Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Collection - JoVE Produced Video
Manuscript Number:	JoVE62671R1
Full Title:	Resin-assisted Capture Coupled with Isobaric Tandem Mass Tag Labeling for Multiplexed Quantification of Protein Thiol Oxidation
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Biochemistry
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Richland,WA,USA
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TITLE:

Resin-assisted Capture Coupled with Isobaric Tandem Mass Tag Labeling for Multiplexed Quantification of Protein Thiol Oxidation

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KEYWORDS:

RAC, TMT, thiol redox proteomics, cysteine, PTM stoichiometry, redox modifications

SUMMARY:

Protein thiol oxidation has significant implications under normal physiological and pathophysiological conditions. We describe the details of a quantitative redox proteomics method, which utilizes resin-assisted capture, isobaric labeling, and mass spectrometry, enabling site-specific identification and quantification of reversibly oxidized cysteine residues of proteins.

ABSTRACT:

Reversible oxidative modifications on protein thiols have recently emerged as important mediators of cellular function. Herein we describe the detailed procedure of a quantitative redox proteomics method that utilizes resin-assisted capture (RAC) in combination with tandem mass tag (TMT) isobaric labeling and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to allow multiplexed stochiometric quantification of oxidized protein thiols at the proteome level. The site-specific quantitative information on oxidized cysteine residues provides additional insight into the functional impacts of such modifications.

The workflow is adaptable across many sample types, including cultured cells (e.g., mammalian, prokaryotic) and whole tissues (e.g., heart, lung, muscle), which are initially lysed/homogenized and with free thiols being alkylated to prevent artificial oxidation. The oxidized protein thiols are then reduced and captured by a thiol-affinity resin, which streamlines and simplifies the workflow steps by allowing the proceeding digestion, labeling, and washing procedures to be performed without additional transfer of proteins/peptides. Finally, the labeled peptides are

eluted and analyzed by LC-MS/MS to reveal comprehensive stoichiometric changes related to thiol oxidation across the entire proteome. This method greatly improves the understanding of the role of redox-dependent regulation under physiological and pathophysiological states related to protein thiol oxidation.

INTRODUCTION:

Under homeostatic conditions, cells generate reactive oxygen, nitrogen, or sulfur species that help to facilitate processes, such as metabolism and signaling[1-3], extending to both prokaryotes and eukaryotes. Physiological levels of these reactive species are necessary for proper cellular function, also known as 'eustress'[1,4]. In contrast, an increase in oxidants that leads to an imbalance between oxidants and antioxidants can cause oxidative stress, or 'distress'[1], which leads to cellular damage. Oxidants transduce signals to biological pathways by modifying different biomolecules, including protein, DNA, RNA, and lipids. In particular, cysteine residues of proteins are highly reactive sites prone to oxidation due to the thiol group on cysteine, which is reactive towards different types of oxidants[5]. This gives rise to a diverse range of reversible redox-based posttranslational modifications (PTMs) for cysteine, including nitrosylation (SNO), glutathionylation (SSG), sulfenylation (SOH), persulfidation (SSH), polysulfidation (SS_nH), acylation, and disulfides. Irreversible forms of cysteine oxidation include sulfinylation (SO_2H) and sulfonylation (SO_3H).

Reversible oxidative modifications of cysteine residues may serve protective roles preventing further irreversible oxidation or serve as signaling molecules for downstream cellular pathways[6,7]. The reversibility of some thiol redox PTMs allows cysteine sites to function as "redox switches"[8,9], wherein changes in the redox state of these sites alter protein function to regulate their role in transient processes. The modulatory effects of redox PTMs[10] have been observed in many aspects of protein function[11], including catalysis[12], protein-protein interactions[13], conformation change[14], metal ion coordination[15], or pharmacological inhibitor binding[16]. Additionally, redox PTMs are involved in cysteine sites of proteins that regulate pathways such as transcription[17], translation[18], or metabolism[19]. Given the impact that redox PTMs have on protein function and biological processes, it is important to quantify the extent of oxidation that a cysteine site undergoes in response to a perturbation of the redox state.

The identification of cysteine sites with altered redox states is focused on the comparison of the oxidation state at the site-specific level between normal and perturbed conditions. Fold change measurements are often utilized to determine what sites are significantly altered as this helps users interpret what cysteine sites may be physiologically significant to the study. Alternatively, stoichiometric measurements of reversible thiol oxidation across a specific sample type give a general picture of the physiological state with respect to cellular oxidation, an important measurement that is often overlooked and underutilized. Modification stoichiometry is based on quantifying the percentage of modified thiol as a ratio to total protein thiol (modified and unmodified)[20,21]. As a result, stoichiometric measurements offer a more precise measurement than fold change, especially when using mass spectrometry. The significance of the increase in oxidation can be more readily ascertained by using stoichiometry to determine the PTM occupancy of a particular cysteine site. For example, a 3-fold increase in thiol oxidation could

result from a transition of as little as 1% to 3% or as big as 30% to 90%. A 3-fold increase in oxidation for a site that is only at 1% occupancy may have little impact on a protein's function; however, a 3-fold increase for a site with 30% occupancy at resting state may be more substantially affected. Stoichiometric measurements, when performed between total oxidized thiols and specific oxidative modifications, including protein glutathionylation (SSG) and nitrosylation (SNO), can reveal ratios and quantitative information with respect to specific modification types.

Because reversible thiol oxidation is typically a low-abundance posttranslational modification, multiple approaches have been developed for the enrichment of proteins containing these modifications out of biological samples. An early approach devised by Jaffrey and others, named the biotin switch technique (BST)[22], involves multiple steps wherein unmodified thiols are blocked through alkylation, reversibly modified thiols are reduced to nascent free thiols, nascent free thiols are labeled with biotin, and the labeled proteins are enriched by streptavidin affinity pulldown. This technique has been used to profile SNO and SSG in many studies and can be adapted to probe for other forms of reversible thiol oxidation[23,24]. While BST has been utilized to probe for different forms of reversible thiol oxidation, one concern with this approach is that enrichment is impacted by the non-specific binding of unbiotinylated proteins to streptavidin. An alternate approach developed in these authors' laboratory, named resin-assisted capture (RAC)[25,26] (**Figure 1**), circumvents the issue of enrichment of thiol groups via the biotin-streptavidin system.

Following the reduction of reversibly oxidized thiols, proteins with nascent free thiols are enriched by the thiolpropyl sepharose 6B resin, which covalently captures free thiol groups, allowing for more specific enrichment of cysteine-containing proteins than BST. Coupling RAC with the multiplexing power of the recent advances in isobaric labeling and mass spectrometry creates a robust and sensitive workflow for the enrichment, identification, and quantification of reversibly oxidized cysteine residues at the proteome-wide level. Recent advances in mass spectrometry have enabled much deeper profiling of the thiol redox proteome, increasing the understanding of both the cause and effect of protein thiol oxidation[27]. The information gained from site-specific quantitative data allows for further studies of the mechanistic impacts and downstream effects of reversible oxidative modifications[28]. Utilizing this workflow has provided insight into the physiological impacts of reversible cysteine oxidation with respect to normal physiological events such as aging, wherein levels of SSG differed with respect to age. The aging effects on SSG were partially reversed using SS-31 (elamipretide), a novel peptide that enhances mitochondrial function and reduces SSG levels in aged mice, causing them to have an SSG profile more similar to young mice[29].

