and protein aggregation. The assay can be employed to streamline protein processing and formulation optimization procedures. Relative to conventional protein aggregation detection dyes, such as Thioflavin T, Enzo's PROTEOSTAT<sup>®</sup> Detection Reagent can detect aggregates from a broader range of proteins, yields a much brighter signal, provides at least 2 orders of magnitude linear dynamic range and offers superior performance across a broad range of pH values (4~10) and buffer compositions. Sensitivity of this assay is in the submicromolar range and less than 1% protein aggregate is detectable in a protein solution. The assay provides a convenient mix and read format and delivers Z' factor scores greater than 0.5. Therefore, the assay is capable of providing quantitative analysis of protein aggregation in a robust and high-throughput fashion. Lyophilized native and aggregated lysozyme is provided as negative and positive controls for monitoring changes in protein aggregation status. To facilitate quantitative analysis of protein aggregation, The PROTEOSTAT<sup>®</sup> Protein Aggregation standards (ENZ-51039) can be used to determine low levels of aggregated protein in a sample by comparing the assay response of a test sample to that of the standard curve; comprised of standards with known concentrations of aggregated IgG.

The PROTEOSTAT<sup>®</sup> Protein Aggregation Assay enables monitoring of protein aggregate formation in solution. This is useful for defining optimal storage formulations for proteins, for screening of compounds that promote or inhibit protein aggregation and, potentially, for the sensitive measurement of molecular chaperone activity.

## II. REAGENTS PROVIDED AND STORAGE

All reagents are shipped on dry ice. Upon receipt, the kit should be stored upright and protected from light at  $\leq -20^{\circ}\text{C}$ . When stored properly, these reagents are stable for at least twelve months. **Avoid repeated freezing and thawing.** The reagents provided in the kit are sufficient for 2 x 96-well microplates or 50 tests

Reagent	ENZ-51023-KP002	ENZ-51023-KP050
PROTEOSTAT <sup>®</sup> Detection Reagent	22 $\mu\text{L}$	5.0 $\mu\text{L}$
PROTEOSTAT <sup>®</sup> Positive Control, Aggregate	300 $\mu\text{g}$ (lyophilized lysozyme)	75 $\mu\text{g}$ (lyophilized lysozyme)
PROTEOSTAT <sup>®</sup> Negative Control, Monomer	300 $\mu\text{g}$ (lyophilized lysozyme)	75 $\mu\text{g}$ (lyophilized lysozyme)
10X Assay Buffer	15 mL	8 mL

## III. OTHER MATERIALS NEEDED

- Calibrated, adjustable precision pipetters, preferably with disposable plastic tips
- Deionized water
- DMSO

### For Microplate Readings:

- Fluorescence microplate reader with a filter set or monochromator setting of Excitation =  $\sim 550$  nm / Emission =  $\sim 600$  nm.
- 96 -well microplate: black wall microplate, preferably with a clear bottom.

### For Flow Cytometry Readings:

- Flow Cytometer
- Optional: DUKE STANDARDS<sup>™</sup> Particle Counter Size Standards, Thermo Scientific.

#### IV. SAFETY WARNINGS AND PRECAUTIONS



Handle  
with care



Protect  
from light

- This product is for research use only and is not intended for diagnostic purposes.
- Some components of this kit may contain hazardous substances. Reagents can be harmful if ingested or absorbed through the skin and may cause irritation to the eyes. Reagents should be treated as possible mutagens and should be handled with care and disposed of properly.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas. All blood components and biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.
- To avoid photobleaching, perform all manipulations in low light environments, in amber microcentrifuge tubes or protected from light by other means.

## V. METHODS AND PROCEDURES FOR MICROPLATES

### A. REAGENT PREPARATIONS

**NOTE:** Allow all reagents to thaw at room temperature before beginning the procedures. Upon thawing, gently hand-mix or vortex the reagents prior to use to ensure a homogenous solution. Briefly centrifuge the vials at the time of first use, to gather the contents at the bottom of the tube.

#### 1. Diluting 10X Assay Buffer

Allow the 10X PROTEOSTAT<sup>®</sup> Assay Buffer to warm to room temperature. Make sure that the reagent is free of any precipitation. Gentle warming may be used to get the solution back into solution. Dilute buffer in deionized water to 1X before use.

