S-Nitrosylated Protein Detection Kit (Biotin Switch)

Item No. 10006518

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TABLE OF CONTENTS

GENERAL INFORMATION
3 Materials Supplied
4 Safety Data
4 Precautions
4 If You Have Problems
5 Storage and Stability
5 Materials Needed but Not Supplied

INTRODUCTION
6 Background
6 About This Assay

PRE-ASSAY PREPARATION
8 Reagent Preparation
10 Sample Preparation

ADHERENT CULTURED CELLS
14 Detection of S-Nitrosylated Proteins
15 Materials Needed but Not Supplied (Cells)
16 Performing the Assay (Cells)

FROZEN TISSUE SECTIONS
18 Detection of S-Nitrosylated Proteins
19 Materials Needed but Not Supplied (Tissue)
20 Performing the Assay (Tissue)

RESOURCES
22 Troubleshooting
23 References
24 Notes
24 Warranty and Limitation of Remedy

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/Size	Storage
10006519	S-Nitrosylation Wash Buffer (10X)	1 vial/25 ml	RT
10006520	S-Nitrosylation Buffer A	1 vial/30 ml	RT
10006521	S-Nitrosylation Buffer B (5X)	1 vial/60 ml	RT
10007142	S-Nitrosylation Blocking Reagent	3 vials	-20°C
10006523	S-Nitrosylation Labeling Reagent	3 vials	-20°C
401147	S-Nitrosylation Reducing Reagent	1 vial	RT
10006524	S-Nitrosylation Detection Reagent I (HRP)	1 vial	-20°C
10006525	S-Nitrosylation Detection Reagent II (Fluorescein)	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.

If You Have Problems

Technical Service Contact Information

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

Email: techserv@caymanchem.com

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 and used before the expiration date indicated on the outside of the box. Once this kit has been opened, store the vials of Reducing Reagent, Wash Buffer, Buffer A, and Buffer B at room temperature. Store the remaining components at -20°C.

Materials Needed But Not Supplied

1. Acetone, *A HAZARDOUS SOLVENT*
2. Dimethylformamide (DMF), *A HAZARDOUS SOLVENT*
3. Pipettes, 1.5 ml microcentrifuge and 15 ml tubes (polypropylene)
4. 10 ml graduated cylinders (2)
5. A Benchtop Centrifuge and Microcentrifuge (at 4°C)
6. Equipment for performing transblotting

INTRODUCTION

Background

Nitric oxide (NO) is produced by three distinct isoforms of nitric oxide synthase and functions as a key signaling molecule in physiology and pathophysiology.[1,2] NO transduces its effects by reacting either directly with heme and non-heme centers of proteins or indirectly *via* further oxidation to various reactive nitrogen species (RNS).[3,4] Nitrosylation refers to the binding of an NO group to a transition metal, as typified in the activation of soluble guanylate cyclase, or to a thiol (SH) group of protein cysteine residues resulting in the formation of an S-NO moiety. This latter reaction, termed S-nitrosylation, is mediated by reactive nitrogen species of higher oxidation states of NO, such as NO_2 and N_2O_3.[5,6] S-nitrosylation is a reversible, and seemingly specific, post-translational modification that regulates the activity of a large number of targets, including metabolic, structural, cytoskeletal, and signaling proteins.[7]

About This Assay

Cayman's S-Nitrosylated Protein Detection Assay Kit (Biotin Switch) employs a modification of the Jaffrey *et al.* "Biotin-switch" method to allow for the direct visualization of S-nitrosylated proteins in whole cells or tissues, as well as by western blot analysis.[8,9] Using this method, free SH groups are first blocked (an addition of 125.1 amu per residue) and any S-NO bonds present in the sample are then cleaved. Biotinylation of the newly formed SH groups (an addition of 523.6 amu per residue) provides the basis for visualization using streptavidin-based colorimetric or fluorescence detection.

