Sulfenylated Protein Cell-Based Detection Kit

Item No. 600320

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1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. For best results, remove components and store as stated below.

Item Number	Item	96 wells Quantity/Size	Storage
600321	Cell-Based Assay DAz-2	1 vial/30 µl	-20°C
600172	Cell-Based Assay Buffer (10X)	1 vial/50 ml	RT
10009899	Cell-Based Assay Fixative	1 vial/10 ml	RT
600173	Cell-Based BSA Blocking Solution	1 vial/10 ml	4°C
600322	Cell-Based Assay Phosphine-biotin	1 vial/50 µl	-20°C
600175	Cell-Based Assay Avidin-FITC Complex	1 vial/1 mg	-20°C
10011096	Cell-Based Assay Epigallocatechin Gallate (EGCG)	1 vial/50 µl	-20°C

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

E-Mail: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section, on page 3, and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. Adjustable pipettes and a repeating pipettor
2. 6-, 12-, 24-, or 96-well plates for culturing cells
3. A plate centrifuge
4. Flow cytometer, fluorescence microscope, or plate reader equipped with laser/fluorescent filters capable of detecting FITC excitation and emission wavelengths of 485 and 535 nm, respectively

INTRODUCTION

Background

Oxidative damage occurs in all living organisms from reactive oxygen species (ROS), which can be a consequence of normal cellular pathways as well as disease processes. ROS react with proteins, resulting in protein modification, such as protein sulfenylation through the reversible oxidation of cysteine residues. Redox-sensitive cysteine residues in proteins can function as sensors of ROS and serve as molecular switches, activating or deactivating proteins, following a change in oxidative state.[1] However, the accumulation of proteins with irreversible cysteine oxidation is a hallmark of stress-induced cellular damage associated with diseases like cancer. Monitoring intracellular sulfenylated protein levels could be very useful in discriminating between normal and pathological conditions in living cells.[2] Furthermore, detection of sulfenylated proteins might help identifying new pathways regulated by sulfenic acid formation.

Previously, detection of sulfenylated proteins has been accomplished by cell destructive methods, including GC/MS, HPLC with various detectors, Western blotting, and ELISA.[3] Recently, a bioorthogonal chemical reporter, DAz-2, which is cell-permeable and chemoselective for sulfenic acids, has been developed,[4,5] making *in situ* detection of protein sulfenylation possible.

About This Assay

Cayman's Sulfenylated Protein Cell-Based Detection Kit employs the newly developed cell-permeable and chemoselective DAz-2 probe to detect sulfenic acid-modified proteins in living cells. Epigallocatechin Gallate (EGCG), a compound shown to react with culture medium to generate hydrogen peroxide (H_2O_2) (see Cayman's booklet, Item No. 600050), is included to be used as a positive control.

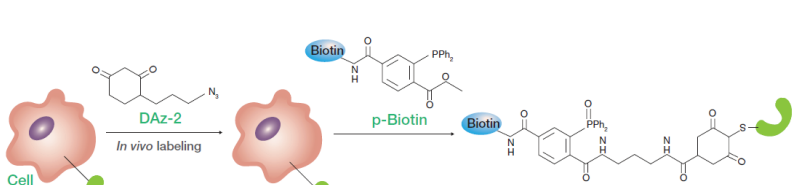


Figure 1. Mechanism of DAz-2 recognition of sulfenylated protein and subsequential binding to phosphine-biotin

PRE-ASSAY PREPARATION

NOTE: Water used to prepare all buffers must be deionized and free of trace organic contaminants ('UltraPure'). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate. UltraPure water may be purchased from Cayman (Item No. 400000).

IMPORTANT

Avidin-FITC is light sensitive. Do not expose to direct intense light.

Reagent Preparation

1. **Assay Buffer Preparation**

   To prepare an Assay Buffer solution, dilute 50 ml of Cell-Based Assay Buffer (10X) (Item No. 600172) with 450 ml of UltraPure water. The diluted Assay Buffer will be stable for six months at room temperature.

2. **DAz-2 Binding Solution Preparation**

   Prior to use, thaw the Cell-Based Assay DAz-2 (Item No. 600321) at room temperature. To prepare a DAz-2 Binding Solution, dilute the reagent 1:200 in the medium you use to culture your cells and mix well. Prepare this solution just before use.

3. **Phosphine-biotin Binding Solution Preparation**

   Prior to use, thaw the Cell-Based Assay Phosphine-biotin (Item No. 600322) at room temperature. To prepare a Phosphine-biotin Binding Solution, dilute the reagent 1:100 in the diluted Assay Buffer and mix well. Prepare this solution just before use.

