PROTEOSTAT® Protein Refolding and Aggregation Sensing Kit

Catalog #: ENZ-51040-KP002
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>2</td>
</tr>
<tr>
<td>Reagents Provided and Storage</td>
<td>3</td>
</tr>
<tr>
<td>Lysozyme Standard, Denaturant and Additives</td>
<td>4</td>
</tr>
<tr>
<td>Other Materials Needed</td>
<td>4</td>
</tr>
<tr>
<td>Safety Warnings and Precautions</td>
<td>5</td>
</tr>
<tr>
<td>Methods and Procedures</td>
<td>5</td>
</tr>
<tr>
<td>Reagent Preparation</td>
<td>5</td>
</tr>
<tr>
<td>Assay Workflow</td>
<td>6</td>
</tr>
<tr>
<td>Solubilization of Inclusion Bodies or Protein</td>
<td>7</td>
</tr>
<tr>
<td>Protein Refolding and Analysis</td>
<td>8</td>
</tr>
<tr>
<td>Proteostat® Protein Aggregations Assay</td>
<td>11</td>
</tr>
<tr>
<td>Expected Results</td>
<td>12</td>
</tr>
<tr>
<td>References</td>
<td>14</td>
</tr>
<tr>
<td>Troubleshooting Guide</td>
<td>15</td>
</tr>
<tr>
<td>Contact Information</td>
<td>18</td>
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INTRODUCTION

The over-expression of eukaryotic proteins in microbial hosts frequently leads to the formation of insoluble protein aggregates within inclusion bodies. Often, it is feasible to solubilize and refold proteins, returning them to a native state. Numerous approaches to protein refolding have been developed, including laboratory scale matrix-assisted refolding and refolding through dilution methods. However, production-level scale-up of these processes is often problematic. Consequently, employment of small molecule buffer additives has been the most common approach for defining refolding conditions due to documented success using a wide range of proteins and the relative simplicity and low-cost nature of the procedure.

The key to successfully refolding a protein is preventing off-pathway products from accumulating. These unwanted species can form aggregates, a process which can be self-nucleating, resulting in poor recoveries of properly folded proteins. Folding intermediates with hydrophobic patches, which become exposed to aqueous solvent, are believed to play a significant role in the formation of off-pathway products. Protein aggregation thwarts attempts to properly refold proteins in this context.

The folding of proteins in solution is affected by a number of physiochemical parameters. These parameters include ionic strength, pH, temperature, oxidation state and protein concentration, as well as the presence of hydrophobic, polar, and chaotropic agents. In order to avoid unwanted off-pathway products, the main strategy applied is to employ a continuous or discontinuous buffer exchange wherein the renaturation buffer is designed to minimize misfolding and aggregation.

Enzo Life Sciences’ PROTEOSTAT® Protein Refolding and Aggregation Sensing Kit provides a concerted assay format employing a design of experiments (DOE) approach that facilitates screening of refolding parameters for a specific protein in different buffers, and a simple, homogenous assay format for monitoring protein aggregation using a proprietary red-emitting aggregation-sensitive molecular rotor dye. Selected samples with low fluorescence readout, thus low aggregation content, can then be validated using traditional enzyme activity-based determination of refolding success.

The PROTEOSTAT® Protein Refolding and Aggregation Sensing Kit permits examination of a wide range of conditions and identifies the critical factors for refolding of a particular protein.
REAGENTS PROVIDED AND STORAGE

All reagents are shipped on dry ice. Upon receipt, the kit should be stored upright and protected from light at ≤ -20°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.

Aggregation Detection Reagents

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<td>PROTEOSTAT® Detection Reagent</td>
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<td>10X PROTEOSTAT® Assay Buffer</td>
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2X Refolding Buffers

