



PROTEOSTAT[®] Thermal Shift Stability Assay Kit

Catalog #: ENZ-51027-K400
for 400 assays

Catalog #: ENZ-51027-K100
for 100 assays



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I INTRODUCTION

Thermal shift analysis procedures utilizing calorimetry, absorbance spectroscopy, extrinsic fluorescence spectroscopy or intrinsic fluorescence spectroscopy have been employed in the assessment of protein stability for well over four decades.^(1,2) Typical applications have included observing the helix-coil transition in proteins upon thermal denaturation, as well as for monitoring the effects that buffers, such as sodium acetate or exogenously added excipients, such as NaCl, have on overall protein thermal stability.^(1,2) The procedures have also been applied to monitoring the binding of small molecule ligands, such as cytidine 2'(3')-phosphate, to active sites on proteins.^(1,2)

Prior to the introduction of the PROTEOSTAT[®] Thermal Shift Stability Assay kit, protein aggregation-based thermal denaturation assays have routinely employed devices to raise the temperature of the protein sample in a stepwise fashion, in conjunction with monitoring of protein aggregation through light scattering or turbidity measurements.⁽¹⁻³⁾ Such approaches typically have required relatively high concentrations of protein, provided a very limited assay dynamic range and/or required specialized equipment not routinely accessible in a standard laboratory environment.

Environmentally-sensitive dyes, such as ANS and SYPRO[®] Orange, have been applied to the detection of protein unfolding in thermal shift assays, by a procedure referred to as the Thermofluor[™] technique.⁽⁴⁾ In the procedure, dyes interact with exposed hydrophobic regions generated by partial or full unfolding of proteins. The PROTEOSTAT[®] Thermal Shift Stability assay provides an improved thermal shift approach for assessment of protein stability through directly monitoring protein aggregation, rather than protein unfolding; minimizing problems encountered with the cited environment-sensitive dyes, such as high background fluorescence from their interaction with detergents, membrane proteins or hydrophobic compounds. The PROTEOSTAT[®] TS dye is a proprietary 488 nm excitable molecular rotor probe that, under standard aqueous conditions, is minimally fluorescent. Upon binding to the surface of aggregated proteins, the dye emits a strong red signal at ~600 nm, thus providing a homogeneous assay for the analysis of protein stability.

The PROTEOSTAT® Thermal Shift Stability Assay Kit includes a fluorescent dye which detects protein aggregation arising from thermally-induced protein denaturation. 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. Conditions that increase the aggregation temperature increase the stability of the protein. The assay facilitates understanding of the underlying mechanisms impacting protein stability, and because it is not dependent upon measuring exposed hydrophobic regions arising from protein unfolding is more tolerant of detergents, micelle formation and certain ligands and proteins possessing hydrophobic characteristics.

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 kits are sufficient for 100 assays (ENZ-51027-K100) or 400 assays (ENZ-51027-K400).

Reagent	ENZ-51027-K400	ENZ-51027-K100
PROTEOSTAT® TS Detection Reagent (1000X)	25 μL	6.25 μL
β -Lactoglobulin Control	16 mg	4 mg
10X Assay Buffer	2 mL	0.5 mL

III ADDITIONAL MATERIALS REQUIRED

- Thermally-controlled fluorescence reading device (such as fluorimeter, real-time PCR instrument or melting temperature analyzer).
- Cuvettes for fluorimeter, optical strip tubes or capillary tubes for thermocycler.
- Calibrated, adjustable precision pipets, preferably with disposable plastic tips.
- Deionized water.



Avoid freeze / thaw cycles

IV SAFETY WARNINGS AND PRECAUTIONS



Handle
with care

- 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. Making 1X Assay Buffer

Allow the 10X Assay Buffer to warm to room temperature. Make sure that the reagent is free of any precipitation before dilution. (Note: Gentle warming may be used to get the solution back into solution). To prepare 1X Assay Buffer dilute 1 part 10X Assay Buffer with 9 parts deionized water.