4. **Avidin-FITC Staining Solution Preparation**

   Prior to use, reconstitute the whole vial of Cell-Based Assay Avidin-FITC Complex (Item No. 600175) with 1 ml of diluted Assay Buffer, prepared above. The reconstituted Avidin-FITC Complex will be stable for one month at 4°C.

   To prepare a Staining Solution, dilute the reconstituted Avidin-FITC Complex prepared above 1:20 in diluted Assay Buffer, prepared above. Mix well and keep on ice. **Protect from light.** Prepare fresh Staining Solution for each assay.

ASSAY PROTOCOL

IMPORTANT

- Avidin-FITC is light sensitive. All staining procedures must be performed without direct exposure to intense light. Incubations should be done in the dark.
- For all assay protocols described below, it is imperative that samples be analyzed immediately following completion of the staining.

Treatment of the Cells and *in situ* labeling

1. Culture cells in a 6-, 12-, 24-, or 96-well plate at a density of 5 x 10^5 cells/ml; grow cells overnight.
2. The next day, treat cells with experimental compounds or vehicle for 24 hours, or for the period of time required for your typical experimental protocol. To use the included Epigallocatechin gallate (EGCG) as a positive control, dilute the Cell-Based Assay EGCG (Item No. 10011096) 1:100-1:200 into your culture medium.
3. Terminate the experiment by aspirating the culture medium.
4. Depending on the size of the vial used, add 1 ml, 500 µl, 250 µl, or 50 µl of DAz-2 Binding Solution prepared above to each well of a 6-, 12-, 24-, or 96-well plate and incubate the cells in a 37°C incubator for 30 minutes to one hour.
5. Detect sulfenylated proteins using the following staining procedure for different applications.

Flow Cytometry

1. Collect the cells in a test tube and centrifuge at 400 x g for three minutes. Aspirate the supernatant.
2. Resuspend the cells at a density of 10^6 cells/ml in Cell-Based Assay Fixative (Item No. 10009899). Mix well to ensure separation of individual cells. Incubate the cells in the Fixative for 10 minutes.
3. Centrifuge the cells at 400 x g for three minutes and aspirate the supernatant.
4. Wash the cells in 2-4 ml of Wash Buffer 2-3 times.
5. Centrifuge the cells at 400 x g for three minutes and aspirate the supernatant.
6. Resuspend the cells at a density of 10^6-10^7 cells/ml in the Cell-Based BSA Blocking Solution (Item No. 600173) and incubate for 30 minutes at room temperature.
7. Centrifuge the cells at 400 x g for three minutes and aspirate the supernatant.
8. Resuspend the cells at a density of 10^6-10^7 cells/ml in the Phosphine-biotin Binding Solution and incubate for 30 minutes to one hour at 37°C.
9. Centrifuge the cells at 400 x g for three minutes and aspirate the supernatant.
10. Wash the cells in 2-4 ml of diluted Assay Buffer 2-3 times.
11. Centrifuge the cells at 400 x g for three minutes and aspirate the supernatant.
12. Resuspend the cells at a density of 10^6-10^7 cells/ml in the Avidin-FITC Staining Solution prepared above and incubate for 30 minutes to one hour at 37°C.
13. Centrifuge the cells at 400 x g for three minutes and aspirate the supernatant.
14. Wash the cells in 2-4 ml of diluted Assay Buffer 2-3 times.
15. Resuspend the cells at a density of 10^5-10^6 cells/ ml in diluted Assay Buffer, depending on the number of cells you have. Mix well to ensure separation of individual cells. The cells must be analyzed immediately. Sulfenylated proteins recognized by DAz-2 and labeled by Avidin-FITC can be detected by flow cytometry with 488 nm excitation and 530 nm band pass filter.

Fluorescence Microscopy

1. Centrifuge the plate at 400 x g for three minutes. Aspirate the supernatant.
2. Add 1 ml, 0.5 ml, 250 µl, or 50 µl of Cell-Based Assay Fixative (Item No. 10009899) to each well of a 6-, 12-, 24-, or 96-well plate, respectively. Incubate the cells in the Fixative for 10 minutes.
3. Centrifuge the plate at 400 x g for three minutes and aspirate the supernatant.
4. Add 2 ml, 1 ml, 500 µl, or 100 µl of diluted Assay Buffer to each well of a 6-, 12-, 24-, or 96-well plate, respectively. Centrifuge the plate at 400 x g for three minutes and aspirate the supernatant.
5. Repeat step 4 two more times.
6. Add 1 ml, 0.5 ml, 250 µl, or 50 µl of the Cell-Based BSA Blocking Solution (Item No. 600173) to each well of a 6-, 12-, 24-, or 96-well plate, respectively, and incubate for 30 minutes at room temperature.
7. Centrifuge the plate at 400 x g for three minutes and aspirate the supernatant.
8. Add 1 ml, 0.5 ml, 250 µl, or 50 µl of the Phosphine-biotin Binding Solution to each well of a 6-, 12-, 24-, or 96-well plate, respectively, and incubate for 30 minutes to one hour at 37°C.
9. Centrifuge the cells at 400 x g for three minutes and aspirate the supernatant.