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</tr>
<tr>
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</tr>
<tr>
<td>Buffer 3: 100 mM Tris, pH 8.3; 40 mM NaCl; 1.6mM KCl</td>
<td>10 mL</td>
</tr>
<tr>
<td>Buffer 4: 100 mM Tris, pH 6.8; 40 mM NaCl; 1.6mM KCl; Guanidine-HCl, 1M</td>
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</tr>
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<td>Buffer 5: 100 mM Tris, pH 7.6; 40 mM NaCl; 1.6mM KCl; Guanidine-HCl, 1M</td>
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<td>Buffer 6: 100 mM Tris, pH 8.3; 40 mM NaCl; 1.6mM KCl; Guanidine-HCl, 1M</td>
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<td>Buffer 7: 100 mM Tris, pH 6.8; 40 mM NaCl; 1.6mM KCl; L-Arginine, 0.8M</td>
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<td>Buffer 15: 100 mM Tris, pH 8.3; 40 mM NaCl; 1.6mM KCl; L-Arginine, 1.6M</td>
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Lysozyme Standard, Detaturant and Additives

<table>
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<td>Lysozyme Standard</td>
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<td>Denaturant: 6 M Guanidine, with 50 mM Tris-HCl,pH 8</td>
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<td>DTT, 100 mM</td>
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<td>CaCl$_2$, 400 mM</td>
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<tr>
<td>MgCl$_2$, 400 mM</td>
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<td>NaCl, 1 M</td>
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<tr>
<td>Tween-20, 0.1%</td>
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<tr>
<td>Sucrose, 1.2 M</td>
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<tr>
<td>α-Cyclodextrin (200 mg/mL)</td>
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</table>

OTHER MATERIALS NEEDED

- Fluorescence microplate reader with monochromator or filter settings of Excitation=550 nm/Emission=600 nm
- 96-well microplate: black wall, preferably with a clear bottom.
- Calibrated, adjustable precision pipetters, preferably with disposable plastic tips
- Deionized water
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.

METHODS AND PROCEDURES

The typical protein refolding workflow is summarized in Figure 1 (page 6). The detailed protocols are described as follows:

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 PROTEOSTAT® 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 PROTEOSTAT® Assay Buffer for the number of samples to be assayed. For each 96-well plate, dilute 1mL of the 10X PROTEOSTAT® Assay Buffer with 9mL of deionized water.

2. PROTEOSTAT® Detection Reagent Loading Solution

   For each 96-well plate, prepare 10 mL of PROTEOSTAT® Detection Reagent Loading Solution as follows: Add 10 µL of PROTEOSTAT® Detection Reagent to 10 mL of 1X PROTEOSTAT® Assay Buffer. Mix well.
3. **α-Cyclodextrin Solution (200 mg/mL)**

   The α-Cyclodextrin may settle out of solution. If so, warm it to 80°C for 5-10 minutes with occasional mixing until no particulate matter is apparent by visual inspection. Aliquot the solution into 100 – 200 µL aliquots and store at –20°C.

4. **100 mM GSH**

   Add 1.5 mL of deionized water to the tube containing 46.1 mg of GSH and with occasional mixing until no particulate matter is apparent by visual inspection. Aliquot the solution into 100 – 200 µL aliquots and store at –20°C.

5. **20mM GSSG**

   Add 1.5 mL of deionized water to the tube containing 18.4 mg of GSSG and mix until no particulate matter is apparent by visual inspection. Aliquot the solution into 100 – 200 µL aliquots and store at –20°C.

### ASSAY WORKFLOW

1. Solubilize Protein in Denaturant

2. Refold Protein (By dilution)

3. Screen for Protein Aggregation (ProteoStat® Dye)

4. Test for Protein Activity - Use samples displaying lowest aggregation in step 3 above. (Employ protein-specific activity assay of choice)

5. Retest the better formulations from steps 3 & 4 above, with additional excipients

6. Rescreen for Protein Aggregation (PROTEOSTAT® Dye)

7. Retest for Protein Activity - Use samples displaying lowest aggregation in step 6 above. (Employ protein-specific activity assay of choice)

8. Dialyze Protein in Optimal Storage Buffer & Retest Activity (Optional)

**Figure 1.** Workflow for refolding proteins using the PROTEOSTAT® Protein Refolding and Aggregation Sensing Kit.
SOLUBILIZATION OF INCLUSION BODIES OR INACTIVATED PROTEIN

1. Resuspend inclusion body pellet (2 mL per gram) or inactivated protein (1 mL per 20 mg) with the Denaturant provided.

To prepare the inclusion bodies from the pellet of a strain in which the target protein was over-expressed, disrupt the cells and wash them in a solution containing 4 M Urea, 0.5M NaCl, 1 mM EDTA, 0.1% Deoxycholate, 50 mM Tris-HCl, pH 8 (urea and salt concentration may be varied for the particular protein of interest). After solubilizing non-aggregated proteins, pellet the inclusion bodies by centrifugation at >12,000 x g for 30 minutes at 4°C. For added purity, the wash step may be repeated. The amount of inclusion body produced can be determined by weighing the tube before adding the sample and again after the inclusion bodies have been isolated.

