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# **ab236207 Biotin Switch Assay Kit (S-Nitrosylation)**

For the measurement of S-Nitrosylated proteins in whole cells and tissues.

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

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## 1. Overview

Biotin Switch Assay Kit (S-Nitrosylation) (ab236207) employs a modification of the Jaffrey et al. 'Biotin-switch' method to allow for the direct visualization of S-NO proteins in whole cells or tissues, as well as by Western blot analysis. 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.

## Analysis by Western blot

Wash and pellet cells by centrifugation



Add Buffer A containing Blocking Reagent and incubate with agitation for 30 minutes at 4°C



Generate clarified lysates by centrifugation



Precipitate proteins with ice-cold acetone



Resuspend protein pellets in Buffer B containing Reducing and Labeling Reagents



Precipitate proteins with ice-cold acetone



Resuspend protein pellets and Western blot



Analyze S-Nitrosylated proteins using HRP or fluorescent detection reagent

## Whole cells

Fix cells with 4% paraformaldehyde



Wash cells



Incubate cells with Buffer B containing 0.1% Triton X-100 and Blocking Reagent for 30 minutes at 4°C



Wash cells



Incubate with Buffer B containing 0.1% Triton X-100, Reducing and Labeling Reagents for 1 hour at room temperature



Wash cells



Incubate with Buffer B containing 0.1% Triton X-100 and Detection Reagent II (Fluorescein)



Wash cells and then analyze by fluorescence microscopy (Ex/Em = 490/520 nm)

## Tissue sections

Use method of choice for tissue embedding, freezing, cutting and slide fixation



Fix cells with 4% paraformaldehyde



Wash sections



Incubate with Buffer B containing 1 % Triton X-100 and Blocking Reagent for 30 minutes at 4°C



Wash sections



Incubate with Buffer B containing 1% Triton X-100, Reducing and Labeling Reagents for 1 hour at room temperature



Wash cells



Incubate with Buffer B containing 1% Triton X-100 and Detection Reagent II (Fluorescein)



Wash cells and then analyze by confocal fluorescence microscopy (Ex/Em = 490/520 nm)

## 2. Materials Supplied and Storage

Store kit at -20°C in the dark immediately on receipt and check below for storage for individual components.

Aliquot components in working volumes before storing at the recommended temperature.

Avoid repeated freeze-thaws of reagents.

<b>Item</b>	<b>Quantity</b>	<b>Storage temperature (before prep)</b>	<b>Storage temperature (after prep)</b>
S-Nitrosylation Wash Buffer (10X)	25 mL	Room temperature	Room temperature
S-Nitrosylation Buffer A	30 mL	Room temperature	Room temperature
S-Nitrosylation Buffer B (5X)	60 mL	Room temperature	Room temperature
S-Nitrosylation Reducing Reagent	1 vial	Room temperature	Do not store
S-Nitrosylation Labeling Reagent	3 vials	-20°C	Do not store
S-Nitrosylation Detection Reagent I (HRP)	1 vial	-20°C	4°C
S-Nitrosylation Detection Reagent II (Fluorescein)	1 vial	-20°C	4°C
S-Nitrosylation Blocking Reagent	3 vials	-20°C	Do not store

### 3. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Acetone (Hazardous)
- Dimethylformamide (DMF; Hazardous)
- Pipettes
- 1.5 mL microcentrifuge tubes
- 15 mL tubes (polypropylene)
- 10 mL graduated cylinders (X2)
- Microcentrifuge (4°C)
- Benchtop centrifuge (4°C)
- Equipment for transblotting
- Embedded frozen tissue samples
- Tissue and histology preparation equipment (tissue cassette, embedding solution, liquid nitrogen, cryostat, microscope slides)
- Paraformaldehyde
- Triton X-100
- Confocal microscope with fluorescence source
- PAP pen (optional)
- Nuclear counter stain (e.g. DAPI or PI)
- 6-, 12-, 24-, or 96-well plates for cell culture
- Humidified chamber

## **4. General guidelines, precautions, and troubleshooting**

Please observe safe laboratory practice and consult the safety datasheet.