10. Add 2 ml, 1 ml, 500 µl, or 100 µl of diluted Assay Buffer to each well of a 6-, 12-, 24-, or 96-well plate, respectively. Centrifuge the plate at 400 x g for three minutes and aspirate the supernatant.
11. Repeat step 10 two more times.
12. Add 1 ml, 0.5 ml, 250 µl, or 50 µl of the Avidin-FITC Staining Solution prepared above to each well of a 6-, 12-, 24-, or 96-well plate, respectively, and incubate for 30 minutes to one hour at 37°C.
13. Centrifuge the cells at 400 x g for three minutes and aspirate the supernatant.
14. Add 1 ml, 0.5 ml, 250 µl, or 50 µl of diluted Assay Buffer to each well of a 6-, 12-, 24-, or 96-well plate, respectively. Examine the staining under a microscope with a fluorescent filter designed to detect flourescein (excitation/emission = 485 nm/535 nm).

Plate Reader Fluorescence Detection

A 96-well clear bottom BLACK culture plate should be used for this method.

1. Centrifuge the plate at 400 x g for three minutes. Aspirate the supernatant.
2. Add 50 µl of Cell-Based Assay Fixative (Item No. 10009899) to each well of the plate, and incubate the cells in the Fixative for 10 minutes.
3. Centrifuge the plate at 400 x g for three minutes and aspirate the supernatant.
4. Add 100 µl of diluted Assay Buffer to each well of the plate. Centrifuge the plate at 400 x g for three minutes and aspirate the supernatant.
5. Repeat step 4 two more times.
6. Add 50 µl of the Cell-Based BSA Blocking Solution (Item No. 600173) to each well of the plate and incubate for 30 minutes at room temperature.
7. Centrifuge the plate at 400 x g for three minutes and aspirate the supernatant.
8. Add 50 µl of the Phosphine-biotin Binding Solution to each well of the plate and incubate for 30 minutes to one hour at 37°C.
9. Centrifuge the cells at 400 x g for three minutes and aspirate the supernatant.
10. Add 100 µl of diluted Assay Buffer to each well of the plate. Centrifuge the plate at 400 x g for three minutes and aspirate the supernatant.
11. Repeat step 10 two more times.
12. Add 50 µl of the Avidin-FITC Staining Solution prepared above to each well of the plate and incubate for 30 minutes to one hour at 37°C.
13. Centrifuge the cells at 400 x g for three minutes and aspirate the supernatant.
14. Add 100 µl of the diluted Assay Buffer to each well of the plate. Centrifuge the plate at 400 x g for three minutes and aspirate the supernatant.
15. Repeat step 15 one or two more times.
16. Add 50 µl of diluted Assay Buffer to each well of the plate. The cells can be analyzed with a fluorescence plate reader and must be analyzed immediately. Sulfenylated protein recognized by DAz-2 and labeled by Avidin-FITC can be measured as fluorescence intensity with excitation and emission wavelengths of 485 nm and 535 nm, respectively.

PERFORMANCE CHARACTERISTICS

Cell Staining

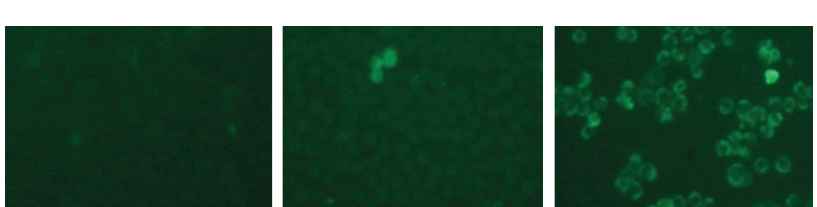


Figure 2. Hydrogen peroxide induces protein sulfenylation in RAW 264.7 cells. RAW 264.7 cells were plated at a density of 2 x 10^4 cells/well in a 96-well plate. The next day, cells were treated with vehicle (control) or 0.25% H_2O_2 for 15 minutes at 37°C. Treatment medium was then removed and replaced with 500 µM DAz-2 in the culture medium (DMEM containing 10% FBS) and incubated at 37°C for 30 minutes. Cells were then processed for staining according to the protocol described above. *Panel A:* cells without DAz-2 loading did not show any fluorescent staining. *Panel B:* cells treated with vehicle (control) had background levels of protein sulfenylation appearing as faint staining. *Panel C:* cells treated with 0.25% H_2O_2 had significantly elevated levels of protein sulfenylation, evidenced by a dramatic increase in staining intensity in the cytoplasm.

