MANUAL

RayBio® S-Nitrosylation Detection Kit for Western Blots

Catalog #: MA-SNO-W

RayBiotech
Empowering your proteomics

ISO 13485

Introduction

Nitric oxide (NO) is a reactive radical species, it is produced by NO synthases (NOS), a family of enzymes encoded by three distinct genes. NO participates in diverse biological process and physiological regulation including gastrointestinal, respiratory, cardiovascular and host defense. These various bioactivities result from the covalent adduction of an NO group to a cysteine thiol of target protein. This process is called S-nitrosylation, a redox-based, reversible, post-translational modification that regulates diverse signaling pathways and implicated in disease states.

RayBio® S-Nitrosylation Detection Kit uses a modified 'biotin-switch' method (Jaffrey *et al*.) to allow for the direct visualization of S-nitrosylated proteins by western blot analysis as well as by antibody array. In this S-nitrosylation biotin switch assay, unmodified free cysteines are first blocked. S-nitrosylated cysteines are then selectively reduced for specific labeling with biotin-maleimide reagents, which irreversibly bind to the cysteine thiol that was S-nitrosylated. Biotinylation of the newly formed thiol groups can then be detected by western blot or antibody array. In addition, avidin resin can be used to selectively enrich S-nitrosylated proteins/peptides labeled with biotin. This procedure also allows for S-nitrosylation site mapping by using mass spectrometry.

Storage

The entire kit may be stored at -20°C for up to 6 months from the date of shipment. For extended storage, it is recommended to store at -80°C. Avoid repeated freeze-thaw cycles. For prepared reagent storage, see table below.

Component	Size / Description	Storage	Storage After Preparation
S-Nitrosylation Buffer A	12 ml	RT	RT
S-Nitrosylation Buffer B	3 ml	RT	RT
S-Nitrosylation Buffer C	5 ml	RT	RT
S-Nitrosylation Buffer D	3 ml	RT	RT
S-Nitrosylation Buffer E	12 ml	RT	RT
S-Nitrosylation Blocking Reagent	2 vials, enough for 2 separate experiments. Crystalline solid.	2–8 °C	Prepare immediately prior to use. Do not store.
S-Nitrosylation Reduction Reagent I	2 vials, enough for 2 separate experiments. Crystalline solid.	RT	Prepare immediately prior to use. Do not store.
S-Nitrosylation Reduction Reagent II	2 vials, enough for 2 separate experiments. Crystalline solid.	RT	Prepare immediately prior to use. Do not store.
S-Nitrosylation Labeling Reagent	2 vials, enough for 2 separate experiments. Crystalline solid.	-20 °C	Prepare immediately prior to use. Do not store.
1000X HRP-Streptavidin	1 vial (50 µl)	-20 °C	2–8 °C (up to 3 months)

RT = room temperature

Additional Materials Required

1. Acetone, ≥ 98% (hazardous)
2. 1.5 mL microcentrifuge tubes
3. 15 mL tubes (polypropylene)
4. 10 mL graduated cylinders (X2)
5. Benchtop centrifuge and microcentrifuge (4°C)
6. Water bath or heat block
7. Distilled or deionized water
8. Precision pipettes to deliver 2 µl to 1 ml volumes
9. Adjustable 1-25 ml pipettes for reagent preparation

Sample Tips and General Considerations

NOTE: Optimal methods of sample preparation will need to be determined by each researcher empirically based on researched literature and knowledge of the samples.

- If not using fresh samples, freeze samples as soon as possible after collection.
- Avoid multiple freeze-thaw cycles. If possible, sub-aliquot samples prior to initial storage.
- It is strongly recommended to add a protease inhibitor cocktail to cell and tissue lysate samples.
- Avoid sonication of 1 ml or less as this can quickly heat and denature proteins.
- Most samples will not need to be concentrated. If concentration is required, a spin column concentrator with a chilled centrifuge is recommended.

Cell lysates can be prepared as follows:

For attached cells, remove supernatant from cell culture, wash cells twice with cold 1X PBS (for suspension cells, pellet the cells by spinning down the cells at 1,000 x g for 10 min) making sure to remove any remaining PBS before adding lysis buffer. Solubilize the cells at $2x10^7$ cells/ml in lysis buffer containing protease inhibitors. Pipette up and down to resuspend cells and rock the lysates gently at 2–8 °C for 30 minutes. Transfer extracts to microfuge tubes and centrifuge at 14,000 x g for 10 minutes.
It is recommended that sample protein concentrations should be determined using a total protein assay. Lysates should be used immediately or aliquot and stored at -70 °C. Thawed lysates should be kept on ice prior to use.
General tips for preparing lysate samples can be viewed on the online Resources page of the website: https://www.raybiotech.com/tips-on-sample-preparation/

Reagent Preparation

NOTE:

- Thaw frozen reagents to room temperature immediately before use. If buffers contain visible crystals, warm to room temperature, and mix gently until dissolved.
- This kit contains enough reagent to label 40 samples containing 100-200 µg of total protein each.

