Guide to Optimizing Protein Characterization

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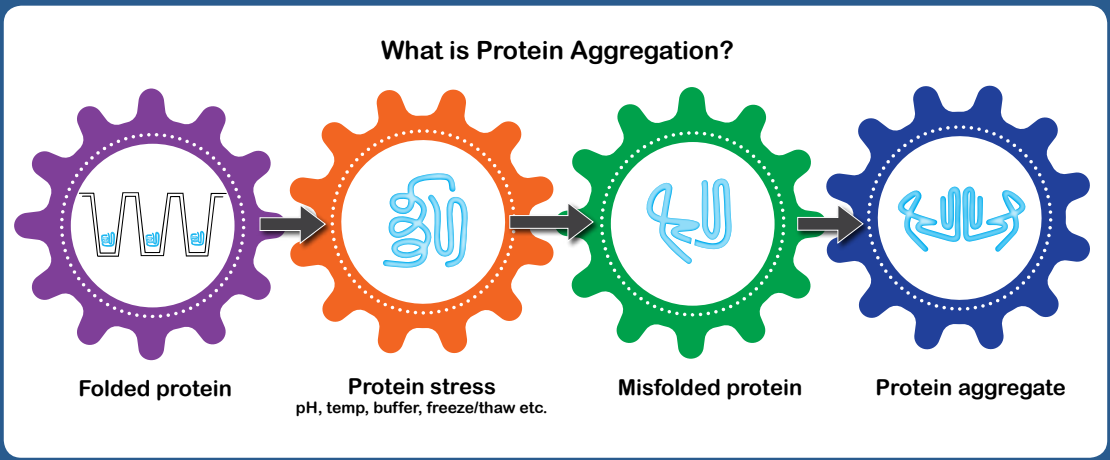
Overview

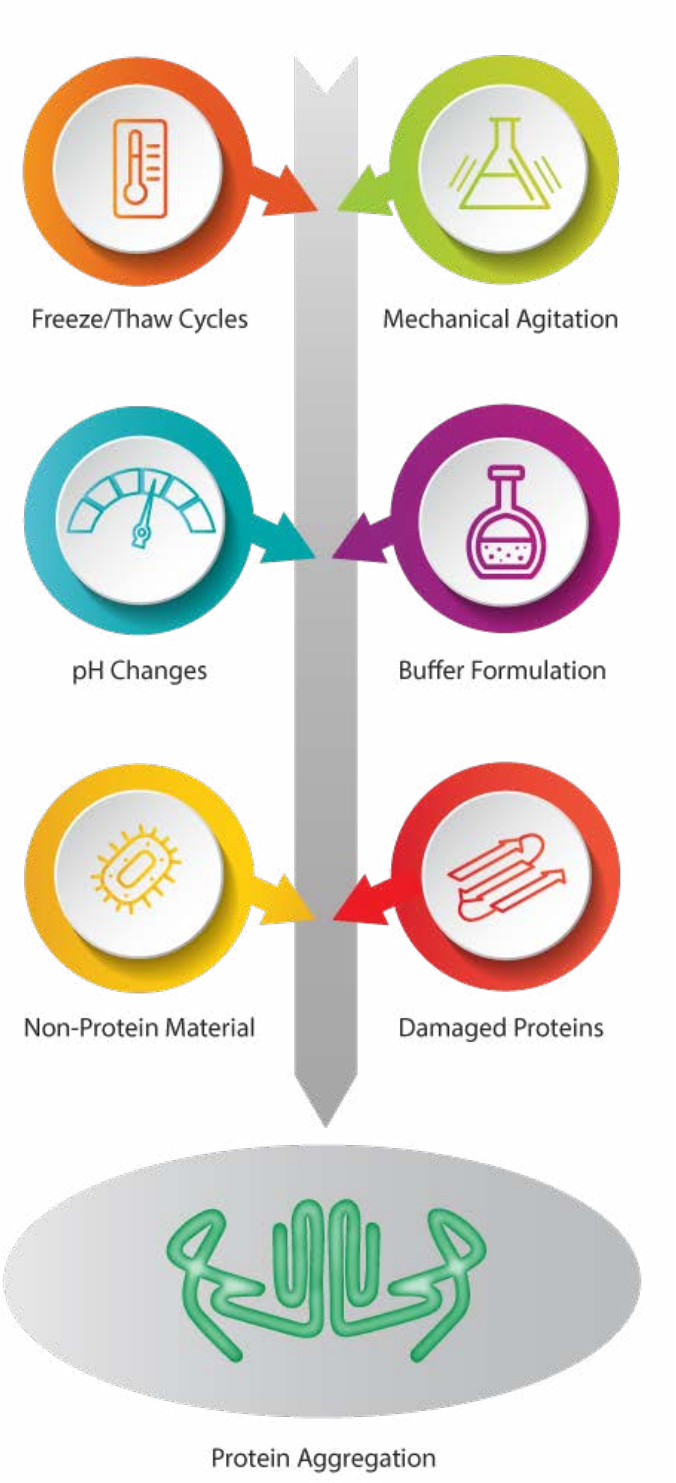
Recombinant proteins have become the frontrunner in biotherapeutics development. A typical example is the growing number of monoclonal antibodies validated for several treatments in which they are used to; induce apoptosis in the targeted cells, block the interaction between a receptor with its ligand, or induce cytotoxicity, etc. The efficient large scale production of these proteins requires careful handling during the process in order to preserve their biological function.

Chemical and physical factors, such as pH, ionic strength, agitation, protein concentration, freeze-thaw cycles, temperature, and packaging agents can compromise protein stability resulting in protein unfolding and aggregate formation. This phenomenon can occur at all stages of the protein manufacturing process including cell culture, purification, formulation, storage, shipping, and handling. Aggregates can be reversible or irreversible, and range in size from subvisible (2-10 µm) to visible (size ≥100 µm). Protein aggregation causes loss of activity and possible alterations of their immunogenic properties, thus representing a major obstacle for pharmacological applications. For this reason, the current FDA (Food and Drug Administration) regulations state that the presence of subvisible aggregates in therapeutic protein products should be assessed from their production over the course of their shelf-life.



Biochemical assays for monitoring protein aggregates rely on ultracentrifugation, size-exclusion chromatography, gel electrophoresis, dynamic light scattering, or turbidity measurements. These techniques are not applicable for every protein, nor are the assays ideal for addressing a wide range of aggregation problems encountered during formulation development and the manufacturing of protein pharmaceuticals. The development of new, efficient, and versatile methods to detect protein aggregation, is therefore extremely relevant.

