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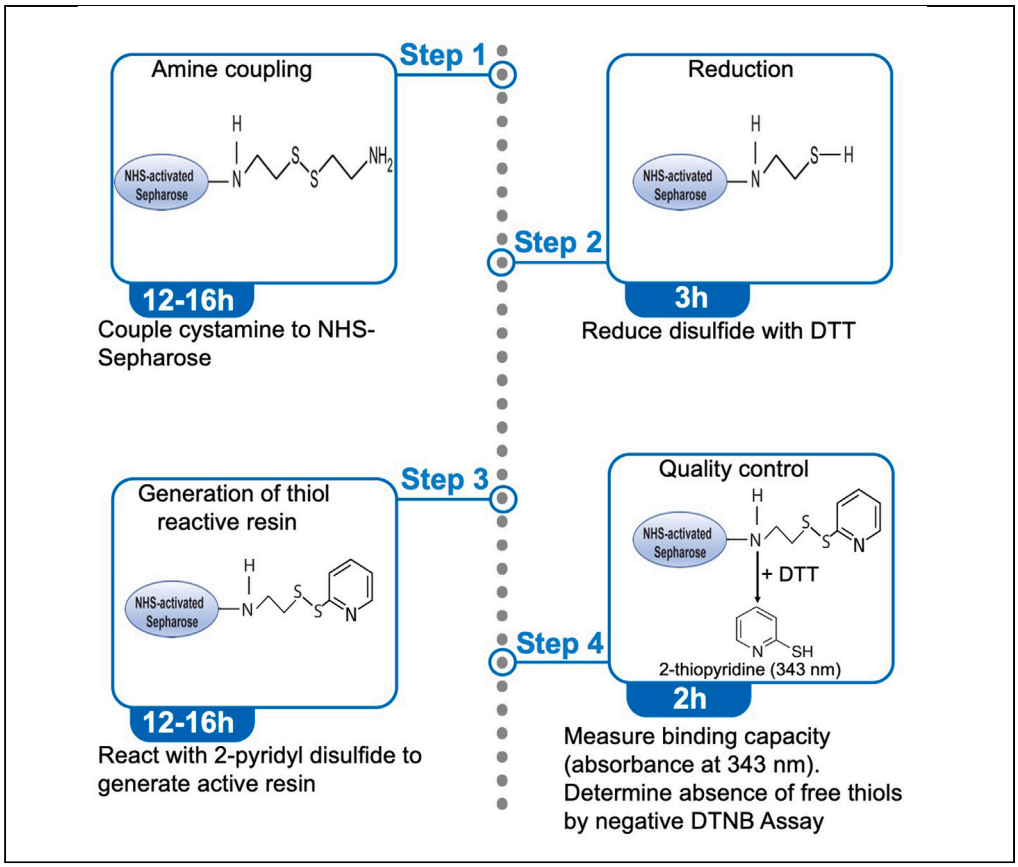
Protocol for preparing Thiopropyl Sepharose resin used for capturing S-nitrosylated proteins



S-nitrosothiol (SNO)-Resin Assisted Capture relies on a Thiopropyl Sepharose resin to identify S-nitrosylated proteins (SNO-proteins) and sites of S-nitrosylation. Here, we present a protocol for preparing Thiopropyl Sepharose resin with efficiency of SNO-protein capture comparable to the discontinued commercial version. We describe steps for amine coupling, disulfide reduction, and generation of thiol reactive resin. We then detail quality control procedures. This resin is also suitable for Acyl-RAC assays to capture palmitoylated proteins.

Puneet Seth, Alfred Hausladen, Richard T. Premont, Jonathan S. Stamler

jss156@case.edu

Highlights

Resin preparation protocol for the capture of S-nitrosylated proteins by SNO-RAC

This resin can be used for the capture of palmitoylated proteins by Acyl-RAC

Protocol includes steps and conditions for the proper storage of this resin

Protocol includes steps to ensure resin quality before its use

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Protocol for preparing Thiopropyl Sepharose resin used for capturing S-nitrosylated proteins

Puneet Seth,[1,2] Alfred Hausladen,[1] Richard T. Premont,[1,2,3] and Jonathan S. Stamler[1,2,3,4,5,*]

[1]Institute for Transformative Molecular Medicine, Case Western Reserve University, Cleveland, OH 44106, USA

[2]University Hospitals, Cleveland, OH 44106, USA

[3]Harrington Discovery Institute, University Hospitals, Cleveland, OH 44106, USA

[4]Technical contact

[5]Lead contact

*Correspondence: jss156@case.edu

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SUMMARY

S-nitrosothiol (SNO)-Resin Assisted Capture (SNO-RAC) relies on a Thiopropyl Sepharose resin to identify S-nitrosylated proteins (SNO-proteins) and sites of S-nitrosylation. Here, we present a protocol for preparing Thiopropyl Sepharose resin with efficiency of SNO-protein capture comparable to the discontinued commercial version. We describe steps for amine coupling, disulfide reduction, and generation of thiol reactive resin. We then detail quality control procedures. This resin is also suitable for Acyl-RAC assays to capture palmitoylated proteins.

For complete details on the use and execution of the SNO-RAC protocol, please refer to Forrester et al.,[1] Fonseca et al.,[2] and Seth et al.[3]

BEFORE YOU BEGIN

Background

The thiols of specific cysteine residues of proteins can be modified by nitric oxide (NO) to form S-nitrosothiols (SNOs). Proteins with this post-translational modification are referred to as S-nitrosylated (SNO-proteins). S-nitrosylation acts at active or allosteric sites to regulate protein function, stability, activity, localization, protein-protein interactions, etc.[4,5] It is estimated that ~70% of the proteome across phylogeny is regulated by S-nitrosylation,[6] representing the prototypic redox-based signaling mechanism under both aerobic and anaerobic conditions.

