ab139486
Aggresome Detection Kit

Instructions for Use

For the quantitative detection of aggresomes by flow cytometry and fluorescence microscopy.

This product is for research use only and is not intended for diagnostic use.

Version 4 Last Updated 19 April 2022

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1. Introduction

In mammalian cells, aggregated proteins may be concentrated by microtubule dependent retrograde transport to perinuclear sites of aggregate deposition, referred to as aggresomes. Aggresomes are inclusion bodies that form when the ubiquitin–proteasome machinery is overwhelmed with aggregation-prone proteins. Typically, an aggresome forms in response to some cellular stress, such as hyperthermia, viral infection or exposure to reactive oxygen species. Aggresomes appear to provide a cytoprotective function by sequestering the toxic, aggregated proteins and may also facilitate their ultimate elimination from cells by autophagy. Certain cellular inclusion bodies associated with human disease are thought to arise from an aggresomal response, including Lewy bodies associated with neurons in Parkinson's disease, Mallory bodies associated with liver cells in alcoholic liver disease and hyaline inclusion bodies associated with astrocytes in amyotrophic lateral sclerosis.

2. Product Overview

Non-physiological protein mutations or genetically engineered cell lines have been developed for assessment of the effects of protein aggregation within cells. Abcam's Aggresome Detection Kit contains a novel 488 nm excitable red fluorescent molecular rotor dye to specifically detect denatured protein cargo within aggresomes and aggresome-like inclusion bodies in fixed and permeabilized cells. The detection reagent supplied in the kit becomes brightly fluorescent upon binding to aggregated proteins within vesicles produced during aggresome formation and has been validated under a wide range of conditions in which the autophagy and proteasome pathways are known to be modulated. MG-132, a proteasome inhibitor, is included as a positive control in the kit. A nuclear counterstain is provided in the kit as well to highlight this organelle. 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. The assay is potentially applicable to the identification of small molecules that inhibit aggresome formation as well as immuno-localization studies between aggregated protein cargo and the various proteins implicated in aggresome formation, such as histone deacetylase 6, parkin, ataxin-3, dynein motor complex and ubiquilin-1.

3. Assay Summary

A. Confocal / Fluorescence Microscopy

Treat cells with experimental test agent of your choice

Prepare positive control by incubating with diluted Proteasome Inhibitor

Fix cells with formaldehyde

Permeabilize cells

Wash cells with PBS

Add Aggresome Detection Reagent

Incubate samples for 30 minutes in dark

Wash cells with PBS

Overlay with coverslip and analyze

B. Flow Cytometry

Treat cells with experimental test agent of your choice

⇩

Prepare positive control by incubating with diluted Proteasome Inhibitor

⇩

Wash twice with PBS

⇩

Fix cells with formaldehyde

⇩

Permeabilize cells

⇩

Wash with PBS

⇩

Strain cells

⇩

Add Aggresome Detection Reagent

⇩

Incubate at RT for 30 minutes in dark

⇩

Analyze immediately (no washing required!)

4. Materials Supplied

A. Kit Contents

Item	Quantity	Storage Temperature
Aggresome Detection Reagent	10 µL	-80°C
Hoechst 33342 Nuclear Stain	50 µL	-80°C
Proteasome Inhibitor (MG-132)	1 Vial	-80°C
10X Assay Buffer	25 mL	-80°C

Reagents provided in the kit are sufficient for at least 200 fluorescence microscopy assays or 100 flow cytometry assays.

5. Storage and Stability

All reagents are shipped on dry ice. Upon receipt, the kit should be stored at -80°C for long term storage. Avoid repeated freezing and thawing. Protect from light.

6. Materials Required, Not Supplied

- CO_2 incubator (37°C).
- Standard fluorescence microscope or flow cytometer equipped with a blue laser (488 nm).
- Calibrated, adjustable precision pipetters, preferably with disposable plastic tips.
- 5 mL round bottom polystyrene tubes for holding cells during flow cytometry.
- Adjustable speed centrifuge with swinging buckets.
- Glass microscope slides.
- Glass cover slips.
- Deionized water.
- Anhydrous DMSO
- Total growth medium suitable for cell type
- Paraformaldehyde
- EDTA, pH 8
- Triton X-100
- 1X Phosphate buffered saline (1X PBS)
- Formalin

Pre-Assay Preparation

NOTE: Allow all reagents to warm to room temperature before starting with the procedures. Upon thawing of solutions, 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, as well as for all subsequent uses, to gather the contents at the bottom of the tube.

