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Empowering your proteomics

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ISO 13485:2016 cGMP

S-Nitrosylated Caspase-3 ELISA Kit

Catalog #: PTE-SNOCASP3

User Manual
Last Revised: July 30, 2024

Introduction

The RayBio® S-Nitrosylated Caspase-3 ELISA Kit uses a modified 'biotin-switch' method to allow for the direct visualization of human and mouse S-nitrosylated Caspase-3 by ELISA. 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 ELISA. An anti-pan Caspase-3 antibody has been coated onto a 96-well plate. Biotinylated samples are pipetted into the wells, and Caspase-3 present in a sample is bound to the wells by the immobilized antibody. The wells are washed, and HRP-conjugated streptavidin is used to detect S-nitrosylated Caspase-3. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of S-nitrosylated Caspase-3 bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

Storage / Stability

The entire kit may be stored at -20°C for up to 6 months from the date of shipment. Avoid repeated freeze-thaw cycles. For prepared reagent storage, see kit contents on the next page.

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Kit Components

Name	Catalog #	Size / Qty	Description	Storage / Stability After Preparation
Anti-Pan-CASP3 Microplate	PTE-SNOCASP3-A	96 wells	Microplate coated with anti-pan Caspase-3 antibody.	1 month at -20°C*
Positive Control	PTE-SNO-PC	1 vial	S-nitrosylated cell lysate	1 week at -20°C
HRP-Streptavidin	EL-HRP	200 µl	300X concentrated HRP-conjugated streptavidin	Do not store and reuse.
Wash Buffer	EL-ITEMB	25 ml	20X concentrated wash buffer	1 month at 4°C
Assay Diluent B	EL-ITEME	15 ml	5X concentrated assay diluent	1 month at 4°C
Lysis Buffer	EL-Lysis	5 ml	2X cell lysate buffer	1 month at 4°C
TMB One-Step Substrate Reagent	EL-TMB	12 ml	3,3,5,5'-tetramethylbenzidine (TMB) in buffer solution	N/A
Stop Solution	EL-STOP	8 ml	0.2 M sulfuric acid	N/A
S-nitrosylation Buffer A	MA-SNO-A	12 ml	S-nitrosylation Buffer A	RT
S-nitrosylation Buffer B	MA-SNO-B	3 ml	S-nitrosylation Buffer B	RT
S-nitrosylation Buffer C	MA-SNO-C	5 ml	S-nitrosylation Buffer C	RT
S-nitrosylation Buffer D	MA-SNO-D	3 ml	S-nitrosylation Buffer D	RT
S-nitrosylation Buffer E	MA-SNO-E	12 ml	S-nitrosylation Buffer E	RT
S-nitrosylation Blocking Reagent	MA-SNO-G	2 vials	Sufficient for two separate experiments. Crystalline solid.	Prepare immediately prior to use. Do not store.

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S-nitrosylation Reduction Reagent I	MA-SNO-H	2 vials	Sufficient for two separate experiments. Crystalline solid.	Prepare immediately prior to use. Do not store.
S-nitrosylation Reduction Reagent II	MA-SNO-I	2 vials	Sufficient for two separate experiments. Crystalline solid.	Prepare immediately prior to use. Do not store.
S-nitrosylation Labeling Reagent	MA-SNO-J	2 vials	Sufficient for two separate experiments. Crystalline solid.	Prepare immediately prior to use. Do not store.

*Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

Additional Materials Required

- Acetone (pre-chilled (-20°C))
- Microplate reader capable of measuring absorbance at 450 nm
- Protease and Phosphatase inhibitors.
- Precision pipettes to deliver 2 µl to 1 ml volumes
- Adjustable 1-25 ml pipettes for reagent preparation
- 100 ml and 1-liter graduated cylinders
- Log-log graph paper or computer and software for ELISA data analysis.
- Absorbent paper
- Tubes to prepare positive control or sample dilutions
- Distilled or deionized water
- Shaker