The S-nitrosothiol Resin Assisted Capture (SNO-RAC) method

The SNO-RAC method specifically captures SNO-proteins.[1] The method entails first blocking free cysteine thiols (i.e., SH groups that are not S-nitrosylated) with an alkylator such as S-Methyl methanethiosulfonate (MMTS) or N-ethylmaleimide (NEM), followed by ascorbate-mediated conversion of S-NO groups to free thiols (denitrosylation). These newly formed free thiols can then be chemically conjugated to a resin via a disulfide bond. After washing to remove non-specific binding of proteins to the resin, only the conjugated SNO-proteins remain bound to the resin. These proteins are subsequently eluted from the resin under reducing conditions and can be run on a polyacrylamide gel or otherwise processed for subsequent analysis. Similarly, hydroxylamine can be used to remove palmitoyl-groups from proteins and the resulting free thiol can be captured on these same beads in an Acyl-RAC protocol.[7]

While other methods are also capable of capturing S-nitrosylated proteins, including the Biotin Switch[8,9] or iodoacetyl-Tandem Mass Tag (TMT)-based immunoprecipitation methods,[10] these are more tedious, require extra steps, and are less efficient at capturing high molecular weight proteins.[1]

As such, numerous groups have adopted SNO-RAC as a means to capture SNO-proteins.[11–14] Additionally, the use of a thiol-reactive resin can avoid false positives in streptavidin-based detection assays that can arise from endogenous proteins binding biotin.[15] Also, the specificity of a thiol-reactive resin in the SNO-RAC method can be immediately visualized or quantified by performing a control lacking ascorbate (see below) and this specificity is particularly advantageous for mass-spectrometry analyses with very high sensitivity.

Widespread use of the SNO-RAC method to capture SNO-proteins is also due to its compatibility with peptide labeling methods including isobaric Tag for Relative and Absolute Quantification (iTRAQ) and TMT-based methods, followed by mass-spectrometry.[3]

Resin for the capture of S-nitrosylated proteins

Having an effective resin with robust binding capacity that can react chemically with free thiols from SNO-proteins is central to a successful SNO-RAC experiment. Until recently, most labs including ours, were routinely using the commercially available Thiopropyl Sepharose 6B resin (GE/Cytiva, Cat #17-0420-01) to capture proteins with free thiols in SNO-RAC and Acyl-RAC assays.[14,16–18] This resin also was widely available through various resellers. Unfortunately, GE discontinued this resin in 2020, and to the best of our knowledge there are no comparable commercial replacements available. In our hands, Acyl-RAC resins and other commercial resins with less than 5 μmol activated thiol/mL have yielded weaker and inconsistent binding of SNO-proteins among replicates, so we have returned to preparing our own SNO-RAC resin and have optimized and streamlined the procedure. The cost of preparing the SNO-RAC resin by this protocol is much lower than that of the discontinued GE resin and compared to commercially available Acyl-RAC resins. (Note that commercially available Acyl-RAC beads appear effective at capturing palmitoylated proteins, but again have proven to be far less efficient at capturing SNO-proteins in our hands.)

Here, we present the detailed protocol for preparing the SNO-RAC resin within the laboratory. In our hands, the SNO-RAC resin prepared by this protocol, when used in the amounts mentioned below, provides results that are comparable to the discontinued commercial resin, as measured by: (i) immunoblotting using antibodies against specific proteins, (ii) running the entire eluted SNO-proteome on a polyacrylamide gel followed by silver staining, or (iii) analysis of the captured SNO-proteome by mass-spectrometry.

This protocol will assist laboratories like ours that utilize the SNO-RAC method in preparing an effective and yet relatively inexpensive "in-house" resin that can be prepared in advance and stored in a refrigerator for a few weeks with no significant loss in ability to bind SNO-proteins. Additionally, we outline the steps for quality control of this resin, by measuring its free thiol binding capacity and for assuring its purity. Furthermore, we outline the steps for optimally equilibrating the stored resin prior to its use in a SNO-RAC reaction for the specific capture of S-nitrosylated proteins from cell or tissue lysates.

The resin prepared using the protocol below is being routinely used by our laboratory for the capture of SNO-proteins from lysates prepared from cultured human or mouse cells, mouse organs, and *C. elegans* after various treatments and conditions. In each case, it has been efficient at capturing the (ascorbate-reduced) S-nitrosylated proteins for further analysis.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal GAPDH (1:1000)	Proteintech 10494-1-AP	RRID:AB_2263076

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
NHS-activated Sepharose 4 Fast Flow	Cytiva	Cat # 17090601
Cystamine dihydrochloride	Sigma-Aldrich	Cat #C121509-100G
Phosphate buffer	Alfa Aesar	Cat #J60825
DTT	VWR	Cat #M109-25G
2-dipyridyl disulfide (Adrithiol™-2)	Sigma-Aldrich	Cat # 143049-25G
Isopropanol	Sigma-Aldrich	Cat #I9516-1L
Methanol	Sigma-Aldrich	Cat # 34860-1L-R
HEPES	Fisher	Cat # BP310-1
EDTA	Fisher	O2793-500
Neocuproine hydrochloride hydrate	Sigma-Aldrich	N1626-5G
SDS	Bio-Rad	Cat # 1610302
5,5′-Dithionitrobis(2-nitrobenzoic acid) [DTNB]	Sigma-Aldrich	Cat #D8130-5G
Reduced L-glutathione	Sigma-Aldrich	Cat #G4251-5G
Experimental models: Cell lines		
HEK 293	ATCC	CRL-1573
Experimental models: Organisms/strains		
Mouse, C57BL/6J (male, age 12 weeks)	The Jackson Laboratory	RRID:IMSR_JAX:000664
Other		
Centrifuge	Beckman Coulter	Allegra X-15A
Swinging bucket rotor	Beckman Coulter	SX4750A
UV-Vis spectrophotometer	Varian	4000
Multi Tube Rotator	VWR	Catalog # 76446-768

MATERIALS AND EQUIPMENT

HEN buffer		
Reagent	Final concentration	Amount
HEPES (1 M) pH 7.5	100 mM	100 mL
EDTA (0.5 M)	1 mM	2 mL
Neocuproine hydrochloride hydrate	0.1 mM	24.5 mg
Milli-Q H_2O	N/A	Up to 1000 mL
Total	**N/A**	**1000 mL**
Stored at 22°C–25°C for up to six months.		