A. Reagent Preparation

1. Positive Control, Proteasome Inhibitor (MG-132)

Reconstitute the lyophilized MG-132 (120 nmol) in 12 µL DMSO for a 10 mM stock solution. To use it as a positive control, dilute the MG-132 to 5-10 µM into your culture medium and grow cells for 6 to 18 hours. The agent has been validated with human cervical carcinoma cell line, HeLa, human T-lymphocyte cell line, Jurkat, and human bone osteosarcoma epithelial cell line, U2OS. Unused stock solution of MG-132 may be stored at -20°C for several weeks.

2. 1X Assay Buffer

Allow the 10X Assay Buffer to warm to room temperature. Make sure that the reagent is free of any crystallization before dilution. Prepare enough 1X Assay Buffer for the

number of samples to be assayed by diluting each milliliter (mL) of the 10X Assay Buffer with 9 mL of deionized water.

3. 4% Formaldehyde Solution

The following procedure is for preparation of 10 mL of 4% formaldehyde solution: Dilute 1.08 mL of 37% formaldehyde to a final volume of 10 mL with 1X Assay Buffer. Mix well. Formalin is a 37% aqueous solution of formaldehyde

4. Permeabilizing Solution

The following procedure is for preparation of 10 mL of permeabilizing solution (0.5% Triton X-100, 3 mM EDTA, pH 8): Add 50 µL Triton X-100 and 60 µL of 0.5M EDTA, pH 8, to 9.89 mL of 1X Assay Buffer.

5. Aggresome Detection Reagent

For optimal staining, the concentration of the Aggresome dye will vary depending upon the application. Suggestions are provided to use as guidelines, though some modifications may be required depending upon the particular cell type employed in the application.

a) For fluorescence microscopy

Prepare a sufficient amount of Dual Detection Reagent for the number of samples to be assayed as follows: For every 2 mL of 1X Assay Buffer or cell culture

medium, add 1 µL of Aggresome Detection Reagent and 2 µL of Hoechst 33342 Nuclear Stain.

NOTE:

a. The dyes may be combined into one staining solution or each may be used separately, if desired.
b. The Hoechst 33342 Nuclear Stain can be diluted further if its staining intensity is much stronger than that of the Aggresome red dye.
c. When staining BFP- or CFP-expressing cells, the Hoechst 33342 Nuclear Stain should be omitted due to its spectral overlap with these fluorescent proteins

b) For flow cytometry

Dilute the Aggresome Detection Reagent 5,000-10,000 fold with 1X Assay Buffer or buffer of choice.

B. Cell Preparations

Cells should be maintained via standard tissue culture practices. Always make sure that cells are healthy and in the log phase of growth before using them for the experiment.

Positive control cells should be pretreated with the MG-132, a cell permeable proteasome inhibitor, for 6~18 hours. Response to MG-132 is time and concentration dependent and may also vary significantly depending upon cell type and cell line. Negative control cells should be treated with a vehicle (DMSO, media or other solvent used to reconstitute or dilute an inducer or inhibitor) for an equal length of time under similar conditions.

7. Assay Protocol

A. Fluorescence/Confocal Microscopy (Adherent Cells)

1. The day before the experiment, seed the cells directly onto glass slides or polystyrene tissue culture plates to ensure ~ 80% confluency on the day of the experiment.
2. Treat the cells with the compound(s) of interest, and negative control cells with vehicle only.
3. Prepare positive control cells by incubating with the diluted Proteasome Inhibitor (5-10 µM MG-132, see Pre Assay Preparation, step 1) for 18 hours under normal tissue culture conditions.
4. Carefully wash the cells twice with 200 µL of 1X PBS per 1 cm^2 surface area for each wash.
5. Carefully remove excess 1X PBS and dispense 200 µL of 4% formaldehyde per 1 cm^2 surface area. Incubate for 30 minutes at room temperature.
6. Carefully wash the cells twice with 200 µL of 1X PBS per 1 cm^2 surface area for each wash.
7. Carefully remove excess 1X PBS and dispense 120 µL of Permeabilizing Solution (see Pre Assay Preparation, step 4)

per 1 cm^2 surface area. Place slides on ice, and gently shake them for 30 minutes.

