User Manual

OxyICC Oxidized Protein Detection Kit

40 reactions utilizing 8-well chamber slides

S7350

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures. Not for Human or Animal Consumption.

Introduction

Oxidative stress has been found to play a key role in a number of pathological disorders. These effects appear to be mediated by reactive oxygen species (ROS) which cause modifications to proteins, lipids, and DNA. In the case of proteins the ROS primarily cause carbonyl derivatives on amino acid sidechains affecting their enzymatic and biochemical functionality. Not surprisingly, carbonyl formation has become an important biomarker for oxidative stress. The OxyICC Kit provides reagents for immunodetection of protein carbonyls by fluorescent immunocytochemistry. The assay procedure is simple and the results obtained are highly sensitive and quantitative.

Background

Reactive oxygen species (ROS) have been implicated in pathological processes including cancer, apoptosis, aging, neurodegenerative diseases, chronic inflammatory diseases, pulmonary diseases, and cardiovascular diseases.[1-4]

Living organisms are continually exposed to potentially harmful oxygen free radicals that are generated through normal cellular functions as well as from environmental factors.[1, 5-7] Yet, these reactive molecules are mitigated by the presence of antioxidants and therefore organisms exist with a delicate balance between oxidants and antioxidants.[7-9] During times of oxidative stress there is an imbalance in favor of the oxidants. The end result of which is that free radicals are able to attack and modify subcellular components including nucleic acids, lipids and proteins.[7, 10-12] In some cases, cells respond to the oxidative stimuli and allow the organism to adapt to the oxidative stress.[13-15]

Proteins are one of the major targets of oxygen free radicals and other reactive species. Oxidation of proteins modifies the side chains of methionine, histidine, and tyrosine and forms cysteine disulfide bonds.[16-19] Metal catalyzed oxidation of proteins introduces carbonyl groups (aldehydes and ketones) at lysine, arginine, proline or threonine residues in a site-specific manner.[16, 20-22] The oxidative modification of proteins can modulate biochemical characteristics of proteins such as enzymatic activity[21-23] DNA binding activities of transcription factors,[24-26] and the susceptibility to proteolytic degradation.[12, 25-28] While a relationship between protein oxidation and aging has been suggested,[29-31] little is known about the importance of oxidative modification of individual proteins in the pathophysiology of free radical mediated processes.

Not surprisingly, carbonyl modification of proteins has become a key biomarker for the identification of oxidative stress. To assist in these research efforts, we now offer the OxyICC Kit which contains the chemical and immunological reagents necessary to detect the carbonyl groups using fluorescent immunocytochemistry. The test method involves chemical derivatization of protein carbonyl groups with 2,4-Dinitrophenylhydrazine (DNPH). This chemical reaction results in proteins being covalently coupled to DNP at their carbonyl sites. The DNP-derivatized proteins are then detected using biotinylated antibodies that are specifically reactive for the DNP moiety. Subsequent incubation with fluorescently conjugated streptavidin enables detection using fluorescence microscopy. The amount of fluorescence detected is correlated to the extent of oxidative stress that the cell was subjected to.

Kit Components

S7350-1: Store at 2-8 °C

- 2,4-Dinitrophenylhydrazine Solution (CS201876): One vial with 1.1 mL
- 10X Wash Buffer (CS201873): One 130 mL bottle
- 5X Blocking Buffer (CS203024): One 10 mL bottle
- Millex® GP Filter Units (0.22 µm) (SLGPR33RB): 3 filters

S7350-2: Store at –20 °C

- Anti-DNP, Biotinylated (mouse monoclonal) Antibody (CS203021): 15 µL of solution
- Streptavidin-Cy3 (CS203022): 15 µL of solution
- DAPI (CS202687): 10 µL of 1 mg/mL solution

Materials Required (Not supplied)

- Tissue culture instruments/supplies (including 37 °C incubator, growth media, flasks/plates, etc.) for cell type of interest
- Microscope and software capable of detecting fluorescence
- Methanol fixative
- 8-Well chamber slides or similar tissue culture vessel
- Mounting media for fluorescent detection
- pH meter
- 5 N NaOH
- General lab supplies such as microcentrifuge tubes, pipets, and pipet tips
- Coverslips

Warnings and Precautions

The OxyICC Kit is designed for research use only and is not recommended for internal use in humans or animals. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.

