



Methods For The Detection of Oxidative Stress

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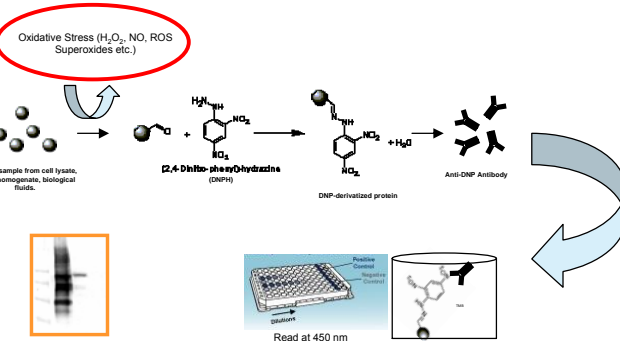
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Introduction

Oxidative stress is a cellular disorder caused by an excess of free radicals which can occur because of increased oxidant levels, decreased anti-oxidant amounts, or failure to repair oxidative damage induced by reactive oxygen species (ROS). This condition has been linked to the pathogenesis of a number of disease states including atherosclerosis, ischemia, cancer, rheumatoid arthritis, and neurodegeneration. ROS are free radicals, reactive molecules containing oxygen, and include superoxides, hydroxyl radicals, hydrogen peroxides, nitric oxides, peroxynitrites, and singlet oxygens. ROS molecules are capable of chemically modifying proteins, lipids, and nucleic acids which potentially can alter or impair their natural biological function. Multiple approaches for detection of modifications are needed due to the variety of ROS molecules that exist as well as the variety of alterations they can impart. The most common approach is to detect a modification or protein involved in processing using an antibody. This is typically done by western blot analysis or IHC. Approaches to detect modifications to DNA (anti-8-hydroxydeoxyguanosine or anti-8-oxoguanine), lipids (anti-neuroketal, anti-4-hydroxynonenal), or proteins (anti-SOD1, anti-nitrotyrosine, anti-nNOS, anti-HIF1 α , and more) are commonly utilized. Another method frequently employed to detect ROS modifications is to chemically attach a molecule to a particular group and subsequently detect this extension. Utilizing this methodology we illustrate detection and quantification of carbonyl modifications using the OxyBLOT™ and OxyELISA™ assays. Results for each of these methodologies are given as well as discussions about advantages and disadvantages of each. This will allow researchers to decide how best to detect ROS-induced modifications based on their particular needs ranging in scope from molecular to single cell to system wide.

Assay Principle:



OxyBLOT™ or OxyELISA™
Detection

Methods

OxyELISA™ Assay: An experiment was carried out to determine the effectiveness of the OxyELISA™ assay. Briefly, HeLa cells were plated in a tissue culture dish. The day after plating the media in each well was replaced with fresh media containing increasing amounts of hydrogen peroxide. The cells were incubated for 30 minutes in the new media and then protein lysates were prepared using the lysis buffer provided in the kit. The lysates were then tested in the OxyELISA™ assay (concurrently with standards provided with the kit) as detailed below:

1. Normalize Protein Lysate Samples to 10ug/ml with Lysis Buffer
2. Prepare Dilutions of Standards (0.0 to 7.2 nmol carbonyl per mg of protein)
3. Absorb 100µl of Samples and Standards to each well of Assay Plate
4. Incubate at 4°C Overnight or 37°C for 1 hr
5. Wash plate 3X with 300µl of Wash Buffer I
6. Add 200µl of Freshly Prepared DNP Derivatization Solution per well
7. Incubate for 45 minutes in the dark at RT
8. Wash with 150µl of Wash Buffer II for 5 minutes with shaking (5X)
9. Wash with 300µl of Wash Buffer I (3X)
10. Add 250µl of 1x Blocking Buffer
11. Incubate 2 hours at RT with Shaking
12. Wash with 300µl of Wash Buffer III (3X)
13. Add 100µl of Diluted anti-DNP Antibody
14. Incubate 1 hour at RT with Shaking
15. Wash with 300µl of Wash Buffer III (7X)
16. Add 200µl of TMB Solution
17. Monitor Color Development and then add 50µl of Stop Solution
18. Read Absorbance at 450nm
19. Create Standard Curve and Calculate Unknowns

OxyBLOT™ Assay: An experiment was carried out to determine the effectiveness of the OxyBLOT™ assay. Briefly, rat dopaminergic cells (N27) were plated in a tissue culture dish. The day after plating the media in each well was replaced with fresh media containing hydrogen peroxide (at 500µM) or media alone. The cells were incubated for 30 minutes in the new medias and then protein lysates were prepared using RIPA buffer. The lysates (as well as standards) were then tested according to the protocol below:

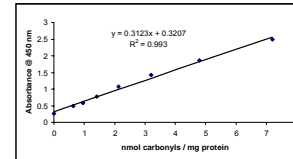
1. Transfer 5µL of each test lysate into 2 tubes (35µg total in each)
2. Add 5µL of 12% SDS to each tube
3. Add 10µL of DNPH solution to one tube and 10µL of negative control solution to the second tube
4. Incubate at RT for 15 minutes
5. Add 7.5µL of Neutralization solution to each tube
6. Load samples onto 4-12% Bis-Tris Gel. Load DNP-derivatized molecular weight standards onto gel as well. Run SDS-PAGE.
7. Transfer proteins from gel to Immobilon® PVDF membrane
8. Incubate the membrane in Blocking Buffer for 1 hour with shaking
9. Add primary antibody (diluted 1:150 with Blocking Buffer)
10. Incubate for 1 hour at RT with shaking
11. Wash membrane with PBS-T
12. Add secondary antibody (diluted 1:300 with Blocking Buffer)
13. Incubate for 1 hour at RT with shaking
14. Wash membrane with PBS-T
15. Detect with Chemiluminescent Reagent

OxyELISA™ Results

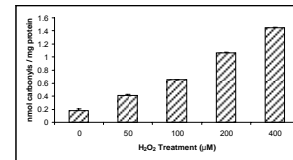
The OD450 absorbance readings for the standards from the OxyELISA™ assay are listed below:

nmol carbonyls/mg protein	STD 1	STD 2	AVG	STD DEV
7.2	2.53	2.45	2.49	0.06
4.8	1.83	1.88	1.86	0.04
3.2	1.41	1.42	1.41	0.01
2.1	1.06	1.06	1.06	0.00
1.4	0.78	0.76	0.77	0.01
0.9	0.58	0.58	0.58	0.00
0.6	0.48	0.5	0.49	0.01
0	0.17	0.35	0.26	0.13

Plotting the average absorbance readings on the Y-axis and the protein carbonylation on the X-axis of a graph allows for the determination of a standard curve.

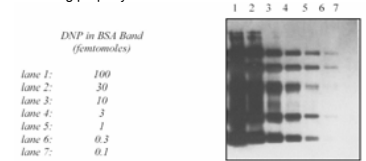


Using the equation for the standard curve allows for determination of the degree of carbonylation of unknown samples. The extent of carbonylation was calculated for each test concentration of hydrogen peroxide and the results are depicted in the following graph.

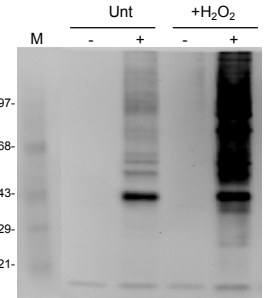


OxyBLOT™ Results

A dilution series of DNP-derivatized molecular weight standard proteins were run to verify the assay conditions being tested were working properly.



Lysates from N27 cells treated with hydrogen peroxide were tested in OxyBLOT™ assays to determine the extent of carbonylation. Negative control reactions (-), which were not treated with DNPH, showed only minimal amounts of background signal. Whereas N27 cells treated with hydrogen peroxide (+H₂O₂) displayed a dramatic increase in the number and degree of oxidized proteins as compared to untreated samples (Unt).



Summary

- Two highly validated approaches for quantitatively and/or qualitatively detecting protein oxidation are presented: OxyELISA™ Assay and OxyBLOT™ Assay.
- The OxyELISA™ Oxidized Protein Quantitation Kit (S7250) utilizes an enzyme linked immunoassay (ELISA) format to quantitate the amount of protein carbonylation present in a cell lysate sample. It has a lower level detection sensitivity of 0.2nmol of carbonyl per mg of protein (data not shown).
- Treatment of HeLa cells with hydrogen peroxide (a known inducer of oxidative stress) followed by subsequent analysis by OxyELISA™ Assay revealed a stepwise increase in the amount of protein carbonylation as the amount of hydrogen peroxide was increased.
- The OxyBLOT™ Protein Oxidation Detection Kit (S7150) utilizes a western blot based system to analyze the oxidation status of proteins. It can detect as little as 1 femtomoles of a DNP-derivatized protein.
- Analysis of cell lysates made from N27 cells treated with hydrogen peroxide revealed a dramatic increase in the degree of protein oxidation as compared to untreated samples. The response observed was nearly completely dependent upon the derivatization reaction with DNPH as the negative control reactions showed little if any signal.
- The OxyELISA™ and OxyBLOT™ kits provide all chemical and immunological reagents necessary to perform quantitative and qualitative detection of carbonyl groups introduced into proteins by oxidative reactions with oxygen free radicals and other reactive species.