8. Carefully wash the cells twice with 200 µL of 1X PBS per 1 cm surface area for each wash.
9. Carefully remove excess buffer and dispense 200 µL of Dual Detection Reagent (see Pre Assay Preparation, step 5a) per 1 cm^2 surface area to cover the monolayer cells.
10. Protect samples from light and incubate for 30 minutes at room temperature.
11. Carefully wash the cells with 200 µL of 1X PBS per 1 cm^2 surface area. Remove excess buffer and place coverslip on microscope slide.
12. Analyze the stained cells by wide-field fluorescence or confocal microscopy (60X magnification recommended). Use a standard Texas Red filter set for imaging the cell aggresome signal and a DAPI filter set (optional) for imaging the nuclear signal.

B. Fluorescence/Confocal Microscopy (Suspension Cells)

1. Cells should be cultured to a density not to exceed 1 x 10^6 cells/mL. Make sure that cells are healthy and in the log phase of growth before starting an experiment.
2. Treat the cells with the compound of interest and the negative control cells with vehicle.
3. Prepare positive control cells by incubating with the diluted Proteasome Inhibitor (5-10 µM MG-132, see Pre Assay Preparation, step1) for 18 hours under normal tissue culture conditions.
4. At the end of the treatment, collect cells to ensure cell count of 2 x 10^5 to 4 x 10^5 cells/sample. Centrifuge suspension for 5 minutes at room temperature at 400 x g.
5. Remove the supernatant and carefully re-suspend the cells in 1 mL of 1X PBS.
6. Centrifuge for 5 minutes at room temperature at 400 x g. Carefully remove most of the supernatant by aspiration. Using a micropipet, gently re-suspend the cell pellet in the remaining 1X PBS (~ 200 µL).
7. Using a micropipet, add drop wise the cell suspension into 1 mL of 4% formaldehyde solution (see Pre Assay Preparation, step 3) contained in a 15 mL conical tube.

Slowly vortex the fixative during addition of the cell suspension. Allow to stand for 30 minutes at room temperature.

8. Collect the fixed cells by centrifugation at 800 x g for 10-15 minutes.
9. Pour out the supernatant. Do not aspirate.
   CAUTION: Fixed cells do not stick to the walls of the tube as tightly as live cells. Be careful not to lose the cell pellet.
10. Re-suspend the cell pellet in the remaining small volume of supernatant using a micropipet. Add 1 mL of 1X PBS to the cells, mix well and centrifuge at 800 x g for 10-15 minutes. Pour out the supernatant, being careful not to lose the cell pellet.
11. Re-suspend the cell pellet in the remaining small volume of supernatant using a micropipet. Using a micropipet, add drop wise the cell suspension into 1 mL of permeabilizing solution (see Pre Assay Preparation, step 4) contained in a 15 mL conical tube. Slowly vortex the permeabilizing solution during addition of the cell suspension. Allow to incubate for 30 minutes on ice.
12. Collect the fixed cells by centrifugation at 800 x g for 10-15 minutes. Pour out the supernatant, being careful not to lose the cell pellet.
13. Re-suspend the cell pellet in the remaining small volume of supernatant using a micropipette and wash again using 1 ml

1X PBS. Then, centrifuge at 800 x g for 10-15 minutes. Pour out the supernatant, being careful not to lose the cell pellet.