2. Reconstitution of Control

The β -Lactoglobulin Control is supplied as a lyophilized powder (16 mg for ENZ-51027-K400 or 4 mg for ENZ-51027-K100).

- a. Immediately before use, reconstitute the control in 500 μ L of 1X Assay Buffer to generate a 32 mg/mL

stock solution (ENZ-51027-K400) or 8 mg/mL stock solution (ENZ-51027-K100).

- b. Gently mix to re-suspend the β -Lactoglobulin in solution. Mix the solution in a rotator for about 20 minutes to completely resuspend the protein.
- c. It is preferable to centrifuge at maximum speed for about 30 seconds to pellet any particulate matter that has not gone into solution.

Do not vortex and avoid formation of bubbles. It is recommended to perform the assay as soon as possible after re-suspending the control.

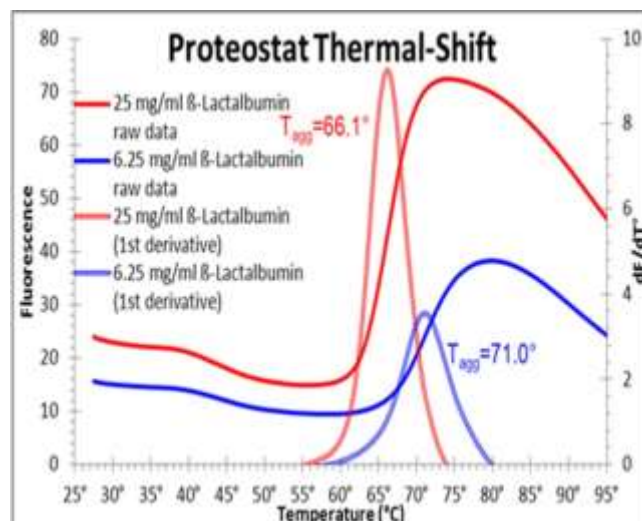
Recommended storage for unused solution of β -Lactoglobulin is 4°C for one month. Freezing should be avoided since this could cause some denaturation.

If storage of β -Lactoglobulin solution for longer periods is necessary, it should be aliquoted in smaller volumes and stored at -20°C. Once thawed, each aliquot should be used in its entirety.

The final concentration of the control used in the assay should be 16mg/mL for ENZ-51027-K400 or 4 mg/mL for ENZ-51027-K100. For most assays, the protein concentration can be within the range of 1-16mg/mL. Ideally, this should be closer to 1mg/mL, and this depends on the protein.

Note that aggregates above 20 mg/mL can saturate the dye.

Please note that additives such as glycerol and different buffer compositions and different protein concentrations can all affect the aggregation temperature.





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3. Preparing 10X ProteoStat® TS Detection Reagent

NOTE: *The PROTEOSTAT® TS Detection Reagent is light sensitive. Avoid direct exposure of the reagent to intense light. Briefly centrifuge the vial to collect all the reagent to the bottom. Store unused reagent at –20°C, protected from light. Avoid repeated freeze/thaw cycles.*

Each sample requires at least 2.5 µL of 10X PROTEOSTAT® TS Detection Reagent. Prepare enough volume of 10X PROTEOSTAT® TS Detection Reagent for the number of samples to be assayed.

To prepare 100 µL of 10X PROTEOSTAT® TS Detection Reagent, add 1 µL of 1000X PROTEOSTAT® TS Detection Reagent to 99 µL 1X Assay Buffer. Mix well.

B. DETERMINATION OF THE AGGREGATION TEMPERATURE

The procedure described below is optimized for the control protein, β-Lactoglobulin, and should be modified to include your protein and buffer of interest.

1. Reaction Set Up Using a Fluorimeter.

- a. For each 100 µl reaction, mix protein of interest (dissolved in 90 µL 1X Assay Buffer or buffer of interest) or 90 µL of the provided β-Lactoglobulin Control (32 mg/mL diluted to 16 mg/mL with 1X Assay Buffer for ENZ-51027-K400 or 8 mg/mL diluted to 4 mg/mL with 1X Assay Buffer for ENZ-51027-K100) and 10 µL of 10X PROTEOSTAT® TS Detection Reagent.