#### 2. Reconstitution of Controls

Prepare the PROTEOSTAT<sup>®</sup> Positive Control (Aggregate) and the PROTEOSTAT<sup>®</sup> Negative Control (Monomer) for monitoring and detection of protein aggregation. Both controls are supplied as lyophilized powder. They should be reconstituted in 500  $\mu$ L for **ENZ-51023-KP002** or 125  $\mu$ L for **ENZ-51023-KP050** deionized water to generate a 40  $\mu$ M stock solution. Gently invert to resuspend the controls. **Do not vortex or cause unnecessary bubbles as this may lead to aggregation which could affect results.** It is strongly recommended to perform the assay with positive (20  $\mu$ M aggregated lysozyme) and negative control (20  $\mu$ M monomeric lysozyme). Unused stock control samples may be stored in aliquots at 4°C for several weeks. Do not centrifuge the positive control, as the aggregates are in suspension, not in solution.

#### 3. Dilution of PROTEOSTAT<sup>®</sup> Detection Reagent

**NOTE:** The PROTEOSTAT<sup>®</sup> Detection Reagent is light sensitive. Avoid direct exposure of the reagent to intense light. Aliquot and store unused reagent at -20°C, protected from light. Avoid repeated freeze/thaw cycles.

**ENZ-51023-KP002**, for each 96-well plate, prepare 200  $\mu\text{L}$  of diluted PROTEOSTAT<sup>®</sup> Detection Reagent as follows: Add 10  $\mu\text{L}$  of PROTEOSTAT Detection Reagent and 20  $\mu\text{L}$  of 10X Assay Buffer into 170  $\mu\text{L}$  water. Mix well.

**ENZ-51023-KP050**, prepare 100  $\mu\text{L}$  of diluted PROTEOSTAT<sup>®</sup> Detection Reagent as follows: Add 5  $\mu\text{L}$  of PROTEOSTAT<sup>®</sup> Detection Reagent and 10  $\mu\text{L}$  of 10X Assay Buffer into 85  $\mu\text{L}$  water. Mix well.

## B. STAINING

1. Dispense 2  $\mu\text{L}$  of the diluted PROTEOSTAT<sup>®</sup> Detection Reagent into the bottom of each well of a 96-well microplate.

**NOTE:** *The PROTEOSTAT<sup>®</sup> detection dye is light sensitive. Be sure to protect samples from light.*

2. Add 98  $\mu\text{L}$  of the protein of interest to each well. The recommended protein concentration range is 1  $\mu\text{g}/\text{mL}$  to 10  $\text{mg}/\text{mL}$ . For higher concentrations of protein ( $\text{mg}/\text{mL}$  range), fluorescence intensity readings in the presence of high amounts of aggregate may become saturated and beyond the linear range of the assay. This should not be a problem when detecting lower aggregation levels (<20% aggregated protein). If desired, higher concentrations of protein may be diluted in 1X Assay Buffer or buffer of interest prior to assaying for protein aggregation. The final concentration of the PROTEOSTAT<sup>®</sup> detection dye is 1000 fold dilution in the assay.

Be certain to run negative and positive control samples, as well as 1X Assay Buffer alone (no protein), as a blank sample.

3. Incubate the microplate containing test samples in the dark for 15 minutes at room temperature.
4. Read generated signal with a fluorescence microplate reader using an excitation setting of about 550 nm and an emission filter of about 600 nm.

**NOTE:** *DO NOT wash the sample after incubation with PROTEOSTAT<sup>®</sup> detection dye. The fluorescence value of 1X Assay Buffer alone should be subtracted from the values for wells containing proteins.*