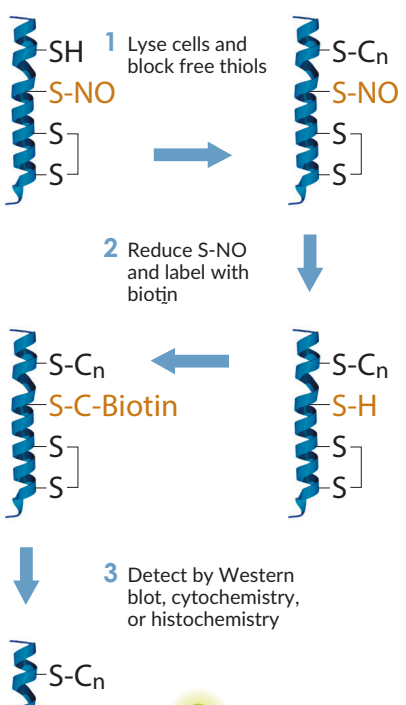

Figure 1. Diagram of the three-step process of protein nitrosothiol (S-NO) derivitization with biotin. Protein free thiols are derivitized with a blocking agent. S-Nitrosothiols are reduced to yield free thiol(s) which are covalently labeled with maleimide-biotin. Subsequent detection by avidin-coupled reagents can be used to localize the biotinylated protein(s).

PRE-ASSAY PREPARATION

Reagent Preparation

1. **S-Nitrosylation Wash Buffer (10X) - (Item No. 10006519)**

   Bring to room temperature. Dilute the contents of the Wash Buffer vial (25 ml) to a final volume of 250 ml with purified water. This should be done before starting the assay(s). *Store this buffer at room temperature.*

2. **S-Nitrosylation Buffer A - (Item No. 10006520)**

   Bring to room temperature. Buffer A is ready to use as supplied. *Store this buffer at room temperature.*

3. **S-Nitrosylation Buffer B (5X) - (Item No. 10006521)**

   Bring to room temperature. Dilute the contents of the Buffer B vial (60 ml) to a final volume of 300 ml with purified water. *Store this buffer at room temperature.*

4. **S-Nitrosylation Blocking Reagent - (Item No. 10007142)**

   Supplied as a crystalline solid. Three vials are provided to allow up to three separate experiments. *Store the Blocking Reagent as supplied at -20°C. Use fresh Blocking Reagent for each experiment. Storage and later use of a reconstituted solution is not advised.*

5. **S-Nitrosylation Labeling Reagent - (Item No. 10006523)**

   Supplied as a lyophilized powder. Three vials are provided to allow for up to three separate experiments. *Store the Labeling Reagent as supplied at -20°C. Use fresh Reducing Reagent for each experiment. Storage and later use of the reconstituted reagent is not advised.*

6. **S-Nitrosylation Reducing Reagent - (Item No. 401147)**

   Supplied as a crystalline solid. A sufficient amount is provided to allow up to three separate experiments. Prior to each experiment, weigh out 60 mg in a 1.5 ml polypropylene vial. *Store the Reducing Reagent as supplied at room temperature. Use fresh Reducing Reagent for each experiment. Storage and later use of the reconstituted reagent is not advised.*

7. **S-Nitrosylation Detection Reagent I (HRP) - (Item No. 10006524)**

   Supplied as a lyophilized powder. Reconstitute the contents of the vial with 400 µl of distilled water and mix gently to dissolve the powder. Use at a 1:75 dilution for blotting overlay experiments or tissue sections. *Store the reconstituted reagent at 4°C for up to six months.*

8. **S-Nitrosylation Detection Reagent II (Fluorescein) - (Item No. 10006525)**

   Supplied as a lyophilized powder. Prepare with 1 ml of Buffer B containing 0.1% Triton X-100 and use at a 1:40 dilution. *Store the reconstituted reagent at 4°C for up to six months.*

Sample Preparation

Sufficent reagents are supplied for three experiments with up to 20 samples per experiment.

Assay Summary	
Cell Washing, Lysis, and Blocking	2-3 hours
Indirect S-NO Labeling	2.5 hours
Total Time	4.5-5.5 hours

NOTE: Steps 1-12 of this procedure are performed under indirect light. This assay is recommended for preparation of up to 20 cell pellets (each ≤0.1 g wet weight).

1. **Substrate-dependent cells**: Wash adherent cultured-cells with three successive aliquots of cold Wash Buffer using a fraction of the last wash to scrape and transfer the cells into a 50 ml conical tube. Generate a cell pellet by centrifugation (500 x g, five minutes) and carefully remove any remaining wash buffer above the cell pellet.