Note: *Assay Buffer is supplied in the kit, but most any buffer between pH 3 and pH 10 will work.*

- b. Set the temperature of the Fluorimeter to 30°C or lower. Then, increase the temperature 5°C. Record the Fluorescence and the temperature at approximately every degree. After the target temperature has been reached, increase the temperature another 5°C and continue recording the temperature and fluorescence. Continue raising the temperature in the same manner until 99°C. Data should be collected using a red fluorescence channel. Excitation from 480 nm to 550 nm will yield

good results. The signal will be stronger when excitation is around 500 nm, but if the non-aggregated sample has background fluorescence, excitation at 550 nm usually reduces the background.

NOTE: The *PROTEOSTAT® TS Detection Reagent* is light sensitive. Be sure to protect samples from light.

2. Reaction Set Up Using a Thermocycler or Melting Temperature Apparatus.

- a. For each 25 μ L reaction, mix protein of interest (dissolved in 22.5 μ L 1X Assay Buffer, or the buffer of choice) or 22.5 μ L of the provided β -Lactoglobulin Control (32 mg/mL diluted to 16 mg/ml with 1X Assay Buffer for [ENZ-51027-K400](#) or 8 mg/mL diluted to 4 mg/mL with 1X Assay Buffer for [ENZ-51027-K100](#)) and 2.5 μ L of 10X *PROTEOSTAT® TS Detection Reagent*.
- b. If using capillary tubes for the reaction, spin the sample to the bottom of the tube following the thermocycler manufacturer's instructions
- c. Program the thermocycler to start at 30°C or lower. Then, as a second step, ramp the temperature up to 99°C, setting acquisition to read the fluorescence continuously. The ramp rate should be set at a low rate, such as 0.05°C/second. For some thermocyclers, it may be necessary to manually program to raise the temperature by one degree or less for each step, and take a reading at every step. Data should be collected using a red fluorescence channel, such as one used for Texas Red. Some machines may require the user to enter the number and location of each sample

NOTE: The *PROTEOSTAT® TS Detection Reagent* is light sensitive. Be sure to protect samples from light.

- d. Load the samples into the thermocycler, and start the reading.

3. Interpreting the Results.

The aggregation temperature is the temperature with the most rapid increase in fluorescence due to aggregated protein.

- a. Examine the data of fluorescence versus temperature.

- b. Many thermocyclers, such as the Roche LightCycler®, have a program built-in to determine melting temperature using the first derivative (slope) of the fluorescent curve (dF/dT°). Most of these programs plot the negative of the first derivative because they are designed to detect loss of fluorescence in the melting of DNA. In this assay, the aggregation temperature is the maximum point in the first derivative plot. For most data, it is best to average over 6°C increments. Using the plot of dF/dT° , or $-dF/dT^\circ$ on most machines, versus temperature find the maximum slope. This is the highest value for dF/dT° , and the lowest for $-dF/dT^\circ$. The temperature at this point is the aggregation temperature.
- c. If the thermocycler does not have the software to plot dF/dT° , the data can be imported into Microsoft Excel®. Within MS Excel®, the slope function can be used to determine dF/dT° .

To use the slope function, start in a cell adjacent to the fluorescence and temperature data. Type “=slope(“ , then select the fluorescence data equal to the first 2° to 4° of data, then type “,” (comma). Select the temperature values adjacent to the fluorescence values just selected, then type “)”

This slope function can then be expanded to include all of the temperatures. Next to the slope data, find the average temperature for each slope value using the “average” function in Excel®. Sample data is shown in Figure 1.