### C. CREATING A STANDARD CURVE

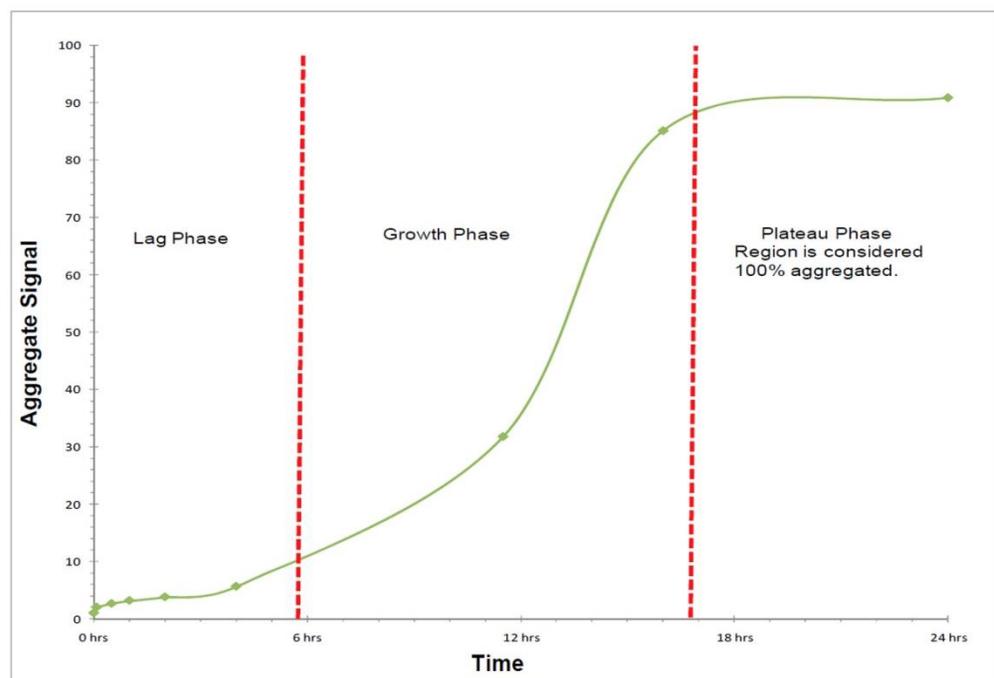
The PROTEOSTAT<sup>®</sup> Protein Aggregation Assay can be used to determine the percentage aggregated protein in a sample by comparing the assay response of a sample to that of a standard whose concentration is known. For convenience, Enzo Life Sciences offers pre-formulated aggregation standards (ENZ-51039). The stabilized, high-quality reference samples allow easy generation of trace protein aggregate levels in concentrated monomeric IgG. However, it is also possible to prepare standards directly in the laboratory. Regardless, protein samples and protein aggregation standards should be analyzed in the same manner by mixing them with detection reagent and using a fluorescence microplate reader to measure the intensity values. As with any protein assay, different protein aggregates will elicit greater or lesser fluorescence intensity response based upon their inherent amino acid composition and sequence. It is recommended that a standard curve be prepared each time the assay is performed.

For best results, the protein aggregation standard should always be prepared in the same buffer as the sample. The best choice for a standard is a highly purified version of the aggregated protein being analyzed in the test samples. If a highly purified version of the protein of interest is not available or if it is too expensive to use as the standard, the alternative is to choose a protein that will produce a similar fluorescence response curve with the PROTEOSTAT<sup>®</sup> Protein Aggregation Assay. For example, when analyzing IgG aggregation, an inexpensive goat anti-mouse antibody may be substituted for generating the standard curve. Once the standard protein is selected, it is necessary to generate a fully aggregated form of it. This is typically achieved by thermal denaturation as described below.

1. Create a fully aggregated standard protein. This is typically performed by heating the protein with shaking in a buffer that promotes aggregation of the protein.
2. Monitor the aggregation process using the PROTEOSTAT<sup>®</sup> dye assay method described in section B at different time points. In the example given in **Figure 1**, different stages are generally observed during the formation of IgG protein aggregates. First, a lag time is observed during which no aggregates are formed. After a certain lag time, nucleation occurs, and aggregates start to form (growth phase). Eventually, the growth rate decreases and becomes zero, indicating a limitation in monomer supply. The protein is considered fully aggregated when the fluorescence intensity no longer increases. Continued incubation may lead to large insoluble aggregates. For this reason it is best to employ the fully aggregated protein as soon

as it reaches the plateau phase for further use.

