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Method Article

# Immunoprecipitation methods to identify S-glutathionylation in target proteins

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S-glutathionylation is a reversible post-translational modification of proteins that generate a mixed disulfide between glutathione to thiolate anion of cysteine residues in target proteins. In the last ten years, S-glutathionylation has been extensively studied since it represents the cellular response to oxidative stress, in physiological as well as pathological conditions. This modification may be a protective mechanism from irreversible oxidative damage and, on the other hand, may modulate protein folding and function.

Due to the importance of S-glutathionylation in cellular redox signaling, various methods have been developed to identify S-gluthationylated proteins.

Herein, we describe two easy methods to recognized S-glutathionylation of a target protein after oxidative stress in cellular extracts based on different immunoprecipitation procedures. The immunoprecipitation assay allows the capture of one glutathionylated protein using a specific antibody that binds to the target protein. The presence of S-glutathionylation in the immunoprecipitated protein is identified using anti-glutathione antibody. The second type of approach is based on the detection of the glutathionylated protein with biotin/streptavidin technique. After different steps of protection of non-oxidized thiolic groups and reduction of S-glutathionylated groups, the newly-formed protein free-thiols are labeled with biotin-GSH. The modified protein can be isolate with streptavidin-beads and recognized using an antibody against target protein.

- S-glutathionylation is a reversible post-translational modification of proteins that recently has been emerged as important signaling in the redox regulation of protein function.
- Both methods to identify glutathionylated proteins are economic, easy and do not require particular equipment.
- The setups of both methods guarantee high reproducibility.



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## Method details

### *Introduction*

Glutathione (GSH) deficiency manifests itself largely through an increased susceptibility to oxidative stress whereas elevated GSH levels observed in many types of cancer cells, generally increase antioxidant capacity. Oxidative stress may cause reversible and/or irreversible oxidative modifications of sensitive proteins that may modulate their activity or function. Mild oxidative stress induces reversible modifications that protect proteins from irreversible damage and module their function. Conversely, excessive oxidative stress triggers irreversible modifications of thiolic groups of proteins generally associated with permanent loss of function, misfolding and aggregation tendency [1].

In mammalian cells, a significant amount of GSH may be reversibly bound to proteins, through the formation of mixed disulfides between GSH and protein sulfhydryl groups. The resulted post-

translational modification, known as S-glutathionylation, not only stores GSH, preventing its loss under oxidative conditions, but also protects sensitive protein thiols from irreversible oxidation [2].

It is important to note that protein S-glutathionylation occurs in pathological as well as physiological conditions, suggesting the possible involvement of this post-translational modification in cellular signaling and redox regulation of protein function [1,2].

Protein sulfhydryls exhibit striking differential susceptibility to S-glutathionylation. Thus, while a protein may contain numerous cysteine residues, only a minority of these will have the chemical properties to function as possible target sites for oxidant.

The two major factors that determine susceptibility of cysteinyl residues to redox reactions are the accessibility of the thiol within the three-dimensional structure of the protein and the cysteine reactivity, which is influenced by the neighboring amino acids. Accessibility is defined by the secondary and tertiary structure of the protein and depends on the relative easiness of the cysteine to be exposed to the solvent. The reactivity of SH groups is more difficult to be predicted. The cysteine sulfhydryls of most cytoplasmic proteins have a pKa value of ~8.5 and, in the normally reducing environment of the cytoplasm at physiological pH, these residues are almost completely protonated. As result, they are unlikely to be S-glutathionylated. However, redox-sensitive proteins have specific cysteine residues localized in a basic microenvironment that shift their pKa to a lower value, near or below 7, at physiological pH. This pKa value keeps the cysteine in the thiolate form and makes it an 'active cysteine', which has enhanced reactivity for GSH [3].