The DNPH solution contains phosphoric acid. Both the DNPH and the acid are harmful if swallowed or inhaled; avoid contact with skin and eyes (wear gloves and eye protection); wash areas of contact immediately with water.

Storage and Stability

The OxyICC Kit requires dual temperature storage. The biotinylated anti-DNP antibody, Streptavidin-Cy3, and DAPI reagents (S7350-2) should be stored at –20 °C. The remaining kit components (S7350-1) should be stored at 2-8 °C. Kit components are stable for a minimum of 6 months from the date of receipt if stored and handled properly.

Protocol

Preparation of DNPH Derivatization Solution

The 2,4-Dinitrophenylhydrazine (DNPH) solution provided in the kit needs to be diluted, adjusted for pH, and filtered prior to use. The preparation of this working solution of DNPH should be completed immediately prior to use.

1. Transfer 333 µL of DNPH solution into 40 mL of Milli-Q® water.
2. Adjust the pH to 6.0-6.5 by drop wise addition of 5 N NaOH.
3. Then add Milli-Q® water to bring the total volume to 50 mL.
4. Filter the solution using the supplied 0.22 µm Millex® syringe filter.

The OxyICC™ Kit is supplied with enough DNPH derivatization solution for up to three independent experiments following the protocol listed above.

Blocking Buffer and Wash Buffer

Please note that the blocking buffer supplied with the kit is a 5X stock and the wash buffer provided is a 10X stock. Dilute each of these with Milli-Q® water to a 1X working concentration prior to use in the assay.

Cell Lines and Treatment:

The OxyICC Kit has been validated to work with numerous cell lines including NIH-3T3, N1E-115, HeLa, 293, A431, and MCF-7 (see figures 1 and 2 and data not shown). It would be expected that most mammalian cells lines would be compatible for testing with this kit since carbonyl formation occurs during oxidative stress regardless of species or cell type. For testing purposes, the cells were grown in their standard media and culture conditions. The cells were then stressed by brief exposure to hydrogen peroxide prior to fixation. Hydrogen peroxide is a chemical that is known to induce oxidative stress in biological systems. Negative control reactions were also performed in which hydrogen peroxide was omitted.