STEP-BY-STEP METHOD DETAILS

⚠ CRITICAL: Use only Milli-Q water with resistance greater than 18.2 MΩ/cm at 25°C (or any commercially available equivalent) for making all reagents and perform all the steps at their indicated temperature. Also, since the resin preparation protocol involves three consecutive days, one must plan accordingly: once the protocol is initiated, we do not recommend storing intermediates beyond the two 12–16 h incubation steps in the detailed protocol.

Amine coupling

Timing: ~ 12–16 h at 4°C

This section accomplishes the coupling of Cystamine to Sepharose.

1. Add 3 mL of NHS-activated Sepharose slurry into a 50 mL tube.

Note: (i) NHS-activated Sepharose comes in a bottle as a ~50% slurry in 100% isopropanol. Shake the NHS-activated Sepharose slurry bottle well to resuspend settled beads. A good way to get an even slurry is by inverting the bottle 10–12 times until the settled NHS-activated Sepharose becomes fully resuspended in the slurry, as seen visually. (ii) Use a clean wide-bore pipette to measure the resuspended slurry and then transfer it to a clean 50 mL tube.

2. Centrifuge the tube at 1000 × *g* for 2 min at 4°C in a swinging-bucket rotor to separate the isopropanol from the beads and carefully remove the supernatant using a pipette or a needle aspirator.

Note: It is a good idea to use a swinging-bucket rotor throughout the preparation of the SNO-RAC resin because such rotors are more efficient at tightly pelleting the NHS-activated Sepharose beads. In comparison, when using a fixed-angle rotor, the NHS-activated Sepharose beads tend to stick to the wall of the tube to a greater degree, leading to greater loss of beads upon aspiration.

3. Rinse the slurry by adding 10 mL of 100 mM phosphate buffer (pH 8.0) to the tube.
   a. Mix by inverting the tube 10–12 times.
   b. Centrifuge at 1000 × *g* for 2 min at 4°C in a swinging-bucket rotor.
   c. Carefully remove the supernatant using a pipette or a needle aspirator.
4. Repeat step 3, for a total of two rinse steps with 100 mM phosphate buffer (pH 8.0).
5. Add 15 mL (5 times the initial 3 mL slurry volume) of freshly prepared 200 mM cystamine in 100 mM phosphate buffer (pH 8.0) and invert the slurry tube for 12–16 h at 4°C.

Washing after amine coupling

Timing: ~30 min at 4°C

This section accomplishes washing off the excess amine and performing buffer exchange in preparation for the steps outlined in the next section.

6. Centrifuge the tube at 1000 × *g* for 2 min in a swinging-bucket rotor and remove the unreacted cystamine supernatant using a pipette or aspirator.
7. Wash the resin thoroughly in the following sequence:
   a. Add 9 mL (3× the initial slurry volume) of 100 mM phosphate buffer (pH 8.0) to the tube.
      i. Resuspend the slurry thoroughly by inverting the tube several times.
      ii. Centrifuge the tube at 1000 × *g* for 2 min at 4°C in a swinging-bucket rotor.
      iii. Remove the phosphate buffer supernatant from the resin using a pipette.
   b. Repeat wash step 7(a) two more times, for a total of three washes with phosphate buffer.
   c. Add 9 mL (3× the initial slurry volume) of milli-Q water to the tube.
      i. Resuspend the beads thoroughly by inverting the tube several times.
      ii. Spin at 1000 × *g* for 2 min at 4°C in a swinging-bucket rotor centrifuge.
      iii. Remove the milli-Q water supernatant from the resin using a pipette or aspirator.
   d. Repeat wash step 7c two more times, for a total of three washes with milli-Q water.
   e. Add 9 mL (3× the initial slurry volume) methanol to the tube.
      i. Resuspend the beads by inverting 10–12 times.
      ii. Spin at 1000 × *g* for 2 min at 4°C in a swinging-bucket rotor centrifuge.
      iii. Remove the supernatant from the resin using a pipette or aspirator.
   f. Repeat wash step 7e two more times, for a total of three washes with methanol, and immediately proceed to the next step.

Note: To ensure minimum carryover of each wash buffer to the next step with a different wash buffer (such as between steps 7b and 7c and between steps 7d and 7e), withdraw as much wash buffer as possible using a 1 mL syringe with a 27 $^{1}/_{2}$ G needle (used due to its narrow lumen) inserted to the bottom of the beads, prior to the addition of the subsequent wash buffer.

Disulfide reduction

Timing: ~3 h, at 22°C–25°C

This section accomplishes the reduction of bound disulfides to free thiols.

Before proceeding to this next step, be sure aspirate out as much methanol as possible from the previous step using a 1 mL syringe with a 27 $^{1}/_{2}$ G needle, as mentioned in the note above, to minimize the carryover into this next step.

8. Reduce the bead-bound disulfides by adding 15 mL (5× the initial slurry volume) of freshly prepared 200 mM dithiothreitol (DTT) in 50 mM phosphate buffer (pH 8.0) and incubating the slurry tube at 22°C–25°C for 3 h with constant inversion using a Mini Tube Rotator (VWR Catalog # 76446-768) set at the rotating speed of 30–40 rpm, or equivalent.

Washing after disulfide reduction

Timing: ~30 min at 4°C

This section accomplishes washing off the excess reducing agent and performing buffer exchange in preparation for the steps outlined in the next section.

9. Centrifuge the reduced slurry at 1000 × *g* for 2 min at 4°C in a swinging-bucket rotor.
   a. Remove the supernatant using a pipette or aspirator.
   b. Then remove any residual DTT supernatant using a 1 mL syringe with a 27 $^{1}/_{2}$ G needle, as mentioned previously after step 7f.
10. Wash the reduced resin thoroughly in the following sequence:
   a. Add 9 mL (3× the initial slurry volume) of 100 mM phosphate buffer (pH 8.0) to the tube.
      i. Mix by inverting the tube a few times to resuspend beads.
      ii. Centrifuge at 1000 × *g* for 2 min at 4°C in a swinging-bucket rotor.
      iii. Remove the supernatant phosphate buffer from the resin using a pipette or aspirator.
   b. Repeat wash step 10a two more times, for a total of three washes with phosphate buffer.
   c. Add 9 mL (3× the initial volume) methanol to the tube.
      i. Mix the beads into a slurry by inverting the tube 10–12 times.
      ii. Centrifuge the tube at 1000 × *g* for 2 min at 4°C in a swinging-bucket rotor.
      iii. Then remove the supernatant methanol from the resin using a pipette or aspirator.
   d. Repeat wash step 10c two more times, for a total of three washes with methanol.

