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ProteoStat® Protein Aggregation Assay

for microplates

Instruction Manual

Cat. No. ENZ-51023-KP002 for 2 x 96-well plates

For research use only.

Rev. 1.4.0 February 2011

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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.^{1,4} 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[®] 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[®] 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 as little as 1-5% 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 proteins are provided as negative and positive controls for monitoring changes in protein aggregation status.

The ProteoStat[®] 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.

Reagent	Quantity
ProteoStat [®] Detection Reagent	22 μL
ProteoStat [®] Positive Control, Aggregate	300 μg (lyophilized powder)
ProteoStat [®] Negative Control, Monomer	300 μg (lyophilized powder)
10X ProteoStat [®] Assay Buffer	15 mL

III. Additional Materials Required

- Fluorescence microplate reader with a filter set or monochromator setting of Excitation = ~550 nm / Emission = ~603 nm.
- 96 -well microplate: black wall microplate, preferably with a clear bottom.
- Calibrated, adjustable precision pipettors, preferably with disposable plastic tips.
- Deionized water.

IV. Safety Warnings and Precautions

- 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

A. REAGENT PREPARATION

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. 1X Assay Buffer

Allow the 10X ProteoStat[®] Assay Buffer to warm to room temperature. Make sure that the reagent is free of any crystallization before dilution. Prepare enough 1X Assay Buffer for the number of samples to be assayed by diluting each milliliter (mL) of the 10X ProteoStat[®] Assay Buffer with 9 mL of deionized water.

2. Controls

Prepare the ProteoStat[®] Positive Control (Aggregate) and the ProteoStat[®] Negative Control (Monomer) for monitoring and detection of protein aggregation. Both controls are supplied as lyophilized powder (300 µg each) and should be reconstituted in 500 µL deionized water to generate a 40 µM stock solution. Gently mix the controls to re-suspend them—do not vortex or cause unnecessary bubbles. It is recommended to perform the assay with positive (20 µM aggregated Lysozyme) and negative control (20 µM native 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. ProteoStat[®] Detection Reagent Loading Solution

NOTE: The ProteoStat[®] 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.

For each 96-well plate, prepare 200 µL of ProteoStat[®] Detection Reagent Loading Solution as follows: Add 10 µL of ProteoStat[®] Detection Reagent and 20 µL of 10X Assay Buffer into 170 µL deionized water. Mix well.

B. STAINING

1. Prepare the 96-well microplate with 98 μL of protein of interest per well. Recommended protein concentration range is 1 μM to 30 μM . Much higher concentrations of protein (mg/ml) can also be employed, but readings in the presence of large amounts of aggregate may be saturated and beyond the linear range of the assay. This should not be a problem when detecting low aggregation levels. If desired, higher concentrations of protein may be diluted in 1X Assay Buffer or buffer of interest.

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

2. Dispense 2 μL of the prepared ProteoStat[®] Detection Reagent Loading Solution (see section A-3, page 3) into each well. The final concentration of the ProteoStat[™] detection dye is about 3 μM .

NOTE: *The ProteoStat[®] detection dye is light sensitive. Be sure to protect samples from light.*

3. Incubate the microplate containing test samples in the dark for 15 minutes at room temperature.
4. Read generated signal with a fluorescent 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[®] 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[®] 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. 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 fluorescence 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. Selection of a protein aggregation standard is potentially the greatest source of error in protein aggregate quantification. 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[®] 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. For example, an IgG antibody should be diluted to 0.9 mg/mL in 100 mM HCl and incubate at 37°C overnight.
2. Monitor the aggregation process using the ProteoStat[®] staining 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 fibrils are formed. After a certain lag time, nucleation occurs, and fibrils 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 cause large insoluble aggregates. For this reason it is best to use 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 μ M, but this may vary significantly, depending upon the needs of the user. 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 μL of 100% aggregated protein and 40 μL of monomeric protein to each well. A 10% aggregated protein can be prepared by mixing 5 μL of 100% aggregated protein and 45 μL 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.

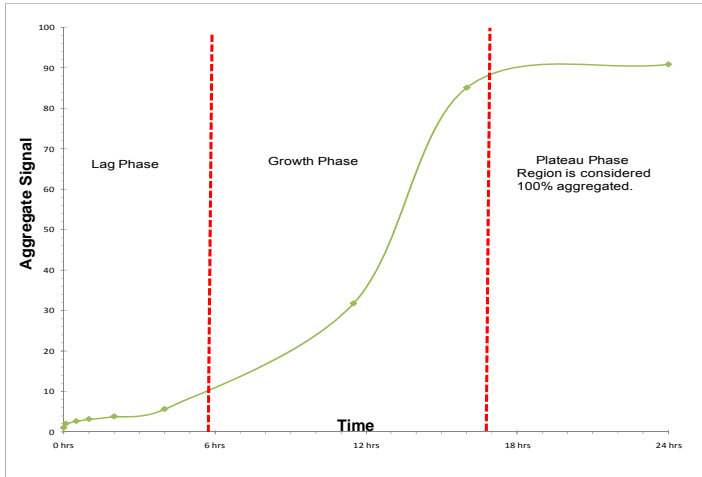


Figure 1. Different stages are generally observed during the formation of IgG protein aggregates. First, a lag time is observed during which no fibrils are formed. Then, nucleation occurs, and fibrils start to form (growth phase). Eventually, the growth rate decreases and becomes zero, indicating a limitation 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. 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 better separation of aggregate from properly folded protein.