3. The standard curve is generated from different concentrations of aggregated protein in monomeric protein. The protein concentration used is typically about 30  $\mu\text{M}$ , but this may vary significantly, depending upon the needs of the investigator. A typical curve can be created using 100%, 80%, 40%, 20%, 10%, 5%, 2.5%, 1.25%, 0.6%, 0.3% and 0% aggregated protein. The different solutions of aggregated protein are prepared by mixing different ratios of aggregated protein and monomeric protein, keeping the total protein concentration the same. For example, a 20% aggregated protein can be prepared by adding 10  $\mu\text{L}$  of 100% aggregated protein and 40  $\mu\text{L}$  of monomeric protein to each well. A 10% aggregated protein can be prepared by mixing 5  $\mu\text{L}$  of 100% aggregated protein and 45  $\mu\text{L}$  of monomeric protein in a well. See **Figure 3** and **Table 1**. Typically, standard curves are constructed using at least three replicates for each point on the curve.



**Figure 1.** Different stages are generally observed during the formation of IgG protein aggregates. First, a lag time is observed during which no fibrils are formed. Then, nucleation occurs, and fibrils start to form (growth phase). Eventually, the growth rate decreases and becomes zero, indicating a limitation in native protein supply. The aggregation process can be accelerated by subjecting the proteins to elevated temperature. Extensive aggregation will cause large insoluble aggregates.

## VI. METHODS AND PROCEDURES FOR FLOW CYTOMETRY

### A. REAGENT PREPARATIONS

**NOTE:** Allow all reagents to thaw at room temperature before beginning the procedures. Upon thawing, gently hand-mix or vortex the reagents prior to use to ensure a homogenous solution. Briefly centrifuge the vials at the time of first use, to gather the contents at the bottom of the tube.

#### 1. 10X Assay Buffer

Allow the 10X PROTEOSTAT<sup>®</sup> Assay Buffer to warm to room temperature. Make sure that the reagent is free of any precipitation. Gentle warming may be used to get the solution back into solution. Dilute the 10X Assay Buffer with deionized water to 1X before use.

#### 2. Dilution of PROTEOSTAT<sup>®</sup> Detection Reagent

**NOTE:** For optimal staining, the concentration of each detection reagent will vary depending upon the application. Suggestions are provided to use as guidelines, though some modifications may be required depending up the particular protein aggregate in the application.

Prepare 3 mL of a 2X PROTEOSTAT<sup>®</sup> Detection Reagent as follows: Add 3  $\mu$ L of PROTEOSTAT<sup>®</sup> Detection Reagent and 300  $\mu$ L of 10X Assay Buffer (other buffers may be substituted) into 2697  $\mu$ L water. Mix well. This is enough for 10 assays.

### B. STAINING

1. Add 300  $\mu$ L of the protein of interest to a 5 mL round bottom tube. The recommended protein concentration range is 2  $\mu$ g/mL to 200  $\mu$ g/mL.
2. To each tube, add 300  $\mu$ L of diluted PROTEOSTAT<sup>®</sup> Detection Reagent. The final concentration of the PROTEOSTAT<sup>®</sup> detection dye is 2000 fold dilution in the assay. It is important to monodisperse the aggregate suspension at this step by gently pipetting up and down repeatedly.
3. Protect the sample from light and incubate for 15 minutes at room temperature.



## C. ANALYSIS

1. Analyze the stained samples in the Side Scatter, Forward Scatter, and FL3 channels of a flow cytometry. No washing is required prior to the flow cytometry analysis.
2. **OPTIONAL:** The size of the detected aggregates can be estimated by comparing the amount of side scatter with the amount of side scatter produced by known size standards<sup>5</sup> (DUKE STANDARDS™ Particle Counter Size Standards, Thermo Scientific) which may be run separately (refer to the application note on the product page, ENZ-51023, [www.enzolifesciences.com](http://www.enzolifesciences.com)).