2. Vortex or homogenize to completely suspend inclusion body pellet or inactivated protein.

3. Add DTT to achieve a 1 mM final concentration. Tween-20 may be added to 0.1% final concentration, if desired to help with the solubilization of some proteins.

4. Incubate the suspension at 4°C overnight or room temperature (~22°C) for 2 hours. Gently rotate the sample to help dissolve the protein.

5. Centrifuge at >12,000 x g for 30 minutes at 4°C to remove any cell debris or other insoluble particulate material. Repeat this step one more time, if necessary.

If the solubilized protein still contains significant amounts of contaminants, which might impact target protein refolding, the target protein may be purified using immobilized metal affinity chromatography (IMAC) or other affinity chromatography, as appropriate. If the protein does not contain a fusion tag, then use a size exclusion column, such as a Sephadex column. Equilibrate the column with a denaturing buffer, such as buffered Denaturant (provided), before applying the protein solution to the column.

Proceed to purifying the protein according to the column or resin manufacturer’s instructions.
PROTEIN REFOLDING AND ANALYSIS

The PROTEOSTAT® Protein Refolding and Aggregation Sensing Kit employs select reagents and conditions that have previously been proven to be effective for high yield refolding of a wide range of proteins. Use of these reagents in a Design of Experiments (DOE) matrix format (e.g. an adjustable fractional factorial matrix) facilitates screening and optimization of protein refolding conditions. A DOE matrix is considered the most efficient approach to screening refolding conditions with a limited number of samples and materials.

The refolding buffers encompass three key factors: pH, Guanidine-HCl concentration and L-arginine concentration. The 4th key factor employed in the refolding process is the redox environment, provided by DTT or the milder GSH/GSSG redox system. A primary screen set-up for examining these factors is illustrated in Table 1 (page 10), and can be used to screen refolding conditions for your protein. Use the DOE matrix as outlined in Table 1 (page 10). If the protein of interest is known to be partially oxidized in the active state (containing critical disulfide bridge(s) involved in protein function), it is recommended to use GSH/GSSG and avoid DTT. Alternatively, if the protein to be refolded is known to be active in the presence of DTT, it is recommended to use DTT and not GSH/GSSH. The total volume of the refolding reaction is 100 µL. Additional factors to be employed in a secondary screen for refolding include PEG, EDTA, CaCl$_2$, MgCl$_2$, NaCl, Tween-20, sucrose and α-cyclodextrin as outlined in Table 2 (page 11).

1. Allow the 2X refolding buffers to warm to room temperature. Make sure that the reagent is free of any crystallization before dilution. Prepare the refolding solutions according to DOE matrix design (Table 1, page 10).
2. Dilute the denatured (and purified, if necessary) protein prepared in page 7, typically adding 5 µL of protein into the refolding buffers in Table 1 (page 10) and incubate overnight at 4°C. If the protein is concentrated, dilute with the Denaturant to adjust the required volume of the protein to 5 µL.
3. Screen for protein aggregation with the PROTEOSTAT® Protein Aggregation Assay (page 11).
4. Select 2~5 samples with the lowest fluorescence intensity and confirm that the protein is refolded in an active conformation using an activity assay for the protein of interest, if one is
available. Identify the buffer conditions that give the highest activity (recommend selecting two or more) for further excipient optimization.

5. Starting with the better conditions determined in step 3 and step 4, (i.e. those with minimal aggregation and maximal protein activity), repeat the refolding assay, with the inclusion of each of the additional excipients MgCl₂, CaCl₂, PEG, EDTA, Sucrose and α-cyclodextrin. Dilute the solubilized denatured protein as shown in Table 2, and incubate overnight at 4°C. Screen for protein aggregation as described in step 3.