Herein, we describe two easy methods developed for identifying glutathionylated proteins that could be used in parallel in order to exclude false positive results. The first one is the immunoprecipitation of target protein followed by electrophoresis under non-reducing condition and western blot analysis with anti-GSH antibody [4,5]. This method remains the most used strategy for the identification of S-glutathionylated proteins. The second method is a modified biotin switch assay proposed as described previously by Jaffrey and Snyder [6] with modifications. This method is based on the use of biotinylated glutathione that can react with sensitive redox thiols group of cysteine residues. The modified protein can be isolate by affinity capture with streptavidin-conjugated media and recognized by western blot [7,8].

The methods proposed in this manuscript were used by the same authors in two previous papers in which the level of S-glutathionylation of STAT3 and STAT1 were analysed in cells after oxidative stress. To identify the modified cysteine residue, oxidized STAT1 and STAT3 were also analysed by mass spectroscopy. The obtained data confirmed the reliability of the proposed methods [7–9].

## *Materials*

### *Buffers*

1 RIPA lysis buffer: 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% (v/v) Igepal (CA-630 I3021, Sigma-Aldrich), 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA), 10% (v/v) glycerol, 100 mM NaF, 1 mM $Na_3VO_4$.
2 Sample buffer: 62.5 mM Tris–HCl pH 6.8, 10% (v/v) glycerol, 5% (w/v) SDS, 0.05% (w/v) bromophenol blue, 5% (v/v) β–mercaptoethanol.
3 Non-reducing Sample buffer: sample buffer without β–mercaptoethanol.
4 Running buffer: 25 mM Tris–HCl pH 8.3, 190 mM glycine, 0.1% (w/v) SDS.
5 Transfer buffer: 25 mM Tris–HCl pH 8.3, 192 mM glycine, 20% (v/v) methanol.
6 TBS buffer: 20 mM Tris–HCl pH 7.5, 150 mM NaCl.
7 Washing buffer: TBS buffer containing 0.1% (v/v) Tween-20 detergent
8 Blocking: washing buffer containing 3% (w/v) BSA.
9 Stripping buffer: 62.5 mM Tris–HCl pH 6.7, 2% (w/v) SDS, 100 mM β–mercaptoethanol.

### *Biotinylated glutathione (BioGSH) synthesis*

Dissolve the water-soluble sulfosuccinimidyl-6-(biotinamido)-hexanoate (sulfo-NHS-biotin) and GSH 1:1 in water and adjust the pH to 7.2 with NaOH. Incubate the reaction at room temperature for 1 h. Unreacted biotin reagent is quenched by the addition of 1 M Tris–HCl pH 7.2.

*Reagents*

1 N-ethylmaleimide (NEM) (E3876, Sigma-Aldrich): Prepare 250 mM NEM in water. This solution is stable for at least 3 months at −20 °C.
2 1,4-Dithiothreitol (DTT) (Sigma-Aldrich): freshly prepare 1 M DTT in water and do not store.
3 Diamide (D3648, Sigma-Aldrich): prepare 100 mM diamide in water. This solution is stable for at least 3 months at −20 °C.
4 Protein A or protein G sepharose: prepare the resin following the manufacturer's instructions, mix thoroughly and equilibrate with RIPA buffer. The slurry is now ready for use. It can be stored at 4 °C for a few days. If using a monoclonal antibody choose protein G-coupled sepharose beads, if using a polyclonal antibody protein A-coupled sepharose beads are usually suitable.
5 Streptavidin-agarose beads (ThermoFisher Scientific): prepare the beads following the manufacturer's instructions, mix thoroughly and equilibrate with washing buffer.
6 Agarose beads (ThermoFisher Scientific): prepare the beads following the manufacturer's instructions, mix thoroughly and equilibrate with washing buffer.
7 Chemiluminescence kit (Immobilon Western, Millipore).
8 Anti-Glutathione monoclonal antibody (ViroGen): dilute antibody 1: 1000 in blocking buffer.
9 Anti-mouse IgG peroxidase-conjugated antibody (Cell Signaling Technology): dilute antibody 1:2000 in blocking buffer.
10 Lowry reagent (Sigma-Aldrich) and BSA standard curve.