## VII. APPENDICES

### A. MICROPLATE SETTING SELECTION

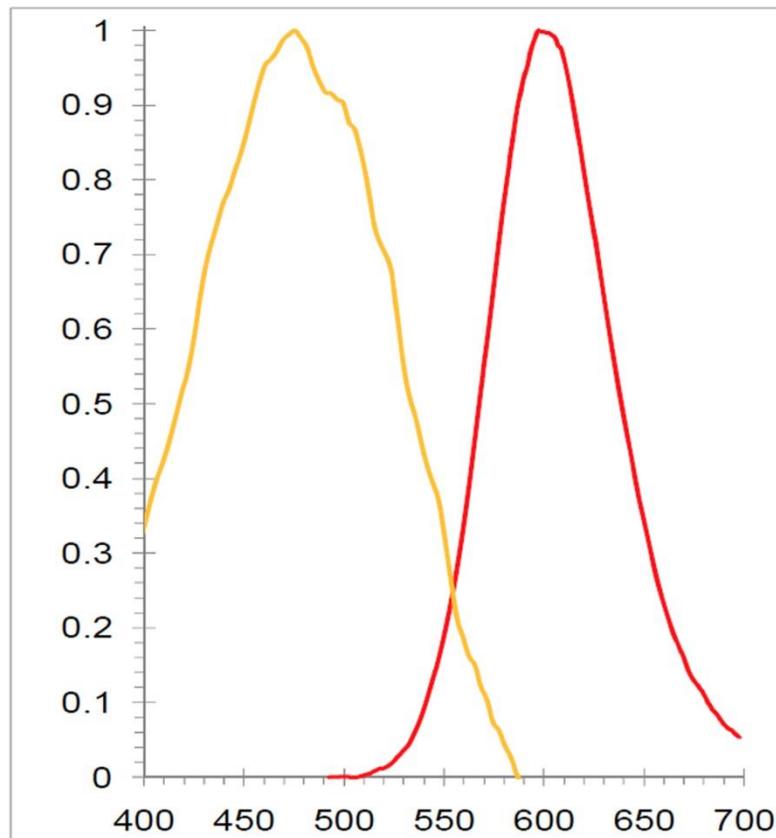
The selection of optimal settings for a fluorescence microplate reader application requires matching the monochromator or optical filter specifications to the spectral characteristics of the dyes employed in the analysis. Please consult your instrument or filter set manufacturer for assistance in selecting optimal filter sets. Pre-designed filter sets for Texas Red should work well for this application. For monochromator-based detection, we recommend a slit width of approximately 9 nm. Excitation around 550 nm gives slightly higher signal for protein aggregates relative to properly folded monomeric protein.

### B. EXPECTED RESULTS

The formulation of protein drugs is a difficult and time consuming process, mainly due to the structural complexity of proteins and the very specific physical and chemical properties they possess. Most protein formulations contain excipients added to stabilize protein structure, such as a particular buffer system, isotonic substances, metal ions, preservatives and one or more surfactants, with various concentration ranges to be tested. The conventional instrumentation-intensive analytical methods are intrinsically low-throughput, and usually require a long period of time to perform, as well as many manual interventions during the evaluation period. The development of new formulations is costly in terms of time and resources. Moreover, even for a known protein formulation, batch to batch quality control analysis is often less than optimal using the current state of the art methods. Therefore, a versatile, reliable, rapid and resource-efficient analytical method is useful for both developing novel protein formulations and identifying protein stability in quality control procedures.

Protein aggregates are characterized by a cross-beta spine quaternary structure.<sup>6</sup> In this structure beta strands of stacked beta sheets, emanating from different protein monomers, are aligned perpendicular to the axis of the fibril. The resulting quaternary structure consists of a double beta sheet, with each sheet formed from parallel segments stacked in register. Side chains protruding from the two sheets form a dry, tightly self-complementing steric zipper, bonding the sheets together. Within each sheet, every segment is bound to its two neighboring segments through stacks of both backbone and side-chain hydrogen bonds. The structure highlights the overall stability of amyloid fibrils, their self-seeding characteristics and their tendency to form polymorphic structures. The PROTEOSTAT<sup>®</sup> Protein Aggregation Assay kit contains a proprietary fluorescent probe that is minimally

fluorescent in the presence of the native form of a protein, but displays a 20~90-fold fluorescence intensity enhancement upon binding to the cross-beta spine quaternary structure of aggregated proteins.