6. Select the samples with the lowest fluorescence intensity and confirm that the protein is refolded in an active conformation using an activity assay for the protein of interest if one is available. Identify the most efficient refolding buffer. The sample can be kept in the refolding buffer or dialyzed into a storage buffer.
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<thead>
<tr>
<th>Tube No.</th>
<th>Refolding Buffer (2X) (50 µL)</th>
<th>DTT 100 mM (Aqueous) (µL)</th>
<th>GSH 100 mM (Aqueous) (µL)</th>
<th>GSSG 20 mM (Aqueous) (µL)</th>
<th>Water (µL)</th>
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</table>

**Table 1.** Primary formulation screen set-up for examining the effect of pH, denaturant concentration and reducing environment on protein refolding.
<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Refolding Buffer (50 µL)</th>
<th>2 µL GSH + 2 µL GSSG or 5 µL DTT</th>
<th>Excipients and Volume (µL)</th>
<th>Water Up to 100 µL</th>
<th>Solubilized Denatured Protein 0.5-20 mg/mL (µL)</th>
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<tbody>
<tr>
<td>A</td>
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<td>From optimal refolding (page 8)</td>
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<td>0</td>
<td>______µL</td>
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<tr>
<td>B</td>
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<td>From optimal refolding</td>
<td>PEG (10 mM)</td>
<td>10</td>
<td>______µL</td>
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<td>C</td>
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<tr>
<td>D</td>
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<td>From optimal refolding</td>
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</tr>
<tr>
<td>F</td>
<td>From optimal refolding</td>
<td>From optimal refolding</td>
<td>NaCl (1 M)</td>
<td>10</td>
<td>______µL</td>
</tr>
<tr>
<td>G</td>
<td>From optimal refolding</td>
<td>From optimal refolding</td>
<td>Tween (0.1%)</td>
<td>5</td>
<td>______µL</td>
</tr>
<tr>
<td>H</td>
<td>From optimal refolding</td>
<td>From optimal refolding</td>
<td>Sucrose (1.2 M)</td>
<td>10</td>
<td>______µL</td>
</tr>
<tr>
<td>I</td>
<td>From optimal refolding</td>
<td>From optimal refolding</td>
<td>α-Cyclodextrin (200 mg/mL)</td>
<td>20</td>
<td>______µL</td>
</tr>
</tbody>
</table>

**Table 2.** Secondary formulation screen set-up for examining the effect of various excipients on protein refolding, based upon the primary screen results.

**PROTEOSTAT® PROTEIN AGGREGATION ASSAY**

1. Prepare a black, clear bottom 96-well microplate with 5 µL of protein of interest per well (after refolding). The recommended protein concentration range is **1 μM to 30 μM**. Higher concentrations of protein can be diluted in 1X PROTEOSTAT® Assay Buffer. Be certain to run negative and
positive controls (if possible), as well as 1X PROTEOSTAT® Assay Buffer alone (no protein), as a blank sample.

2. Dispense 95 µL of the prepared PROTEOSTAT® Detection Reagent Loading Solution (see section 2, page 5) into each well. The final concentration of the dye is 3 µM.

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

3. Incubate the microplate containing test samples in the dark for 10 minutes at room temperature.

4. Read the fluorescence intensity 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 wells after PROTEOSTAT® detection dye loading. The fluorescence value of 1X assay buffer alone should be subtracted from the values for wells containing proteins.

**EXPECTED RESULTS**

Protein refolding based on buffer exchange typically generates 30~80% yield, depending upon the individual protein. Since successful protein refolding has no universally applicable rules, not all proteins are guaranteed to be successfully refolded with components provided within this kit.

For downstream applications, it is often required to exchange the refolding buffer into stabilizing buffer for long-term storage of the refolded protein. Care must be taken to ensure that the protein is stable in the storage solution.

Low fluorescence intensity does not necessarily indicate proper refolding. The protein may be in an incorrectly folded yet soluble state, or in a stable molten globule intermediate. Therefore, it is strongly recommended to run an activity assay on several samples if possible.

Tables 1 and 2 illustrate the refolding process using PROTEOSTAT® dye as an evaluation tool to simplify the refolding process.

