-SulfoBiotics-
Protein S-Nitrosylation Monitoring Kit

Technical Manual

Technical Manual (Japanese version) is available at http://www.dojindo.co.jp/manual/sb14.pdf

General Information

Modification of protein thiol is one of the most important post-translational modifications and it occurs depending on the redox state in cells. Protein *S*-nitrosylation is NO (nitric oxide)-dependent modification of protein thiols and is crucial for regulation of cellular functions such as transcription, protein expression, and signal transduction.

-SulfoBiotics- Protein *S*-Nitrosylation Monitoring Kit allows to detect *S*-nitrosylated proteins by gel-electrophoretic analysis. This kit contains chemical reagents for blocking of free thiols on proteins, reducing of *S*-nitrosylated thiols, and labeling of the reduced thiols. After blocking free thiols of protein, *S*-nitrosylated thiols are selectively reduced by the reducing agent, and labeled with Protein-SHifter Plus, which is a novel maleimidyl compound consisted of a high molecular weight. When one molecule of Protein-SHifter Plus binds to a thiol group of protein, a mobility shift corresponding to about 15 kDa of molecular mass is observed by the gel-electrophoretic analysis. Thus, the number of *S*-nitrosylated thiol group on a protein can be clearly identified by SDS-PAGE through the mobility shift assay. In addition, the Protein-SHifter Plus moiety can be cleaved from the labeled protein in a gel with UV irradiation after gel-electrophoresis because Protein-SHifter Plus has a UV photocleavable moiety in the molecule. Therefore, the protein treated with UV irradiation can be transferred from the gel to PVDF membrane and detected on the membrane similar to the unlabeled protein by a specific antibody.

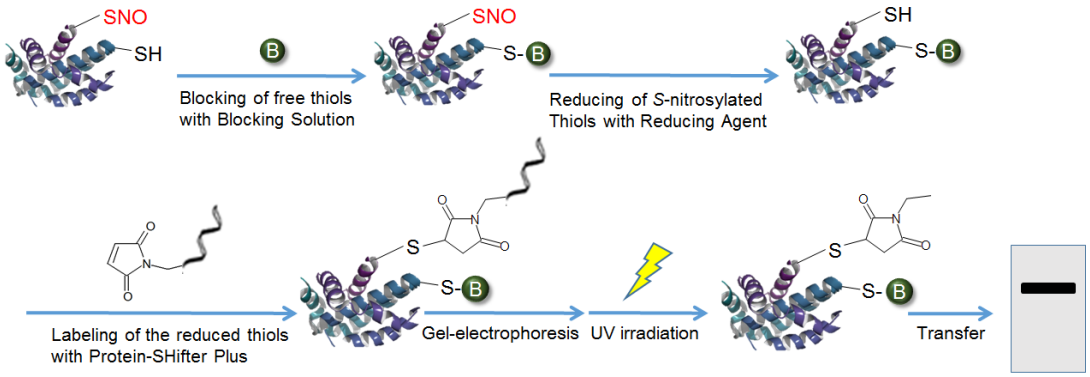

Figure 1 Schematic Protocol of Protein *S*-Nitrosylation Monitoring

Kit Contents

- Protein-SHifter Plus	x 20
- Reaction Buffer A	x 1
- Reaction Buffer B	x 1
- Lysis Buffer	x 1
- Blocking Stock Solution	x 1
- Reducing Agent	x 5

Storage Condition

Store at 0 - 5 °C

Required Equipment and Materials

- 10 µl and 100-200 µl pipettes
- Centrifuge
- 70 (v/v) % ethanol solution
- Incubator (37°C)
- Acetone
- HBSS or PBS
- Electrophoresis reagents: Gel, Loading buffer, Protein staining reagents, e.g. Coomassie Brilliant Blue
- Western Blotting reagents: Transfer system, PVDF (polyvinyl difluoride) membrane, Transilluminator

Precaution

- Centrifuge the tube (Protein-SHifter Plus) briefly before opening the cap because the content may be on the tube wall or on the ceiling of the cap.
- If a precipitate is ocurred in Reaction Buffer B, dissolve the precipitates by warming the solution to 40-50°C.
- This kit is optimized for mammalian cells.

General Protocol

Preparation of Blocking Solution

Add 100 µl of Blocking Stock Solution to 900 µl of Lysis Buffer to prepare Blocking Solution.

※ Defoam bubbles by centrifugation at 7,000 x *g* for 1 - 2 minutes if foaming occurs.

※ Add a protease inhibitor as necessary.