   Cell pellet from suspension culture or isolated from tissue: Add Wash Buffer and resuspend the pellet. Invert the tube to mix and generate a cell pellet by centrifugation (500 x g, five minutes). Decant the supernatant and resuspend cells in another aliquot of Wash buffer and generate a cell pellet as before. Repeat the process once more.
2. Carefully remove any residual Wash Buffer with a pipette. Keep all samples on ice.
3. Dissolve the contents of 1 vial of Blocking Reagent with 100 µl DMF and then add 900 µl of Buffer A to the vial. Close the vial and mix well by inversion. Add 1 ml of the reconstituted reagent to 9 ml Buffer A and mix well; this is now **Buffer A containing Blocking Reagent**.
4. To each cell pellet add 5 volumes (up to 0.5 ml total volume) of **Buffer A containing Blocking Reagent** and tap the side of each tube to resuspend the cells. Transfer the samples to prelabeled microcentrifuge tubes. Place the samples on a shaker or rocker (with gentle agitation) for 30 minutes at 4°C.
5. Generate clarified lysates by centrifugation of the samples for 10 minutes at 4°C with a tabletop microcentrifuge.
6. Transfer the supernatants into separate cold, pre-labeled 15 ml conical polypropylene tubes and add 4 volumes of ice-cold acetone to each sample.
7. Mix each sample thoroughly by inversion followed by incubation at -20°C for one hour.
8. Remove the samples from -20°C and produce a compact protein pellet by centrifugation at 3,000 x g for 10 minutes at 4°C. (Proceed to step 9 during centrifugation.)

9. During centrifugation, dissolve 60 mg of S-Nitrosylation Reducing Reagent with 1 ml of Buffer B. Also, dissolve the contents of 1 vial of S-Nitrosylation Labeling Reagent with 100 µl of DMF followed by 900 µl of Buffer B, mixing well. When each reagent is fully dissolved, add 0.5 ml of the Reducing Reagent and 0.5 ml of the Labeling Reagent into a graduated cylinder containing 4 ml of Buffer B. This is called **Buffer B containing Reducing and Labeling Reagents**. Mix it well by inversion.
10. Remove the samples from the centrifuge and decant the supernatants from each tube (containing primarily acetone) into the appropriate organic solvent waste container. Set the protein pellet-containing tubes on ice. Remove residual acetone from pellets with a pipette.
11. Resuspend each protein pellet with 0.5 ml of **Buffer B containing Reducing and Labeling Reagents**.

Control Sample: Contribution of signal from endogenously biotinylated proteins can be observed by resuspension of control cell pellets in Buffer B alone.

12. Incubate these samples for one hour at **room temperature**.
13. Add 4 volumes (2 ml) of ice-cold acetone to each sample, mixing each thoroughly by inversion followed by incubation at -20°C for one hour.
14. Remove the samples from -20°C and produce a compact protein pellet by centrifugation at 3,000 x g for 10 minutes at 4°C.
15. Decant the acetone-containing supernatant into a solvent waste container and set the protein pellet samples on ice.
16. Resuspend each of the protein pellets in a minimal volume (100-200 µl suggested) of cold Wash Buffer. Reserve a small volume of each for protein determination and store the rest at -20°C for analysis by western blotting, immunoprecipitation of a specific protein, or anti-biotin immunoprecipitation for concentration of nitrosylated proteins.

Avidin Overlay/Western Blotting Notes:

1. Prepare protein samples for SDS-PAGE as is done for western blotting; add Laemmli buffer, mix, and incubate samples in a boiling water bath for five minutes followed by five minutes on ice prior to loading. The biotin label is covalently bound.
2. Do not use milk products for blocking unoccupied surfaces of the transfer-membranes because the endogenous biotin content may cause high background signal or no signal from experimental samples when probing with the avidin detection reagents. Solutions of 2% bovine serum albumin (BSA) in PBS or TBS buffers are acceptable for blocking membranes and dilution of Detection Reagent I (HRP) in this assay.
3. Reconstitute the contents of the **S-Nitrosylation Detection Reagent I (HRP)** vial with 400 µl of distilled water. Use at a 1:75 dilution for overlay experiment (similar to western blotting). Develop the membrane(s) with Enhanced Chemiluminescence (ECL) Reagents or other peroxidase compatible substrate. Alternatively, S-Nitrosylation Detection Reagent II (Fluorescein) may be used in place of Detection Reagent I (HRP) if the appropriate detection equipment is available.

ADHERENT CULTURED CELLS

Detection of S-Nitrosylated Proteins

Sufficient reagents are supplied for three experiments utilizing a culture-dish of cells; either 6-, 12-, 24-, or 96-well plates work well.