*Immunoprecipitation method*

1 Seed $5 \times 10^5$–$1 \times 10^6$ cells in culture dish and culture them until confluence. Treat cells with appropriate oxidant. For example, 1 mM diamide for 30 min. Not treated cells are used as control.
2 Place culture dishes on ice and wash the cells with ice-cold PBS. Add new ice-cold PBS and scrape adherent cells off the dish using a cold plastic cell scraper then gently transfer the cell suspension into a pre-cooled microcentrifuge. Centrifuge samples at 4 °C and discard the supernatants.
3 Lyse cells in RIPA buffer supplemented with 50 mM NEM and with protease inhibitors (10 μg/mL antipain, 5 μg/mL pepstatin, 1 mM phenylmethylsulfonyl fluoride) for 30 min on ice.
4 Centrifuge samples at ≈14,000 × *g* for 30 min at 4 °C to pellet the cell debris.
5 Gently transfer supernatants to new Eppendorf tubes.
6 Quantify total proteins content using Lowry reagent and BSA standard curve.
7 Clarify 1 mg of proteins from cell lysates using 40 μL of slurry of protein A- or G-sepharose in Eppendorf tubes. Incubate at 4 °C for 1 h under gentle rotation. Pre-clearing the lysate can help to reduce non-specific binding and reduce background.
8 Centrifuge samples at ≈1000 × *g* at 4 °C for 1–2 min.
9 Gently transfer supernatants to new Eppendorf tubes.
10 Incubate equal amounts of proteins from the clarified cell lysates with the antibody against the target protein at 4 °C for 12 h, preferably under gentle rotation. The length of the incubation period depends on the amount of protein and affinity properties of the antibody. Check the antibody datasheet for recommended antibody concentration. As a guideline use 1 μg of affinity purified polyclonal antibody.
11 Centrifuge samples at ≈1000 × *g* at 4 °C for 1–2 min.
12 Add 60 μL of slurry of protein A or G sepharose and mix at 4 °C under gentle rotation for 2 h.
13 Centrifuge samples at ≈1000 × *g* at 4 °C for 1–2 min.
14 Collect the beads and discard the supernatants. Add 200 μL of RIPA buffer to the tube with the beads and gently mix. Repeat this wash twice.
15 Add 30 μL of 2X non-reducing Sample buffer to the collected beads and heat the samples at 100 °C for 3 min.
16 Centrifuge samples at ≈1000 × *g* at 4 °C for 1–2 min and collect the supernatants for western blot analysis.
17 Separate Protein A or G pull-down samples on a 5–10% SDS–polyacrylamide gel in running buffer following the manufacturer's instructions of the electrophoresis apparatus.
18

Transfer proteins to PVDF membrane using transfer buffer following the manufacturer's instructions of the transfer apparatus.

19 Block non-specific binding on the membrane by incubation in blocking buffer at room temperature for 1 h under gentle agitation.

20 Probe membrane with monoclonal antibody against GSH at 4 °C overnight under gentle agitation.

21 Wash the membrane in washing buffer for 5 min under gentle agitation. Repeat wash three times.

22 Incubate blots with anti-mouse IgG peroxidase-conjugated antibody at room temperature for 1 h under gentle agitation.

23 Wash the membrane in washing buffer for 5 min under gentle agitation. Repeat wash three times.

24 Detect protein-antibody reactions with chemiluminescent detection reagent following the manufacturer's instructions. Acquire images with an automated image acquisition system.

To check the immunoprecipitated protein, remove primary and secondary antibodies from the membrane and re-probe it with the primary antibody against the targeted protein.

