FlowCellect™ Oxidative Stress Characterization Kit

25 Tests

Cat. No. FCCH025111

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Not for use in diagnostic procedures.

USA & Canada Phone: +1 (800) 437-7500 Fax: +1 (951) 676-9209
Australia +61 3 9839 2000
www.millipore.com

Introduction

Oxidative stress has been found to play a key role in a number of pathological disorders. To aid researchers studying this phenomenon Millipore has developed the FlowCellect™ Oxidative Stress Characterization Kit. This kit contains the chemical and immunological reagents necessary to detect carbonyl groups introduced onto proteins by reactive oxygen species (ROS). The test method involves 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 incubated with a FITC conjugated monoclonal antibody that specifically binds to the DNP moiety. Subsequent detection with a bench-top Guava EasyCyte™ Plus flow cytometer allows quantification of the extent of oxidative stress within cells. Although the assay and all of the kit components were optimized using a Guava instrument, any flow cytometer can be utilized provided it contains a blue laser (488 nm) light source.

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 (for reviews, see ref. 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).

Proteins are one of the major targets of reactive species. Oxidation of proteins causes modifications to 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 (32).

To assist in these research efforts Millipore now offers the FlowCellect Oxidative Stress Characterization Kit. This kit enables detection of oxidative stress by flow cytometry analysis. The procedure is relatively simple and permits sensitive and specific immunodetection of protein carbonyls groups, a known biomarker for oxidative stress.

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Kit Components

FCCH025111-1 (*Store at 2-8°C*)

1. 10X Wash Buffer: (Part No. CS202123) 1 bottle containing 13 mL
2. 5X Assay Buffer: (Part No. CS202124) 1 bottle containing 55 mL
3. DNPH Solution: (Part No. CS204896) 1 bottle containing 2.5 mL
4. Derivatization Control Solution: (Part No. CS204897) 1 bottle containing 2.5 mL

FCCH025111-2 (*Store at -20°C. Note: this component is shipped on ice, at 2-8°C*)

5. FITC Conjugated Anti-DNP Antibody: (Part No. CS204988) 1 vial containing 10 µL

Materials Not Supplied

1. Guava EasyCyte™ Plus Flow Cytometry System or any flow machine capable of exciting and detecting FITC
2. Guava™ Instrument Cleaning Fluid (ICF) (Catalog # 4200-0140)
3. ViaCount reagent (Catalog No. 4000-0041)
4. Tissue culture instruments, supplies, and cells
5. General lab supplies such as test tubes, pipets, and pipet tips
6. Tabletop centrifuge
7. Vacuum pump
8. 96-well v-bottom plate- **Recommended but not required if performing assay in a plate method*
9. Hydrogen Peroxide
10. 100% Methanol

Precautions

- All kit components should be considered potentially harmful and good laboratory practice should be followed.
- The DNPH Solution and the Derivatization Control Solution both contain hydrochloric acid (HCl). These solutions are harmful if swallowed or inhaled. Avoid contact with skin and eyes (wear gloves, eye protection, and other personal protective equipment). Wash areas of skin contact immediately with water.
- The FITC conjugated anti-DNP antibody is light sensitive. Store it in the dark and limit its exposure to light during testing.
- During storage and shipment, the product supplied in the vials may condense within the cap. For maximum recovery of product, centrifuge prior to removing cap.

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Storage

The FlowCellect Oxidative Stress Characterization Kit is shipped on ice. Upon arrival the FITC conjugated anti-DNP antibody should be transferred to a -20°C freezer. The remaining kit components (FCCH025111-1) should be stored at 2 - 8ºC.

All kit components are stable for up to six (6) months from date of receipt if stored and handled appropriately.

Preparation of Reagents

10X Wash Buffer

The Wash Buffer is supplied as a 10X concentrate and should be diluted to 1X with MilliQ™ water prior to use. It may be necessary to pre-warm the 10X Wash Buffer to room temperature prior to use as precipitate may form at colder temperatures. The 1X Wash Buffer should be stored at 2 - 8ºC until ready for use and is stable for up to 6 months.

5X Assay Buffer

The Assay Buffer is supplied at 5X concentration and should be diluted to 1X with MilliQ™ water prior to use. After dilution the 1X Assay Buffer should be stored at 2 - 8ºC until ready for use and is stable for up to 6 months.

Assay Procedure

For optimal kit performance, please follow the instructions provided below. Keep in mind that a minimum of approximately 250,000 cells are needed for Guava based flow analysis and often four times that amount is needed for other flow cytometers. Care should be taken to keep all cell concentrations as constant as possible among all test samples during flow analysis.

This procedure has been successfully validated with several different cell types including HeLa, N1E-115, and PC12, at a concentration of 1 x 10^6 cells per well. Most cell types should be suitable for testing with this kit but some optimization may be necessary. Be sure to include proper controls for instrument set up to validate results.