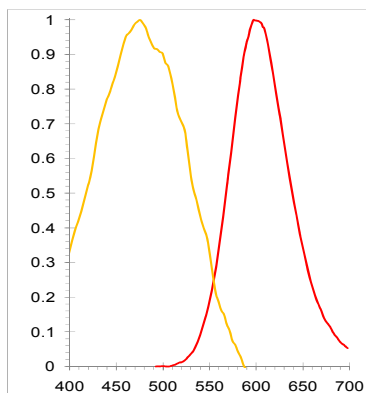


Figure 2. Absorption and fluorescence emission spectra for ProteoStat™ Detection Reagent. All spectra were determined in 1X Assay Buffer.

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, often referred to as amyloid fibrils, are characterized by a cross-beta spine quaternary structure.⁵ 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[®] 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 20~60-fold fluorescence intensity enhancement upon binding to the cross-beta spine quaternary structure of aggregated proteins.

Figure 3 demonstrates the linear dynamic range of detection for goat anti-mouse IgG aggregate using ProteoStat[®] 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,

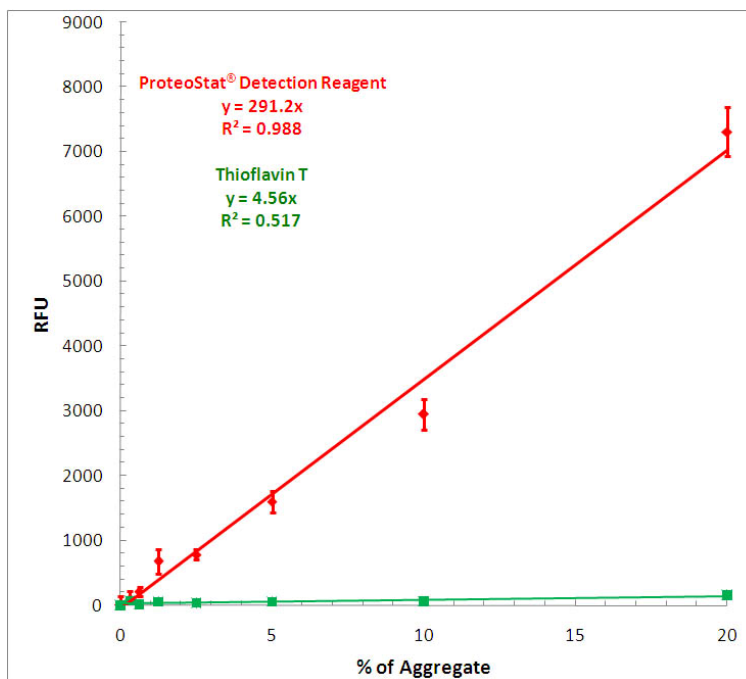


Figure 3. Effective linear dynamic range for antibody aggregate detection using ProteoStat[®] Detection Reagent compared with Thioflavin T.

such that the total IgG concentration remained 60 µg/mL protein. The readings were taken in 50 mM potassium phosphate, pH 7, containing either 3 µM ProteoStat® reagent or 3 µM Thioflavin T. Protein was incubated with the dye for 15 minutes prior to determining the fluorescence using a BioTek Synergy Mx plate scanner, with excitation setting at 500 nm and emission setting at 603 nm, both with a 9 nm slit-width for ProteoStat® 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® µClear black, clear bottom 96-well microplate. As demonstrated in Figure 3, the signal generated from the ProteoStat® 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® Detection Reagent is able to reliably detect as low as 0.3% aggregated IgG in concentrated IgG sample (*i.e.*, 0.19 µg/mL of the 60 µg/mL total IgG concentration).

Aggregated Antibody Conc.	% of Aggregated Proteins	ProteoStat® (3µM)				Thioflavin T (3µM)			
		Avg. Intensity	St Dev	S/N	Z'	Avg. Intensity	St Dev	S/N	Z'
0.00 µg/ml	0%	0	+/-140			0	+/-24		
0.19 µg/ml	0.3%	100	+/-120	0.7	1.08	11	+/-40	0.5	1.04
0.38 µg/ml	0.6%	212	+/-75	1.5	1.17	0	+/-67	0.0	0.84
0.75 µg/ml	1.3%	679	+/-196	4.8	1.55	0	+/-47	0.0	1.31
1.50 µg/ml	2.5%	787	+/-77	5.6	1.64	0	+/-42	0.0	0.82
3.00 µg/ml	5.0%	1,600	+/-168	11.4	2.29	3	+/-40	0.1	1.14
6.00 µg/ml	10.0%	2,952	+/-1,592	21.0	3.81	4	+/-34	0.2	0.91
12.00 µg/ml	20.0%	7,313	+/-575	52.1	6.92	102	+/-36	4.2	1.52

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

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 list 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.

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.*

Table 2. Substances tested for compatibility with the ProteoStat® Protein Aggregation Assay.

VII. References

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4. Arakawa T, Philo JS, Ejima D, Sato H, Tsumoto K (2007), "Aggregation analysis of therapeutic proteins, part 3". *Bioprocess International* 5 (10) : 52-70
5. Nelson R, Sawaya MR, Balbirnie M, Madsen AØ, Riekel C, Grothe R, Eisenberg D. (2005) "Structure of the cross-beta spine of amyloid-like fibrils." *Nature*. 2005 435(7043):773-778.

VIII. Troubleshooting Guide

Problem	Potential Cause	Suggestion
Poor staining observed in the positive control	Stained reagents have 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 fluorescent probes.	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™ dye concentration	Follow the procedures provided in this manual.
	The aggregated protein is not in solution. Extended centrifugation will cause the aggregate to precipitate	Do not centrifuge the aggregated protein (sample or control).
High fluorescent background in the well without protein sample	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.
	Dust or solid material in well	Make certain all solutions and 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.



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