25 Incubate membrane in stripping buffer at 50 °C for 30 min under gentle agitation.

26 Check the efficiency of stripping by incubating the membrane with chemiluminescent detection reagent.

27

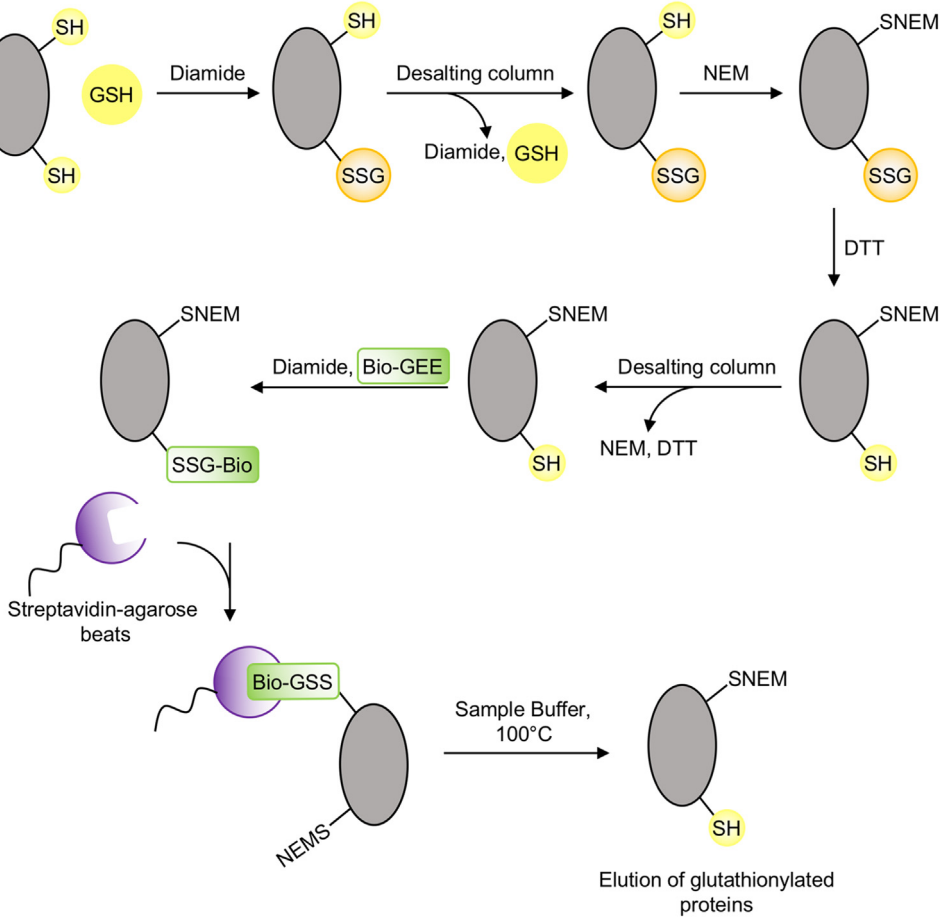


**Fig. 1.** Modified biotin switch assay.

If stripping is judged to be satisfactory, rinse the membrane several times with washing buffer, then block with blocking buffer, 1 h under gentle agitation.

28 Probe the membrane with antibody against target protein. Check the antibody datasheet for recommended antibody concentration.