1. Plate cells in T75 flasks with 15 mL of media. Cells should be plated such that they are approximately 70-80% confluent when harvested. **Note:** *Typically at least two separate flasks are needed for each experiment- one for treatment with a stress inducer (i.e. H_2O_2) and one without treatment.*
2. Incubate cells in a tissue culture incubator.
3. Treat cells with any drugs or chemicals as needed (e.g. H_2O_2).
4. Aspirate media and/or chemicals then wash with PBS (~10 mL). **Note:** *For suspension cell lines collect cells by centrifugation, wash with PBS, and then skip to step 8.*
5. Aspirate PBS then add 3 mL of pre-warmed Accutase (Catalog #: SCR005) per flask.
6. Incubate ~5 minutes at 37°C.
7. Add 7 mL of media per flask to quench. Pipette up and down gently to dislodge all cells.
8. Transfer cells to conical tubes.
9. Count the cells.

10. For each treatment condition aliquot $1x10^6$ cells into two different 15 mL tubes. Thus, a minimum of two tubes will be prepared and labeled as follows:

    a. - Treatment

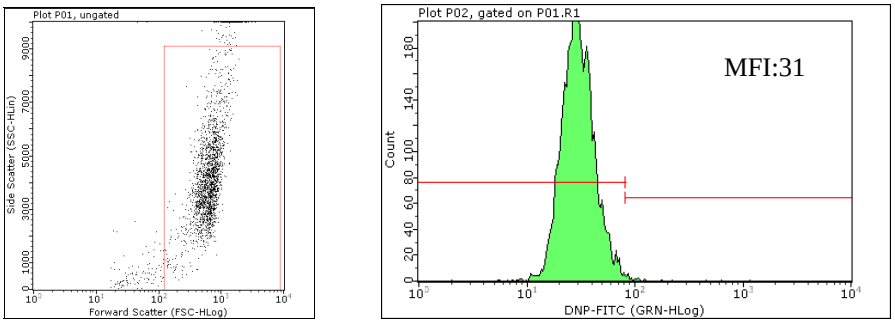
    b. + Treatment (e.g. H_2O_2)

    Note: *Additional reactions may also be set up at this time. They may include negative control tests where the FITC conjugated anti-DNP antibody is omitted or reactions without DNPH treatment.*

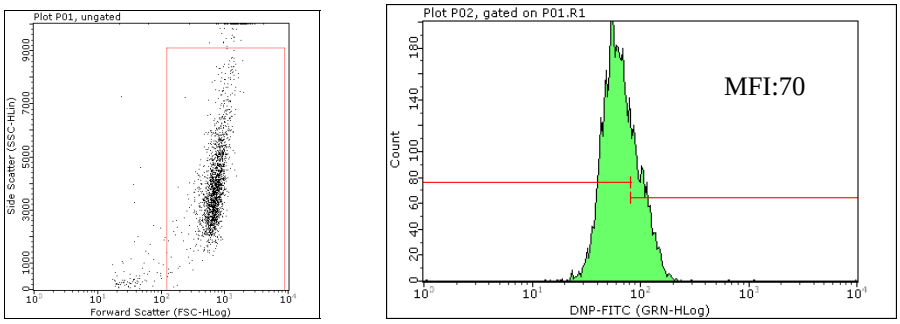
11. Centrifuge the cells at 670 x *g* for 5 minutes.
12. Carefully discard the supernatants without disturbing the cell pellets.
13. Gently resuspend the cells with 0.5 mL of ice cold 1X Wash Buffer per tube.
14. Centrifuge the cells at 670 x *g* for 5 minutes.
15. Carefully discard the supernatants without disturbing the cell pellets.
16. Add 0.5 mL of ice cold 100% methanol to each tube and gently mix.
17. Fix the cells by incubating for 5 minutes on ice. **Note:** *If necessary the cells can be stored temporarily (3-4 days) at 4°C at this point. If storing cells, add additional methanol (10 mL/tube) to avoid sample evaporation.*
18. Centrifuge the cells at 670 x *g* for 5 minutes.
19. Carefully discard the supernatants without disturbing the cell pellets.
20. Gently wash the cell pellets two times with ice cold 1X Wash Buffer (0.5 mL each).
21. Carefully discard the supernatants without disturbing the cell pellets.
22. Add 100 µL of DNPH Solution to each test sample. **Note:** *If running a "no DNPH treatment" negative control, add 100 µL of Derivatization Control Solution in place of the DNPH Solution*.
23. Mix gently then incubate for 45 minutes in the dark.
24. Wash four times with ice cold 1X Wash Buffer (0.5 mL each)
25. Carefully discard the supernatants without disturbing the cell pellets.
26. Wash with ice cold 1X Assay Buffer (0.5 mL).
27. Add 100 µL of freshly diluted FITC conjugated anti-DNP antibody per tube (the antibody must be diluted 1:1000 in ice cold 1X Assay Buffer prior to use).
28. Gently pipet the cells and antibody solution to mix.
29. Incubate on ice for one hour in the dark with occasional gentle mixing.
30. Wash cells with ice cold 1X Assay Buffer (0.5 mL) two times. Be sure to spin the cells for 5 minutes at 670 x *g* between each wash.
31. Resuspend cells in 0.5 mL of ice cold 1X Assay Buffer.
32. Analyze 200 µL (approximately 300 to 500 cells/µL for 96 well format) or 500 µL (single tube format) using a Guava EasyCyte™ Plus flow cytometer or like machine. **Note:** *Cells should be analyzed by flow cytometry shortly after completion of this protocol for optimal results.*