Note: As described above earlier (in the note under step 7f), in order to ensure minimum carryover of the wash buffer to the next wash step with a different wash buffer, withdraw the previous wash buffer as much as possible after step 10b, using a 1 mL syringe with a 27 $^{1}/_{2}$ G needle (used due to its narrow lumen) inserted to the bottom of the beads, prior to the addition of the subsequent wash buffer in step 10c.

Generating the activated thiol resin

Timing: ~12–16 h at 4°C

This section accomplishes the reaction of the Sepharose-bound thiols with 2-dipyridyl disulfide to generate the final SNO-RAC resin.

11. Add 15 mL (5× the initial slurry volume) of freshly prepared 200 mM 2-dipyridyl disulfide dissolved in methanol to the resin.

a. Mix by inversion by hand 10–12 times.
b. Rotate the tube in the dark at 4°C for 12–16 h.

Note: It is advisable to wrap the tube in aluminum foil to protect the resin from light.

Washing after generating the activated thiol resin

Timing: ~30 min at 4°C

This section accomplishes the removal of the excess 2-dipyridyl disulfide and performs buffer exchange in preparation for storage of the active resin.

12. Centrifuge the activated thiol resin at 1000 × *g* for 2 min at 4°C in a swinging-bucket rotor and remove the supernatant using a pipette or aspirator.

Note: Beyond this point, keep the resin protected from light throughout by keeping it wrapped in aluminum foil when outside the centrifuge and performing all the subsequent steps in a dimly lit part of the laboratory.

13. Wash the resin thoroughly in the following sequence:
    a. Add 9 mL (3× the initial slurry volume) of methanol to the resin.
        i. Resuspend the beads by inverting the tube 10–12 times.
        ii. Centrifuge at 1000 × *g* for 2 min at 4°C in a swinging-bucket rotor.
        iii. Remove the supernatant methanol from the resin using a pipette or aspirator.
    b. Repeat wash step 13a two more times, for a total of three washes with methanol.
    c. Add 9 mL (3× the initial slurry volume) of milli-Q water to the tube.
        i. Mix by inverting the tube 10–12 times.
        ii. Spin the tube at 1000 × *g* for 2 min at 4°C in a swinging-bucket rotor.
        iii. Remove the supernatant milli-Q water from the resin using a pipette or aspirator.
    d. Repeat wash step 13c two more times, for a total of three washes with water.
    e. Add 9 mL (3× the initial slurry volume) of Isopropanol to the tube.
        i. Resuspend the beads thoroughly by inverting the tube 10–12 times.
        ii. Spin at 1000 × *g* for 2 min at 4°C in a swinging-bucket rotor(iii) Remove the supernatant isopropanol from the resin using a pipette or aspirator.
    f. Repeat wash step 13e two more times, for a total of three washes with isopropanol.

Note: As described above earlier (under step 7f), in order to ensure minimum carryover of each wash buffer to the next wash step with a different wash buffer, aspirate out any residual wash buffer after the steps 13b and 13d (i.e., prior to steps 13c and 13e), using a 1 mL syringe with a 27 $^{1}/_{2}$ G needle (used due to its narrow lumen) inserted to the bottom of the beads, prior to the addition of the subsequent wash buffer in steps 13c and 13e.

Resin storage

Timing: ~2 min

This section accomplishes preparing the SNO-RAC resin for storage under the specified conditions.

14. After the final wash in step 13f, add a volume of isopropanol equal to the final bead volume to make a ~50% slurry. Ideally this is 1.5 mL (for 3 mL final volume of slurry), but any accumulated losses during pipetting/aspirating will reduce this.

Note: SNO-RAC resin is now ready and can be used within four weeks without significant reduction in its ability to bind S-nitrosylated proteins. This resin can be stored in the dark in a foil-wrapped tube at 4°C for future use, for up to 4 weeks.

⚠ CRITICAL: Since the resin beads will settle down, always remember to resuspend the beads in the isopropanol by tapping and gently inverting the tube before each use.

Quality control – Quantifying the thiol-binding capacity of the prepared resin

Timing: ~30 min

This section accomplishes the estimation of Binding Capacity of this SNO-RAC resin.

15. Estimation of the Binding Capacity of the prepared resin.
    a. Prepare 1 ml of 100 mM DTT in 100 mM Phosphate buffer, pH 8.0 and keep aside at room temperature.
    b. Resuspend prepared resin to a 50% slurry by gentle swirling/mixing.
    c. Using wide orifice tips take out 150 µL of the 50% slurry and transfer it to a microfuge tube.
    d. Spin at 1000 × *g* for 1 min at 4°C.
    e. Remove the supernatant by aspiration.
    f. Add 200 µL of 100 mM Phosphate buffer and spin at 1000 × *g* for 1 min at 4°C.
    g. Repeat the above step 15f two more times for a total of three washes with 100 mM Phosphate buffer.
    h. Remove the supernatant and add visually an equal volume of 100 mM Phosphate buffer as the packed bead volume to get a ~50% resin slurry in 100 mM Phosphate buffer.
    i. In a fresh microfuge tube, add 50 µL of the 50% resin slurry to 450 µL of 100 mM DTT solution that has been freshly prepared in 100 mM Phosphate buffer (pH 8.0; step 15a) and to a separate tube containing 450 µL of 100 mM Phosphate buffer (pH 8.0) as negative control add 50 µL of 50% slurry as negative control.
    j. Incubate both tubes for 5 min at 22°C–25°C with intermittent shaking.
    k. Centrifuge for 1 min at 1000 × *g* at 22°C–25°C.
    l. Add 100 µL of the supernatant to 900 µL 100 mM phosphate buffer (pH 8.0). Discard the remaining supernatant and keep the resin pellet at 22°C–25°C for use in subsequent step 16.
    m. Measure the Absorbance at 343 nm (ε_{343} = 8.08 mM^{-1} cm^{-1} for 2-thiopyridine) using a UV-Visible Spectrophotometer.
    n. Calculate the binding capacity, accounting for the 100-fold dilution, using the following formula:

$$\text{Binding capacity } (\mu\text{Mol} / \text{mL}) = (\text{Abs343} \times 100) / \varepsilon_{343}$$