Pathophysiological conditions attributed to nanoparticle exposure have been shown to involve SSG in a mouse macrophage model. Using RAC coupled with mass spectrometry, the authors showed that SSG levels were directly correlated to the degree of oxidative stress and impairment of macrophage phagocytic function. The data also revealed pathway-specific differences in response to different engineered nanomaterials that induce different degrees of oxidative stress[30]. The method has also proven its utility in prokaryotic species, where it was applied to

study the effects of diurnal cycles in photosynthetic cyanobacteria with respect to thiol oxidation. Broad changes in thiol oxidation across several key biological processes were observed, including electron transport, carbon fixation, and glycolysis. Furthermore, through orthogonal validation, several key functional sites were confirmed to be modified, suggesting regulatory roles of these oxidative modifications[6].

Herein, we describe the details of a standardized workflow (**Figure 1**), demonstrating the utility of the RAC approach for the enrichment of total oxidized cysteine thiols of proteins and their subsequent labeling and stoichiometric quantification. This workflow has been implemented in studies of the redox state in different sample types, including cell cultures[27,30] and whole tissues (e.g., skeletal muscle, heart, lung)[29,31-33]. While not included here, the RAC protocol is also easily adapted for the investigation of specific forms of reversible redox modifications, including SSG, SNO, and S-acylation, as previously mentioned[25,29,34].

PROTOCOL:

All procedures described in the protocol related to animal or human samples/tissues were approved by and followed the institutional guidelines of the human and animal research ethics committee.

1. Sample homogenization/lysis

1.1. Frozen tissue samples

1.1.1. Mince frozen tissue (~30 mg) on a glass microscope slide on dry ice using a prechilled razor blade and forceps. Transfer the minced tissue to a prechilled 5 mL round-bottom polystyrene tube containing 700 µL of buffer A (see **Table 1**) and incubate on ice for 30 min, protected from light.

1.1.2. Disrupt the tissue for 30 s or until completely homogenized with a hand-held tissue homogenizer. Place the samples on ice and allow the foam to subside for another 10 min.

NOTE: An aluminum baking sheet placed on dry ice provides a stable working surface and platform for the initial processing/mincing of the tissue.

1.2. Alternatively, use adherent cell cultures in 100 mm culture dishes as the starting material.

1.2.1. Keep the cells on ice and use a serological pipette to rinse the cells twice with 10 mL of ice-cold PBS containing 100 mM NEM.

1.2.2. Lyse the cells by adding 1 mL of cold homogenization/lysis buffer and scraping vigorously with a rigid cell scraper. Transfer the lysate to a 2 mL centrifuge tube using a micropipette.

NOTE: Rinsing buffer and lysis buffer may be scaled accordingly to different size culture vessels.

Typically, 2–5 million cells are required; however, this varies depending on the lysis efficiency and protein yield for specific cell types. Homogenization buffer may be prepared without NEM for samples being analyzed for total thiols.

1.3. Transfer the resulting homogenate (step 1.1.2 or 1.2.2) to a 2 mL centrifuge tube using a micropipette, and centrifuge at full speed (≥16,000 × *g*) and 4 °C for 10 min.

1.4. Transfer the supernatant (~700 µL or ~1 mL for cell culture) using a micropipette to a 5 mL conical microcentrifuge tube and incubate for 30 min at 55 °C in the dark with shaking at 850 rpm.

1.5. Using a glass serological pipette, add 4 mL of ice-cold acetone to the samples and incubate at -20 °C overnight for precipitation of protein and removal of excess *N*-ethylmaleimide.

2. Resin-assisted capture

2.1. Wash the precipitated protein pellets twice with acetone by centrifuging at 20,500 × *g* and 4 °C for 10 min, decanting the acetone, removing any remaining acetone using a micropipette, and adding 3 mL of fresh, ice-cold acetone using a glass serological pipette. Invert several times to mix. After the second wash, allow the pellets to air-dry for 1–2 min, being careful not to over-dry as resuspension may become difficult.

2.2. Using a micropipette, add 1 mL of buffer B (see **Table 1**) and solubilize the protein using repeated sonication for 15–30 s at a time using a bath sonicator with an output of 250 W and brief vortexing. Measure the protein concentration using the bicinchoninic acid (BCA) assay according to the manufacturer's protocol.

2.3. To standardize the protein concentrations across samples for further processing and ensure complete removal of NEM, transfer 500 µg of protein to a 0.5 mL 10 kDa centrifugal filter using a micropipette and adjust to a final volume of 500 µL with resuspension buffer.

2.4. Centrifuge at 14,000 × *g* at room temperature until the volume in the centrifugal filter is less than 100 µL. Collect the samples by inverting the filter in a collection tube. Centrifuge at 1,000 × *g* for 2 min and adjust to a final volume of 500 µL using buffer C (see **Table 1**).

2.5. Reduce the protein thiols by adding 20 µL of 500 mM dithiothreitol (DTT) using a micropipette to a final concentration of 20 mM and incubating the samples for 30 min at 37 °C while shaking at 850 rpm.

2.6. After reduction, transfer the samples using a micropipette to 0.5 mL 10 kDa centrifugal filters and centrifuge for 15 min at 14,000 × *g* at room temperature or until the volume in the centrifugal filter is less than 100 µL. Add buffer D (see **Table 1**) to make up the volume in the centrifugal filter to 500 µL.

2.6.1. Repeat the centrifuging and addition to 500 µL in step 2.6 three times, and after the fourth centrifugation, collect the samples by inverting the filter in a collection tube and centrifuging at 1,000 × *g* for 2 min.

2.7. Measure the protein concentration using the BCA assay according to the manufacturer's protocol.

2.8. During this buffer exchange, prepare the thiol-affinity resin by weighing the appropriate amount of resin (30 mg/sample) using a microbalance and transferring it to a 50 mL centrifuge tube. Then, using a serological pipette, add water for a final concentration of 30 mg/mL resin and incubate at room temperature for 1 h with agitation for proper hydration of the resin.

NOTE: The thiol-affinity resin mentioned above has been discontinued by the manufacturer. A possible replacement for this thiol-affinity resin is commercially available. However, this replacement has a nearly 5-fold less binding capacity (see **Supplemental Information**). Alternatively, the thiol-affinity resin can be synthesized using 2-(pyridyldithio) ethylamine hydrochloride and *N*-hydroxysuccinimide-activated resin (see **Supplemental Information**).