Reagent Preparation

1. Bring all reagents and samples to room temperature (18 - 25ºC) before use.
2. S-nitrosylation Blocking Reagent: Make fresh. Spin briefly, add 50µl S-nitrosylation Buffer B, vortex until all crystals are dissolved completely, then transfer everything into 5 ml S-nitrosylation Buffer A, mix well.
3. S-nitrosylation Reduction Reagent I: Make fresh. Spin briefly, add 800 µl S-nitrosylation Buffer C, vortex until all crystals are dissolved completely.
4. S-nitrosylation Reduction Reagent II: Make fresh. Spin briefly, add 500 µl S-nitrosylation Buffer D, vortex until all crystals are dissolved completely.
5. S-nitrosylation Reducing Buffer: Add 4 µl of dissolved S-nitrosylation Reduction Reagent II into 800 µl of dissolved S-nitrosylation Reduction Reagent I, mix well.

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6. S-nitrosylation Labeling Reagent: Make fresh. Spin briefly, add 100µL dH2O, vortex until all crystals are dissolved completely.
7. Acetone (not included): pre-chilled (-20°C).
8. 4:1 acetone/water mixture: 4 parts acetone mixed with 1 part dH2O, pre-chilled (-20°C).
9. Assay Diluent B should be diluted 5-fold with deionized or distilled water before use.
10. Lysis Buffer should be diluted 2-fold with deionized or distilled water (for cell lysate and tissue lysate). We also recommend the addition of protease and phosphatase inhibitors (not included) to the lysis buffer prior to use.
11. Preparation of Positive Control: Briefly spin the Positive Control vial. Add 250 µl of prepared 1X Assay Diluent B to prepare Positive Control solution. Gently mix the powder to allow it to dissolve thoroughly.
12. If the Wash Buffer concentrate contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer concentrate into deionized or distilled water to yield 400 ml of 1X Wash Buffer.
13. Preparation of HRP-Streptavidin: Briefly spin the vial of HRP-Streptavidin concentrate before use. HRP-Streptavidin should be diluted 300-fold with 1X Assay Diluent B and used in step 7 of the Assay Procedure.

Assay Procedure

A. Biotinylation of S-nitrosylated cysteines.

This kit contains sufficient reagents to label 40 samples containing 100-200 µg of total protein each.

1. Prepare 100 µl sample with a total protein concentration of 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 before use) into each sample. Incubate the samples in the dark at 50 °C on a shaker with gentle rocking for 30 minutes.
3. Precipitate protein by adding 1200 µl of pre-chilled (-20°C) acetone to each sample. Mix thoroughly by inversion, then incubate 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.

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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 the 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 before use), along with 3 µl S-nitrosylation Labeling Buffer (use fresh reagent, prepared immediately before 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 the pellet, or it may not be dissolved properly.
12. Reconstitute each protein pellet in 100 µl S-nitrosylation Buffer E. The sample is now ready for the ELISA.

B. ELISA Procedure.

1. Bring all reagents and samples to room temperature (18 - 25ºC) before use. It is recommended to run all samples in at least duplicate.
2. Label removable 8-well strips as appropriate for your experiment.
3. Add 100 µl sample and Positive Control (see Reagent Preparation step 11) into the appropriate wells. Cover the wells and incubate for 2.5 hours at room temperature or overnight at 4ºC with gentle shaking.
4. Discard the solution and wash 4 times with 1X Wash Solution. Wash by filling each well with Wash Buffer (300 µl) using a multi-channel pipette or auto-washer. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
5. Add 100 µl of prepared HRP-Streptavidin solution (see Reagent Preparation step 13) to each well. Incubate for 1 hour at room temperature with gentle shaking.
6. Discard the solution. Repeat the wash as in step 4.
7. Add 100 µl of TMB One-Step Substrate Reagent to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.

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8. Add 50 µl of Stop Solution to each well. Read at 450 nm immediately.

Assay Procedure Summary

1. Prepare all reagents, samples and Positive Control as instructed.
2. Block unmodified free cysteines using the prepared S-nitrosylation Blocking Buffer at 50 °C with gentle rocking.
3. Precipitate proteins with ice-cold acetone.
4. Wash the protein pellet twice with cold acetone/water mixture.
5. Resuspend the protein pellets in buffer containing Reducing and Labeling Reagents.
6. Precipitate proteins with ice-cold acetone.
7. Resuspend protein pellets.
8. Add 100 µl of sample and Positive Control to each well. Incubate for 2.5 hours at room temperature or overnight at 4ºC with gentle shaking.
9. Add 100 µl prepared HRP-Streptavidin solution. Incubate for 1 hour at room temperature with gentle shaking.
10. Add 100 µl TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.
11. Add 50 µl Stop Solution to each well. Read at 450 nm immediately.

