S-Nitrosothiol Assay Kit (Thiosulfonate Switch)

Item No. 601210

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GENERAL INFORMATION

Materials Supplied

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

Item Number	Item	Quantity/Size	Storage
601211	TST Assay Buffer	1 vial/35 ml	4°C
601212	TST S-Phenylsulfonylcysteine	1 vial/lyophilized	-20°C
601213	TST Sodium Benzenesulfinate	1 vial/lyophilized	-20°C
601214	TST Z-Rhodamine-SH	1 vial/lyophilized	-20°C
601215	TST MMTS	1 vial/5 µl	-20°C
601216	TST Positive Control BSA	1 vial/20 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.

NOTE: It is recommended that proper safety precautions be taken when working with isolated mitochondria and mitochondrial inhibitors.

If You Have Problems

Technical Service Contact Information

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

Fax: 734-971-3641

Email: 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.

Materials Needed But Not Supplied

1. Amber and natural microcentrifuge tubes (1.5 ml)
2. ~0.5 ml desalting columns (for up to 100 µl sample size)
3. Microcentrifuge capable of 1,500 x g
4. Non-Reducing SDS-PAGE sample buffer (electrophoresis)
5. Fluorescence Imaging System (UVP, LI-COR, GE, Kodak, etc.)

INTRODUCTION

Background

Nitric oxide (NO) is produced by three distinct isoforms of NO synthase and functions as a key signaling molecule.[1,2] Protein S-nitrosation is a post-translational modification occurring as a product of NO reactivity with protein free thiol or thiyl radical groups.[3,4] Development of the Thiosulfonate Switch Technique (TST) provides a method for the fluorescent detection of protein S-nitrosothiols as mixed disulfides from protein thiosulfonates at pH 4.0 and ambient room temperature.[5,6] This format eliminates the need for the use of ascorbate and biotin, which are known to produce false positive signals from instances of protein-disulfide reduction and endogenous mitochondrial carboxylase enzymes, respectively.[7-9] The TST is a method complementary to the biotin switch technique and may be more suitable for analysis of select protein samples.

About This Assay

Cayman's S-Nitrosothiol Assay Kit (TST) provides a fast, simple method for detection of protein S-nitrosation *in vitro*. There are sufficient reagents to perform 18 reactions, including a positive control protein for generation of a standard curve. S-Nitrosothiols may be generated in cell culture or directly from a protein source such as cell or tissue lysates by the addition of a NO donor. Reaction products can be analyzed directly by proteolysis and MS, SDS-PAGE and imaging, or by MS after band extraction from a gel. The bovine serum albumin rhodamine mixed disulfide positive control provided in the kit can be used to determine the relative quantity of protein(s) in samples after SDS-PAGE using fluorescence imaging and densitometry.

Features of this kit include:

- Prepare samples for SDS-PAGE in under four hours.
- Detect endogenous or NO donor-generated protein S-nitrosothiols.
- Identify proteins with S-nitrosothiols by fluorescence immediately after electrophoresis.
- Generate an in-gel standard curve for relative quantitation.
- Prepare samples by TST and proceed directly to proteomic analysis.

PRE-ASSAY PREPARATION

Reagent Preparation

Sample composition

Use 200-500 µg of protein sample per tube. Dilution of samples in HEN buffer (250 mM HEPES, pH 7.7, 1.0 mM EDTA, and 0.1 mM neocuproine) is suggested for use with most NO donor treatments.

TST Assay Buffer (Item No. 601211) - *is ready to use as supplied*

The TST Assay Buffer is used for dissolution or dilution of the kit components according to the table below. Save the remaining TST Assay Buffer to prime desalting columns as needed.

Item No.	Item Name	Add TST Assay Buffer (µl)
601212	TST S-Phenylsulfonylcysteine	400
601213	TST Sodium Benzenesulfinate	120
601214	TST Z-Rhodamine-SH	66
601215	TST MMTS	495
601216	TST Positive Control BSA	None; ready to use as supplied at 0.1 mg/ml in SDS-PAGE sample loading buffer. Briefly centrifuge the capped tube to ensure contents are all at the bottom before use.

ASSAY PROTOCOL

CAUTION: It is recommended to test your protein sample for potential losses from the pH 4.0 desalting step. Alternative desalting conditions may be warranted if excess protein sample losses occur. Adding sucrose (to 0.4 M) to the TST Assay Buffer may increase the desalting yields of certain proteins. Make sure to reserve at least 4 ml of TST Assay Buffer for dissolution of the lyophilized kit reagents. Alternatively consider treating cells or tissues with NO donors prior to lysis, wash excess donor away and lyse cells directly with TST Assay Buffer containing 0.1 mM N-octyl-glucoside.

Negative controls beyond vehicle treated protein samples may be desired. Denitrosation reactions may be generated from an additional NO donor treated sample. For example:

- Desalt the negative control sample in parallel with other samples but into HEPES buffer instead of TST Assay Buffer. Transfer the desalted sample into a natural (non-colored) 1.5 ml tube and expose it to a strong UV source (312 nm, five minutes without heating).