2.8.1. After hydration of the resin, place the spin columns on a vacuum manifold and transfer 500 µL of the resin slurry using a micropipette to each column. Apply vacuum for removal of water; repeat this step once to obtain a total of 30 mg of resin per column. Alternatively, centrifuge at 1,000 × *g* for 2 min instead of using the vacuum manifold for this and all the resin washing and elution steps.

NOTE: Cutting the end of a 1000 µL pipette tip to increase the bore size helps with the transfer of the resin. It is important to triturate between pipetting to ensure that the resin remains suspended and homogeneous and equal amounts of resin are transferred to each column.

2.8.2. Wash the resin by adding 500 µL of ultrapure water with a micropipette and applying vacuum for removal of the water; repeat this 5 times. Then, wash the resin 5 times with 500 µL of buffer E (see **Table 1**).

NOTE: Alternatively, centrifugation at 1,000 x *g* for 2 min may be used in place of a vacuum manifold for all subsequent wash steps. All the proceeding wash steps are performed with a volume of 500 µL. When adding wash buffers to the column, carefully add with enough force to fully resuspend the resin while avoiding splashing and loss of resin; this allows for complete and efficient washing of resin.

2.9. Using a micropipette, transfer 150 µg of protein from each reduced sample to a new tube and adjust to a final volume of 120 µL of buffer C (see **Table 1)**. Transfer the protein solution using a micropipette to a plugged spin column containing the resin, place the cap on the column, and incubate for 2 h at room temperature with shaking at 850 rpm.

2.10. Wash the resin five times with 25 mM HEPES, pH 7.0; 8 M urea; followed by five times with

2 M NaCl; followed by five times with 80% acetonitrile (ACN) with 0.1% trifluoroacetic acid (TFA); and finally five times with 25 mM HEPES, pH 7.7, as described in step 2.8.2 and replace the plug.

NOTE: Samples may be eluted here for analysis at the protein level (e.g., SDS-polyacrylamide gel electrophoresis (SDS-PAGE), western blot) as described in step 4.1.

3. On-resin tryptic digestion and TMT labeling

3.1. Prepare enough sequencing-grade modified trypsin solution for 6–8 µg per sample by solubilizing it at a concentration of 0.5 µg/µL in buffer C (see **Table 1**) so that the final volume allows for at least 120 µL per sample. Using a micropipette, add 120 µL of this trypsin solution to the samples and incubate overnight at 37 °C with shaking at 850 rpm.

NOTE: To increase the digestion efficiency, an additional digestion step can be included the next day by removing the trypsin solution and replacing it with fresh solution, and continue the digestion for 2 h.

3.2. Wash the resin five times with 25 mM HEPES, pH 7.0; followed by five times 2 M NaCl; followed by five times with 80% ACN with 0.1% TFA; followed by three times with 25 mM HEPES, pH 7.7. Finally, wash the resin two times with 50 mM triethyl ammonium bicarbonate buffer (TEAB) and replace the plug.

3.3. Prepare TMT labeling reagents by first allowing them to warm to room temperature before spinning down briefly using a centrifuge at 16,000 × *g*. Add 150 µL of anhydrous ACN to each vial of TMT labeling reagent using a micropipette. Incubate the vials at room temperature on a thermomixer set to 850 rpm for 5 min to solubilize the reagent completely. Briefly vortex and spin down at 16,000 × *g* to collect the reagent.

3.4. Using a micropipette, add 40 µL of 100 mM TEAB to the washed resin, then add 70 µL of the dissolved TMT reagent and incubate for 1 h at room temperature with shaking at 850 rpm. Store any remaining TMT reagent at –80 °C.

NOTE: Take note of the individual TMT labels assigned to each biological sample (**Figure 1**).

3.5. Wash the resin five times with 80% can with 0.1% TFA, three times with 100 mM ammonium bicarbonate buffer (ABC), pH 8.0, and twice with water as previously described and replace the plug.

4. Peptide elution

4.1. Elute the labeled peptides by adding 100 µL of 20 mM DTT in 100 mM ABC, pH 8.0, to each column using a micropipette and incubate at room temperature for 30 min on a thermomixer set to 850 rpm.

NOTE: After the addition of DTT, the resin will clump. The resin can be disrupted with a pipette tip to break up clumps and ensure the complete elution of the peptides.

4.2. After this incubation, place the column on a vacuum manifold intended for solid-phase extraction (SPE), apply vacuum, and elute the samples into a 5 mL microcentrifuge tube. Repeat this step once.

4.3. Finally, add 100 µL of 80% ACN with 0.1% TFA, incubate for 10 min at room temperature, and elute into the same 5 mL centrifuge tube. Collect all the fractions in the same 5 microcentrifuge tube.

NOTE: To prevent sample loss, low protein-/peptide-binding tubes must be used for elution, and volumes must be kept at or below a volume of 4.0 mL (10 samples) for a single 5 mL tube.

4.4. Place the eluted samples in a vacuum concentrator until dry. Store the dry peptides at –80 °C and resuspend them later.

NOTE: Samples may also be eluted separately, and an aliquot may be removed and analyzed by SDS-PAGE for analysis at the peptide level before combining the samples.

5. Peptide alkylation and desalting/clean-up

5.1. Resuspend the dried peptides by adding a small volume of 100 mM ABC buffer, pH 8.0 (no greater than 500 µL), using a micropipette. Use repeated sonication for 15–30 s at a time using a bath sonicator with an output of 250 W and vortex to solubilize and transfer to a 2 mL tube.

NOTE: The volume of 100 mM ABC, pH 8.0, to be added is based on the volume needed to resuspend the DTT at a molarity of 150 mM. Users will need to determine the amount of DTT present in their sample based on what was added originally in step 4.1.

5.2. Add enough concentrated stock solution (600 mM) of iodoacetamide (IAA) dissolved in ABC using a micropipette to achieve a 1:4 molar ratio of DTT:IAA and incubate the samples at RT for 1 h with shaking at 850 rpm.

5.3. Acidify the samples to pH < 3 by adding concentrated TFA (10%) using a micropipette and perform sample desalting using reverse-phase clean-up according to the manufacturer's instructions.

5.4. Place the clean peptides in a vacuum concentrator until dry. Store the dry peptides at –80 °C until further analysis.

6. Liquid chromatography-tandem mass spectrometry

6.1. Resuspend the dried peptides by repeated sonication for 15–30 s at a time using a bath

sonicator with an output of 250 W and vortexing in 20–40 µL of water containing 3% ACN. Determine the peptide concentration by performing a BCA assay according to the manufacturer's protocol.

6.2. Separate the samples by reversed-phase LC and MS/MS as previously described[6] and record the MS1 spectra over the *m/z* range of 400–2000. Ensure high-energy collisional dissociation (HCD) to obtain reporter ion intensity information for the analysis of isobarically labeled peptides. See the methods sections of previous reports for more details about instrument run conditions[27,30] and the analysis of MS data[27,31].

NOTE: Different LC-MS/MS systems or settings can be used to analyze the peptide samples. The coverage and sensitivity of peptide identification will depend on the particular system and settings used.

