Detection of bacterial aggregation by flow cytometry

Jack Coleman[1] and Melis McHenry[2]
[1]Enzo Life Sciences, Farmingdale NY, USA, [2]Beckman Coulter Inc., Miami FL, USA

PROTEOSTAT® Aggresome Detection Kit (ENZ-51035)

BACKGROUND

Since the advent of recombinant DNA technology, bacteria have been used often to express foreign proteins [1]. Soluble proteins expressed by bacteria can be expressed either in the cytoplasm or they are secreted to the periplasm or outside the cell. In general proteins expressed in the cytoplasm are expressed to higher levels than if they are secreted[1]. Many hydrophobic and highly expressed proteins in the cytoplasm form inclusion bodies of aggregated protein that are difficult to solubilize [1]. Proteins targeted to the periplasm or outside of the cell often do not all get secreted and form insoluble aggregates [1, 2]. Different growth and induction conditions for the expressing bacteria can sometimes allow for soluble expression of a previously insoluble protein [2].

RESEARCH APPLICATIONS

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. A recently developed dye, PROTEOSTAT®, is now available (Enzo Life Sciences Cat. # ENZ-51035) that specifically stains amyloid type aggregates. The PROTEOSTAT® dye has been used to stain aggregates formed by overexpressed proteins in bacteria [3]. In this application note, a simple and rapid method to identify aggregation in bacterial cells, showcased using a CytoFlex flow-cytometer, is described.

STANDARD PROCEDURE

Prior to staining the bacterial culture for aggregate detection, MG-132, a proteasome inhibitor, is used as a positive control to identify aggresome detection in mammalian cell culture. These results identifying aggresomes in induced mammalian cells are analogous to identifying aggregates in bacteria. PROTEOSTAT® Aggresome Detection kit (Enzo Life Sciences, Farmingdale, NY) is used for this assay as per manufacturer's recommendations. Briefly, Jurkat cells were mock-induced with 0.2% DMSO or induced with 5 µM MG-132 for overnight 18 hours at 37°C. After treatment, cells were fixed and incubated with PROTEOSTAT® dye, then acquired using a CytoFlex Flow Cytometer (Beckman Coulter, Miami, FL) without washing. Results are analyzed with CytExpert 1.1 Software (Beckman Coulter, Miami, FL) and presented as histogram overlays.

The bacterial culture for aggregate detection assay is performed as follows:

- A culture of Escherichia coli BL21 with a plasmid (pET151-Klenow) was inoculated and grown overnight with shaking (320 RPM) at 37ºC in Terrific Broth (SIGMA, St. Louis, MO) containing ampicillin. This strain produces DNA Polymerase I Klenow fragment [4] from a T7 RNA polymerase promoter controlled with a *lac* operator (induced with IPTG).
- After 24 hour incubation, two tubes containing Terrific Broth and ampicillin were inoculated with one tenth volume of the overnight culture.

- After 30 minutes of shaking at 37ºC, IPTG (1 mM final concentration) was added to one of the tubes to induce overexpression of DNA Polymerase I, Klenow fragment. Growth was continued for 5 hours with shaking at 37º. The OD_{550} was about 1.
- After growth, the cells are placed on ice for 5 minutes. One mL of culture is removed to a Microcentrifuge tube, and the cells are pelleted by centrifugation for one minute at 16,000 x g.
- The supernatant is removed, and the cells are resuspended in 1 mL 1x Assay Buffer (supplied with PROTEOSTAT® dye, Enzo Life Sciences, Farmingdale, NY).
- The cells are again pelleted by centrifugation at 16,000 x g for 1 minute.
- After removal of the supernatant, the cells are resuspended in 1 mL 1x Assay Buffer containing 10 % Formalin (Sigma, St. Louis, MO.).
- The cells remain in the formalin at room temperature for 30 minutes.
- The fixed cells are again pelleted by centrifugation at 16,000 x g for 1 minute, and washed once with 1x Assay Buffer.
- After the wash, the cells are resuspended in a minimal volume (50 µL) of 1 x Assay Buffer and 1 ml of permeabilizing solution (0.5 % Triton™ X-100, 3 mM EDTA in 1X Assay Buffer) was added.
- The cells are then mixed by inversion 5-6 times and incubate on ice for 30 minutes.
- The cells are collected by centrifugation for 1 minute at 16,000 x g.
- After removing the supernatant, the cells are resuspended in 1 mL 1x Assay Buffer.
- When the starting OD_{550} was approximately 1, 40 µL of culture is pipetted to a fresh microcentrifuge tube.
    - Staining solution is prepared by adding 1 mL of 1x Assay buffer – 2 µL of Hoechst (included with PROTEOSTAT® dye kit Enzo Life Sciences, Farmingdale, NY) and 4 µL PROTEOSTAT® dye (if using ENZ 51035-0025, or 1 µL if using from ENZ 51035-K100).
- 400 µL of the staining solution is added to the cells, stained for 30 minutes at room temperature, followed by centrifugation at 16,000 x g for 1 minute.
- The cells are resuspended in 1 mL 1x Assay Buffer and subsequently analyzed by using a CytoFlex Flow Cytometer (Beckman Coulter, Miami, FL). The data is analyzed using CytExpert Software (Beckman Coulter Inc, Miami FL). Fluorescence is quantitated by mean peak channel fluorescence.