Figure 3. EGCG induces protein sulfenylation in RAW 264.7 cells. RAW 264.7 cells were plated at a density of 2 x 10^4 cells/well in a 96-well plate and treated with vehicle or different doses of EGCG. The next day, treatment medium was replaced with 500 µM DAz-2 in the culture medium (DMEM containing 10% FBS) and incubated at 37°C for 30 minutes. Cells were then processed for staining according to the protocol described above. *Panel A:* cells treated with vehicle (control) had little protein sulfenylation as shown by a background level of fluorescence intensity. *Panel B:* cells treated with 100 µM EGCG had an increased level of protein sulfenylation as shown by sparse staining of cells. *Panel C:* cells treated with 200 µM EGCG had a significantly increased level of protein sulfenylation as shown by abundant staining of cells.
NOTE: Overnight treatment of cells with EGCG causes protein sulfenylation in both cytoplasm and nuclei, as shown in Figure 3. This is in contrast to the acute treatment of cells with H_2O_2 in which protein sulfenylation mainly occurs in the cytoplasm, as shown in Figure 2.

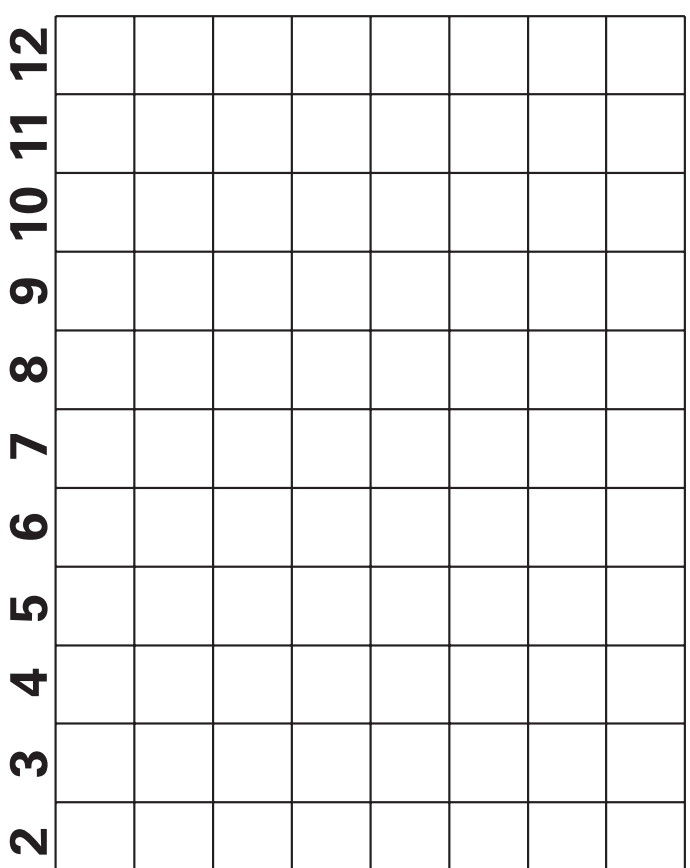
RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
No staining, including positive control	Reagents were not added in correct order	Add reagents in the correct order
Control cells without treatment show strong staining	Control cells are not healthy	Use only healthy cells

References

1. Leonard, S.E., Reddie, K.G., and Carroll, K.S. Mining the thiol proteome for sulfenic acid modifications reveals new targets for oxidation in cells. *ACS Chem. Biol.* **4(9)**, 783-799 (2009).
2. Roos, G. and Messens, J. Protein sulfenic acid formation: From cellular damage to redox regulation. *Free Radic. Biol. Med.* **51(2)**, 314-326 (2011).
3. Hawkins, C.L., Morgan, P.E., and Davies, M.J. Quantification of protein modification by oxidants. *Free Radic. Biol. Med.* **46(8)**, 965-988 (2009).
4. Seo, Y.H. and Carroll, K.S. Facile synthesis and biological evaluation of a cell-permeable probe to detect redox-regulated proteins. *Bioorg. Med. Chem. Lett.* **19**, 356-359 (2009).
5. Reddie, K.G., Seo, Y.H., Muse, W.B.I., *et al.* A chemical approach for detecting sulfenic acid-modified proteins in living cells. *Mol. BioSyst.* **4**, 521-531 (2008).



NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.