Note:

i. In our hands the total binding capacity is generally ~7 µmol disulfide/mL of resin slurry. If the binding capacity of the prepared resin is less than 5 µmol disulfide/mL of resin, please refer to the troubleshooting section below.
ii. Even after proper storage of the resin in dark at 4°C, the binding capacity of the resin was reduced by ~60% after six months and was found to be 2.6 µmol disulfide/mL. Therefore, we do not recommend long term storage of the resin. If the resin that was prepared more than four weeks ago and stored optimally at 4°C in the dark still needs to be used for a SNO-RAC experiment due to time constraints, please refer to the troubleshooting section below.

Quality control – Confirming lack of free thiols in resin

Timing: ~ 1 h

This section accomplishes performing a quality control test of the freshly prepared resin by confirming that no free thiols are present in the SNO-RAC resin.

16. Confirm that no free thiols are present in the resin (the DTNB assay for free thiols should be negative).
    a. Wash the DTT treated and the untreated control resin bead pellets from step 15l that had been stored at 22°C–25°C, with 0.5 mL of 100 mM Phosphate buffer (pH 8.0), and centrifuge for 1 min at 1000 × *g* at 22°C–25°C.
    b. Repeat step 16a four more times, for a total of five washes with 0.5 mL of 100 mM Phosphate buffer (pH 8.0).
    c. Aspirate the supernatant.
        i. Add 0.5 mL 100 mM Phosphate buffer (pH 8.0) plus 5 μL of 10 mM 5,5′-Dithionitrobis(2-nitrobenzoic acid) [DTNB] freshly prepared in methanol, to each resin pellet.
        ii. Mix gently using your fingers to tap the tube.
        iii. Incubate for 5 min at 22°C–25°C with intermittent gentle inversion by hand.
    d. Centrifuge for 1 min at 1000 × *g* at 22°C–25°C.
    e. Add 100 μL supernatant to 900 μL 100 mM Phosphate buffer (pH 8.0), mix and add to a cuvette.
    f. Read the absorbance at 412 nm.
    g. Compare the reading to reduced glutathione (GSH) standards from 0 to 100 μM, that have also been similarly treated with 10 μL/mL of 10 mM DTNB. These GSH standards are prepared by creating two-fold serial dilutions of 100 μM GSH (serial dilutions range from 100 μM to 3.125 μM) in 100 mM Phosphate buffer (pH 8.0) and adding 5 μL of 10 mM DTNB to each serial dilution, gently mixing by hand, and incubating for 5 min at 22°C–25°C.

Note: Using this protocol for resin preparation, we do not detect any free thiol remaining on the resin. The absorbance of DTT-treated resin and the untreated resin (disulfide form) at 412 nM after DTNB treatment should be similar to absorbance reading of the 0 μM GSH background. If free thiols are detected in the prepared SNO-RAC resin, then please refer to the troubleshooting section below.

Resin equilibration for use

Timing: ~90 min

This section accomplishes the equilibration of the stored resin just before its use in a SNO-RAC experiment.

17. Equilibration of stored resin before using it in a SNO-RAC experiment.

Note: This needs to be performed on the same day as the SNO-RAC experiment.

    a. Take 250 μL of the resin for each SNO-RAC sample pair (i.e. sample and its minus ascorbate control) in the following way.
        i. Gently mix by tapping the storage tube with your finger until the stored resin (i.e. stored as 50% slurry in isopropanol) is resuspended uniformly in the isopropanol.
        ii. Using a wide-bore (or cut) pipette tip, transfer 250 μL of the well-mixed slurry to a microfuge tube for each sample pair in your SNO-RAC assay.

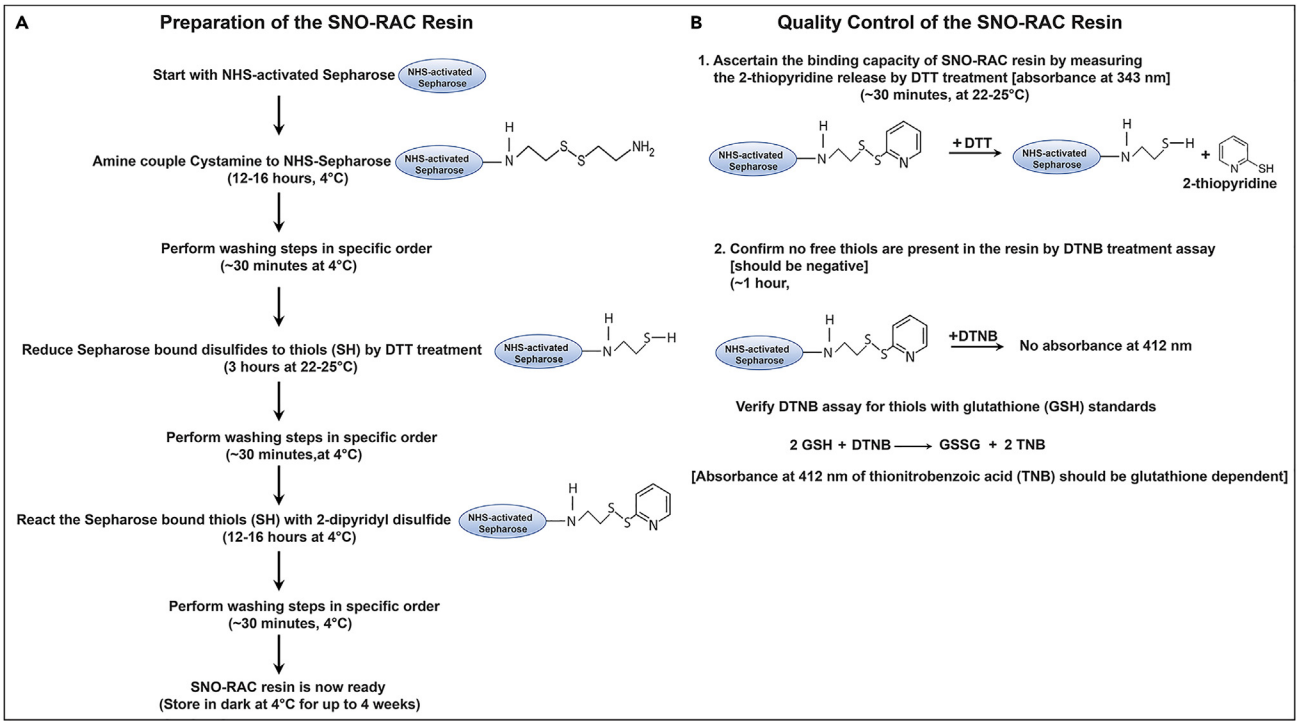
Note: For each biological sample comprising of 1–2 mg of total protein lysate prepared from cultured cells, mammalian tissues or *C. elegans*, SNO-RAC will be performed using 100 μL of the equilibrated resin as a 50% slurry in one tube with the added ascorbate to remove the SNO-moiety and generate free thiols, and in a second tube with 100 μL of the equilibrated resin as a 50% slurry but without any added ascorbate (as the negative control). Therefore, it is best to begin here with 250 μL of slurry to account for loss of volume in subsequent equilibration steps.