REPRESENTATIVE RESULTS:

Completion of the protocol will result in highly specific enrichment of formerly oxidized cysteine-containing peptides, often with >95% specificity[27,35,36]. However, several key steps of the protocol require special attention, e.g., the initial blocking of free thiols prior to sample lysis/homogenization, which prohibits artificial oxidation and non-specific enrichment of artificially oxidized thiols[25]. Samples may be analyzed at several stages of the protocol and by different methods, including SDS-PAGE analysis of both proteins and peptides. SDS-PAGE allows for qualitative analysis of samples wherein total-thiol samples enable ratio-metric comparisons between samples for determining different levels of oxidation due to treatments/stimuli (**Figure 2A**). To further investigate the oxidation levels of individual proteins, SDS-PAGE gels may be subjected to western blotting[36] (**Figure 2B**). This enables the model system to be analyzed in greater detail, generating supporting data and further hypotheses about networks and biological pathways. These methods/supporting data can also be utilized as quality control to confirm the expected responses before further in-depth analysis such as LC-MS/MS. Reporter ion intensities of cysteine-containing peptides analyzed by LC-MS/MS can be used to quantify thiol oxidation stoichiometry at individual Cys site levels (**Figure 2C,D**).

FIGURE AND TABLE LEGENDS:

Figure 1: Sample processing workflow. The sample processing workflow is adaptable for investigating thiol oxidation in various sample types and biological systems. The workflow allows for investigation of oxidation at both the protein and peptide levels (e.g., SDS-PAGE, western blot) as well as deep coverage for quantitative, site-specific identification of individual cysteine sites using HPLC coupled with mass spectrometry. Sample processing can be completed in as little as three days, including the completion of several critical steps for the generation of quality, consistent data. Sample multiplexing via TMT labeling allows for the processing of multiple samples in parallel at the same time. The representative 10-plex TMT labeling scheme illustrates how samples can be arranged considering the potential crosstalk from the total-thiol channel. With the isotopic impurities of the TMT reagents, the signal intensity of one channel with high intensity (such as total-thiol) can contribute to another channel with low signal intensity and

influence its quantification[37]. In the scheme, a pooled total-thiol channel (a combination of control and experimental samples) is expected to contain high levels of Cys-peptides and is labeled with 131N, which will have a signal in channel 130N. Thus, channel 130N is not used in the experiment. The amount of channel crosstalk created by TMT labels can be found in the manufacturer's certificates of analysis for a corresponding batch of the reagent. This figure has been modified from [25]. Abbreviations: NEM = *N*-ethylmaleimide; DTT = dithiothreitol; SDS-PAGE = sodium dodecylsulfate polyacrylamide gel electrophoresis; SPE = solid-phase extraction; LC-MS/MS = liquid chromatography-tandem mass spectrometry; TMT = tandem mass tag.

Figure 2: Analysis of peptides from RAC enrichment. (**A**) SDS-PAGE analysis of oxidized peptides from RAW 264.7 cells treated with the chemical oxidant, diamide, for 30 min at increasing concentrations (0.1 and 0.5 mM) and total peptide thiols. This sub-figure has been modified from [25]. Peptides were visualized by silver staining. (**B**) RAW 264.7 cells were treated with exogenous oxidants (hydrogen peroxide and diamide) at increasing concentrations. The resulting SSG-enriched protein eluate was separated by SDS-PAGE and subsequently probed by western blot for individual proteins (GAPDH, TXN, PRDX3, and ANXA1). This figure has been modified from [36]. (**C**) Representative MS/MS spectrum data of a cysteine-containing peptide viewed in Xcalibur software. The inset MS/MS image shows the corresponding reporter ion intensities for the same peptide in each TMT channel. In this experiment, the total-thiol sample was assigned to the TMT label 131N, which has the highest intensity of all channels used in the experiment. (**D**) Stoichiometry of iTRAQ-labeled, enriched, oxidized peptides as measured by LC-MS/MS. The total-thiol channel was used as a reference to calculate the stoichiometry of oxidation based on the ratio of reporter ion intensity of each sample compared to that of the total-thiol channel. Abbreviations: RAC = resin-assisted capture; SDS-PAGE = sodium dodecylsulfate polyacrylamide gel electrophoresis; Ctrl = control; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; TXN = thioredoxin; PRDX3 = thioredoxin-dependent peroxide reductase; ANXA1 = annexin A1; LC-MS/MS = liquid chromatography-tandem mass spectrometry; MS/MS = tandem mass spectrometry; TMT = tandem mass tag; iTRAQ = isobaric tag for relative and absolute quantitation.

DISCUSSION:

Resin-assisted capture has been utilized across a variety of sample types and biological systems for the investigation of oxidative modifications of cysteine residues[25,29,30]. This method allows for the evaluation of samples at multiple levels and readouts, including proteins and peptides using SDS-PAGE and western blot analysis, as well as individual cysteine sites using mass spectrometry. Regardless of the sample type or the final endpoint, the method ultimately allows for highly efficient and specific enrichment of cysteine-containing proteins and peptides[38]. Using RAC, we have identified changes in the oxidation state of up to several thousands of cysteine sites in different model systems following a perturbation.

In mice subjected to fatiguing muscle contractions, 2,200 *S*-glutathionylation sites were identified, with more than half having significantly altered levels of *S*-glutathionylation[32]. RAC has also been used to profile reversible thiol oxidation of >2,100 sites in cyanobacteria following exposure to a photosynthesis inhibitor or different light conditions[6]. Recently, we profiled total

reversible thiol oxidation and *S*-glutathionylation of >4,000 cysteine sites in RAW 264.7 macrophage cells under resting conditions[27]. Similarly, Behring et al. quantified the oxidation of ~4,200 cysteine sites in A431 cells following epidermal growth factor stimulation[39]. These studies from this laboratory demonstrate the robustness of RAC to identify many cysteine sites (at least several thousand) that undergo reversible thiol oxidation. Additionally, the fractionation of samples can enhance the coverage of peptides recovered from an experiment.

Outside of experimental controls, wherein either positive or negative control samples may be employed to confirm the biological responses of the model system, total-thiol enrichment may be performed in parallel with oxidation of thiols. This total-thiol sample provides both stoichiometric comparisons and a baseline from which experimental or treated samples may be compared. In short, this total-thiol sample provides a measurement of the total number of cysteine thiols for a given Cys site in a given sample. This concept was also adopted by the OxiTMT method, which generates samples that contain totally reduced thiols that represent "total cysteine content" for comparison against oxidized cysteines[40].