COMPONENT	PREPARATION
S-Nitrosylation Buffer A	No preparation
S-Nitrosylation Buffer B	
S-Nitrosylation Buffer C	
S-Nitrosylation Buffer D	
S-Nitrosylation Buffer E	
S-Nitrosylation Blocking Reagent	Make fresh. Spin briefly, add 50 µl S-Nitrosylation Buffer B, vortex until all crystals are dissolved completely, then transfer everything into 5 ml S-Nitrosylation Buffer A, mix well.
S-Nitrosylation Reduction Reagent I	Make fresh. Spin briefly, add 800 µl S-Nitrosylation Buffer C, vortex until all crystals are dissolved completely.
S-Nitrosylation Reduction Reagent II	Make fresh. Spin briefly, add 500 µl S-Nitrosylation Buffer D, vortex until all crystals are dissolved completely. Take 4 µl dissolved S-Nitrosylation Reduction Reagent II into 800 µl dissolved S-Nitrosylation Reduction Reagent I, mix well, this is S-Nitrosylation Reducing Buffer.
S-Nitrosylation Labeling Reagent	Make fresh. Spin briefly, add 100 µL dH_2O, vortex until all crystals are dissolved completely
1000X HRP-Streptavidin	Recommended dilution: 1000-fold for western blot
Acetone (not included)	pre-chilled (-20°C)
4:1 acetone/water mixture	4 parts acetone mixed with 1 part dH_2O, pre-chilled (-20°C)

Assay Procedure

1. Prepare 100 µl sample with total protein concentration at 1-2 mg/ml. It is recommended to label samples with equivalent protein concentrations.
2. Add 200 µl prepared S-Nitrosylation Blocking Buffer (use fresh reagent, prepared immediately prior to use) into each sample. Incubate the samples in dark at 50 °C on a shaker with gentle rocking for 30 minutes.
3. Precipitate protein by adding 1200 µl pre-chilled (-20°C) acetone for each sample. Mix thoroughly by inversion followed by incubation at -20°C for 1 hour.
4. Centrifuge at 14,000 × g for 10 minutes at 4°C.
5. Carefully dispose of the supernatant, without dislodging the protein pellet.
6. Add 500 µl pre-chilled 4:1 acetone/water mixture to wash the pellet. Repeat steps 4 and 5.
7. Repeat step 6 to wash the pellet one more time.
8. Allow the acetone to evaporate from the uncapped tube at room temperature for 30 minutes. Do not over-dry pellet, or it may not be dissolved properly.
9. Reconstitute the pellet in 100 µl S-Nitrosylation Buffer E.
10. Add 22 µl S-Nitrosylation Reducing Buffer (use fresh reagent, prepared immediately prior to use), along with 3 µl S-Nitrosylation Labeling Buffer (use fresh reagent, prepared immediately prior to use) to the reconstituted sample and incubate for 2 hours at room temperature with gentle rotation.
11. Repeat steps 3-5. Allow the acetone to evaporate from the uncapped tube at room temperature for 30 minutes. Do not over-dry pellet, or it may not be dissolved properly.
12. Reconstitute each protein pellet in 40 µl S-Nitrosylation Buffer E. The sample is now ready for analysis by western blot or antibody array. HRP-Streptavidin concentrate provided in this kit may be used for either application. The labeled sample can be stored at -20°C for future analysis.

Notes:

1. For western blot, do not use milk products for blocking transfer membranes because endogenous biotin 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 (HRP) in this assay.
2. The S-Nitrosylation Detection Reagent (HRP), may be used at 1:1000 dilution. Develop the membrane(s) with ECL reagents or other peroxidase compatible substrate.

Assay Procedure Summary

1. Prepare all reagents and samples as instructed.
2. Block unmodified free cysteines using prepared S-Nitrosylation Blocking Buffer at 50 °C with gentle rocking.
3. Precipitate proteins with ice-cold acetone.
4. Wash protein pellet twice with cold acetone/water mixture.
5. Resuspend protein pellets in Buffer containing Reducing and Labeling Reagents.
6. Precipitate proteins with ice-cold acetone.
7. Resuspend protein pellets.

Troubleshooting Guide

Problem	Possible Cause	Solution
S-Nitrosylation signal not detected	Reducing or Labeling reagent were not added	Add reducing reagent followed by labeling reagent
	Incomplete removal of blocking reagent	Add more washes after acetone precipitation
	S-NO levels were too low	Add positive and negative control for further analysis
	S-NO was labile	Protect samples from light until labeling reagent addition
		Avoid using reducing reagent in sample preparation
High background in Western Blot	Free thiols were not sufficiently blocked	Increase blocking incubation time
	Detection reagents were excessively used	Increase dilution factor of detection reagent
	Insufficient/incorrect membrane blocking or washing	Use 2% BSA in PBS as blocking buffer and wash more times

References

Jaffrey, S.R., *et al.* (2001). The biotin switch method for the detection of S-nitrosylated proteins. *Sci STKE*. **86**:p11.

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