Common Causes of Protein Aggregation



Current Food and Drug Administration Guidelines

Assessment should be made of the range and levels of subvisible particles (2–10 microns) present in therapeutic protein products initially and over the course of the shelf life. Several methods are currently qualified to evaluate the content of subvisible particulates in this size range (Mahler and Jiskoot 2012). As more methods become available, sponsors should strive to characterize particles in smaller (0.1–2 microns) size ranges.

Strategies to minimize aggregate formation should be developed as early as feasible in product development. This can be done by using an appropriate cell substrate, selecting manufacturing conditions that minimize aggregate formation, employing a robust purification scheme that removes aggregates to the greatest extent possible, and choosing a formulation (see section V.B.7) and container closure system (see section V.B.8) that minimize aggregation during storage.

Methods that individually or in combination enhance detection of protein aggregates should be employed to characterize distinct species of aggregates in a product…Constant improvement and development of these methods should be considered in choosing one or more appropriate assays. Assays should be validated for use in routine lot release and stability evaluations, and several of them should be employed for comparability assessments.

Animal studies may be useful in identifying aggregate species that have the potential to be immunogenic, although additional considerations (amount and types of aggregates, route of administration, etc.) may determine the extent to which such aggregate species pose clinical risk.

For the link to this guideline, go to: https://www.fda.gov/media/85017/download

How to Determine Protein Stability

Large scale production of protein-based pharmaceuticals goes hand-in-hand with stability concerns. Destabilized proteins are susceptible to both chemical and physical alterations. Chemical alterations are mainly due to covalent modifications such as oxidation and disulfide bond shuffling while physical changes may include protein unfolding, binding to surfaces, and aggregation. The latter impacts product quality significantly in terms of biological activity, pharmacokinetics, pharmacodynamics, and immunogenicity. As mentioned earlier, protein aggregation can occur at all stages of the manufacturing process including cell culture, purification, formulation, packaging, storage, shipping, and handling. Understanding the fundamental mechanisms of aggregation is valuable for identifying the factors underlying the issue. The pharmaceutical industry, therefore, requires a large panel of methods to detect, monitor, and quantify factors governing aggregation during manufacturing.

Size-exclusion Chromatography

Size-exclusion chromatography (SEC), also referred to as gel filtration, is an effective method used for the analysis of proteins. It is performed principally to verify the quality of the sample, the ability to form complexes, or the propensity of a protein to aggregate. SEC separates molecules by differences in size as they pass through a resin made of a porous matrix of spherical particles (or beads) packed in a column. Depending on their sizes, molecules can penetrate the pores at varying degrees.

Molecules larger than the pores of the resin are unable to diffuse into the beads and are eluted first. Molecules smaller than the pores can enter the total pore volume and are eluted last. The choice of SEC resin is based on its fractionation range. A fractionation range between 100 and 7000 Da is adapted to the separation of peptides while a fractionation range between 100 and 300 kDa is more suited to the analysis of large proteins such as antibodies.

Unlike other chromatographic techniques such as affinity chromatography or ion exchange chromatography, there is no binding involved in the process of SEC and the molecules only pass through the beads. The composition of the buffer is, therefore, not so critical to the resolution of the method. Other factors such as particle size, column dimension, flow rate,

and packing of the column come into the equation in order to achieve high resolution (i.e. narrow peaks). Optimization of the chromatography system (e.g. HPLC, FPLG, or UPLC) can be done via a monitor to follow the separation and a detector to confirm the elution of molecules (Fig. 1).

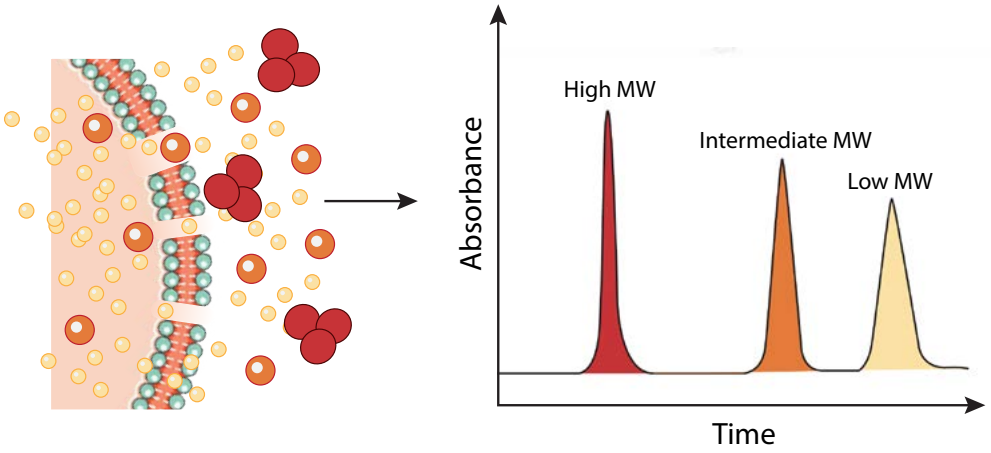


Figure 1. Principle of size-exclusion chromatography

Ultra-violet (UV) is the predominant mode of detection. Measurements can be done at low wavelengths (214 or 220 nm) for improved sensitivity or at longer wavelengths (280 nm) for a greater linear dynamic range. These two wavelengths can be combined together to cumulate these advantages and respectively allow the detection of low abundant species (e.g. protein aggregates) and a higher linear range for more abundant species (e.g. monomers). The wavelength ratio can then be used to determine purity as well as levels of aggregates against monomers.

While many techniques have been developed for the detection of protein aggregates, SEC is considered the standard method for evaluating protein aggregates of up to 100 nm in size due to its speed and reproducibility.

Dynamic Light Scattering

Dynamic light scattering (DLS) is used to ascertain the size of particles and macromolecules. DLS measures the Brownian motion of particles in a dispersion. Brownian motion is the random movement of particles, which results from the collisions with solvent molecules such as water. As particles get bigger, they move or diffuse more and more slowly. The rate of Brownian motion is quantified as the translational diffusion coefficient (i.e. “Dt”). DLS uses this information to determine the hydrodynamic size, which is defined as the size of a sphere that diffuses at the same rate as the particle being measured.