b. Centrifuge at 1000 × *g* at 4°C for 1 min using a swinging bucket rotor.
c. Carefully aspirate away the isopropanol without disturbing the resin, by tilting the tube at an angle of ~45° and then aspirating out the supernatant using a pipette tip.
d. Add 500 μL of HEN buffer (100 mM HEPES buffer pH 7.5, 1 mM EDTA, 0.1 mM Neocuproine,) to the resin, and mix by constant inversion at 4°C for 1 h.
e. Centrifuge at 1000 × *g* at 4°C for 1 min.
f. Carefully aspirate the supernatant with a pipette tip.
g. Add an equal volume of fresh HEN Buffer to the resin pellet (i.e., add 125 μL of HEN Buffer to the 125 μL packed volume of resin from the original 250 μL of 50% slurry, per sample pair) and keep on ice and covered from light until it is ready to be added to the lysate samples.

Note: The equilibrated resin should be used within 2–4 h.

h. Mix the beads gently and using a wide-bore pipette tip, add 100 μL of resuspended, equilibrated resin to each of the paired SNO-RAC samples immediately after the addition of the ascorbate to the SNO-RAC tubes (or after omitting the addition of the ascorbate in the negative control tubes). See Seth et al.[3] for a detailed SNO-RAC procedure.

Note: Using 100 μL of this resin per individual sample in our SNO-RAC experiments has given us results that are similar to those obtained with the discontinued GE resin,[2,3] as seen by Silver-staining of the total SNO-proteome or by immunoblotting with protein specific antibodies (Figures 1 and 2).