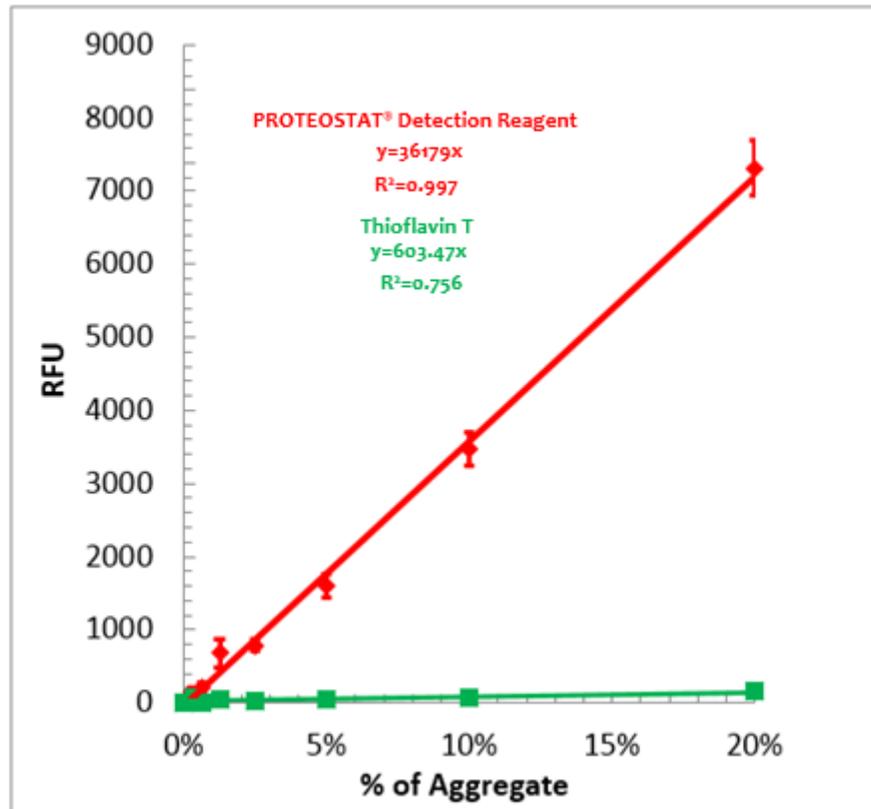


**Figure 2.** Absorption and fluorescence emission spectra for PROTEOSTAT Detection Reagent. All spectra were determined in 1X Assay Buffer.

**Figure 3** demonstrates the linear dynamic range of detection for goat anti-mouse IgG aggregate using PROTEOSTAT<sup>®</sup> Detection Reagent, as compared with Thioflavin T. Purified rabbit anti-goat IgG (4.26 mg/mL) was incubated in 100 mM HCl, pH 2.7, at 80°C for 90 minutes to form aggregates. The signal from the aggregate was determined after mixing aggregated with monomeric proteins at different ratios, such that the total IgG concentration remained 60 µg/mL protein. The readings were taken in 50 mM potassium phosphate, pH 7, containing either PROTEOSTAT reagent or Thioflavin T. Protein was incubated with the dye for 15 minutes prior to determining the fluorescence using a BioTek Synergy Mx microplate reader, with excitation setting at 500 nm and emission setting at 603 nm, both with a 9 nm slit-width for PROTEOSTAT<sup>®</sup> reagent, while Thioflavin T was detected with excitation setting at 435 nm and emission setting at 495 nm, both with a 9 nm slit-width. Readings were taken in at least triplicate in a Greiner<sup>®</sup> µClear black, clear bottom 96-well microplate. As demonstrated in **Figure 3**, the signal generated from the PROTEOSTAT<sup>®</sup> Detection Reagent is ~100-fold brighter

than Thioflavin T, and the concentration response curve is more linear.

In **Table 1**, the limit of antibody aggregate detection using PROTEOSTAT Detection Reagent compared with Thioflavin T is shown. PROTEOSTAT<sup>®</sup> Detection Reagent is able to reliably detect as little as 0.3% aggregated IgG in concentrated IgG sample (i.e., 0.19 µg/mL of the 60 µg/mL total IgG concentration).



**Figure 3.** Effective linear dynamic range for antibody aggregate detection using PROTEOSTAT<sup>®</sup> Detection Reagent compared with Thioflavin T. Relative fluorescence unit values (RFUs) may differ depending upon the microplate reader employed for the analysis.