The measurement of the diffusion rate is done by illuminating particles with a laser. They scatter some of the light that hits them. In a dispersion, diffusion causes the intensity of scattered light to fluctuate over time. The detected light scattered from lots of randomly diffusing particles combines to create a fluctuating intensity signal. The intensity changes over time as the particles continue to diffuse. The speed of these intensity fluctuations depends on these particles' diffusion rates. Small particles diffuse quickly, which translates to more rapid fluctuations in scattered light (Fig. 2).

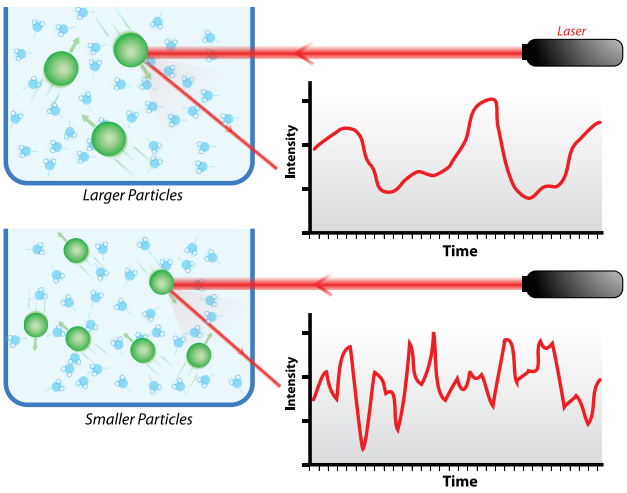


Figure 2. Principle of dynamic light scattering.
Credit: Mike Jones, https://commons.wikimedia.org/w/index.php?curid=10502233

Snapshots of a light scattering signal are taken rapidly one after another, always comparing these to the original signal measures. Between consecutive snapshots, which are on a scale of nanoseconds or microseconds, the intensity signals are very similar or well correlated. Between snapshots that are further apart in time, similarity or correlation begins to decrease. Eventually, intensity signals change completely. There is no longer any correlation with the original signal. The larger the particle being measured (e.g. protein aggregates), the more slowly they diffuse and the longer it takes for the correlation signal to decay.

This process is called auto-correlation and it enables the determination of "Dt". This value is used in the Stokes-Einstein Equation to obtain size information. Variables such as solvent viscosity and temperature affect the particles' diffusion rate and need to be added to the equation for accurate sizing. Using DLS, protein aggregates of up to 1 µm in size can be estimated in a sample. DLS has been used to investigate and improve vaccine and drug formulations.

Analytical Ultracentrifugation

Analytical ultracentrifugation (AUC) allows the quantitative analysis of macromolecules in solution. Two methods of AUC can be distinguished: sedimentation velocity (SV-AUC) and sedimentation equilibrium (SE-AUC).

In SV-AUC, a centrifugal force is used to separate macromolecules in solution. They settle at a specific sedimentation velocity, which depends mainly on instrument settings (i.e. angular

velocity), the molecule itself (i.e. density, mass, and shape), and the solution (i.e. density and viscosity). A boundary layer is formed between the top phase which is totally depleted and the bottom phase which contains all the macromolecules. As the centrifugation occurs, the depleted area inflate and the boundary layer shifts towards the bottom. The distance that the boundary layer travels over time depends on the mass, shape, and size of the macromolecules being deposited. The shape of the boundary layer is also indicative of the distribution of differently sized molecules in solution (e.g. monomers and protein aggregates).

In SE-AUC, a lower centrifugation speed is applied to macromolecules in solution. This approach allows for sufficient back diffusion from the bottom of the tube and leads to a stable concentration profile once all the particle movements have reached equilibrium. As shape-related parameters are not so essential to reach sedimentation equilibrium, SE-AUC is particularly useful to determine the molecular weight of macromolecules in homogeneous solutions but it can also be used with heterogeneous solutions to examine oligomerization as well as protein-protein interactions.

Absorbance, interference, or fluorescence measurements are performed over the course of the run to measure the sedimentation of macromolecules (Fig. 3). Unlike SEC, AUC allows the detection of aggregates of up to 100nm in size without any change in solution conditions. AUC and SEC can be performed in parallel to ensure the accurate representation of the aggregate population present in solution.

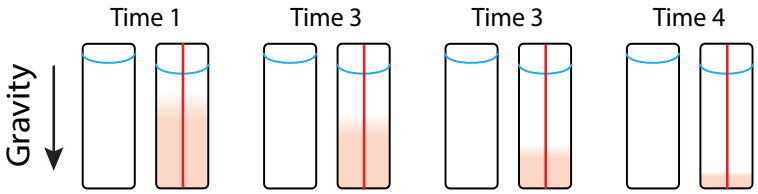


Figure 3. Fluorescence measurement at different time-points over the course of the ultracentrifugation process

Microscopy

While large aggregates can be seen with the naked eye, microscopy can be used to detect smaller aggregates. Aggregates bigger than 1 µm can be detected using standard bright-field or even fluorescence microscopy. With the help of fluorescent dyes specific to protein aggregates such as Congo Red, Nile Red, or Thioflavin T (ThT), greater details can be achieved using fluorescence microscopy. Although this approach offers limited ability to differentiate between different contaminants routinely found in therapeutic formulations, observations about shape and size of the protein aggregates are acquired in addition to counting. Microscopy also has the advantage of relying on basic instruments that can be found in most laboratories. They represent a very convenient and easy way to start characterizing protein aggregates and generate results. More complex systems such as atomic

force microscopy or electron microscopy can be combined with brightfield or fluorescence microscopy to detect protein aggregates of different but not overlapping sizes (i.e. typically smaller) and produce finer details as well as structural information.

Assay-based Kits

The aforementioned techniques are not applicable for every protein, nor are the assays ideal for addressing a wide range of aggregation problems encountered during formulation development and the manufacturing of protein pharmaceuticals. There is also no accepted method to effectively and simultaneously measure size, enumerate, and classify particles within the critical size range of 1-100 µm.

Molecular probes such as 8-Anilinonaphthalene-1-sulfonic acid (ANS), Congo Red, or Thioflavin T (ThT) can be used to monitor the aggregation process in real time. The exact mechanism of action of these dyes is currently debated. However, depending on the concentration they are used at, it has been suggested that they reduce aggregation. Similar to microscopy, assay-based kit utilize instruments (such as plate readers) that are commonly found in most laboratories. They offer a high-throughput method for the screening of many conditions. However, scientists can encounter numerous issues such as low brightness, low sensitivity, and poor specificity.