Assay Summary	
Wash cells 3X and Fix in Wash Buffer + PFA (4%)	35 minutes
Wash cells 3X and incubate with Blocking Buffer	45 minutes
Wash cells 3X and incubate with Labeling Buffer	75 minutes
Wash cells 3X and incubate with ICC/IHC Detection Reagent	75 minutes
Wash cells 3X and incubate with Nuclear Stain (Optional)	45 minutes
Wash cells 3X and observe reactivity by fluorescence microscopy	>15 minutes
Total Time	5 hours

Materials Needed But Not Supplied (Cells)

1. 6-, 12-, 24-, or 96-well plates for cell culture
2. Humidified chamber
3. Paraformaldehyde
4. Dimethylformamide (DMF), *A HAZARDOUS SOLVENT*
5. Triton X-100
6. Confocal microscope with fluorescence source
7. AquaPoly/Mount (optional)
8. Nuclear counterstain (optional); for example, Propidium Iodide (PI) or 4'6-Diamidino-2-phenylindole (DAPI), *Caution: these chemicals are probable MUTAGENS*

Performing the Assay (Cells)

NOTE: Perform steps 2-11 of this procedure are done under indirect light at room temperature unless otherwise stated.

1. Prepare enough Wash Buffer containing 4% paraformaldehyde to fix the cells but not more than needed to allow for the washing steps and separate experiments.
2. Remove media from culture wells and rinse each briefly with **Wash Buffer** three times**.**
3. Fix the cells by incubation with **Wash Buffer** containing 4% paraformaldehyde for 20 minutes.
4. Wash cells 3 x 5 minutes with **Wash Buffer**. Let the last wash remain on the cells and proceed to step 5.
5. Prepare the buffer for step 6 immediately before use. To make **Buffer B containing Blocking Reagent and 0.1% Triton X-100**, dissolve the contents of one vial of blocking reagent with 100 µl of DMF, and then add 900 µl of Buffer B. Transfer the dissolved Blocking reagent into a 20 ml graduated cylinder, add 10 µl Triton X-100 and bring to 10 ml final volume with Buffer B. Mix this buffer well by inversions.
6. Remove Wash Buffer from the cells, and incubate cells with **Buffer B containing Blocking Reagent and 0.1% Triton X-100** for 30 minutes at 4°C.
7. Wash cells 3 x 5 minutes with Buffer B containing 0.1% Triton X-100. Let the last wash remain on the cells and proceed to step 8.
8. Prepare the Reducing/Labeling buffer immediately before use by dissolving 60 mg of S-Nitrosylation Reducing Reagent with 1 ml of Buffer B containing 0.1% Triton X-100. Also, dissolve the contents of 1 vial of S-Nitrosylation Labeling Reagent with 100 µl of dimethylformamide (DMF) followed by 900 µl of Buffer B containing 0.1% Triton X-100, mixing well. When each reagent is fully dissolved, transfer all of the Reducing Reagent and all of the Labeling Reagent into a graduated cylinder containing 8 ml of Buffer B containing 0.1% Triton X-100. This is called **Buffer B containing 0.1% Triton X-100, Reducing and Labeling Reagents**. Mix it well by inversion. Remove the Wash Buffer from the cells and add enough **Buffer B containing 0.1% Triton X-100, Reducing and Labeling Reagents** to cover the cells. Incubate for one hour at room temperature.
9. Wash cells 3 x 5 minutes with Buffer B containing 0.1% Triton X-100.
10. Remove the last wash and incubate the cells with **Buffer B containing 0.1% Triton X-100 and 1:40 dilution of Detection Reagent II (Fluorescein)** for one hour in a humidified chamber.
11. Wash cells 3 x 5 minutes with Buffer B containing 0.1% Triton X-100. The cells may be observed by fluorescence microscopy (λ_{EX} = 490 nm, λ_{EM} = 520).

Optional technique: Stain nuclei with propidium iodide (10 µg/ml solution) for 30 minutes in a humidified chamber. Wash away excess propidium iodide with Buffer B containing 0.1% Triton X-100 and examine by fluorescence microscopy.

FROZEN TISSUE SECTIONS

Detection of S-Nitrosylated Proteins

Sufficient reagents are supplied for three separate experiments of up to 25 samples.