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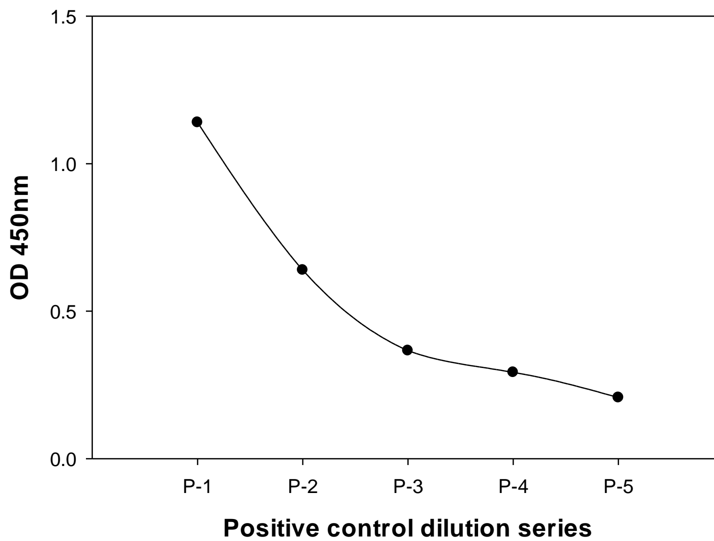
Typical Data

Calculate the mean absorbance for each set of duplicate positive controls, and samples.

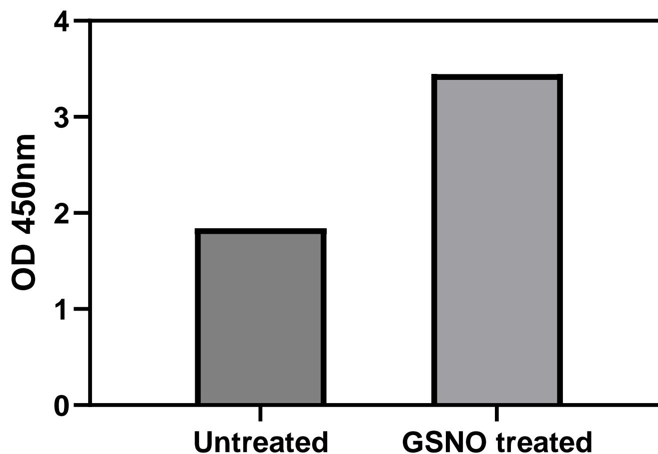
A. Positive Control

Three-fold serial dilutions of the Positive Control were analyzed in this ELISA.



B. S-Nitrosoglutathione (GSNO) Stimulation of Cell lysate

Cell lysates were treated with or without GSNO and then analyzed using this ELISA.



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Troubleshooting Guide

Problem	***Cause***	***Solution***
Low signal in samples	• Sample concentration is too low • Improper preparation of detection antibody • Too brief incubation times • Inadequate reagent volumes or improper dilution	• Increase sample concentration. Briefly spin down vials before opening. Dissolve the powder thoroughly. • Ensure sufficient incubation time; Assay Procedure, B. ELISA Procedure, step 3 may be done overnight. Check pipettes and ensure correct preparation
High signal in samples	• Sample concentration is too high	• Reduce sample concentration
Large CV	• Inaccurate pipetting • Air bubbles in wells	• Check pipettes • Remove bubbles in wells
High background	• Plate is insufficiently washed • Contaminated wash buffer	• Review the manual for proper washing. If using a plate washer, ensure that all ports are unobstructed. • Make fresh wash buffer
Low sensitivity	• Improper storage of the ELISA kit • Stop solution • Improper primary or secondary antibody dilution	• Store your positive control at <- 70ºC after reconstitution, others at 4ºC. Keep substrate solution protected from light. • Add stop solution to each well before reading plate • Ensure correct dilution