To address this need, scientists at Enzo developed the PROTEOSTAT® reagent, a fluorescent dye that facilitates detection and quantification of aggregated proteins in solution. This dye is an example of a molecular rotor-type fluorochrome, which exhibits a significant increase in fluorescence quantum yield in the presence of protein aggregates. The PROTEOSTAT reagent allows scientists to evaluate the propensity of a protein to aggregate by determining its temperature of aggregation in a thermal shift set-up. This information is complementary to the temperature of melting, which can be determined with the same approach using a dye like SYPRO® Orange, that binds to the hydrophobic regions of a protein. The combination of the two allows a better characterization of the stability of a protein in solution.

Additionally, the versatility of the PROTEOSTAT reagent can easily be adapted to the high-throughput screening and quantification of protein aggregation in a microplate format as well as the optimization of different protein drug formulations with sensitivity and specificity while ensuring the production of the most stable and aggregate-free formulations.

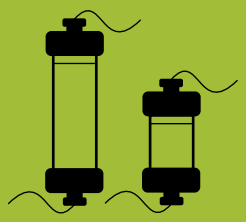
Methods for Detecting **Protein Aggregates**

PROS & CONS OF COMMON METHODS

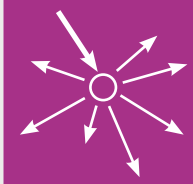
Size-exclusion Chromatography

Pro: In order to detect low levels of protein aggregates, a small amount of starting material is required.

Con: Nonspecific interactions with the column material.



Dynamic Light Scattering



Pro: Samples with different concentrations can be analyzed directly. Other advantages include quick analysis, low sample volume requirement, and information on protein shape.

Con: Temperature and solvent viscosity may alter results. Other disadvantages include low resolution, high dilution of samples required, and difficulty to analyze heterogeneous samples.

Analytical Ultracentrifugation

Pro: The experiments are performed in free solution, there are no complications due to interactions with matrices or surfaces.

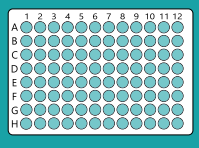
Con: The precision and accuracy for detecting low levels of protein aggregates are not well established.

Microscopy

Pro: Provides particle size and concentration information.

Con: Typically one sample analyzed at a time.

Fluorescence-based Assay Solutions



Pro: They allow high-throughput screening of many conditions.

Con: Needs set of standards or samples of known concentration for quantitative determination of protein aggregation.

Development and Specificity of the PROTEOSTAT® Dye

Plate-based assay methods using fluorescent dyes is a widely used technique for the characterization of proteins and their stability. Previously, many environmentally-sensitive dyes have been used to assess the thermostability of proteins by measuring the unfolding temperature, the challenge of looking at the propensity of a protein to aggregate still remained. The development of the PROTEOSTAT dye sought to resolve this issue. Its development was based on Thioflavin T (ThT), a molecular rotor dye with high affinity for protein aggregates (Fig. 1A). The PROTEOSTAT dye spins like a propeller in solution and simply intercalates in the cross-beta spline of stacked β-sheets characteristic of the quaternary structure of protein aggregates (Fig. 1B). Upon binding to aggregated proteins, the dye locks itself, stops rotating, and starts emitting a strong red fluorescence signal (Fig. 1C).

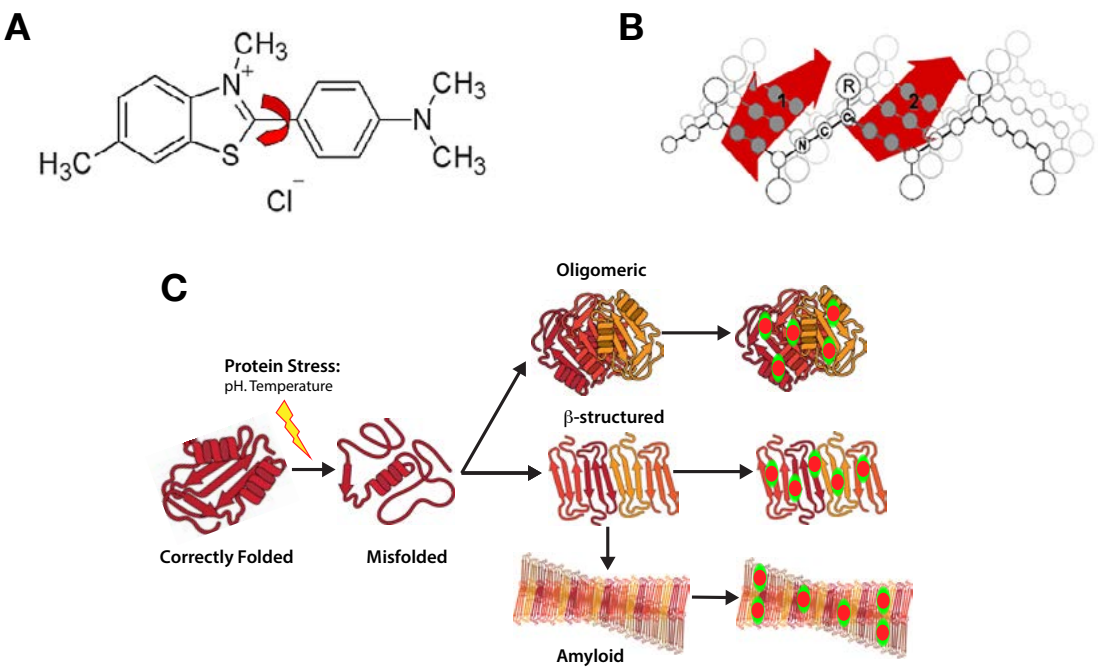


Figure 1. Characteristic structure of molecular rotor dye that rotates around a single bond (red arrow) in the absence of protein aggregates (A). Binding to stacked β-sheets in protein aggregates and immobilization (B). Molecular rotor dye emitting fluorescence upon binding and immobilization to protein aggregates (C).

The PROTEOSTAT dye does not detect natural or native dimerization, oligomerization or multimerization of peptides/proteins. It is specific for misbehaving peptides/proteins as shown notably by size-exclusion chromatography (Fig. 2) and has been validated with oligomeric aggregates, amorphous aggregates, and amyloid fibrils. Aggregates from a broad range of proteins can be detected by this dye (Table 1).