Note: If higher concentrations of cells are used, the concentration of dye may have to be increased. The Hoechst dye is used to validate that bacteria are present, and can be used to gate on the bacterial population.

RESULTS

Insoluble aggregates often form in bacterial cells overexpressing non-native proteins for pharmaceutical or therapeutic purposes. The PROTEOSTAT® dye is immobilized when it binds the aggregated protein, which causes a significant increase in fluorescence, therefore makes it a simple method to detect aggregates via flow cytometry. Growth of cells under different conditions, such as growth at a different temperature or inducing expression of the protein of interest with different concentrations of the inducer can affect the amount of aggregate formed [3]. Uninduced control and 5 µM MG-132-treated Jurkat cells were used to show the typical results of flow cytometry based analysis of cell populations using the PROTEOSTAT® aggresome red detection reagent. After 18 hours treatment, cells were loaded with PROTEOSTAT® reagent, and then analyzed without washing by flow cytometry. Results are presented by histogram overlays (Figure 1). Control cells display low fluorescence. In the samples treated with 5 µM MG-132 for 18 hours, the PROTEOSTAT® aggresome red dye (excited with 488nm blue laser and collected with emission filter 610/20 nm) signal increases over 2-fold, indicating that MG-132 induced aggresome formation in Jurkat cells.

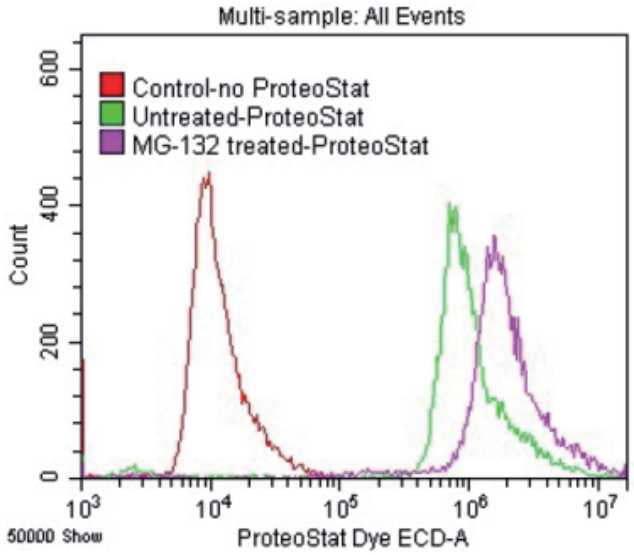

Figure 1. Flow cytometry-based cell aggresome analysis of unstained control, untreated control and MG-132 treated Jurkat cells. MG-132 treated cells show 2-fold signal increase over untreated cells.

The *E.coli* bacterial cells were first identified in a FSC vs SSC plot as shown in Figure 2. The Hoechst dye is used to identify the nucleated cells vs. the debris. The 405nm violet laser is used to excite the Hoechst dye which is then collected with 450/45BP filter. When the samples are overlayed in a histogram plot, the Hoechst stained cells are significantly brighter than unstained cells (Figure 3).

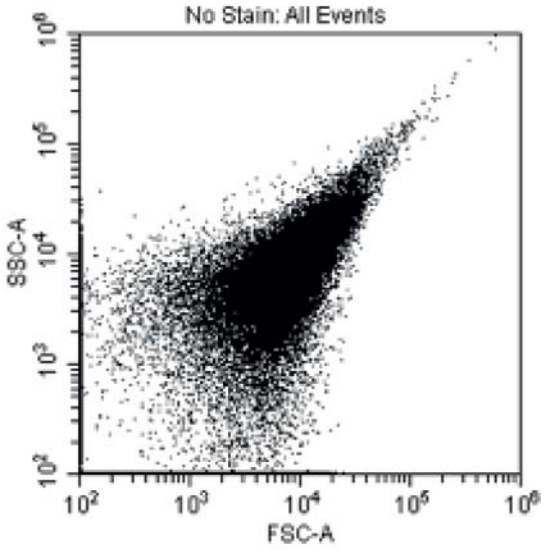

Figure 2. Shows the FSC vs SSC plot for the *E.coli* sample. The plot is in logarithmic scale for both axes.