For general guidelines, precautions, limitations on the use of our assay kits and general assay troubleshooting tips, particularly for first time users, please consult our guide:

[www.abcam.com/assaykitguidelines](http://www.abcam.com/assaykitguidelines)

For typical data produced using the assay, please see the assay kit datasheet on our website.

## 5. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

### 5.1 S-Nitrosylation Wash Buffer (10X)

1. Bring to room temperature.
2. Dilute contents of the bottle to a final volume of 250 mL with purified water (do this before starting the assays).
3. Store 1X S-Nitrosylation Wash Buffer at room temperature.

### 5.2 S-Nitrosylation Buffer A

1. Bring to room temperature.
2. Ready to use as supplied.

### 5.3 S-Nitrosylation Buffer B (5X)

1. Bring to room temperature.
2. Dilute the contents of the bottle to a final volume of 300 mL.
3. Store 1X S-Nitrosylation Buffer B at room temperature.

### 5.4 S-Nitrosylation Blocking Reagent

1. Supplied as a crystalline solid. Three vials are provided to allow for up to three separate experiments.
2. Use fresh reagent for each experiment. Do not store reconstituted reagent.
3. Prepare immediately prior to use.
4. See individual protocols for reconstitution details.

### 5.5 S-Nitrosylation Labeling Reagent

1. Supplied as a lyophilized powder. Three vials are provided to allow for up to three separate experiments.
2. Use fresh reagent for each experiment. Do not store reconstituted reagent.
3. Prepare immediately prior to use.
4. See individual protocols for reconstitution details.

### 5.6 S-Nitrosylation Reducing Reagent

1. 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.

2. Use fresh reagent for each experiment. Do not store reconstituted reagent.
3. Prepare immediately prior to use.
4. See individual protocols for reconstitution details.

### **5.7 S-Nitrosylation Detection Reagent I (HRP)**

1. Supplied as a lyophilized powder.
2. Reconstitute the contents of the vial with 400  $\mu$ L of distilled water and mix gently to dissolve the powder.
3. Use at a 1:75 dilution for blotting overlay experiments or tissue sections.
4. Store reconstituted reagent at 4°C for up to 6 months.

### **5.8 S-Nitrosylation Detection Reagent II (Fluorescein)**

1. Supplied as a lyophilized powder.
2. Prepare with 1 mL Buffer B containing 0.1% Triton X-100.
3. Use at 1:40 dilution.
4. Store reconstituted reagent at 4°C for up to 6 months.

## 6. Sample Preparation

### **General sample information:**

We recommend that you use fresh samples for the most reproducible assay (cultured cells or tissue sections).

## 7. Assay Procedure

### Analysis by Western blotting

**ΔNote:** Perform all steps up to 7.3.10 under indirect light.

**ΔNote:** The assay is recommended for up to 20 cell pellets (each ≤ 100 mg wet weight).

#### 7.1 Adherent cells:

1. Wash adherent cultured cells with three successive aliquots of cold 1X S-Nitrosylation Wash Buffer using a fraction of the last wash to scrape and transfer the cells to a 50 mL conical tube.
2. Generate a cell pellet by centrifugation (500 x g for 5 minutes).
3. Carefully remove any remaining buffer above the cell pellet.

#### 7.2 Suspension cells or cells isolated from tissue:

1. Pellet cells by centrifugation (500 x g for 5 minutes).
2. Add 1X S-Nitrosylation Wash Buffer and resuspend the cell pellet. Mix by inversion.
3. Generate a cell pellet by centrifugation (500 x g for 5 minutes).
4. Decant the supernatant and resuspend the cells in another aliquot of 1X S-Nitrosylation Wash Buffer. Generate a cell pellet as before.
5. Repeat the resuspension/centrifugation process once more.