14. Re-suspend the cell pellet in 100 µL of Dual Detection Reagent (see Pre Assay Preparation, step 5a). It is important to achieve a monodisperse cell suspension at this step by gently pipetting up and down repeatedly.
15. Protect samples from light and incubate for 30 minutes at room temperature.
16. Resuspend the cells in 1 mL of 1X PBS, centrifuge them at 800 x g for 10 - 15 minutes and remove the supernatant.
17. Resuspend the cells in 100 µL of 1X PBS and apply a 20 µL aliquot of the cell suspension, sufficient for 2 x 10^4 cells, onto a microscope slide. Immediately overlay the cells with a cover slip.
18. Analyze the stained cells by wide-field fluorescence or confocal microscopy (60X magnification recommended). Use a standard Texas Red filter set for imaging the cell aggresome signal and a DAPI filter set (optional) for imaging the nuclear signal.

C. Flow Cytometry (Both Suspension and Adherent Cells)

1. Cells should be maintained via standard tissue culture practice in a humidified incubator at 37°C, 5% CO_2. Make sure that cells are healthy and in the log phase of growth before starting an experiment.
2. Treat cells with compound of interest and negative control cells with vehicle.
3. Prepare positive control cells by incubating with the diluted Proteasome Inhibitor (5-10 µM MG-132, see Pre Assay Preparation, step 1) for 18 hours under normal tissue culture conditions.
4. At the end of the treatment, trypsinize (adherent cells), or collect cells (suspension cells). Samples may contain 1 x 10^6 to 2 x 10^6 cells per mL.
5. Centrifuge at 400 x g for 5 minutes to pellet the cells. Carefully resuspend and wash the cells with 1 mL of 1X PBS.
6. Remove the supernatant and carefully re-suspend the cells in 1 mL 1X PBS.
7. Centrifuge for 5 minutes at room temperature at 400 x g. Carefully remove most of the supernatant by aspiration. Using a micropipet, gently re-suspend the cell pellet in the remaining 1X PBS (~ 200 µL).
8. Using a micropipet, add drop wise the cell suspension into 1 mL of 4% formaldehyde solution (see Pre Assay

Preparation, step 3) contained in a 15 mL conical tube. Slowly vortex the fixative during addition of the cell suspension. Allow to stand for 30 minutes at room temperature.

9. Collect the fixed cells by centrifugation at 800 x g for 10-15 minutes.
10. Pour out the supernatant. Do not aspirate.
    CAUTION: Fixed cells do not stick to the walls of the tube as tightly as live cells. Be careful not to lose the cell pellet.
11. Re-suspend the cell pellet in the remaining small volume of supernatant using a micropipet Add 1 mL of 1X PBS to the cells, and centrifuge at 800 x g for 10-15 minutes. Pour out the supernatant, being careful not to lose the cell pellet.
12. Re-suspend the cell pellet in the remaining small volume of supernatant using a micropipet. Using a micropipet, add drop wise the cell suspension into 1 mL of permeabilizing solution (see Pre Assay Preparation, step 4) contained in a 15 mL conical tube. Slowly vortex the permeabilizing solution during addition of the cell suspension. Allow to incubate for 30 minutes on ice.
13. Collect the fixed cells by centrifugation at 800 x g for 10-15 minutes. Pour out the supernatant, being careful not to lose the cell pellet.
14. Wash the cells by re-suspending the cell pellet with 1 mL 1X PBS using a micropipet. Then, transfer the cell suspension through a cell strainer to remove cell debris. Then,

centrifuge the tube at 800 x g for 10-15 minutes. Remove the cell strainer and carefully pour out the supernatant, being careful not to lose the cell pellet.

15. Re-suspend the cell pellet in 500 µL of freshly diluted (5,000-10,000-fold) Aggresome Red Detection Reagent (see Pre Assay Preparation, step 5b). It is important to achieve a monodisperse cell suspension at this step by gently pipetting up and down repeatedly.
16. Protect samples from light and incubate for 30 minutes at room temperature.
17. Analyze the samples in the FL3 channel of a flow cytometer. No washing is required prior to the flow cytometry analysis.

8. Data Analysis

A. Filter Set Selection

The selection of optimal filter sets for a fluorescence microscopy application requires matching the optical filter specifications to the spectral characteristics of the dyes employed in the analysis (see Figure 1). Consult the microscope or filter set manufacturer for assistance in selecting optimal filter sets for your microscope. Predesigned filter sets for Texas Red should work well for this application. For flow cytometry, fluorescence channel FL-3 is recommended for analysis of the Aggresome red dye staining using a 488 nm laser source.