Preparation of RA Solution

Add 2 ml of Reaction Buffer A to Reducing Agent, and mix with inversion to RA Solution.

※ RA Solution should be applied for electrophoretic analysis immediately.

Example of sample preparation for mammalian cells

1. Prepare 5-10 x10^5 cells for each assay.
2. Wash the cells twice with 500 µl HBSS or PBS.
3. Add 200 µl of Blocking Solution and incubate at 37°C for 10 minutes.
4. Lyse the cells by pipetting, and transfer the cell lysate to a 1.5 ml micro tube.
5. Add 1 ml of cold acetone to each tube and centrifuge 12,000 x *g* for 3 minutes and remove the supernatant.
6. Repeat Step 5.
7. Add 1 ml of cold 70% ethanol solution to each tube and centrifuge 12,000 x *g* for 3 minutes and remove the supernatant.
8. Add 10-50 µl of Lysis Buffer to each tube and dissolve the cell pellet by sonication.
9. Centrifuge 12,000 x *g* at 4°C for 3 minutes and use the supernatant as a sample solution.

※ Proceed to the following "Labeling and Analysis" immediately.

Labeling and Analysis

1. Add 4 µl of RA Solution to Protein-SHifter Plus and mix by pipetting.
2. Add 2 µl of the sample solution and 4 µl of Reaction Buffer B to the solution of Step1, and mix by pipetting.
   ※ Dissolve precipitates completely by warming the solution to 40-50℃ when precipitation occurs .
   ※ Defoam bubbles by centrifugation at 7,000 x *g* for 1 - 2 minutes if foaming occurs.
3. Incubate at 37℃ for 30 minutes.
   ※ Proceed to Step 4 immediately.
4. Add a Loading buffer to the solution of Step 3 and apply the solution to gel electrophoresis.
   ※ This kit does not contain a Loading buffer. When using (5x) Loading buffer, add 2 µl of (5x) Loading buffer to 10 µl of the solution of Step 3 and apply all of the solution to gel electrophoresis.
5. Expose the gel to UV rays (302 nm) with a transilluminator for 10 minutes.
   ※ During this step, keep the gel in the glass plates to avoid drying it.
6. Transfer proteins from the gel to PVDF membrane, and detect a target protein by a specific antibody.

Experimental Example

Analysis of GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) *S*-Nitrosylation in HeLa cell

1. HeLa cells were seeded on a 24-wells plate at the concentration of 5 x 10^5 cells/well and cultured overnight at 37℃ in a 5% CO_2 incubator (culture media : MEM).
2. The cells were washed using HBSS (500 µl) twice, and two different concentrations of *S*-nitrosocysteine solutions (1 mmol/l and 100 µmol/l) in PBS (500 µl) was added to each well.
3. The cells were incubated at 37℃ for 45 minutes.
4. After the cells were washed using HBSS (500 µl) twice, Blocking Solution (200 µl) was added to each well. Then, the cells were dissolved by pipetting.
5. The cell lysate was transferred to each tube, and incubated at 37℃ for 10 minutes.
6. Cold acetone (1 ml) was added to each tube, and the supernatants were removed after centrifugation of the tubes at 12,000 x g for 3 minutes.
7. Step 6 was repeated.
8. Cold 70% EtOH solution (1 ml) was added, and the supernatants were removed after centrifugation of the tubes at 12,000 x g for 3 minutes.
9. Lysis Buffer (20 µl) was added, and the cell pellet was dissolved by vortex and sonication.
10. RA Solution (4 µl) was added to Protein-SHifter Plus and mixed by pipetting.
11. The solution (2 µl) of Step 9 and Reaction Buffer B (4 µl) were added to the tube of Step 10, and the solution was mixed by pipetting.
12. The tube of Step 11 was incubated at 37 ℃ for 30 minutes.
13. Loading Buffer ([10 (w/v) % sodium dodecyl sulfate, 50 (v/v) % glycerol, 0.2 mol/l Tris-HCl (pH 6.8) , 0.05 (w/v) % bromophenol blue], 2 µl) was added to the tube of Step 12 and mixed by pipetting.
14. The solution of Step 13 was used for SDS-polyacrylamide gel (10-20%) electrophoresis.
15. The gel was exposed with UV rays(302 nm) using a transilluminator for 10 minutes.
16. The separated proteins in the gel were electrophoretically transferred onto a PVDF membrane.
17. The GAPDH on the membrane was detected with anti-GAPDH antibody, HRP labeled secondary antibody, and luminol substrate.

Figure 2 Analysis of GAPDH *S*-Nitrosylation in HeLa cells

References

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