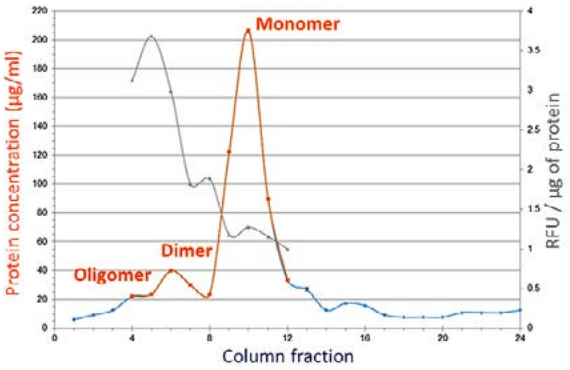


Figure 2: Size-exclusion chromatography of an IgG sample (orange line) demonstrates specificity of the PROTEOSTAT dye for protein aggregates (gray line).

ANTIBODY		
Sample	**Reference**	**Link**
ADC, ADCETRIS®	F. Lhospice, *et al.* (2015)	https://www.ncbi.nlm.nih.gov/pub med/25625323
Anti-PA biligand	M.B. Coppock, *et al.* (2017)	https://www.ncbi.nlm.nih.gov/pubmed/27539157
Anti-PA mAb	M.B. Coppock, *et al.* (2017)	https://www.ncbi.nlm.nih.gov/pubmed/27539157
IgG	A.D. McConnell, *et al.* (2013)	https://www.ncbi.nlm.nih.gov/pubmed/23173178
IgG	H. Uehara, *et al.* (2015)	https://www.ncbi.nlm.nih.gov/pubmed/25182973
IgG	A.D. McConnell, *et al.* (2016)	https://www.ncbi.nlm.nih.gov/pubmed/25517312
IgG	S. Murakami, *et al.* (2019)	https://www.ncbi.nlm.nih.gov/pubmed/30679500
IgG	C. Probst (2020)	https://www.ncbi.nlm.nih.gov/pubmed/31136765
mAbs	G.L. Lin, *et al.* (2016)	https://www.ncbi.nlm.nih.gov/pubmed/26891116
mAbs	A. Teplyakov, *et al.* (2018)	https://www.ncbi.nlm.nih.gov/pubmed/29283291
PEGylated (Fab')	C. Roque, *et al.* (2015)	https://www.ncbi.nlm.nih.gov/pubmed/25548945
Trastuzumab	S. Murakami, *et al.* (2019)	https://www.ncbi.nlm.nih.gov/pubmed/30679500

PROTEINS		
Sample	**Reference**	**Link**
α-synuclein fibrils	Y. Watanabe, *et al.* (2012)	https://www.ncbi.nlm.nih.gov/pubmed/23300799
α-synuclein fibrils	A. Tsujimura, *et al.* (2015)	https://www.ncbi.nlm.nih.gov/pubmed/25466281
β-amyloid	C. Regitz, *et al.* (2014)	https://www.ncbi.nlm.nih.gov/pubmed/24909620
β-amyloid	E. Vion, *et al.* (2014)	https://www.ncbi.nlm.nih.gov/pubmed/29223600
β-amyloid	N. Chaudhary, *et al.* (2019)	https://www.ncbi.nlm.nih.gov/pubmed/30704121
β-glucosidase	H. Uehara, *et al.* (2015)	https://www.ncbi.nlm.nih.gov/pubmed/25182973
DHFR variants	S. Bershtein, *et al.* (2013)	https://www.ncbi.nlm.nih.gov/pubmed/23219534
Endoglucanase	G. Bayram, *et al.* (2015)	https://www.ncbi.nlm.nih.gov/pubmed/25784767
Hemagglutinin	K.M. Holz, *et al.* (2014)	https://www.ncbi.nlm.nih.gov/pubmed/25540031
Hexapeptides	J. Berteen, *et al.* (2015)	https://www.ncbi.nlm.nih.gov/pubmed/25600945

HK2 variants	M.H. Nawaz, *et al.* (2018)	https://www.ncbi.nlm.nih.gov/pubmed/29298880
HSP70 variants	J.H. Seo, *et al.* (2016)	https://www.ncbi.nlm.nih.gov/pubmed/27708256
Lysozyme	C. Probst (2020)	https://www.ncbi.nlm.nih.gov/pubmed/31136765
Memb. proteins	I. Vandecaetsbeek, *et al.* (2016)	https://www.ncbi.nlm.nih.gov/pubmed/26695024
Diphteria toxin	S.M. McClure, *et al.* (2018)	https://www.ncbi.nlm.nih.gov/pubmed/29508082
NBD1 ΔF508 CFTR	W.M. Rabeh, *et al.* (2012)	https://www.ncbi.nlm.nih.gov/pubmed/22265408
STING proteins	J. Conlon, *et al.* (2013)	https://www.ncbi.nlm.nih.gov/pubmed/23585680
Tau fibrils	J.O. Esteves, *et al.* (2015)	https://www.ncbi.nlm.nih.gov/pubmed/25545358
Transferrin	H. Uehara, *et al.* (2015)	https://www.ncbi.nlm.nih.gov/pubmed/25182973

Table 1: List of antibodies and proteins tested with the PROTEOSTAT dye and cited in literature.

Performance of the PROTEOSTAT Dye

Relative to conventional protein aggregation detection dyes such as Nile Red or ThT, the PROTEOSTAT dye yields a much brighter signal, provides at least two orders of magnitude linear dynamic range and offers superior performance across a broad range of pH (4~10). Its sensitivity is in the sub-micromolar range and it can detect as low as 0.1% aggregate in a concentrated monomer solution (Fig. 3A). It also measures aggregates from 100 nm to 1 µm range (Fig. 3B). Finally, it is compatible with numerous chemicals including detergents such as CHAPS, Tween®-20, or Triton™ X-100 making it ideally suited for the optimization and the screening of drug formulations (Table 2).