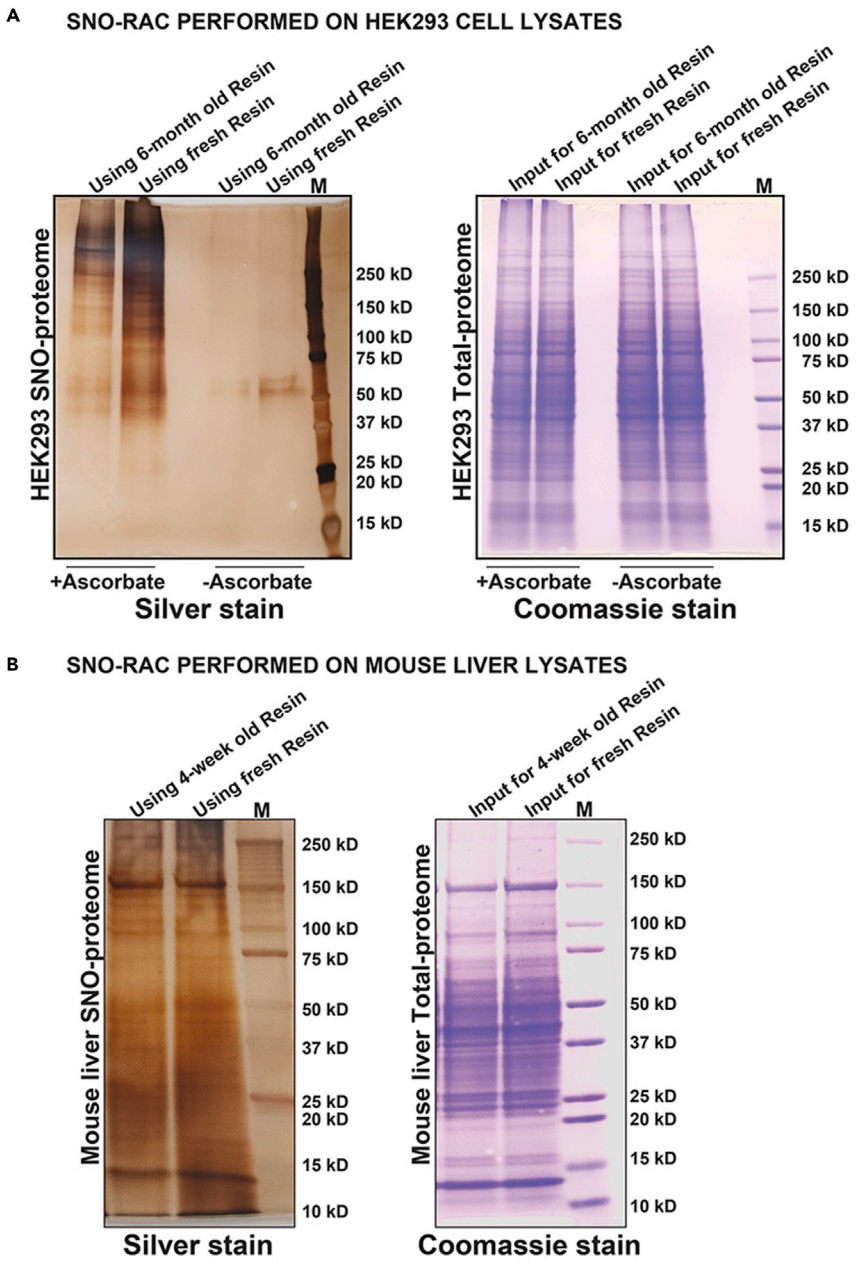


Figure 1. Using the prepared resin in a SNO-RAC experiment and subsequent SNO-proteome visualization by silver stain

(A) Comparison of HEK 293 cell total SNO-proteome under baseline conditions, using SNO-RAC resin that had been prepared six-months prior to use in this experiment versus freshly prepared resin. The left panel establishes the specificity of assay for S-nitrosylated proteins (SNO-proteome) by their presence in the Ascorbate-treated lanes (+Asc), and their absence in the Ascorbate-untreated lanes (-Asc), by protein visualization by silver staining. M = Molecular weight marker. The right panel shows the input HEK cell lysate by Coomassie staining, for comparison with their respective SNO-proteomes in the left panel. 1.5 mg total protein lysate from HEK cells was used for the SNO-RAC experiment and 25 μg of the input was loaded for the Coomassie staining.

(B) Left panel shows the silver-stained gel comparing the mouse liver total SNO-proteome captured using either the SNO-RAC resin that had been prepared four weeks prior to its use and stored appropriately at 4°C versus freshly prepared resin. M = Molecular weight marker. Right panel shows the input mouse liver total lysates by Coomassie staining, for comparison with their respective SNO-proteomes in the left panel. 1.5 mg total protein lysate from mouse liver was used for the SNO-RAC experiment and 25 μg of the input was loaded for the Coomassie staining.

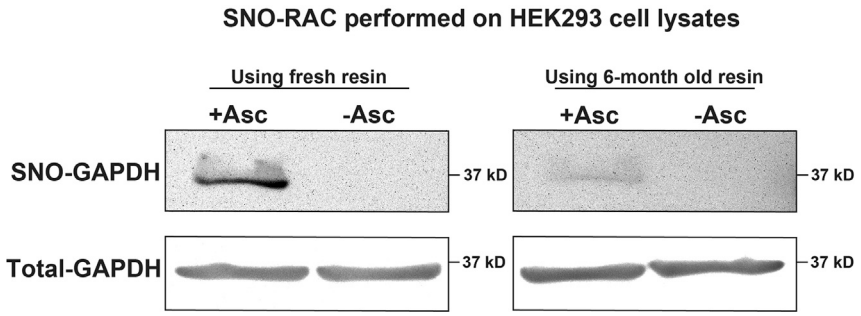


Figure 2. Using the prepared resin in a SNO-RAC experiment and subsequent specific protein visualization by immunoblotting

Comparison of a specific S-nitrosylated protein (GAPDH) by immunoblotting performed either on the SNO-proteome (top panel) or total proteome/lysate (bottom panel) from HEK 293 cells lysates that had been cultured under baseline conditions, using either freshly prepared SNO-RAC resin (top left) or six-month old resin (top right) that had been stored appropriately at 4°C. 1.5 mg total protein lysate from HEK cells was used for the SNO-RAC experiment (top panels) and 60 μg of the total protein lysate was used for total-GAPDH analysis by Western Blotting (bottom panels).

EXPECTED OUTCOMES

This protocol describes the preparation of the Thiopropyl Sepharose resin that is routinely used in a SNO-RAC reaction to selectively capture the S-nitrosylated proteins from lysates.[2] Since the single-sourced commercial option has now been discontinued, this detailed method of resin preparation and quality control offers a substitute that has performed, in our hands, comparably to the discontinued commercial resin.

The S-nitrosylated proteins isolated using this resin in a SNO-RAC reaction can then be run on a polyacrylamide gel, and either visualized directly using a standard protein staining method like Coomassie blue or silver staining. Figure 1 demonstrates that the resin prepared by this protocol is efficient at capturing the S-nitrosylated proteins at baseline in lysates prepared from HEK 293 cells, which have been previously shown to express endothelial NO Synthase (NOS3) at baseline.[19] Alternatively, the proteins can be transferred out of the SDS-PAGE gel onto a nitrocellulose membrane and probed using specific antibodies in a standard Western Blot (Figure 2).

We find that it is best to use this SNO-RAC resin within 4 weeks of preparation, even with storage at 4°C. Longer term storage results in decreased binding capacity of the resin, as shown for specific proteins in Figure 2.

We have found that the S-nitrosylated proteins captured by this resin are readily amenable for analysis by Mass Spectrometry (MS), following standard MS techniques for the identification of proteins and peptides (data not shown). Additionally, these captured S-nitrosylated proteins are compatible with specific mass-tags, including isobaric tags for relative and absolute quantification (iTRAQ) or tandem mass tag (TMT) reactions, allowing relative quantitation of S-nitrosylated proteins and peptides under different conditions.

LIMITATIONS

Binding capacity: As noted previously,[3] the discontinued GE resin had a binding capacity of ~18 μmol activated thiol/mL swollen beads, while the resin prepared by this method has a binding capacity of ~7 μmol activated thiol/mL swollen beads. We have successfully overcome this binding capacity limitation by using twice as much of the resin in each SNO-RAC reaction (i.e., for each sample in a SNO-RAC reaction, we use 100 μL of the 50% slurry prepared by this method, as opposed to using 50 μL of 50% slurry of the discontinued commercial GE resin). This modification provides us with robust capture of the S-nitrosylated proteins from total lysates as seen by silver staining and

by immunoblotting using a specific antibody, without notable increase in non-specific binding to the beads as seen in controls lacking ascorbate (Figures 1 and 2, respectively).