In contrast to OxiTMT, RAC is not constrained by the plex number of iodoTMT and thus can incorporate more total thiol channels to better represent the thiol content of multiple sample types used in a study. Additionally, preparing a global sample (not subject to enrichment) in parallel with the thiol redox proteomics workflow may be needed to check whether protein abundances are altered in different sample types. As the method is adaptable to multiple types of redox modifications, proper controls for the specific modification of interest must be considered. For example, ultraviolet light and mercury chloride are both effective at cleaving SNO from proteins, creating an effective negative control for the measurement of SNO[7,25]. An effective control for investigating SSG-modified proteins is the omission of the glutaredoxin enzyme from the reduction cocktail during the reduction step. Owing to the relatively high specificity of glutaredoxin, its omission eliminates the reduction of SSG-modified proteins and prevents them from undergoing disulfide exchange and ultimately from being enriched in the final analysis[36].

There are several steps within the workflow for RAC that are fundamental for the generation of quality, reproducible data. One of the first crucial steps, and the most important, is the alkylation/blocking of free thiols using the membrane-permeable alkylating agent, *N*-ethylmaleimide (NEM), which reacts rapidly across a broad pH range[41,42]. This step prohibits nascent thiols from being oxidized during sample processing and mitigates non-specific enrichment of these artificially oxidized thiols during disulfide exchange enrichment. Inadequate alkylation will result in an increased background and false-positive signal, as was identified in a previous report[6].

However, because of its high reactivity and ability to block free thiols, caution must also be taken to ensure its complete removal from samples prior to enrichment, where any remaining NEM can bind and interfere with resin coupling, ultimately resulting in the loss of signal due to a decrease in protein binding. This is accomplished by performing acetone precipitation and several rounds of buffer exchange using molecular weight cut-off filters. Monitoring and maintaining proper pH throughout the protocol is also crucial. A pH of 6.0 is maintained to

mitigate disulfide shuffling and the formation of mixed disulfides prior to enrichment. Other steps where extra care must be taken include the enrichment and elution steps, wherein pH values of 7.7 and 8.0 are required for proper enrichment and elution, respectively. Erroneous pH values during these steps will result in a decrease or loss of signal.

To date, there are many chemistry-based methods aside from RAC that are widely used for studying cysteine thiol oxidation, including the most used method, the biotin switch technique[43,44]. One thing that all these methods have in common, including RAC, is that they are based on indirect methods for the detection of oxidized cysteines. They rely on chemically modified intermediates of the original oxidized thiol for detection. However, a key attribute that sets RAC apart from other methods is the ability to collect multiplexed, quantitative data on specific cysteine sites of oxidized thiols.

The method is performed with proteins/peptides covalently bound to the resin, which allows proceeding steps (e.g., reduction, labeling, washing, digestion) to be carried out without further handling. By performing LC-MS/MS on multiplexed samples, datasets are generated that enable proteome-wide discoveries. The effects of a specific treatment or stimuli across multiple sample groups are observed at a global level, which enables the discovery of novel mechanisms and pathways. The fundamental workflow is highly adaptable to the end-users' specific needs and areas of interest. Orthogonal validation of findings observed in mass spectrometry data remains a challenge. Site-directed mutagenesis of a specific site and utilization of assays that investigate the consequent effects is a common but labor-intensive approach.

To screen candidate sites that may be biologically significant, bioinformatic studies may be used to learn more about the characteristics of a site with a high level of oxidation, such as a site's proximity to an active site or secondary structure[27]. Molecular dynamics simulations may prove to be of great value in future studies as they can model the effects of redox modifications on protein structure and provide insight into how a protein's function may be affected[13,45]. By implementing this robust strategy, we hope the scientific community will benefit by adapting this method to their own unique model system and expand the current knowledge of redox biology across many different models and biological systems.

ACKNOWLEDGMENTS:

Portions of the work were supported by NIH Grants R01 DK122160, R01 HL139335, and U24 DK112349

DISCLOSURES:

The authors declare no conflicts of interest, financial or otherwise.

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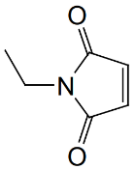
Fig. 1

Sample Processing Workflow

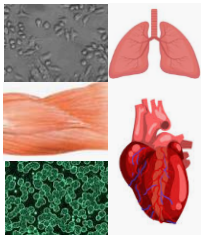
Parallel Processing of Samples from Different Conditions

Initial blocking of free thiols with lysis buffer containing NEM



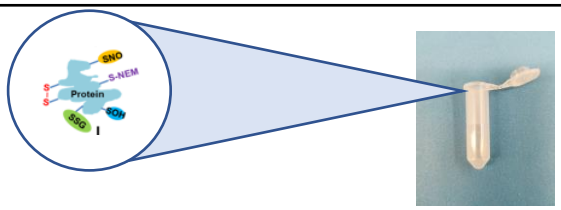
NEM

Penetration



! Critical Step
Block Free Thiols

Sample homogenization/cell lysis, protein extraction, precipitation, and resuspension

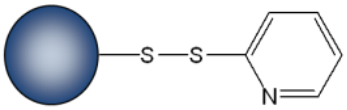


! Critical Step
Mitigate Oxidation
-pH (<7.0)
-temperature

Reduction of oxidative modification of interest (e.g. total oxidation)

DTT Reduction

Thiol-affinity enrichment



-S-S- Protein -NEM

On-resin tryptic digestion

Trypsin

-S-S- Peptide

On-resin multiplex isobaric labeling

TMT

-S-S- Peptide — Label

Elute peptides off resin

DTT elute

HS- Peptide — Label

-Combine
-Alkylation
-SPE cleanup

LC–MS/MS analysis

Protein/peptide level elution

SDS-PAGE, Western Blot

10-Plex TMT Labeling Scheme:

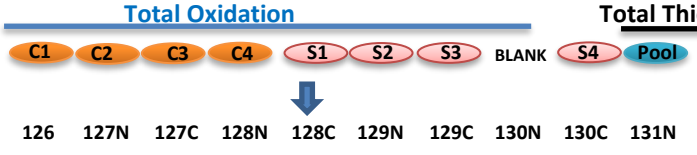


Fig. 2

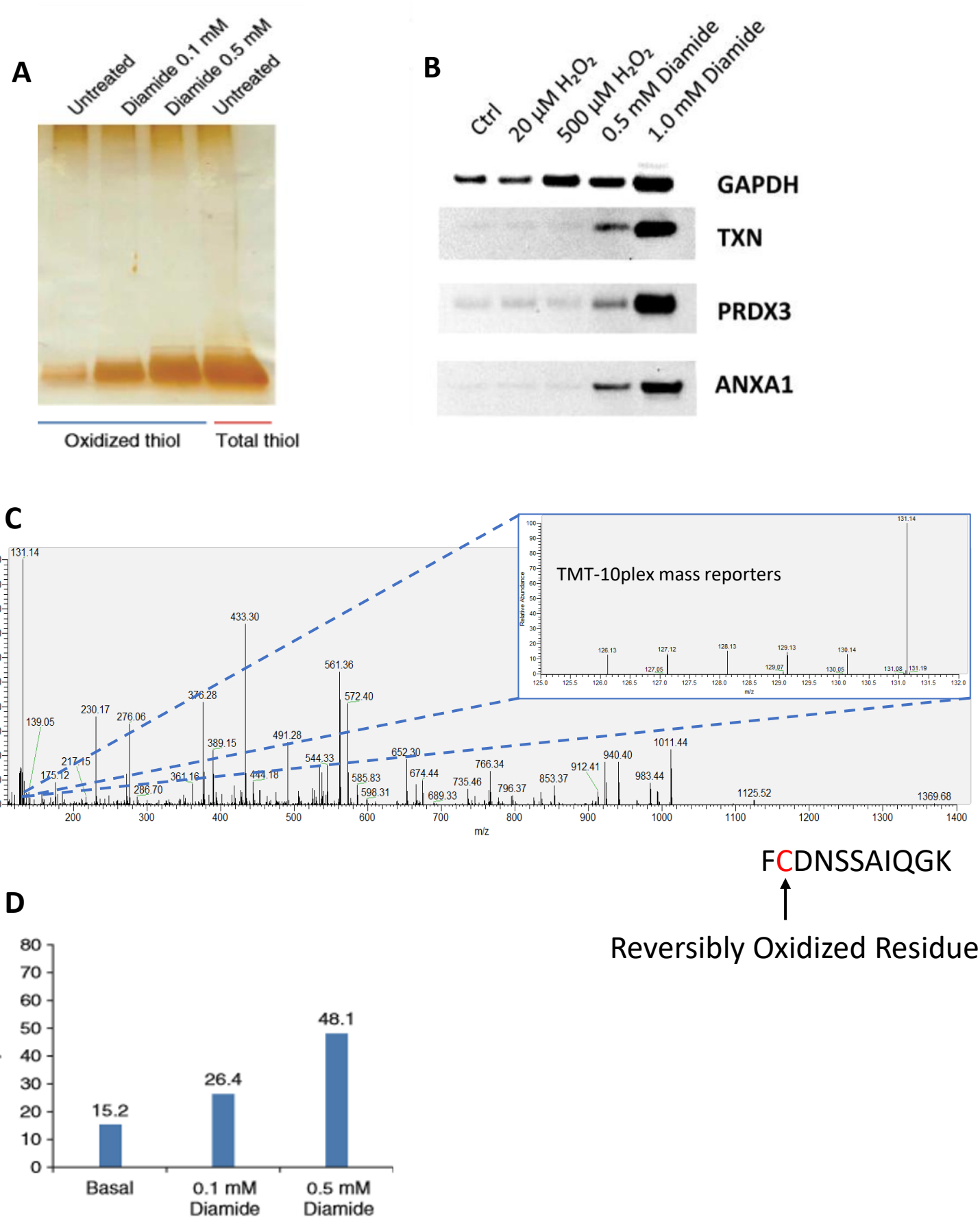


Table 1. List of buffers

Buffer name	Purpose
Buffer A	Lysis/homogenization
Buffer B	Resuspension following protein precipitation and first buffer exchange
Buffer C	Reduction/ Enrichment/ Digestion of Cysteine-containing proteins
Buffer D	Second buffer exchange following reduction
Buffer E	Washing the resin after hydration

Contents

250 mM 2-(*N* -morpholino)ethanesulfonic acid (MES), pH 6.0; 1% sodium dodecylsulfate (SDS); 1% Triton X-100; and 100 mM *N* -ethylmaleimide (NEM)
250 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.0; 8 M urea; 0.1% SDS

25 mM HEPES, pH 7.7; 1 M urea; 0.1% SDS
25 mM HEPES, pH 7.0, 8 M urea; 0.1% SDS
25 mM HEPES, pH 7.7

Click here to access/download

Table of Materials

RAC_JoVE_Materials_revision_v2.xls

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: We have proofread the manuscript.

2. Please provide an email address for each author.

Response: Email addresses of co-authors were added after the corresponding author.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Corning, Falcon, Tissue Tearor tissue homogenizer (Biospec Products), (Eppendorf), (Thermo Fisher), (Millipore Sigma), Sepharose4 Fast Flow resin (Sigma Aldrich), Pierce spin columns (Thermo Fisher), Qiavac 24 Plus vacuum manifold (Qiagen), Promega, LoBind, etc.

Response: Commercial language has been removed.

4. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human/animal research ethics committee.

Response: We have included a statement as a note at the beginning of the protocol section to inform users that their samples must be collected ethically based on IACUC standards.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

Response: The protocol section has been revised such that it is written in the imperative tense.

6. The Protocol should contain only action items that direct the reader to do something.

Response: The protocol has been revised to contain only action items.

7. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

Response: Protocol steps have been revised to reduce the number of actions per step.

8. Only one note can follow one step. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

Response: The number of notes per step has been edited in accordance with this policy.

9. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

Response: Protocol steps have been edited to include more details to inform readers of how the procedure should be completed.

10. What kind of tissues/cells are used in the study? what is the origin of the tissues?

Response: An additional statement in the final paragraph of the introduction has been included to elaborate on the different sample types that have been investigated using this workflow. The associated citations can direct readers to a more in-depth explanation of their origins. This builds upon the findings described in the second to last paragraph of the introduction, which describes some findings from several applications of the workflow for different sample types and are also brought up again in the discussion. Additionally, the data presented in figure 2 demonstrates the application of the workflow for different sample types as well. The reader is directed to the origins of the data/sample via citations in

the figure legend.
11. 6: For this step please include button clicks and knob turns to show how the step is performed.
Response: Depending on the make and model of an LC-MS/MS system, the "button clicks" and "knob turns" will vary. For this reason, we do not include a step by step procedure of this part of the protocol, but rather mention a few critical details of parameters that are necessary for a successful run. Users of this protocol that are unfamiliar with operation of an LC-MS/MS system should consult a trained LC-MS/MS operator on how to run their sample on the instrument. Additionally, we have included a citation with more details of the LC-MS/MS run that will guide an operator on how to run the instrument in a similar fashion as is done at our institution.
12. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Response: We have highlighted the part of the protocol that we think is necessary/essential content for readers to observe in the film.
13. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
Response: The copyright permissions have been uploaded. And the figure has been cited in the figure legend accordingly.
14. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Response: The content of discussion section addresses these points.
15. Please sort the materials table in alphabetical order.
Response: The table of materials has been re-sorted by alphabetical order.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This is a protocol by Gaffrey et. al describing an RAC approach to assess thiol oxidation of proteins in both cell lines and tissue samples. The assay is coupled with quantitative redox proteomics and the outlined protocol allows not only identification of oxidized proteins but also to determine stoichiometry of oxidation. Given the physiological significance of protein oxidation, this protocol could be a valuable addition to JoVE library. However, some issues are needed to be addressed.

Major Concerns:

1. Currently, Thiopropyl Sepharose 6B resin has been discontinued by the manufacturer and the protocol for in-house synthesis has not been published. However, Activated Thiol-Sepharose® 4B is still

commercially available (Sigma, T8512). It needs to be discussed if 4B resin can be used in the assay and if it is suitable, this needs to be validated experimentally.