29 Repeat step 19–22 for the detection of the protein.

*Modified biotin switch assay method*

1 Cells are lysed in RIPA buffer supplemented with protease inhibitors (10 μg/mL antipain, 5 μg/mL pepstatin, 1 mM phenylmethylsulfonyl fluoride) on ice for 30 min.
2 Centrifuge samples at ≈14,000 × *g* at 4 °C for 30 min to pellet the cell debris.
3 Transfer supernatants to new Eppendorf tubes.
4 Quantify total proteins content using Lowry reagent and BSA standard curve.
5 Incubate 1 mg of proteins from cell lysates with 1 mM diamide or other oxidizing agent on ice for 30 min. For the control sample, incubate 1 mg of proteins from cell lysates without oxidant agents and follow the same procedure.
6 Transfer the samples in Amicon® Ultra spin desalting column (Millipore) and follow the manufacturer's instructions to remove cellular GSH and the oxidants in excess.
7 Transfer the collected desalted samples in new Eppendorf tubes.
8 Add 50 mM NEM and keep on ice for 20 min to stably alkylate the free thiols.
9 Reduce the thiol groups that are not alkylated by NEM with 60 mM DTT keeping the solution on ice for 20 min.
10 Transfer the samples in Amicon® Ultra column following the manufacturer's instructions to remove free NEM and DTT.
11 Transfer each sample in new Eppendorf tube.
12 Oxidize again the free thiol groups with 1 mM diamide or other oxidizing agents on ice for 30 min.
13 Incubate the samples with 1 mM BioGSH on ice for 30 min. Otherwise, the commercially available Biotinylated Glutathione ethylene ester (BioGEE, Molecular Probes, ThermoFisher Scientific) can be used to label the redox sensitive cysteine.
14 Transfer the samples in Amicon® Ultra column and follow the manufacturer's instructions in order to remove cellular GSH and the oxidants in excess.
15 Replace the buffer with 400 μL cold RIPA buffer supplemented with protease inhibitors (10 μg/mL antipain, 5 μg/mL pepstatin, 1 mM phenylmethylsulfonyl fluoride) and transfer each sample in new Eppendorf tube.
16 Quantify proteins content using Lowry reagent and BSA standard curve
17 Incubate 1 mg of proteins with 100 μL of slurry agarose beads in Eppendorf tubes at 4 °C for 1 h under gentle rotation. This pre-clearing step can help to reduce non-specific binding and background.
18 Centrifuge samples at ≈1000 × *g* at 4 °C for 1–2 min.
19 Transfer supernatants to new Eppendorf tubes and add 100 μL of streptavidin-agarose beads. Mix under rotation at 4 °C for 2 h.
20 Centrifuge samples at ≈1000 × *g* at 4 °C for 1–2 min.
21 Collect the beads and discard the supernatants. Add 200 μL of RIPA buffer to the tube with the beads and gently mix. Centrifuge samples at ≈1000 × *g* at 4 °C for 1–2 min and discard the supernatants. Repeat this wash twice.
22 Add 30 μL of 2X Sample buffer to the collected beads and heat the samples at 100 °C for 3 min.
23 Centrifuge samples at ≈1000 × *g* at 4 °C for 1–2 min and collect the supernatants for SDS-PAGE and Western Blot analysis.
24 Separate Streptavidin pull-down samples on a 5–10% SDS–polyacrylamide gel (SDS-PAGE) in running buffer following the manufacturer's instructions of the electrophoresis apparatus.
25 Transfer proteins to PVDF membrane using transfer buffer following the manufacturer's instructions of the transfer apparatus.
26

Block non-specific binding on the membrane by incubation in blocking buffer at room temperature for 1 h under gentle agitation.

27 Probe the membrane with primary antibody against target protein overnight at 4 °C under gentle agitation using the recommended dilution in blocking solution. Check the antibody datasheet for recommended antibody dilution.

28 Wash the membrane in washing buffer for 5 min under gentle agitation. Repeat wash three times.

29 Incubate the membrane with secondary antibody peroxidase-conjugated for 1 h at room temperature under gentle agitation using the recommended dilution in blocking solution. Check the antibody datasheet for recommended antibody dilution.

30 Wash the membrane in washing buffer for 5 min under gentle agitation. Repeat wash three times.

31 Detect protein-antibody reactions with chemiluminescent detection reagent following the manufacturer's instructions. Acquire images with an automated image acquisition system.

A workflow of representation of modified biotin switch procedure for the identification of S-glutathionylated protein is reported in Fig. 1.

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