#### 7.3 All cell types:

1. Carefully remove any residual buffer from the cell pellet with a pipette.
2. Dissolve 1 vial of S-Nitrosylation Blocking Reagent with 100  $\mu$ L DMF and then add 900  $\mu$ L of Buffer A to the vial. Mix well by inversion. Add 1 mL of the reconstituted reagent to 9 mL Buffer and mix well. This is designated Buffer A containing Blocking Reagent.
3. To each pellet add 5 volumes (up to 0.5 mL total volume) of S-Nitrosylation Buffer A containing Blocking Reagent and resuspend the pellet by tapping the side of the tube. Transfer the

- samples to pre-labeled microcentrifuge tubes. Place tubes on a rocker or shaker (with gentle agitation) for 30 minutes at 4°C.
4. Generate clarified lysates by centrifugation for 10 minutes at 4°C in a microcentrifuge.
  5. Transfer the supernatants into separate cold, pre-labeled 15 mL conical polypropylene tubes and add 4 volumes of ice-cold acetone to each sample.
  6. Mix thoroughly by inversion followed by incubation at -20°C for 1 hour.
  7. Remove tubes from -20°C and produce compact protein pellets by centrifugation at 3,000 x g for 10 minutes at 4°C. (Proceed to step 8 during centrifugation)
  8. During centrifugation, reconstitute the contents of 1 vial 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 DMF followed by 900 µL Buffer B, mixing well. When each reagent is fully dissolved add 0.5 mL of each reconstituted reagent into a graduated cylinder containing 4 mL of 1X S-Nitrosylation Buffer B. Mix well by inversion. This is designated Buffer B containing Reducing and Labeling Reagents.
  9. Remove the sample from the centrifuge and decant the supernatants (containing primarily acetone) into the appropriate waste solvent container. Place the pellet-containing tubes on ice. Remove residual acetone from the pellets with a pipette.
  10. Resuspend each protein pellet with 0.5 mL of Buffer B containing Reducing and Labeling Reagents.

**ΔNote:** Control sample. The contribution of signal from endogenously biotinylated proteins can be observed by resuspension of control pellets in Buffer B alone.

11. Incubate the samples for 1 hour at room temperature.
12. Add 4 volumes (2 mL) of ice-cold acetone to each sample, mixing thoroughly by inversion followed by incubation at -20°C for 1 hour.
13. Remove the samples from -20°C and produce compact protein pellets by centrifugation at 3,000 x g for 10 minutes at 4°C.
14. Decant the acetone-containing supernatant into a solvent waste container and place the protein pellet samples on ice.

15. Resuspend each protein pellet in a minimal volume of (100 – 200  $\mu$ L) of cold 1X S-Nitrosylation 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 S-nitrosylated proteins.

#### **7.4 Avidin overlay / Western blot analysis:**

1. Prepare samples for Western blotting by adding Laemmli buffer, mixing and incubating in a boiling water bath for 5 minutes followed by 5 minutes on ice prior to loading. The biotin label is covalently bound.

**ΔNote:** Do not use milk products for blocking 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% BSA in PBS or TBS buffers are acceptable for blocking of membranes and for dilution of S-Nitrosylation Detection Reagent I (HRP) in this assay.

2. Reconstitute S-Nitrosylation Detection Reagent I (HRP) (see Section 5.7). Use at 1:75 dilution for overlay experiments (similar to Western blotting). Develop the membrane(s) with ECL reagents or other peroxidase compatible substrate. Alternatively, S-Nitrosylation Detection Reagent II (Fluorescein) may be used instead of S-Nitrosylation Detection Reagent I (HRP) if appropriate detection equipment is available.

#### **Performing the assay with cells:**

##### **7.5 Cell protocol**

**ΔNote:** Perform steps 2-11 (below) under indirect light at room temperature unless stated otherwise.

1. Prepare enough 1X S-Nitrosylation 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 the media from culture wells and rinse briefly with 1X S-Nitrosylation Wash Buffer three times.