Response: We indeed performed experiments where a side-by-side comparison of the binding capacity of Activated Thiol-Sepharose® 4B versus Thiopropyl Sepharose 6B resin was conducted. The major caveat of 4B resin was the observed nearly five-fold decreased binding capacity based on the total amount of enriched peptides. This information is noted in the revised text and provided in the supplemental information.

2. Other BST techniques employ more selective approach to thiol reduction to detect specific cysteine modifications. In this protocol, 20 mM DTT does not discriminate between many cysteine PTMs. In particular, DTT will likely cleave the thioester bond between cysteine and a fatty acid moiety. Do authors expect to detect S-palmitoylated proteins by this method as well? If yes, this needs to be discussed.

Response: Indeed, different types of modifications can be selectively reduced by different reductants (as reported in our previous manuscripts). In this case, DTT is used as a general reductant for reducing and enriching all reversible oxidative modifications within a sample. If other types of modifications, including S-palmitoylation, are of interest, different reductive strategies can be applied using a similar protocol.

Minor Concerns:
1. 165-166 Please specify the centrifugal force (x g)
Response: The centrifugal force has been included in the text.
2. 170 Please explain why acetone is added (i.e. to precipitate the protein)
Response: An explanation of acetone usage has been included in the protocol.
3. 174 Please note that pellets are being washed not just "samples"
Response: The word usage has been corrected.
4. 177 Please specify "briefly".
Response: We have elaborated on the length of time for drying the pellet
5. 177 For frequently used buffers, names can be designated (for example Buffer A or resuspension buffer).
Response: We have included designations where possible.
6. 178 What are sonication conditions?
Response: We have included sonication conditions where they are necessary.
7. 182 Please explain the purpose of the step, i.e. is it concentration, purification etc.
Response: We have elaborated on the purpose of this step in our revision and find it important for standardizing all samples before moving on to the next step in the protocol.
8. 187 Please specify the concentrations of stock DTT. What is the recommended final volume? How much DTT would you add based on the provided example (500 ul)?
Response: We have included the stock DTT molarity info in the revised manuscript.
9. 232 What is recommended elution procedure/buffer?
Response: Elution must be performed with 2 aliquots of 100 µl of 100 mM ammonium bicarbonate buffer; pH 8.0 containing 20 mM DTT followed by one aliquot of 80% acetonitrile with 0.1% TFA as described in step 4.

Reviewer #2:

Manuscript Summary:
The manuscript "Resin Assisted Capture Coupled with Isobaric Tandem Mass Tag Labeling for Multiplexed Quantification of Protein Thiol Oxidation" is a detailed description of the experimental procedures to perform the quantitative thiol redox proteomics analysis according to the already published method based on the resin assisted capture (RAC) published in 2014 by the same authors. The manuscript is well written and sufficient details are furnished to reproduce the workflow. However, some improvement still can be performed.

Major Concerns:
1) It is not clear enough how the total thiol sample is produced. Are the protein extracts obtained from the same cell dish of the oxidized thiol sample? If not, the total thiol sample is not the same of the oxidized thiols thus what is calculated is not the real stoichiometry of the sample. Similarly, how does the protocol work for tissues? Two different tissues of the same experimental condition could be very different.
Response: The total thiol sample is a representation of all the cysteine thiols of any given biological system or specific sample type (e.g. cell line, heart tissue, skeletal muscle) regardless of any treatment or physiological perturbance. The total thiol sample is generated by omitting N-Ethylmaleimde (NEM) from the lysis buffer constituents and leaving all thiols in that sample un-blocked/alkylated. In the case of a cell line or cell culture a separate sample can be used to represent the total thiol content of all the samples as all samples will have the same total thiol profile regardless of treatment or perturbance. For whole tissues or studies where true biological replicates are used (e.g. human, animal, etc.) the total thiol sample is generated by combining aliquots from a homogenous mixture of minced tissue from each sample prior to homogenization/lysis to mitigate biological variability between samples.

2) I think that the data mining process is part of the workflow and it is missing. The manuscript will be dramatically improved if supplemented with this section. In particular the authors should pay attention to how the ratios of the peptides carrying more than one cysteine in different redox states (NEM and IAA alkylated) are treated. In this case the partially modified peptide (1 NEM /1 IAA) and its fully DTT reduced form (IAA/IAA) have not the same mass and thus the TMT quantification of the site occupancy is not possible. How redundant data coming from the ratios of the peptides carrying the same cysteine but with different modifications (methionine oxidation, deamidation…) or with different trypsin miss cleavages are treated.
Response: The purpose of this manuscript was to provide an update and visual demonstration of the RAC method. We agree that data analysis will be important, and we anticipate releasing a more detailed description of the data analysis procedure in a future publication. To address this reviewer's concern here, in the note that follows the last step in the protocol, we provide citations of our previous works that contain more information about how analysis can be completed. To address the reviewer's concern about a cysteine site in different redox states, we perform our analysis using parameters that treat IAA and NEM as dynamic modifications, which allows us to quantify and compare the different states of the site.

3) The authors correctly highlight the underestimated challenge of the reduced thiol saturation. However, they didn't furnish sufficient evidences that 100 mM of NEM can saturate reduced thiols neither in this manuscript and in the previous paper. An example of reduced thiol saturation test in literature was showed by Shakir et al. in Anal Bioanal Chem. 2017 Jun;409(15):3821-3830. doi: 10.1007/s00216-017-0326-6. I think that a such kind of control is necessary in any thiol redox proteomics workflow.

Response: We appreciated the reviewer's comment on this important blocking step. We have demonstrated nearly complete blocking of all free thiols by using 100 mM NEM concentration in our previous work. The excessive amount of NEM plus its rapid reactivity to alkylate thiols should be sufficiently alkylated/blocked. Examples include nearly no signal in the untreated channel after blocking (Figure 3a, Guo et al, Nat. Protocols, 2014); and very little signals in pre-Ascorbate treatment channels (Figure 4A, Su. et al., FRBM, 2013), confirming the efficient blocking of free thiols by NEM.
We also agree that this will be a good control to be included to make sure the efficient blocking.

Paulech J, Solis N, Cordwell SJ. Characterization of reaction conditions providing rapid and specific cysteine alkylation for peptide-based mass spectrometry. Biochim Biophys Acta. 2013 Jan;1834(1):372-9. doi: 10.1016/j.bbapap.2012.08.002. Epub 2012 Aug 15. PMID: 22910378.
Guo J, Gaffrey MJ, Su D, Liu T, Camp DG 2nd, Smith RD, Qian WJ. Resin-assisted enrichment of thiols as a general strategy for proteomic profiling of cysteine-based reversible modifications. Nat Protoc. 2014 Jan;9(1):64-75. doi: 10.1038/nprot.2013.161
Su D, Shukla AK, Chen B, Kim JS, Nakayasu E, Qu Y, Aryal U, Weitz K, Clauss TR, Monroe ME, Camp DG 2nd, Bigelow DJ, Smith RD, Kulkarni RN, Qian WJ. Quantitative site-specific reactivity profiling of S-nitrosylation in mouse skeletal muscle using cysteinyl peptide enrichment coupled with mass spectrometry. Free Radic Biol Med. 2013 Apr;57:68-78. doi: 10.1016/j.freeradbiomed.2012.12.010. Epub 2012 Dec 28.

