

Differential *in vivo* Thiol Trapping with N-ethylmaleimide (NEM) and 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS)

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[Abstract] This protocol is used to compare the *in vivo* redox status of *Escherichia coli* and *Vibrio cholerae* protein before and after HOCl treatment. For example, I examined whether the EF-Tu protein is reduced or oxidized in the referenced publication. This protocol should work for other proteins and other oxidative stress treatments. You will need the antibody for your protein to visualize your protein on western blot.

Materials and Reagents

1. Bacteria (this protocol works for *E. coli* MG1655 and *Vibrio cholerae* O395 strains, I didn't try other bacteria)
2. HOCl (Sigma-Aldrich)
3. Trichloroacetic acid (TCA)
4. Urea
5. Tris-HCl
6. EDTA
7. SDS
8. N-ethylmaleimide (NEM)
9. DTT
10. 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (AMS)
11. Polyclonal antibodies against your protein and appropriate secondary antibodies
12. LB media (see Recipes)
13. DAB buffer (see Recipes)

Equipment

1. Centrifuges
2. Western blotting equipment
3. Spectrometer

Procedure

1. Cultivate bacterial strains in LB media at 37 °C, shake 250 rpm, until OD₆₀₀ of 0.4-0.5 was reached.
2. Collect 1 ml cells as untreated control, 2 tubes of 1 ml cells as labeling controls (see step 13 for detail) and then add 3 mM HOCl (final concentration) to rest of culture directly and continue incubation at 37 °C, shake 250 rpm.
3. After the 20 min of HOCl stress treatment, collect 1 ml of treated cells.
4. Acidify collected samples and precipitate proteins with TCA to a final concentration of 10%. This step also prevents any further thiol oxidation.
5. Incubate samples 30 min on ice, pellet precipitated proteins by centrifugation [13,000 rpm (15.7 x g), 20 min, 4 °C].
6. Rinse protein pellet (not resuspend) with 100 µl 5% TCA, remove TCA with centrifugation [13,000 rpm (15.7 x g), 2 min, 4 °C]. Carefully remove all TCA, residual acid will interfere subsequent labeling.
7. Resuspend the protein pellet in 50 µl DAB buffer supplemented with 100 mM NEM to irreversibly alkylate all *in vivo* reduced cysteines.
8. Incubate samples for 30 min at 25 °C, with 1,300 rpm shaking.
9. Precipitate the proteins again with TCA to remove any unbound NEM, and pellet by centrifugation.
10. Resuspend protein pellets in 50 µl DAB buffer supplemented with 10 mM DTT to reduce all *in vivo* oxidized cysteines, and incubate for 1 h at 25 °C, with 1,300 rpm shaking.
11. Remove excess DTT by TCA precipitation and centrifugation.
12. For differential thiol trapping with AMS, modify all newly accessible cysteines (previously oxidized) with 10 mM of the thiol-specific alkylation reagent AMS, which adds 500 Da mass to every modified cysteine.
13. As controls, we also prepared fully NEM-labeled proteins (represent the fully reduced species) and fully AMS-labeled proteins (represent the fully oxidized protein species).
 - a. Take untreated cell aliquots, precipitate with TCA and resuspend in 50 µl DAB buffer supplemented with 10 mM DTT to reduce all *in vivo* thiol modifications.
 - b. After incubation of the samples for 1 h at 25 °C, precipitate proteins with TCA to remove DTT, centrifuge and resuspend in 50 µl DAB buffer supplemented with either 100 mM NEM or 10 mM AMS.
14. Separate proteins on SDS-PAGE and visualize your protein by western blot analysis mass addition significantly slows the migration of AMS-labeled proteins and allows direct visualization of the *in vivo* redox status of proteins.

Recipes

1. LB media
2. DAB buffer
 - 6 M Urea
 - 200 mM Tris-HCl (pH 8.5)
 - 10 mM EDTA
 - 0.5% w/v SDS

Acknowledgments

The protocol described here is adapted from one reported previously (Wholey *et al.*, 2012).

References

1. Wholey, W. Y. and Jakob, U. (2012). [Hsp33 confers bleach resistance by protecting elongation factor Tu against oxidative degradation in *Vibrio cholerae*](#). *Mol Microbiol* 83(5): 981-991.