Assay Summary and Timeframe	
Tissue Preparation	up to 2 hours
Wash/Fix Sections	45 minutes
Block/Label S-Nitrosylation	90 minutes
Detection Reagent Incubation	75 minutes (or overnight)
Counterstain/Wash	60 minutes
Total Time	<5 hours

Materials Needed But Not Supplied (Tissue)

1. Embedded frozen tissue samples
2. Tissue and histology preparation equipment (Tissue cassette, Embedding Solution, liquid nitrogen, cryostat, microscope slides)
3. Paraformaldehyde
4. Triton X-100
5. Dimethylformamide (DMF), *Caution: HAZARDOUS SOLVENT*
6. Confocal microscope with fluorescence source
7. PAP Pen (optional)
8. Nuclear counterstain (optional); for example, Propidium Iodide (PI) or 4'6-Diamidino-2-phenylindole (DAPI), *Caution: these chemicals are probable MUTAGENS*

General Information

- Perform all steps of this procedure under indirect light only.
- Preparation of fresh tissue sections is highly recommended due to the transient nature of the S-nitrosothiol moiety. However, if tissue sections have been prepared previously, skip ahead to step 3.

Performing the Assay (Tissue)

1. Infuse or rinse organ tissues with cold **Wash Buffer** for 10 minutes.
2. Use your standard protocol to perform tissue embedding, freezing, section cutting, and slide fixation.
3. Wash sections 3 x 5 minutes with **Wash Buffer**. Circumscribe tissues with PAP Pen if desired.
4. Fix sections in **Wash Buffer containing 4% paraformaldehyde** for 30 minutes at room temperature.
5. Wash sections 3 x 5 minutes with Wash Buffer containing 1% Triton X-100.

   Prepare the buffer for step 6 immediately before use by dissolving the contents of one vial of Blocking Reagent with 100 µl of dimethylformamide (DMF) followed by 900 µl Wash Buffer mixing well. Transfer the dissolved Blocking Reagent into a 20 ml graduated cylinder along with 100 µl of Triton X-100 and bring to a final volume of 10 ml with Wash Buffer. Mix well by inversions. This buffer is called Wash Buffer containing Blocking Reagent and 1% TritonX-100.
6. Incubate sections with **Wash Buffer containing Blocking Reagent and 1% Triton X-100** for 30 minutes at 4°C.
7. Wash sections 3 x 5 minutes with Wash Buffer containing 1% Triton X-100.

   Prepare the buffer for step 8 immediately before use by dissolving 60 mg of S-Nitrosylation Reducing Reagent with 1 ml of Buffer B containing 1% Triton X-100. Also, dissolve the contents of one vial of S-Nitrosylation Labeling Reagent with 100 µl of dimethylformamide (DMF) followed by 900 µl of Buffer B containing 1% Triton X-100, mixing well. When each reagent is fully dissolved, add 0.5 ml of the Reducing Reagent and 0.5 ml of the Labeling Reagent into a graduated cylinder containing 4 ml of Buffer B containing 1% Triton X-100. This is called **Buffer B containing 1% Triton X-100, Reducing and Labeling Reagents**. Mix it well by inversion.
8. Incubate sections in **Buffer B alone** (as the endogenous biotinylated protein control) or **Buffer B containing 1% Triton X-100, Reducing and Labeling Reagents** for one hour at room temperature.

   If not already done, prepare the Buffer for step 10 during this time; resuspend the contents of one vial of S-Nitrosylation Detection Reagent II (Fluorescein) with 1 ml of Buffer B containing 1% Triton X-100. Once dissolved keep this reagent on ice.
9. Wash the sections 3 x 5 minutes with Buffer B containing 1% Triton X-100.
10. Use the **S-Nitrosylation Detection Reagent II (Fluorescein)** at a 1:40 dilution in Buffer B containing 1% Triton X-100. Incubate the sections for one hour at room temperature or overnight at 4°C (wrapped in foil or other dark container).
11. Wash the sections 3 x 5 minutes with Buffer B containing 1% Triton X-100.

    Optional: Counterstain the sections (nuclei) with DAPI or PI as suggested by the product manufacturer. Wash away excess counterstain with Buffer B containing 1% Triton X-100.
12. View stained sections by confocal fluorescence microscopy (λ_{EX} = 490 nm, λ_{EM} = 520).

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
High background	A. Insufficient blocking of free thiols B. Excessive detection reagent for the test	A. Increase the incubation time of the blocking step B. Increase the dilution factor of the detection reagent to 1:1,000-1:2,000
Nothing detected	A. Low levels of endogenous S-NO may not be detected B. Poor protein recovery	A. Comparison of results to positive and negative controls will provide evidence for further investigation into the source of the problem B. More protein may need to be loaded in gels

References

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NOTES

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