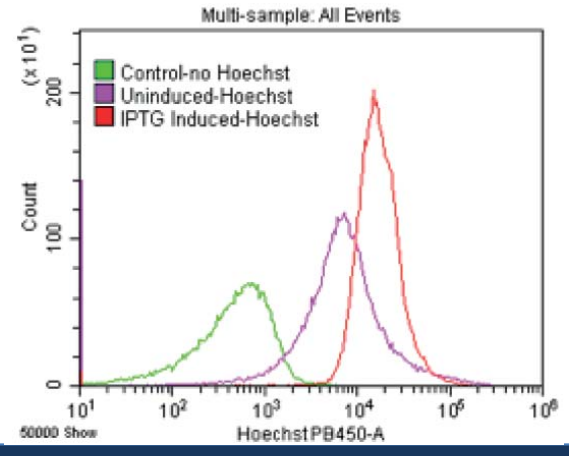

Figure 3. Histogram overlay for Hoechst fluorescence shows the no staining control (green), IPTG uninduced cells (purple) and IPTG induced cells (red).

In Figure 4, we show that the induction of bacterial cells BL21/pET151-Klenow with 1 mM IPTG for 5 hours significantly increases the signal from the PROTEOSTAT® dye, indicating protein aggregates have formed. There appears to be a small population of cells in the induced cells that do not have aggregates. These cells may no longer be overexpressing the Klenow protein, or may have developed some method to prevent aggregate formation through random mutation.

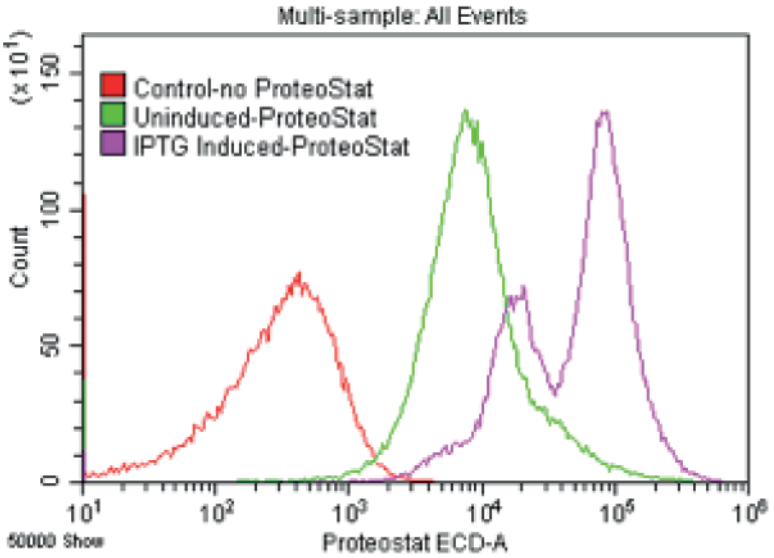

Figure 4. The histogram overlay for PROTEOSTAT® fluorescence shows the induction of aggregate formation in *E.coli* cells induced with IPTG (purple) compared to uninduced cells (green). Unstained control cells are also shown (red).

In figure 5 we see that the mean fluorescence intensity (PROTEOSTAT® dye) of the population of IPTG induced cells increases dramatically compared to the uninduced cells.

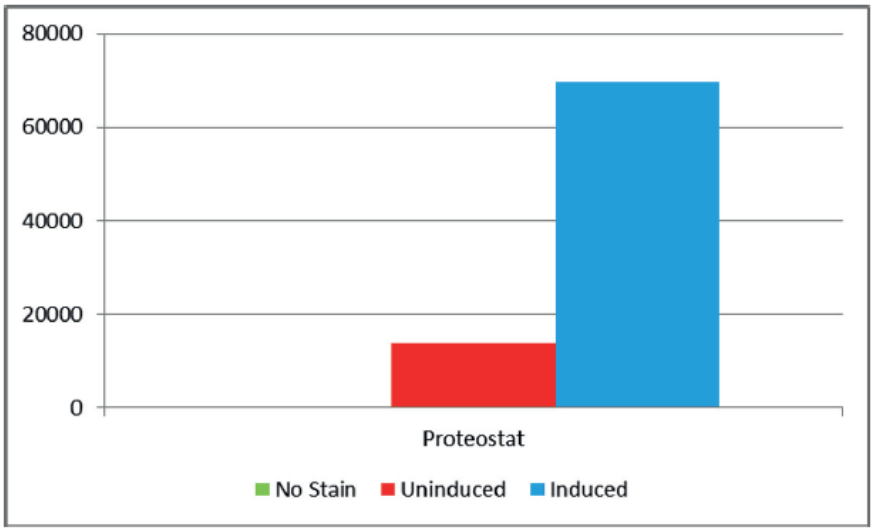

Figure 5. Shows the increase in mean fluorescence intensity in the induced cell population (blue) compared to uninduced (red) cells. The green bars show the signal for unstained control cells.

These results demonstrate that PROTEOSTAT® dye using the CytoFlex flow cytometer can be used to screen for methods of growing bacteria to produce soluble protein. Conditions such as different growth media, different temperatures, different lengths of induction, and different amounts of inducer can all be tested in one simple screen for protein aggregate formation.

References

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The results demonstrated in this application sheet represent those generated on the Beckman Coulter CytoFlex Flow Cytometer. As differences exist in the performance between analyzers, the author cannot guarantee a similar appearance with the use of other flow cytometers.