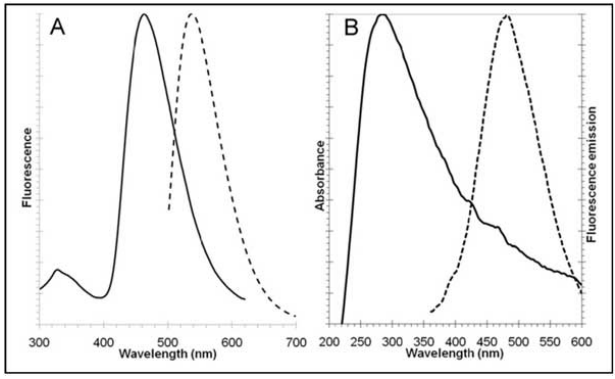

Figure 1. Excitation and fluorescence emission spectra for Aggresome Detection Reagent dye, ex/em 500/600 nm (panel A) and Hoechst 33342, ex/em 350/461 nm (panel B). All spectra were determined in 1X Assay Buffer.

B. Anticipated Results (Fluorescence Microscopy)

Selective degradation of intracellular targets, such as misfolded proteins and damaged organelles, is an important homeostatic function that autophagy has acquired in addition to its more general role in restoring the nutrient balance during stress and starvation. Although the exact mechanism underlying selection of autophagic substrates remains the subject of much study, ubiquitinylation is now appreciated to be a signal for autophagic degradation of aggregated proteins. In order to better understand the nature and contribution of the pathway modulators to the specific targeting of protein cargo for degradation, the Aggresome red fluorescent molecular rotor dye has been developed specifically devised to detect aggregated proteins within the aggresome and aggresome-like structures. This detection reagent detects protein cargo accumulation within these structures, as observed by fluorescence microscopy (see Figure 2).

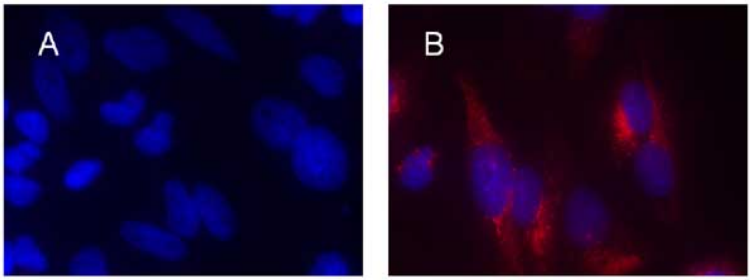

Figure 2. Aggresome Detection reagent detects protein accumulation within aggresomes, as observed by fluorescence microscopy. HeLa cells were mock-induced with 0.2% DMSO (panel A) or induced with 5 µM MG-132 panel B) for 12 hours at 37°C. After treatment, cells were incubated with aggresome detection reagent for 30 minutes.

The ability to detect aggresomes was demonstrated using various potent, cell permeable and selective proteasome inhibitors: Lactacystin, Epoxomicin and Bortezomib (US FDA-approved drug Velcade®), as shown in Figure 3.

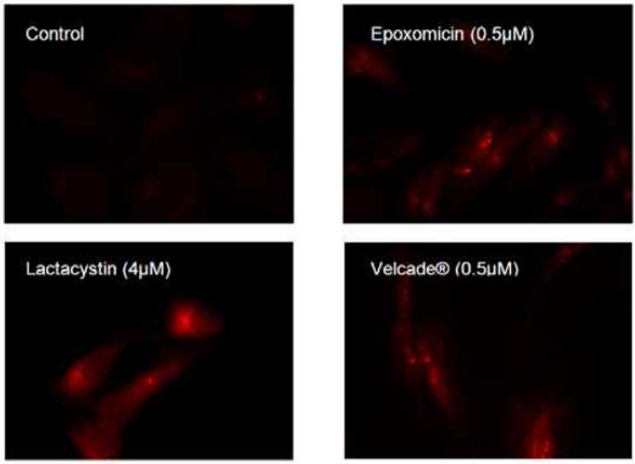

Figure 3. Aggresomes detected by aggresome dye in HeLa cells after overnight incubation with various proteasome inhibitors, as observed by fluorescence microscopy.