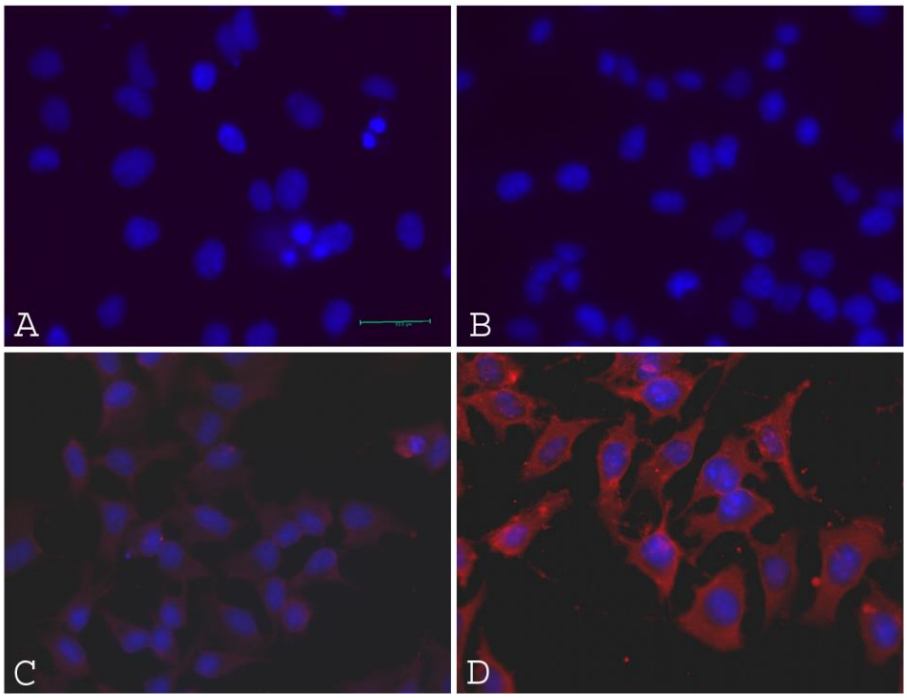

Figure 1: OxyICC Detection of Oxidative Stress. HeLa cells were cultured with or without hydrogen peroxide (H_2O_2; 400 µM) for 30 minutes. The cells were then analyzed for oxidative stress following the OxyICC protocol. Control reactions without DNPH treatment were also run to assess background. The test reactions were as follows: **(A)** H_2O_2/-DNPH **(B)** +H_2O_2/-DNPH **(C)** -H_2O_2/+DNPH and **(D)** +H_2O_2/+DNPH. Cell nuclei are blue due to DAPI staining whereas DNP signal is red. Virtually no staining was observed in the control reactions A and B which lack DNPH treatment. Minimal signal was observed in reaction C which is indicative of basal levels of oxidation that is present under normal cellular conditions. Treatment of cells with hydrogen peroxide in reaction D induces a strong fluorescent signal indicating extensive oxidative stress.

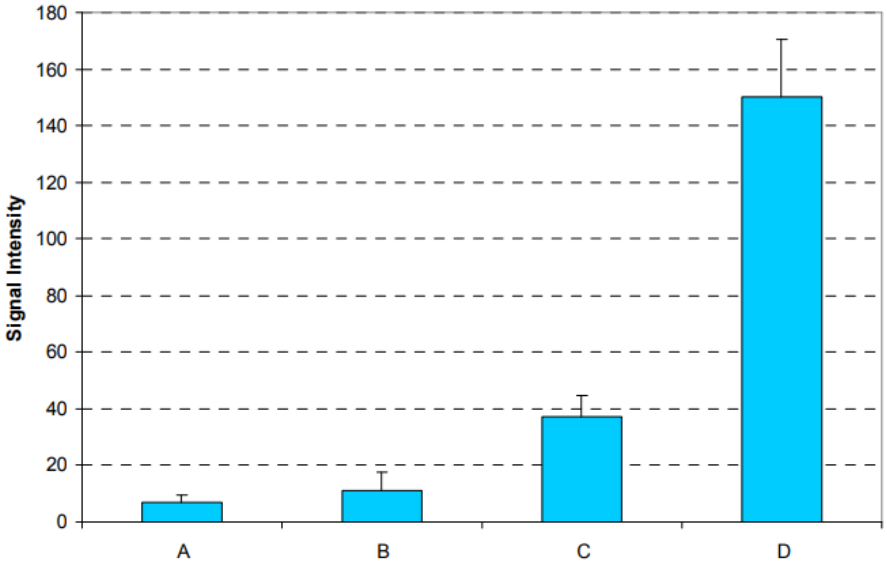

Figure 2: Quantitation of OxyICC Analysis. Three independent experiments were performed as in **Figure 1** using HeLa cells and hydrogen peroxide (H_2O_2) treatment. As before the test reactions were: **(A)** -H_2O_2/-DNPH **(B)** +H_2O_2/-DNPH **(C)** -H_2O_2/+DNPH and **(D)** +H_2O_2/+DNPH. For each test reaction the Cy3 fluorescence pixel intensity was quantified for ten randomly chosen cells using Leica® LAS AF software. The signals obtained were averaged and then normalized to background to give a signal intensity measurement for each experiment. The data for the three experiments was then averaged and is depicted above with error bars. The quantitative measurement shows that the +H_2O_2/+DNPH reaction **(D)** had over a four-fold signal intensity increase versus basal levels found in the -H_2O_2/+DNPH sample **(C)**.

OxyICC Assay Protocol

Ideally each experiment should include several control experiments to verify any results obtained using the OxyICC Kit. Reactions with and without DNPH as well as plus and minus oxidative stress treatment (using a known stress inducer such as H_2O_2) should be included in every test that is performed. Additional tests that may also be run include omitting antibody and/or streptavidin to verify specificity.