Protocol duration: It takes three days to prepare the SNO-RAC resin using this procedure, although the hands-on time is relatively short. We have not assessed the effects of stopping midway through the procedure, so optimal synthesis requires good planning. Additionally, the three days that are needed to prepare the SNO-RAC resin, need to be consecutive days. Since we have not assessed the effects of increasing the duration of two 12–16 h incubations steps to longer periods (say, over the weekend), we do not recommend it.

Light sensitivity: After step 11, the resin needs to be protected from light, by covering the tube with aluminum foil and performing the subsequent steps in a dimly lit part of the laboratory. This does make these subsequent steps more cumbersome.

Long term storage: We do not recommend using the SNO-RAC resin prepared by this method beyond four weeks, even when stored in the dark at 4°C, as its ability to capture S-nitrosylated proteins diminishes over time (Figures 1 and 2). The binding capacity can be verified at any age using the procedure in step 15.

TROUBLESHOOTING

Problem 1

Using this method, the binding capacity should be > 5 μmol disulfide/mL of resin. In our hands, resins with less than 5 μmol disulfide/mL have yielded inconsistent data. In case the binding capacity of the SNO-RAC resin is less than 5 μmol disulfide/mL of resin, we do not recommend using it.

Potential solution

- Prepare the resin again, using fresh reagents and ensuring minimal carry-over between different wash buffers (as mentioned in detail after step 7f).
- Additionally, in step 8 that involves a 3-h incubation with DTT at 22°C–25°C with constant inversion, the concentration of DTT can be increased up to 400 mM as it may improve the binding capacity of the resin.
- Also, ensure that the methanol carry-over from washing step 7f to the subsequent DTT treatment (step 8) is minimal, by aspirating out the methanol with a 27 $^{1}/_{2}$ G needle until the beads appear dry prior to proceeding with the DTT treatment as shown in Figure 3.
- Alternately, if preparing fresh resin is not an option due to time constraints, one can increase the amount of resin used (to 1.5 times the suggested amount) for use in subsequent SNO-RAC reactions.

Problem 2

Loss of binding capacity due to prolonged storage (of more than 4 weeks).

Potential solution

- We suggest that freshly prepared resin be used within four weeks of its preparation.
  - If using resin beyond the recommended four-week period, we suggest that its binding capacity be measured again before use, by the method outlined under step 15. If its binding capacity is found to be < 5 μmol disulfide/mL of resin, then see potential solutions to problem 1 of the troubleshooting section.

Problem 3

Free thiols are present in the prepared resin as indicated by quality control step 16.

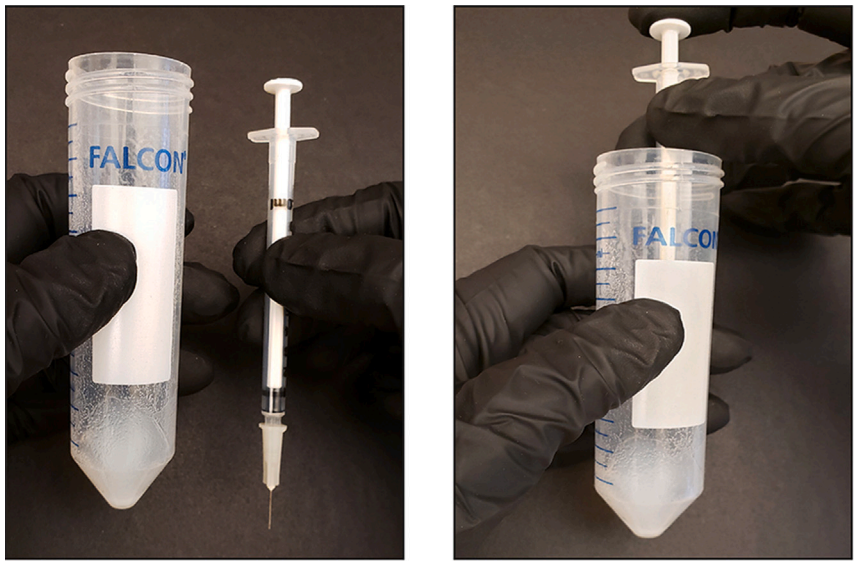


Figure 3. Ensuring minimal buffer carry over to the next step during resin preparation

Image showing the 1 mL syringe and 27 $^{1}/_{2}$ G needle (with a narrow lumen) inserted to the bottom of the beads for withdrawing the remaining methanol from the resin, to ensure minimal carry over to the next step as mentioned after step 7(f).

Potential solution

- The presence of free thiols suggests that the reaction of the Sepharose-bound thiols with 2-dipyridyl disulfide in step 11 was incomplete. This is most likely due to incomplete removal of excess DTT in steps 9 and 10. Do not use this resin for SNO-RAC.
- Resin preparation should be initiated again from the beginning, with fresh reagents, especially ensuring that the washing in steps 9 and 10 after the DTT treatment in step 8 are performed with minimal carryover of buffers from one wash step to the next, by aspirating out the buffer with a 27 $^{1}/_{2}$ G needle until the beads are visibly dry prior to the addition of the next buffer, as has been described in detail earlier (in the note after the step 7f).
- Alternatively, the prepared resin that failed the quality control test of step 16 may be made usable by repeating the steps from step 9 onwards, while ensuring minimal carry over of buffers from one wash step to the next and then performing the quality control step again as described in step 16.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jonathan S. Stamler (jss156@case.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate/analyze datasets or code.

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AUTHOR CONTRIBUTIONS

P.S. and A.H. conducted the experiments. P.S., R.T.P., and J.S.S. designed the study. A.H., R.T.P., and J.S.S. provided technical inputs toward optimizing the protocol. P.S., A.H., R.T.P., and J.S.S. wrote the manuscript.

DECLARATION OF INTERESTS

J.S.S. is a founder of and has equity interest in SNO bio, a company developing nitrosylation-related therapeutics, and NNOXX, a company developing NO-based device technology. CWRU and UHCMC are aware of these conflicts, and appropriate management plans are in place.

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