3. Fix the cells by incubating with 1X S-Nitrosylation Wash Buffer containing 4% paraformaldehyde for 20 minutes.
4. Wash cells 3 x 5 minutes with 1X S-Nitrosylation Wash Buffer. Let the last wash remain on the cells and proceed to step 5.
5. Prepare the buffer for this step immediately prior to use. To make Buffer B containing Blocking Reagent and 0.1% Triton X-100, dissolve the contents of 1 vial of S-Nitrosylation Blocking Reagent with 100  $\mu$ L DMF, and then add 900  $\mu$ L of 1X S-Nitrosylation Buffer B. Transfer the dissolved Blocking Reagent to into a 20 mL graduated cylinder, add 10  $\mu$ L Triton X-100 and bring to 10 mL final volume with 1X S-Nitrosylation Buffer B. Mix well by inversion.
6. Remove Wash Buffer from the cells, 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 prior to use by reconstituting 1 vial 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 in 100  $\mu$ L of DMF followed by 900  $\mu$ L 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 designated Buffer B containing 0.1% Triton X-100, Reducing and Labeling Reagents. Mix well by inversion. Remove the Wash Buffer from the cells and add enough designated Buffer B containing 0.1% Triton X-100, Reducing and Labeling Reagent to cover the cells. Incubate for 1 hour at room temperature.
9. Wash cells 3 x 5 minutes with Buffer B containing 0.1% Triton X-100.
10. Remove the wash and incubate the cells with Buffer B containing 0.1% Triton X-100 and 1:40 dilution of S-Nitrosylation Detection Reagent II (Fluorescein) for 1 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 (Ex/Em = 490/520 nm).

- Optional: Stain nuclei with propidium iodide (10  $\mu\text{g}/\text{mL}$  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.

## Performing the assay with tissue samples:

### 7.6 Tissue protocol

**ΔNote:** Perform all steps under indirect light only.

**ΔNote:** Preparation of fresh tissue sections is highly recommended due to the transient nature of the S-nitrosyl moiety. However, if tissue sections have been prepared previously, skip to step 3.

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

Prepare the buffer for step 6 immediately prior to use by dissolving 1 vial of S-Nitrosylation Blocking Reagent with 100  $\mu\text{L}$  DMF, followed by 900  $\mu\text{L}$  1X S-Nitrosylation Wash Buffer mixing well. Transfer the dissolved Blocking Reagent to into a 20 mL graduated cylinder along with 100  $\mu\text{L}$  of Triton X-100 and bring the final volume to 10 mL with Wash Buffer. Mix well by inversion. This is designated Wash Buffer containing Blocking Reagent and 1% Triton X-100.

- Incubate sections with Wash Buffer containing Blocking Reagent and 1% Triton X-100 for 30 minutes at 4°C.
- Wash sections 3 x 5 minutes with Wash Buffer containing 0.1% Triton X-100.

Prepare the buffer for step 8 immediately prior to use by reconstituting 1 vial 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  $\mu\text{L}$

of DMF followed by 900  $\mu$ L of Buffer B containing 0.1% Triton X-100, mixing well. When each reagent is fully dissolved, add 0.5 mL of Reducing Reagent and 0.5 mL of Labeling Reagent into a graduated cylinder containing 4 mL of Buffer B containing 1% Triton X-100. This is designated Buffer B containing 1% Triton X-100, Reducing and Labeling Reagents. Mix well by inversion.

8. Incubate in Buffer B alone (as the endogenous biotinylated protein control) or in Buffer B containing 1% Triton X-100, Reducing and Labeling Reagents for 12 hours at room temperature.

**ΔNote** If not already done, prepare the buffer for step 10 during this time. Resuspend the contents of 1 vial of S-Nitrosylation Detection Reagent II (Fluorescein) in 1 mL of Buffer B containing 1% Triton X-100. Once dissolved, keep this reagent on ice.

9. Wash 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 sections for 1 hour at room temperature or overnight at 4°C (wrapped in foil or other dark container).
11. Wash sections 3 x 5 minutes with Buffer B containing 1% Triton X-100.  
Optional: Counter stain sections with DAPI or PI according to the manufacturer's instructions. Wash away excess counter stain with Buffer B containing 1% Triton X-100.
12. View stained sections by confocal microscopy (Ex/Em = 490/520 nm).

## 8. FAQs / Troubleshooting

General troubleshooting points are found at [www.abcam.com/assaykitguidelines](http://www.abcam.com/assaykitguidelines)

<b>Problem</b>	<b>Possible causes</b>	<b>Recommended solutions</b>
High background	A: Insufficient blocking of free thiols.  B: Excessive detection reagent used	A: Increase incubation time of the blocking step B: Increase the dilution factor of the detection reagent to 1:1000 to 1:2000
Nothing detected	A: Low levels of endogenous S-NO may not be detected  B: Poor protein recovery	A: Comparison of results of positive and negative controls will provide further evidence as to the nature of the problem B: More protein may need to be loaded on gels





## 9. Notes



## **Technical Support**

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