**Table 1.** Detection limit comparison between PROTEOSTAT® Detection Reagent and Thioflavin T.

Aggregated Antibody Conc. (µg/mL)	% of Aggregated Proteins	PROTEOSTAT®				Thioflavin T			
		Average Intensity	St. Dev (+/-)	S/N	S/B	Average Intensity	St. Dev (+/-)	S/N	S/B
0	0	0	140			0	24		
0.19	0.3	100	120	0.7	1.08	11	40	0.5	1.04
0.38	0.6	212	75	1.5	1.17	0	67	0	0.84
0.75	1.3	679	196	4.8	1.55	0	47	0	1.31
1.5	2.5	787	77	5.6	1.64	0	42	0	0.82
3	5	1600	168	11.4	2.29	3	40	0.1	1.14
6	10	2952	236	21	3.81	4	34	0.2	0.91
12	20	7313	380	52.1	6.92	102	36	4.2	1.52

Thus, the kit provides an ideal analytical method enabling sensitive, accurate, and linear detection of protein aggregates over a broad concentration range. Additionally, the assay is resistant to sample-matrix interference and compatible with high throughput screening workflows.

## C. REAGENT COMPATIBILITY WITH ASSAY

The following table summarizes a sampling of reagents and their concentrations that have been demonstrated to be compatible with the PROTEOSTAT® Protein Aggregation Assay. The values represent the highest concentration of reagent tested with the protein sample. Reagents were tested individually in the protein samples and were considered compatible if the monomer signal didn't increase by more than 2-fold. This information is only provided as a guideline and actual samples, which normally contain different buffers and other components, may behave differently.

**Table 2.** Substances tested for compatibility with the PROTEOSTAT<sup>®</sup> Protein Aggregation Assay.

Tested Reagents and Validated Concentrations	
Sodium Chloride, up to 1 M	Ascorbic Acid, up to 1 mM
Calcium Chloride, up to 200 mM	Dithiothreitol, up to 1 mM
Ammonium Sulfate, up to 300 mM	Triton X-100, up to 0.01%*
Sorbitol, up to 600 mM	Tween-20 up to 0.01%*
Mannitol, up to 600 mM	Arginine, up to 500 mM
Trehalose, up to 600 mM	Glycine, up to 2%
Lactose, up to 300 mM	Ethanol, up to 20%

*Higher concentrations of certain detergents, such as 0.2% Tween-20, may produce an increase in background signal with the monomeric protein. However, the protein aggregate signal typically remains substantially larger*

## VIII. REFERENCES

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## IX. TROUBLESHOOTING GUIDE

Problem	Potential Cause	Suggestion
Poor fluorescent signal observed in the positive control	Detection reagent has been exposed to strong light.	Protect samples from exposure to strong light and analyze them immediately after staining.
	Kit reagent has degraded.	Verify that the reagents are not past their expiration dates before using them.
	Band pass settings are too narrow or not optimal for the fluorescent probe.	Use correct monochromator setting or filter set for the fluorophore. Check Methods and Procedures section of this manual and Appendix A for recommendations.
	Insufficient PROTEOSTAT <sup>®</sup> dye concentration	Follow the procedures provided in this manual.
High fluorescence background in the well without protein sample	The aggregated protein is not in solution. Extended centrifugation will cause the aggregate to precipitate	Do not centrifuge the aggregated protein (sample or control).
	Inappropriate dye dilution	Follow the procedures provided in this manual. It is important to make certain that there are no particles in the dye. Centrifuge well before use.
High fluorescence background in the well without protein sample	Dust or solid material in well	Make certain all solutions, micro-plates and tubes are clean and free of dust and dirt.
	Protein signal is saturated.	The concentration from protein sample is too high. Dilute the sample further with 1X Assay Buffer.
High fluorescence signal observed in negative control	Sample contains interfering substances.	The assay is compatible with commonly used buffers (PBS, Tris, HEPES) and excipients (trehalose and sucrose), but not with high concentration of Tween 20 (e.g., >0.2%)
There is no apparent protein in the controls.	Sometimes, the dried protein is difficult to see.	Resuspend the protein as recommended.



# Product Manual

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