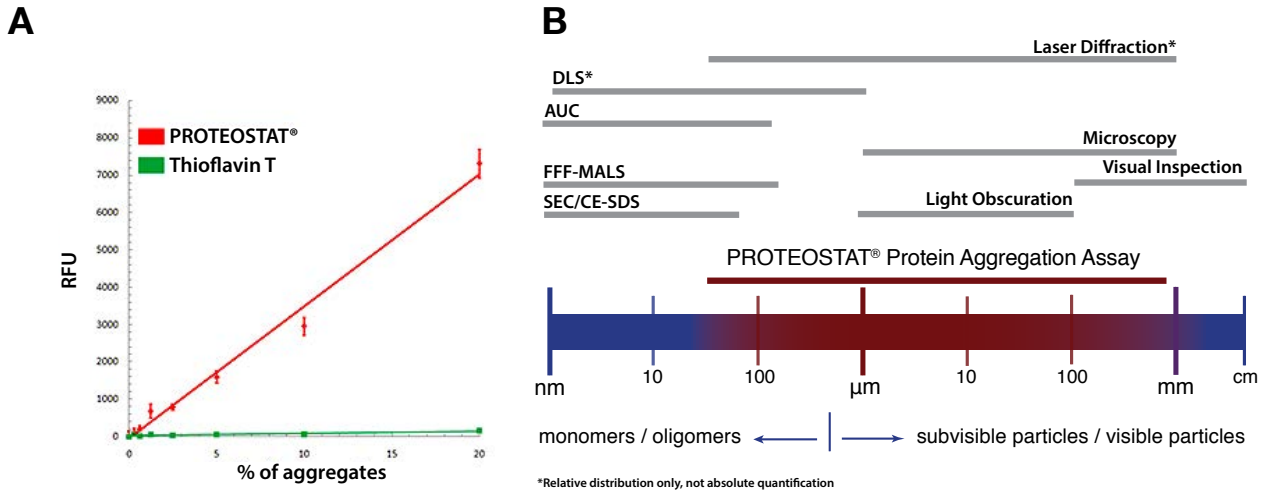


Figure 3: Sensitivity (A) and size range (B) of the PROTEOSTAT dye.

TESTED CHEMICALS AND VALIDATED CONCENTRATIONS	
Sodium chloride, up to 1M	Arginine, up to 500mM
Calcium chloride, up to 200mM	Glycine, up to 2%
Ammonium sulfate, up to 300mM	Glycerol, up to 50%
Sorbitol, up to 600mM	Urea, up to 6M
Mannitol, up to 600mM	Dithiothreitol, up to 1mM
Trehalose, up to 600mM	CHAPS, up to 0.5%
Lactose, up to 300mM	Tween-20, up to 0.01%
Ascorbic acid, up to 1mM	Triton X-100, up to 0.01%

Table 2: Compatibility of the PROTEOSTAT dye with chemicals commonly used in drug formulation.

Main Solutions for the Study of Protein Aggregates with the PROTEOSTAT Dye

The PROTEOSTAT Protein Aggregation Assay Kit (ENZ-51023) enables monitoring of protein aggregate formation in solution using a fluorescence microplate reader. This assay is useful for determining storage formulations for proteins, screening compounds that promote or inhibit protein aggregation, and potentially for the sensitive measurement of molecular chaperone activity. It provides a convenient and robust mix-and-read format and delivers Z' factor scores greater than 0.5. It is capable of providing quantitative analysis of protein aggregation in a robust and high-throughput fashion. It can be used alongside our PROTEOSTAT Protein aggregation standards (ENZ-51039) for accurate quantification of protein aggregation in solution.

The PROTEOSTAT Thermal Shift Stability Assay Kit (ENZ-51027) provides an improved thermal shift approach for assessment of protein stability through directly monitoring protein aggregation. From the thermal shift assay, a temperature at which the bulk of the protein becomes aggregated can readily be identified. The aggregation temperature is an indicator of protein stability and can be used to optimize conditions that enhance protein stability as well as to identify ligands or drugs that bind and confer structural stability to a protein of interest. It also minimizes problems encountered with other dyes such as high background fluorescence from their interaction with detergents, membrane proteins or hydrophobic compounds.

Finally, the PROTEOSTAT Aggresome Detection Kit (ENZ-51035) allows the detection of denatured protein cargo within aggresomes and aggresome-like inclusion bodies in fixed and permeabilized cells. This kit can be used to facilitate understanding of the basic molecular processes involved in the four key steps of autophagosome-dependent

degradation, namely induction or cargo packaging, vesicle formation and completion, docking and fusion, and vesicle breakdown. It is potentially applicable to screen for methods of growing cells to produce soluble recombinant proteins. Conditions such as different growth media, different temperatures, different lengths of induction, and different amounts of inducers can all be tested in one simple screen for protein aggregate formation in both bacteria and mammalian systems.

Using PROTEOSTAT® for Rapid Detection and Characterization of Protein Aggregates by Flow Cytometry

Proteins are an important class of therapeutic drugs. Protein aggregates form in a range of sizes. Larger visible aggregates are usually easily detectable and can be eliminated from formulations through means of ultracentrifugation, size exclusion chromatography, or gel electrophoresis. Smaller sub-visible aggregates, between 0.1-50 µm, are more difficult to detect especially if there are not appropriate means of detection already set in place.

These small aggregates are concerning because they possess a high potential of triggering an immune response due to their size and closely spaced repetitive epitopes on their surfaces, which can be similar to highly immunogenic viruses and bacteria.

Besides protein aggregates, therapeutic protein formulations may be contaminated with particles such as silicone oil droplets. Hydrophobic contaminants require a completely different means of elimination than protein aggregates. Therefore, it is crucial to have reliable distinction between oil droplets and protein aggregates for quality control.

In the experiment described below, a dye specific to protein aggregates, PROTEOSTAT, and to silicone oil droplets, Bodipy, were used in conjunction with flow cytometry techniques to analyze therapeutic protein formulations for contaminant particles, and

“Smaller subvisible aggregates, between 0.1-50 µm, are more difficult to detect.”

to distinguish between oil droplets and protein aggregates. In Figure 1, protein aggregates were identified by fluorescence upon binding of the PROTEOSTAT protein aggregation dye. Oil droplets were identified upon binding to the Bodipy dye.

