S-Glutathionylated Protein Detection Kit

Item No. 10010721

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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	Quantity	Storage
10010726	PSSG Assay Buffer (10X)	1 vial	RT
10010727	PSSG Blocking Reagent	3 vials	-20°C
10010728	PSSG Lysis Buffer	1 vial	RT
10010729	PSSG Reduction Reagent	3 vials	-20°C
10010731	PSSG Labeling Reagent	3 vials	-20°C
10010732	PSSG Detection Reagent I (HRP)	1 vial	-20°C
10010733	PSSG Detection Reagent II (FITC)	1 vial	-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.

This kit is intended for the detection of S-glutathionylated proteins within intact (permeabilized) cells by flow cytometry or fluorescence microscopy. Cell lysates may also be generated for use with an biotin-avidin blotting overlay assay, avidin-affinity purification, or immunoprecipitation.

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 and used before the expiration date indicated on the outside of the box. Once the kit has been opened, store the vials of PSSG Assay Buffer (10X) and PSSG Lysis Buffer at room temperature. Store the remaining components at -20°C.

Materials Needed But Not Supplied

1. A flow cytometer or microscope capable of measuring fluorescence at an excitation wavelength of 488 nm and an emission wavelength of 518-535 nm.
2. Adjustable pipettes and a repeating pipettor.
3. Cells and cell culturing equipment and media.
4. Waterbath or cell culture incubator set at 37°C.
5. Tabletop centrifuge capable of 500 x g to collect cells.
6. PBS, pH 7.2, with and without 3.7 % paraformaldehyde.
7. Dimethylformamide (DMF), *A hazardous solvent*, for reagent dissolution.
8. 1.5 ml microcentrifuge tubes for sample processing.
9. Electrophoresis and immunoblotting equipment (optional technique).
10. Nuclear counterstain (optional); for example, Propidium Iodide (PI) or 4'6-Diamidino-2-phenylindole (DAPI), *CAUTION: These chemicals are probable mutagens.*
11. Avidin-linked agarose or similar media for purification of labeled proteins (optional).

INTRODUCTION

Background

Glutathione (GSH) is a tripeptide (γ-glutamylcysteinylglycine) widely distributed in both plants and animals.[1,2] GSH is involved in maintenance of protein sulfhydryl reduction status.[3,4] The concentration of GSH ranges from a few micromolar in plasma to several millimolar in tissues such as liver.[5,6] Mixed protein glutathionyl disulfides are a post translational protein modification of growing interest.[7-9] Protein-S-glutathionylation may modify the activity of a large number of cell proteins, including metabolic, structural, cytoskeletal, and signaling proteins.[10] PSSG detection methods can employ GSH adduct antibodies, GSH derivatives, and differential labeling systems based on the 'Biotin-Switch' method.[9,11,12]

About This Assay

Cayman's S-Glutathionylated Protein Detection Assay Kit provides a convenient method for the direct visualization of S-glutathionylated proteins in whole (permeabilized) cells by flow cytometry and microscopy as well as avidin overlay analysis.[13] This cell-based assay starts with the modification of protein free-thiols groups followed by enzymatic cleavage of any protein-S-glutathione (PSSG) adducts present in the sample. Biotinylation of the newly-formed protein free-thiols provides the basis for visualization using streptavidin-based colorimetric or fluorescence detection. Reagents are provided to test three sets of 10 samples (most convenient) or up to thirty samples total at once if desired.

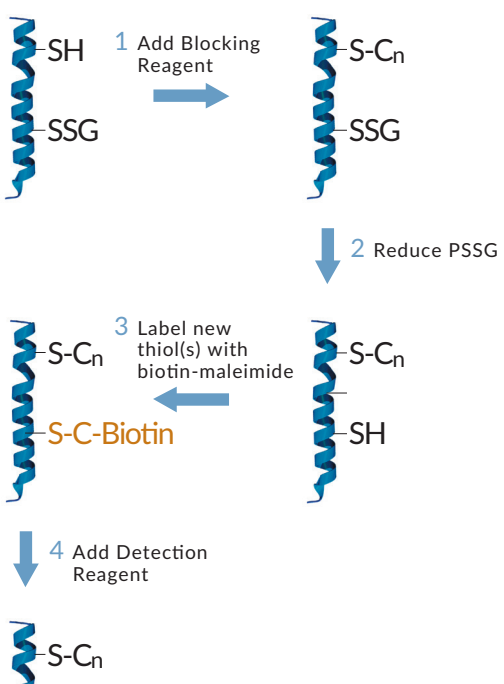


Figure 1. The four-step process of Protein-S-Glutationylation labeling. Permeabilized whole cells are treated with a blocking agent to derivatize existing protein-free thiols. S-glutathionylated protein sites are enzymatically reduced to new free thiols which are then labeled with a thiol-specific biotinimide. Subsequent detection or purification by avidin-coupled reagents can be used to localize or purify the labeled protein(s).