1. Plate cells in 8-well chamber slide with 300 µL of media. Cells should be plated such that they are less than 80% confluent the following day. For most cell lines about 30,000 cells per well is sufficient.
2. Incubate cells overnight at 37 °C in a tissue culture incubator.
3. Treat cells with any drugs or chemicals as needed (for example, H_2O_2).
4. Gently wash the cells twice with 1X PBS (300 µL/well).
5. Fix cells with ice cold 100% methanol for 5 minutes (300 µL/well). Do not use formaldehyde fixative as this may interfere with DNPH reaction.
6. Gently wash the cells three times with 1X PBS (300 µL/well).
7. Add 300 µL of freshly diluted DNPH solution (see above for details) per well. For negative control reactions add 300 µL of Milli-Q® water or 1X PBS per well.
8. Incubate for 45 minutes in the dark.
9. Gently wash four times with 1X PBS (300 µL/well).
10. Add 300 µL of 1X blocking buffer solution per well.
11. Incubate for 1 hour at 37 °C or overnight at 4 °C.
12. Remove blocking buffer.
13. Add 300 µL per well of Biotinylated anti-DNP antibody pre-diluted 1:1000 in 1X blocking buffer.
14. Incubate 1 hour at 37 °C or overnight at 4 °C.
15. Gently wash three times with 1x PBS (300 µL/well).
16. Add 300 µL per well of Streptavidin-Cy3 pre-diluted 1:1000 in 1X blocking buffer.
17. Incubate for 1 hour at 37 °C or overnight at 4 °C.

18. Gently wash three times with 1X PBS (300 µL/well).
19. Add 300 µL per well of DAPI diluted 1:1000 in 1X PBS.
20. Incubate for 5 minutes at room temperature.
21. Gently wash twice with 1X PBS (300 µL/well).
22. Remove wells from slide, add mounting media, and then coverslip.
23. Examine cells using a fluorescent microscope with the appropriate filter sets. The Cy3 conjugate is brighter, more photostable, and gives less background than most other fluorophores. It can be maximally excited at 550 nm with a peak of emission at 570 nm. For fluorescence microscopy, it can be visualized using traditional tetramethyl rhodamine isothiocyanate (TRITC) filter sets since the excitation and emission spectra are nearly identical to those of TRITC. As for DAPI it can be maximally excited at 350 nm with a peak of emission at 460 nm.

Quick Reference for OxyICC Procedure

Day 1:

1. Plate the cells at ~30,000 cells/well of an 8-well chamber slide.
2. Incubate overnight at 37 °C.

Day 2:

3. Treat cells with chemical (For example, H_2O_2).
4. PBS wash (2X)
5. Methanol fix the cells for 5 min.
6. PBS wash (3X)
7. Incubate with freshly prepared DNPH solution for 45 min in dark.
8. PBS wash (4X)
9. Add 1X blocking buffer and incubate 1 hr at 37 °C.
10. Add anti-DNP antibody (1:1000) and incubate 1 hr at 37 °C.
11. PBS wash (3X)
12. Add Streptavidin-Cy3 (1:1000) and incubate 1 hr at 37 °C.
13. PBS wash (3X)
14. Add DAPI (1:1000) and incubate for 5 min at RT.
15. PBS wash (2X)
16. Add mounting medium.
17. Examine with fluorescent microscope.

Troubleshooting

Problem	Cause
Poor positive staining or No positive staining with little or no background Staining	• Anti-DNP antibody was omitted, used at the wrong concentration, or used in the wrong order. • Streptavidin-Cy3 reagent was omitted, used at the wrong concentration, or used in the wrong order. • Use a longer incubation time for the Anti-DNP Antibody. • Try longer exposure setting on microscope to see if signal appears. • Do not let slides dry out! Keep wet at all times during the testing procedure. • Cell fixation failed. Ensure methanol was used for testing and not formaldehyde. Possibly try other fixative solutions such as methacarn (60% methanol, 30% chloroform, and 10% acetic acid). • Was oxidative stress positive control (+H_2O_2) included in testing? • Protocol procedure was not followed correctly, or steps were omitted.
High Background Staining	• Anti-DNP antibody was not diluted in blocking buffer or was used at the wrong concentration. • Streptavidin-Cy3 was not diluted in blocking buffer or was used at the wrong concentration. • Increase the volume, time, and/or the number of washes between steps.

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