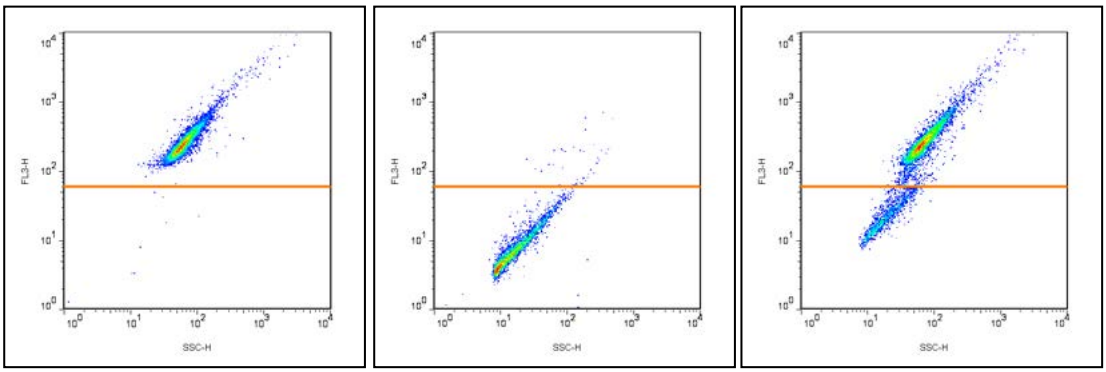


Figure 1. Side Scatter vs. PROTEOSTAT fluorescence of IgG aggregates (A), silicon oil droplets (B), and a mixture of silicon oildroplets and IgG aggregates (C).

Particle size standards (2.0µm, 5µm, and 10µm) were used for comparison with flow cytometry analysis. A mixture of 100% aggregated sheep IgG and native sheep IgG was used to create an unknown solution. A second solution was created using IgG aggregates and silicone oil mixture. After, PROTEOSTAT reagent and Bodipy dye were added to both. Flow cytometry was performed with the samples and analyzed. The size of the detected aggregates in the unknown IgG solution was estimated by comparing the amount of side scatter with the amount of side scatter of the known size standards.

	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%

Figure 2. Size range of the IgG aggregate standards. .

Utilizing the PROTEOSTAT dye allowed for clear differentiation (distinction) between the protein aggregates and the silicone oil droplets. More than 97% of aggregates from the IgG aggregate standard were less than 2 µM in size. Non-protein aggregate particles treated with PROTEOSTAT did not fluoresce in the absence of protein but did when treated with the Bodipy dye. PROTEOSTAT dye combined with Bodipy dye allows for distinction between oil droplets and true protein aggregates using two-channel flow cytometry.

By utilizing this method, bioprocessing facilities can be better equipped to identify protein aggregates and other contaminants. This would allow them to develop steps in their workflow to limit or prevent protein aggregation and as a result develop safer and more effective protein drugs. Analysis utilizing flow cytometry is advantageous in multiple ways. It is rapid, requires a small sample volume, and provides quantitative results regarding the nature and size of particles in solution. The addition of the red fluorescing PROTEOSTAT dye distinguishes protein aggregates from other particles in the solution. Another dye in the green channel, like Bodipy, can be utilized to detect oil droplets that frequently arise in bio-formulations. Using these dyes allows for time-effective analysis of parameters impacting the protein stability (pH, ionic strength, cryoprotectants, and excipients) of monoclonal antibody and recombinant protein products. This high-throughput screening capability allows for optimization of different protein drug formulations at one time, ensuring production of stable and particle free formulations.


APPLICATION NOTE

Rapid Detection and Characterization of Protein Aggregates by Flow Cytometry

For more information, download the complete Application Note.

Detection of Bacterial Aggregation by Flow Cytometry

Since the advent of recombinant DNA technology, bacteria have been used to express foreign proteins. Soluble proteins expressed by bacteria can be expressed either in the cytoplasm or secreted to the periplasm, the area outside the cell. In general, proteins expressed in the cytoplasm are expressed at higher levels than if they are secreted. Many hydrophobic and highly expressed proteins in the cytoplasm form inclusion bodies of aggregated proteins that are difficult to solubilize. Proteins that are targeted to the periplasm may not all get secreted and can form insoluble aggregates.

The most common method of aggregate detection in a bacterial culture involves isolating the cells, disrupting them by sonication, separating soluble from insoluble proteins by centrifugation, and finally identifying the location of the protein of interest using polyacrylamide gel electrophoresis. With the development of the PROTEOSTAT® dye, it can specifically stain amyloid type aggregates without the hassle of the previous steps mentioned, making it a simple method to detect aggregates via flow cytometry, such as the CytoFlex (Beckman Coulter Inc.). The PROTEOSTAT dye can be used to stain aggregates formed by overexpressed proteins in bacteria.

Prior to staining with the PROTEOSTAT dye, mammalian cells can be treated with MG-132, a proteasome inhibitor usually used as a positive control to induce protein aggregation. In the performed experiment, Jurkat cells were either mock-induced using 0.2% DMSO or induced using 5 µM MG-132 for 18 hours at 37°C. Following treatment, cells were fixed, permeabilized, incubated with the PROTEOSTAT dye, and analyzed with a flow cytometer. Typical results are illustrated by histogram overlays in Figure 1. Control cells displayed low fluorescence. MG-132-treated cells displayed over a two-fold increase of the fluorescence signal generated by the PROTEOSTAT dye when compared to untreated cells indicating that MG-132 induced the formation of protein aggregates in Jurkat cells.

In this study, a culture of *E.coli* BL21 containing a pET151-Klenow plasmid was used. This strain produces a DNA polymerase I Klenow fragment from a T7 RNA polymerase promoter controlled with a lac operator. The cells were either left un-induced or induced with 1mM IPTG for five hours at 37°C to promote the over-expression of the Klenow protein. Control cells displayed low fluorescence. The induction with IPTG increased dramatically the signal from the PROTEOSTAT dye when compared to non-induced cells suggesting that protein aggregates have formed. There also appeared to be a small population of cells in the induced cells that did not have aggregates. It can be hypothesized that these cells may either no longer be overexpressing the Klenow protein or have developed some method to prevent aggregate formation through random mutation.

The results from this experiment demonstrate that PROTEOSTAT dye using a flow cytometer can be used to screen for methods of growing bacteria to produce soluble protein. Differences in growth media, temperature, lengths of induction, and amount of inducer can all be tested in one simple screen for protein aggregate formation.