PRE-ASSAY PREPARATION

Reagent Preparation

1. **PSSG Assay Buffer (10X) - (Item No. 10010726)**

   Prior to use, bring to room temperature. Dilute the contents of the Assay Buffer vial (20 ml) to 200 ml with purified water. This buffer may be stored at room temperature for up to six months.

2. **PSSG Blocking Reagent - (Item No. 10010727)**

   Each vial contains crystalline solid. Three vials are provided to allow for up to three separate experiments. Reconstitute the contents of a vial as needed (see **Performing the Assay** step 2, on page 11) by the addition of 100 µl of DMF to the lyophilized solid followed by mixing and dilution to 10 ml final volume with 1X PSSG Assay Buffer. Store the Blocking Reagent as supplied at -20°C. Use fresh Blocking Reagent for each experiment. Storage and later use of the solution is not advised.

3. **PSSG Lysis Buffer - (Item No. 10010728)**

   Prior to assaying, bring to room temperature. This buffer is ready for use as supplied. This buffer may be stored at room temperature for up to six months.

4. **PSSG Reduction Reagent - (Item No. 10010729)**

   Each vial contains a lyophilized powder. Three vials are provided to allow for up to three separate experiments. Reconstitute the contents of each vial with 1.1 ml water immediately prior to use (see **Performing the Assay** step 3, on page 11). Store the Reduction Reagent as supplied at -20°C. Use fresh Reduction Reagent for each experiment. Storage and later use of the solution is not advised.

5. **PSSG Labeling Reagent - (Item No. 10010731)**

   Each vial contains a lyophilized solid. Three vials are provided to allow for up to three separate experiments. Reconstitute the contents of each vial as needed (see **Performing the Assay** step 4, on page 11) by the addition of 10 µl of DMF to the lyophilized solid followed by mixing and dilution to 1.1 ml final volume with 1X PSSG Assay Buffer. Store the Labeling Reagent as supplied at -20°C. Use fresh Labeling Reagent for each experiment. Storage and later use of the solution is not advised.

6. **PSSG Detection Reagent I (HRP) - (Item No. 10010732)**

   This vial contains a lyophilized powder. Reconstitute the contents of the vial with 400 µl water. Use at a 1:75 (or up to 1:1,000) dilution for blotting overlay. Store the reconstituted reagent at 4°C for up to six months.

7. **PSSG Detection Reagent II (FITC) - (Item No. 10010733)**

   This vial contains a lyophilized powder. Reconstitute the contents of the vial with 1 ml of 1X PSSG Assay Buffer and use at a 1:50 dilution. Store the reconstituted reagent at 4°C for up to six months.

ASSAY PROTOCOL

General Information

- *NOTE: All cell viability tests should be carried out before starting the S-Glutathionylated Protein Detection Assay due to the presence of a cell permeabilization agent in the PSSG Assay Buffer.*
- All steps are carried out at room temperature unless otherwise stated.
- To multiplex this assay with other biomarkers, add other cell marker detection reagents relevant to your experiment at the Detection Reagent II step of the assay.

Performing the Assay

Whole cell staining for Flow Cytometry or Fluorescence Microscopy

1. One sample = 5,000-15,000 cells/well (if adherent culture) or 5,000-15,000 cells/1.5 ml microcentrifuge tube (if suspension culture). Wash cells twice with PBS. (Wash cells by momentary gentle tapping of each cell sample in 100 µl of Buffer followed by 500 x g centrifugation to collect cells. Decant the wash gently as not to disturb the cell pellet, then gently tap the pellet to resuspend cells in the residual buffer. This equals one “wash”.) Fix cells with 3.7% formaldehyde in PBS for 10 minutes at room temperature. Wash cells once more with PBS alone.
2. Block protein free-thiols in each sample with 100 µl freshly prepared PSSG Blocking Reagent for 30 minutes. Wash each sample twice with 1X Assay Buffer.
3. Add 100 µl of freshly reconstituted PSSG Reduction Reagent to each sample and incubate for 15 minutes at 37°C. Wash each sample two times with 1X Assay Buffer.
4. Add 100 µl of freshly reconstituted PSSG Labeling Reagent to each sample and incubate for one hour. Wash samples three times with 1X Assay Buffer.
5. Add PSSG Detection Reagent II (FITC) at a 1:50 dilution in 1X Assay Buffer to each sample (for example add 2 µl of reconstituted PSSG Detection Reagent II per 100 µl 1X Assay Buffer) and incubate one hour. Wash each sample two times again with 1X Assay Buffer and collect data with flow cytometer or microscope.