Results for refolding lysozyme using a redox environment are shown on page 13 in Figures 2 and 3.
Figure 2. Refolding Lysozyme using a Redox Environment. The experiment was designed according the DOE matrix in Table 1. In detail, 20mg/ml lysozyme was first denatured in the 6 M Guanidine solution at 4°C for 18 hours. Then, the denatured lysozyme was diluted 1: 20 into the various refolding buffers and incubated overnight at 4°C. For the PROTEOSTAT® Protein Refolding and Aggregate Sensing assay, a 1:20 dilution of the refolding solutions was added directly to PROTEOSTAT® Assay Buffer containing dye and fluorescence intensity was determined (Ex = 550 nm, Em = 610 nm). The enzymatic activity of the lysozyme in the refolding buffers was measured using 20 µg/mL Micrococcus lysodeikticus cell walls that had been excessively labeled with fluorescein, which caused dye quenching. When the lysozyme acts on this substrate, unquenched dye-labeled fragments of cell wall are released. The fluorescence intensity increase was determined (Ex/Em 490/520 nm). Native protein diluted in PROTEOSTAT® Assay Buffer was used as a reference for both the aggregation and activity assays. The refolding solutions with high aggregation signal typically had low enzymatic activity and thus should be avoided. 1A-15A: with DTT; 1B-15B: with GSH/GSSG.

Figure 3. Refolding buffer 13B was determined to have a high activity and low fluorescence, as shown in Figure 2, and was selected to evaluate excipient effects on lysozyme refolding. Excipients were individually added into the refolding buffer with GSH/GSSG environment, and analyzed for aggregation and activity, as in Figure 2. The results indicated that MgCl₂ was the best at promoting refolding in refolding buffer 13B, with minimal tendency to aggregate.
REFERENCES


<table>
<thead>
<tr>
<th>Problem</th>
<th>Potential Cause</th>
<th>Suggestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limited fluorescence variation in PROTEOSTAT® dye signal among refolding buffers</td>
<td>The denaturant is still binding to the protein to prevent aggregation.</td>
<td>Dialyze against refolding buffer without denaturant to remove denaturant, then run fluorescence assay to detect aggregates.</td>
</tr>
<tr>
<td>Weak PROTEOSTAT® dye fluorescence intensity obtained from sample with low “activity”</td>
<td>Protein aggregate size is too big, causing significant amount of protein precipitation.</td>
<td>Shake the microplate for a longer time period before reading.</td>
</tr>
<tr>
<td></td>
<td>Amorphous protein aggregate is formed.</td>
<td>Use a higher amount of protein in the analysis.</td>
</tr>
<tr>
<td></td>
<td>The protein may be in an incorrectly folded yet soluble state, or has formed a stable molten globule intermediate.</td>
<td>Run activity or ligand binding assay if the sample’s fluorescence intensity is within 2-fold of the sample with minimum fluorescence intensity.</td>
</tr>
<tr>
<td>Poor PROTEOSTAT® dye fluorescence signal observed in the positive control</td>
<td>Detection reagent has been exposed to strong light.</td>
<td>Protect samples from exposure to strong light and analyze them immediately after staining.</td>
</tr>
<tr>
<td></td>
<td>Detection reagent has degraded.</td>
<td>Verify that the reagents are not past their expiration dates before using them.</td>
</tr>
<tr>
<td></td>
<td>Band pass filters are too narrow or not optimal for the detection reagent.</td>
<td>Use correct monochromator setting or filter set for the fluorophore.</td>
</tr>
<tr>
<td></td>
<td>Insufficient PROTEOSTAT® dye concentration was used.</td>
<td>Follow the procedures provided in this manual.</td>
</tr>
<tr>
<td>High fluorescence background in the well without protein sample</td>
<td>Inappropriate dye dilution</td>
<td>Make certain that there are no particles in the dye. Centrifuge well before use.</td>
</tr>
<tr>
<td>PROTEOSTAT® dye fluorescence signal is saturated.</td>
<td>The protein sample concentration is too high.</td>
<td>Dilute the sample further with 1X PROTEOSTAT® Assay Buffer.</td>
</tr>
</tbody>
</table>
NOTES