Minor Concerns:
1) Reduced thiol alkylation with NEM is performed at pH 6 while oxidized thiol alkylation with IAA is performed at pH 8. Despite NEM can react with thiols also at pH lower than 7, thiol reactivity is strongly influenced by the pH and reduced thiols could not be alkylated by NEM at pH 6 while alkylated by IAA at pH 8 generating false positives. This different reactivity could be enhanced by the fact that reduced thiols are alkylated at protein level, and thus partially buried, while oxidized thiols are alkylated at peptide level, and thus more accessible. Authors should discuss about this point.
Response: During the lysis and homogenization steps pH 6.0 is maintained to mitigate disulfide shuffling/exchange between intramolecular disulfides. Sodium dodecyl sulfate is added to assist in denaturization of proteins in addition to an incubation at 55°C for further denaturization of protein and complete alkylation, as observed in our mass spectrometry data. Alkylation of free thiols must be performed immediately to inhibit further oxidation during processing and enrichment of artificially oxidized thiols, therefore initial alkylation is required at the protein level. The second alkylation performed with IAA is done after enrichment and elution and is solely for analytical purposes. There are several studies displaying the broad range of pH in which NEM can react with little effect when pH is less than 7.0 is used. Alkylation reactions using iodoacetamide are commonly carried out at pH 8.0.
Bednar, R. A. (1990). Reactivity and pH dependence of thiol conjugation to N-ethylmaleimide: detection of a conformational change in chalcone isomerase. Biochemistry, 29(15), 3684-3690. doi:10.1021/bi00467a014
Paulech J, Solis N, Cordwell SJ. Characterization of reaction conditions providing rapid and specific cysteine alkylation for peptide-based mass spectrometry. Biochim Biophys Acta. 2013 Jan;1834(1):372-9. doi: 10.1016/j.bbapap.2012.08.002. Epub 2012 Aug 15. PMID: 22910378.
Suttapitugsakul S, Xiao H, Smeekens J, Wu R. Evaluation and optimization of reduction and alkylation methods to maximize peptide identification with MS-based proteomics. *Mol Biosyst*. 2017;13(12):2574-2582. doi:10.1039/c7mb00393e

2) I agree with the authors that the calculation of the site occupancy is more informative than the fold changes for the quantification of PTMs. However, my opinion is that proteomics analysis of the PTMs is

incomplete if the PTM quantification is not associated to the quantification of the protein level. For example, a decrease of the thiol oxidation occupancy could be the result of an increased biosynthesis of the protein reduced form instead of an oxidation/reduction event. This observation has an important impact on data interpretation. Performing the thiol enrichment at protein level, RAC approach avoids protein level quantification. Other methods such the OxiTMT strategy mentioned above (Shakir S. et al.) overcome this issue with an approach very similar to the here presented RAC. It would be very useful for the readers to learn about this aspect in the introduction.

Response: We introduced the OxiTMT method in our revision and found it more appropriate in the discussion section. We mention it as another method that also emphasizes the importance of including a sample that represents the "baseline" of all thiol present in a sample to be able to make the necessary percent oxidation calculations.

3) I think that authors are too optimist considering SDS PAGE more resolutive than mass spectrometry to study thiol oxidations. The validation of thiol oxidations is, at my opinion, one of the most difficult challenges of this field. Many reversible oxidations could not affect the protein electrophoretic mobility (in monodimensional SDS PAGE) and thus be not detectable. The oxidation of one cysteine in protein with a high number of this residue could have negligible effects. The use of MalPEG alkylating agent was introduced to improve the SDS separations between oxidized and reduced protein forms and to estimate the number of oxidized cysteines. However also this approach is very difficult to reproduce with contrasting results. It will be more useful to inform the readers about the challenge in the validation of the thiol redox proteomics data

Response: To clarify, our viewpoint is that mass spectrometry is the ideal method for studying thiol oxidation. However, SDS-PAGE can be used as an orthogonal method to evaluate broad changes in thiol oxidation in response to a perturbation or exposure to an oxidant (such as diamide, a shown in Figure 2A). We have changed our wording in the representative results section to avoid confusion with the readers. We thank this reviewer for raising the concern about validation of data and have added several sentences towards the end of our discussion to make readers aware of this challenge.

Supplemental Information:

Because the thiopropyl-sepharose 6B resin utilized in the protocol has been discontinued by the manufacturer, we have provided two alternative options.

First, while there is a commercially available alternative (activated thiol-sepharose 4B), preliminary data revealed nearly a five-fold decrease in binding capacity compared to the thiopropyl-sepharose 6B resin (**Figure S1**). Thus, the 4B resin needs to be used with consideration of its decreased binding capacity.

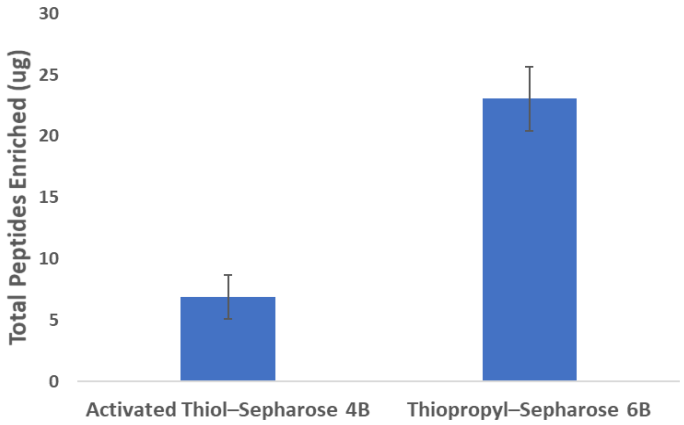


Figure S1. Comparison of binding capacity between Activated Thiol–Sepharose 4B Thiopropyl–Sepharose 6B resin. 200 µg proteins from RAW 264.7 cell lysate were used to enriched total cysteine-containing peptides. Error bars are representative of standard deviation of three independent replicates (n =3).

The second alternative is to synthesize the resin in-house using 2-(pyridyldithio) ethylamine hydrochloride and N-hyroxysuccinimide (NHS)-activated resin (NHS-Activated Sepharose 4 Fast Flow). This synthesized resin performs similarly to the 6B resin. A detailed procedure and assessment of the in-house resin synthesis and its performance, including supporting data, will be described elsewhere (Li et al., manuscript in preparation).

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Resin-assisted enrichment of thiols as a general strategy for proteomic profiling of cysteine-based reversible modifications

Author: Jia Guo et al

Publication: Nature Protocols

Publisher: Springer Nature

Date: Dec 12, 2013

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