The p62 protein (also called sequestosome 1 (SQSTM1)) is an ubiquitin-binding scaffold protein that co-localises with ubiquitinylated protein aggregates in, for example, many neurodegenerative diseases and proteinopathies of the liver. The protein is able to polymerise via an N-terminal PB1 domain and can interact with ubiquitinylated proteins via its C-terminal UBA domain. Also, p62 binds directly to LC3 and GABARAP family proteins via a specific sequence motif. The protein is itself degraded by autophagy and serves to link ubiquitinylated proteins to the autophagic machinery in order to enable their degradation in the lysosome. Since p62 accumulates when autophagy is inhibited, and decreased levels can

be observed when autophagy is induced, p62 may be used as a marker to study autophagic flux. Figure 4 shows the use of a fluorescein conjugated antibody directed to p62, in concert with the aggresome detection reagent, in co-localizing these elements of the autophagic process in intact cells. It is clear that p62 co-localizes with aggresomes.

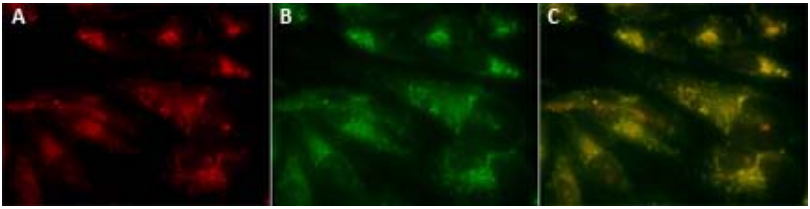

Figure 4. Aggresomes within HeLa cells, previously treated for 12 hours with 5 µM MG-132, detected by Aggresome detection reagent dye (A) co-localization with fluorescein-p62 antibody (B) composite image (C) as observed by fluorescence microscopy.

C. Flow Cytometry Data Analysis and Anticipated Results

1. Obtain the mean fluorescence intensity (MFI) values for treated and untreated sample.
2. Calculate the aggresome propensity factor (APF) value using the following formula:

$$APF = 100 \times (MFI\ treated - MFI\ control)/MFI\ treated$$

The expected APF value using the control MG-132 is > 25.

Figure 5 shows the typical results of flow cytometry-based analysis of cell populations using the Abcam Aggresome Detection Kit (ab139486). Uninduced control and 5 µM Proteasome inhibitor MG-132-treated Jurkat cells (T-Cell leukemia) were used. After 18 hours treatment, cells were loaded Aggresome red Detection Reagent, then analyzed without washing by flow cytometry. Results are presented by histogram overlays. Control cells were stained as well but display low fluorescence. In the samples treated with 5 µM MG-132 for 18 hours. The aggresome red dye signal increases over 2-fold, indicating that MG-132 induced aggresome formation in Jurkat cells. The APF value is about 66.

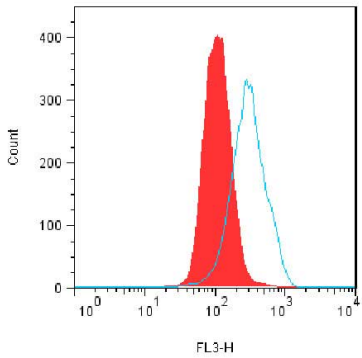

Figure 5. Flow cytometry-based cell aggresome analysis: Jurkat cells were mock induced with 0.2% DMSO or induced with 5 µM MG-132 for overnight hours at 37°C. After treatment, cells were fixed and incubated with Aggresome Detection Reagent then analyzed by flow cytometry without washing using a 488 nm laser with fluorescence detection in the FL3 channel. Results are presented as histogram overlays. In MG-132 treated cells, fluorescent green signal increases over 2-fold. The described assay allows assessment of the effects of protein aggregation.

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