OR

- Desalt into HEPES buffer instead of TST Assay Buffer and spike the desalted sample with add sodium ascorbate (to 10-20 mM) and copper I chloride (to 10 µM) for one hour at ambient temperature.[7] Negative control samples will need to be desalted into TST Assay Buffer before continuing with the assay (at step 4 on page 10).

Performing the Assay

Protein denitrosation can occur under direct light. Keep all samples under cover.

1. Treat protein samples (200-500 µg, usually 100 µl or less per sample) with and without NO donor (1 mM GSNO in HEN buffer, pH 7.7, for example) for one hour at ambient room temperature or according to your scheme.
2. Prime 0.5 ml desalting columns for each sample using the TST Assay Buffer.
3. Desalt the samples by centrifugation collecting the products into pre-labeled amber 1.5 ml tubes.
4. Add TST reagents sequentially to each sample for the appropriate incubation time according to the table below.
5. Dilute MMTS treated samples with **non-reducing** SDS-PAGE sample buffer prior to protein electrophoresis.
6. SDS-PAGE: Load the TST Positive Control BSA into one to four wells (3-4 wells for standard curve generation).
7. Analyze gel after electrophoresis by rhodamine fluorescence imaging (excitation 532 nm, emission 580 nm).

Item Name	µl/sample	Reaction Time (minutes)
TST S-Phenylsulfonylcysteine	20	60
TST Sodium Benzenesulfinate	6	30
Determine protein concentrations*		
TST Z-Rhodamine-SH	3	10
TST MMTS	4	10

*Optional step but highly recommended if any sample losses occur during the assay or multiple sources of lysates or protein mixtures are being used. Take a minimal volume (5 µl) per sample for quantitation before the addition of Z-Rhodamine-SH.

ANALYSIS

Performance Characteristics

Fluorescence analysis

Detect labeled proteins using a fluorescence imager capable of rhodamine analysis.

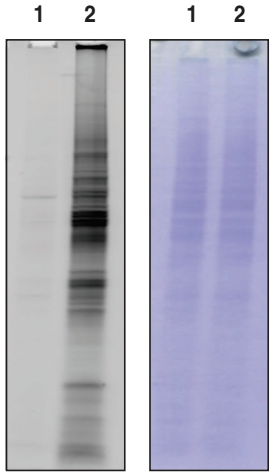

Figure 1. In-gel detection of protein S-nitrosothiols. Equal parts lysate were treated with vehicle (1) or NO donor (2) respectively, desalted, and treated with TST assay reagents according to the assay protocol. Left side image is TST rhodamine fluorescence and the right side image is the same gel post Coomassie Blue destaining. Image generated by UVP imager; four second capture with ethidium bromide filter, coupled with UV light source.

Calculations

Use ImageJ software or other appropriate software to generate densitometry values and plot them against known amounts of TST Positive Control BSA.

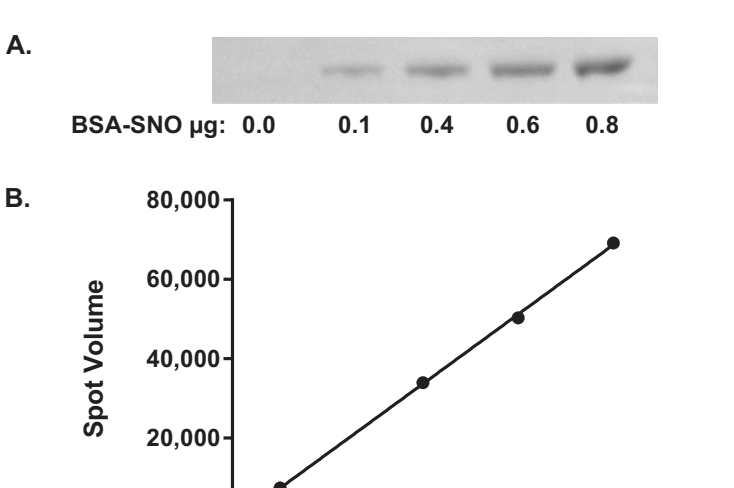

Figure 2.

A. Representative TST Positive Control BSA rhodamine fluorescence.
B. Plot of relative band density *versus* known concentrations of BSA mixed disulfide.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
No fluorescent signal from protein sample	A. Loss of proteins during desalting 50% or more may occur due to incompatibility with TST Assay Buffer B. Light exposure too great during the assay C. Incorrect filter or λex, λem	A. Prime desalting columns with TST Assay Buffer containing additives B. Minimize sample exposures to light C. Rhodamine acquisition or UV source with ethidium bromide filter for image capture
Excessive fluorescence at the bottom half of the gel	Some gradient gels may retain more free rhodamine-S-S-CH_3 than non-gradient or fixed percentage gels	A. Run the dye front off the end of gel or use a fixed 10 or 12% acrylamide resolving gel B. Desalt excess fluorophore from samples by treatment with ≥3 volumes of ice cold acetone, set at -20°C for one hour, and pellet proteins by 3,000 x g centrifugation; Decant acetone and dissolve the protein pellet in small volume of TST assay buffer

References

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9. Kallakunta, V.M., Staruch, A., and Mutus, B. Sinapinic acid can replace ascorbate in the biotin switch assay. *Biochim. Biophys. Acta.* **1800**, 23-30 (2010).

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.