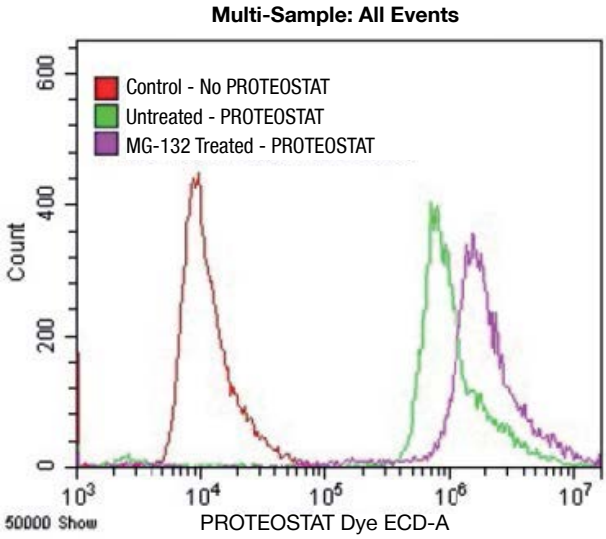


Figure 1. Flow cytometry analysis of protein aggregation in un-stained control, untreated control and MG-132-treated Jurkat cells.

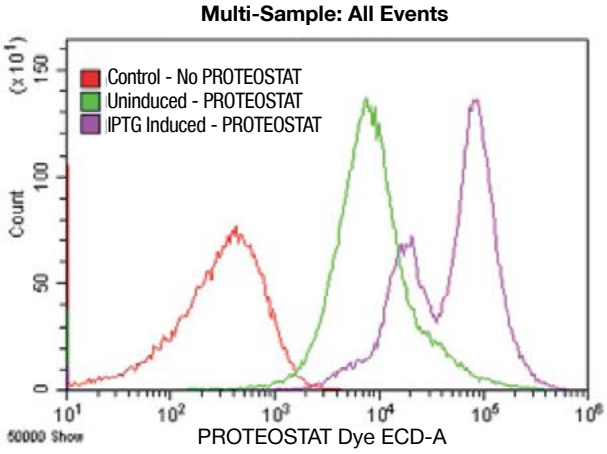


Figure 2. Flow cytometry analysis of protein aggregation in unstained control, non-induced control, IPTG-induced *E.coli.*


Enzo APPLICATION NOTES

Detection of bacterial aggregation by flow cytometry

For more information, download the complete Application Note.

Using PROTEOSTAT® Reagents to Predict Aggregation Propensity and Monitor Aggregation of Antibody-Drug Conjugates (ADC)

Like a Trojan horse, antibody-drug conjugates (ADC) are monoclonal antibodies engineered to go unnoticed, delivering cytotoxic drugs to cells expressing the antigen target. Successful development of an ADC requires optimization of several elements including the antibody, the potency of the cytotoxic drug, the stability of the linker, the site of conjugation and the stoichiometry of the resulting adducts.

ADCs are difficult to develop because multiple components must be optimized. A major factor in ADC performance is the efficiency of drug linkage. Unlinked antibodies as well as variations in the number of drugs linked to an ADC can have severe effects on the final drug's efficacy, stability, target-specificity, and toxicity.

Innate Pharma, a biopharmaceutical company that develops novel immuno-therapy drugs used to treat cancer and inflammatory diseases, developed a new ADC-coupling technology. In order to verify the technology for clinical applications, the stability of the ADC products was assessed by measuring the aggregation propensity and physical and chemical stability of ADC products. The data was collected utilizing the PROTEOSTAT dye were correlated with results acquired by size exclusion-high performance liquid chromatography (SE-HPLC) and liquid chromatography-mass spectrometry (LC-MS).

MATERIAL	TYPE	MANUFACTURERS
SGN30 S-sp1a-Click-sp2-vcMMAE	ADC Product	Innate Pharma
SGN30 Q-sp1a-Click-sp2-vcMMAE	ADC Product	Innate Pharma
SGN30 S-sp1b-Click-sp2-vcMMAE	ADC Product	Innate Pharma
SGN30 Q-sp1b-Click-sp2-vcMMAE	ADC Product	Innate Pharma
SGN30 S-sp1b-vcMMAE	ADC Product	Innate Pharma
SGN30 Q-sp1b-MMAE	ADC Product	Innate Pharma
ADCETRIS®	Reference Control	Seattle Genetics
ADCETRIS® DIL	Diluent	Innate Pharma

Figure. 1: ADC Products and ADCETRIS®

The PROTEOSTAT Protein Aggregation Assay was used with the ADC products to measure and compare the soluble/non-soluble and non-covalent aggregates levels. The PROTEOSTAT Thermal Shift Stability Assay was used to determine the temperature of aggregation (T_{agg}) of the ADC products. T_{agg} is related to propensity for aggregation in that a low T_{agg} value correlates to a greater aggregation propensity. The PROTEOSTAT Protein Aggregation Assay results correlated with the results acquired by SE-HPLC and LC-MS.

PRODUCTS	T_{Agg} (°C)
SGN30 Q-sp1a-Click-sp2-vcMMAE	64.82
SGN30 Q-sp1b-vcMMAE	65.68
SGN30 Q-sp1b-Click-sp2-vcMMAE	65.85
ADCETRIS®	66.10
SGN30 S-sp1b-Click-sp2-vcMMAE	67.93
SGN30 S-sp1a-Click-sp2-vcMMAE	67.97
SGN30 S-sp1b-vcMMAE	69.38

Figure. 2: Aggregation propensity of ADC products developed by Innate Pharma and ADCETRIS®. Arrow going from green (higher Tagg) to red (lower Tagg). The lower the temperature, the greater is the propensity to aggregate, hence red.

The data illustrated that the PROTEOSTAT dye aided in correctly predicting the aggregation propensity of ADC products and validated that Innate Pharma's new ADC-coupling technology is suitable for the development of stable ADC products. This positions the PROTEOSTAT dye as an orthogonal method for predicting protein aggregation propensity of ADC products in the fight against cancer.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APPLICATION NOTES
Prediction of Aggregation Propensity and Monitoring of Aggregation of Antibody-Drug Conjugates (ADC) using Proteostat® Reagents

For more information, download the complete Application Note.

PROTEOSTAT® Protein Aggregation Assay

Orthogonal High Throughput Method for Monitoring Protein Aggregation

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Small Molecule Activators & Inhibitors

Targeted Compound Screening Libraries

Optimize Protein Characterization

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PROTEOSTAT® Thermal Shift Stability Assay

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Host Cell Protein ELISAs

Protein A ELISA

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