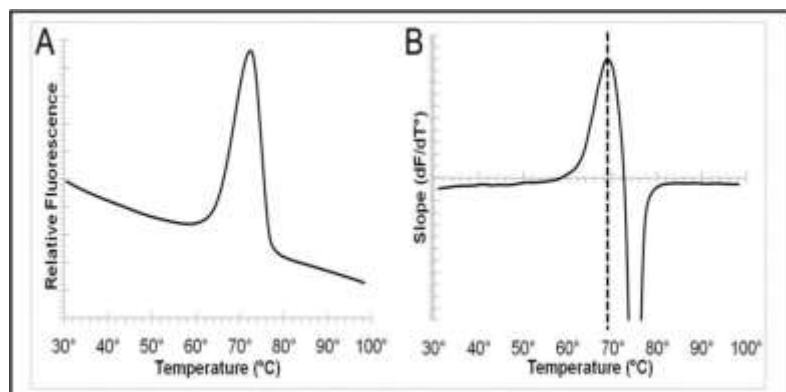


Figure 1. Typical data for the determination of the aggregation temperature of IgG (goat anti-mouse, 5.6mg/ml). Panel A is the raw data from the thermocycler. Panel B is a plot of dF/dT° or slope of the data shown in panel A. The dashed line indicates the aggregation temperature.

VI APPENDICES

A. FILTER SETTING SELECTION

The selection of optimal settings for a fluorescence reading device 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. Predesigned filter sets for Texas Red should work well for this application.

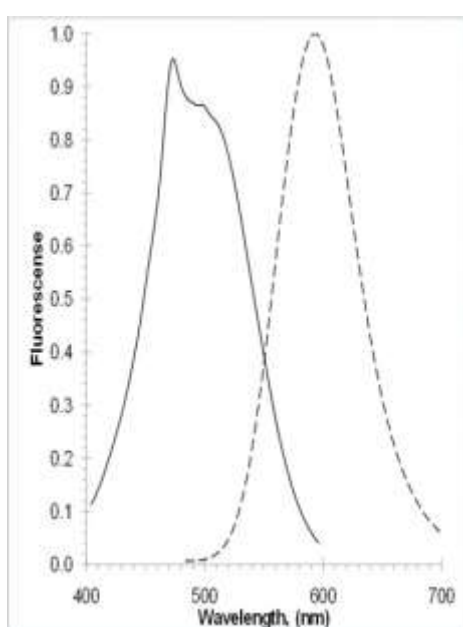


Figure 2.

Fluorescence excitation and emission spectra for PROTEOSTAT[®] Thermal Shift Detection Reagent. Spectra were determined in 1X Assay Buffer.

B. COMPATIBILITY

Tested Reagents	Validated Concentrations
Sodium Chloride	up to 1000 mM
Calcium Chloride	up to 200 mM
Ammonium Sulfate	up to 300 mM
Sorbitol	up to 600 mM
Mannitol	up to 600 mM
Trehalose	up to 600 mM
Lactose	up to 300 mM
Ascorbic Acid	up to 1 mM
Triton X-100	up to 0.01%
Arginine	up to 500 mM
Glycine	up to 2%
Tween 20	up to 0.01%*
Dithiothreitol	up to 1 mM

Table I: Substances tested for compatibility with the PROTEOSTAT[®] Protein Aggregation Assay. Note: Higher concentrations of some reagents, such as 0.2% Tween-20, may produce an increase in

background signal with the monomeric protein, however, the protein aggregate signal is typically still substantially larger.

C. EXPECTED RESULTS

The fluorescence-vs-temperature graph (see Figure 1A, page 9), generated from a protein thermal denaturation experiment, should show a rapid increase in fluorescence at the temperature that the protein of interest begins to aggregate. This is often followed by a decrease in fluorescence due to precipitation of the aggregated protein. The position of maximum slope is determined by plotting dF/dT° versus temperature. The position of highest value is the aggregation temperature. The temperature of aggregation is dependent upon protein concentration, buffer conditions and the presence of ligands or drugs. 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 being 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 and resource-efficient analytical method is useful for both developing novel protein formulations and identifying protein stability in quality control procedures.

Protein aggregation is not equivalent to protein unfolding. Some conditions will cause a reversible unfolding of a protein that do not lead to irreversible protein aggregation.

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® Thermal Shift 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.

VII REFERENCES

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