Avidin Overlay Protocol

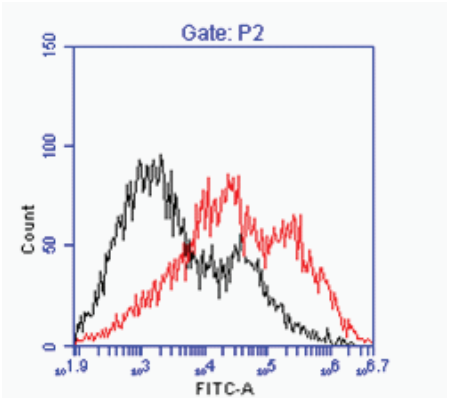
1. Perform steps 1-4 from Whole cell staining for Flow Cytometry or Fluorescence Microscopy on page 11.
2. Prepare cell lysates for avidin affinity techniques. Lyse cell samples by adding 50 µl of PSSG Lysis Buffer to each tube, followed by a 30 minute incubation on ice. Use clarified lysates as desired with a variety of avidin affinity techniques. For the avidin overlay assay, process samples with electrophoresis sample buffer (reducing or nonreducing as desired). Separate proteins by electrophoresis and transfer to membrane, block free sites on the membrane with 0.1% bovine serum albumin (BSA) in TBS or PBS for one hour at room temperature. Add Detection Reagent 1 (HRP) diluted in a fresh aliquot of the membrane-blocking buffer (BSA in buffer), incubate with gentle agitation for 40 minutes. Wash membrane 3 x five minutes with membrane-blocking buffer, one minute with distilled water, then apply chemiluminescent HRP substrate to membrane and develop film according to your protocol (5-10 minute exposure to start). A normal cell sample will possess multiple cellular proteins with S-glutathionyl residues, thereby detection of a range of proteins is expected.

ANALYSIS

Fluorescence Microscopy

Figure 2. Typical immunofluorescence images using 10,000 mouse monocytes per sample. *Panel A:* Cells stained by the standard method with omission of Reduction Reagent generated no fluorescence. *Panel B:* Cells stained by the method as written reveal S-glutathionylated proteins. *Panel C:* Cells treated by the method with omission of free-thiol Blocking Reagent reveals labeling of all accessible protein thiols.

Flow Cytometry



Black = cells treated with omission of Reducing Reagent (microscopy image A)

Red = standard assay conditions (microscopy image B)

Figure 3. Parallel samples of mouse monocytes (10,000 cells each) were analyzed by flow cytometry with detection of fluorescein-labeled proteins (FL1). The figure presents only data gated (P2) to exclude debris from the initial FSC *versus* SSC plot. Significant fluorescence was detected from cells stained by the standard method over cells stained after omission of Reduction Reagent (compare to microscopy images B and A, respectively).

Avidin-HRP Overlay

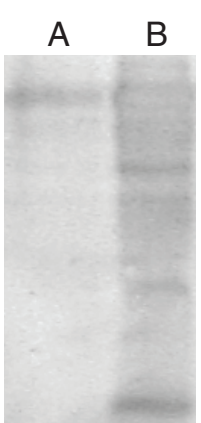


Figure 4. Parallel samples of murine monocytes (10,000 cells each) treated by the standard method using avidin-overlay detection. Sample A represents cells treated with omission of the PSSG Reduction Reagent as a background control showing all protein free-thiols (except endogenously biotinylated proteins) were completely blocked and no false positive signal were generated. Sample B represents cells treated by the method as written to reveal multiple S-glutathionylated proteins (equal to microscopy image B and flow cytometry Red data).

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
High Background or apparent false positive in all samples	Too many cells used per sample	The 5-15,000 cells per sample as suggested in the assay method may exceed the capacity of the blocking reagent to modify all protein free thiols for some cell types; prepare a series of cell aliquots (2, 5, 10, or 20 thousand cells per 1.5 ml tube) treated with equal amounts of blocking reagent in assay buffer, 30 minutes at room temperature; wash the cells twice as described in the assay method; prepare cell lysates for electrophoresis and overlay assay; overlay results will reveal the maximum number cells that are effectively blocked; optimize assay conditions by using the maximum number of cells fully blocked with PSSG blocking reagent; alternatively increase the time and temperature of PSSG blocking reagent incubations to modify protein free thiols and monitor the reaction with the avidin overlay assay as described
No PSSG was detected	Protein mixture was not prepared correctly	Comparison of results to positive and negative controls will provide evidence for further investigation into the source of the problem

References

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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.