Sample Data

Two flasks of HeLa cells were grown as detailed above. One flask was treated with 400 µM of hydrogen peroxide for 30 minutes to induce oxidative stress whereas the other flask was left untreated. Cells from the two flasks were subsequently harvested and processed as detailed in the protocol above. The cells were then analyzed using a bench-top Guava EasyCyte Plus flow cytometer. The untreated (negative control) cells were assayed first and the machine was calibrated to them such that they displayed fluorescence intensity readings between 10^1 and 10^2 with the DNP-FITC channel. The hydrogen peroxide treated cells were then run using the same instrument settings. The results of the experiment are detailed below in Figure 1.



Panel A: Untreated Cells



Panel B: H_2O_2 Treated Cells

Figure 1: Detection of Oxidative Stress. HeLa cells treated with or without hydrogen peroxide were processed using the FlowCellect Oxidative Stress Characterization Kit then analyzed on a Guava EasyCyte Plus flow cytometer. Panel A above displays test results of untreated cells and Panel B displays test results of cells treated with hydrogen peroxide. The figure on the left in each panel is a scatter plot (SSC) from the Green channel (log) and is typically used as a counting gate to eliminate debris. The figure on the right in each panel is a histogram which displays the fluorescence intensity of the cells as detected by the DNP-FITC channel. For each experiment the mean fluorescence intensity (MFI) was calculated and is noted above. An increase in oxidative stress is detected as a right shift in MFI from untreated sample to the treated sample. In this example, the hydrogen peroxide treated cells (Panel B) showed a 2.26 fold increase (from 31 to 70) in mean fluorescence intensity (MFI) as compared to the untreated samples (Panel A)

Technical Hints

- The buffers and reagents used during testing (1X Wash Buffer, 1X Assay Buffer, DNPH Solution, Derivatization Control Solution, and diluted antibody) should be kept at 2 - 8ºC prior to and during use.
- The FITC Conjugated Anti-DNP Antibody should be stored at -20ºC and multiple freeze/thaw cycles should be avoided.
- Mix samples thoroughly before use; avoid excessive bubbling.
- If minor precipitate is detected in the 10X Wash Buffer, place it in a warm water bath for 30 minutes. Once it is heated mix it using a mechanical vortex prior to dilution to 1X.
- For cellular staining and analysis to be most effective, make sure that test cells have good viability prior to use.
- Depending upon the cell type, the cell pellet may become hazy or transparent following the fixation step making it difficult to see. If sampling a small collection of cells for flow analysis, it is recommended to perform all steps in a smaller collection tube (e.g. micro-centrifuge tube).

Troubleshooting

Potential Problem	Experimental Suggestions
Acquisition rate decreases dramatically Instrument clogging Too many cells	• Too many cells per microliter. Decrease the number of cells by diluting the sample to 300–500 cells per microliter. The Guava EasyCyte™ Plus flow cytometer gives the most accurate data when the cell density is less than 500 cells per microliter. • Run three quick cleans to rinse out the flow cell. This procedure can be performed during or after an assay. This will wash away any material forming within the glass capillary walls.
Too few cells	• Spin down cells and resuspend in a smaller volume. The optimal cell density range is between 100-500 cells/μL in the final test sample run on the Guava instrument. However, cell loss is a common occurrence during washing steps and a substantial decrease in cell numbers can lead to difficulty in adjusting settings. Be sure to leave the cell pellet intact during washes. If the cells are not generating a compact pellet after centrifugation, increase the time and/or increase the speed until a compact and visible cell pellet forms.
Background staining and/or non-specific staining of cells	• Although the assay procedure has been optimized to function utilizing many different cell types, further antibody titrations may be necessary for some cell types to capture the ideal staining concentration. Non-specific staining and background may indicate that less antibody will need to be used during the staining procedure.

Variability in day to day experiments	• Monitor experimental cell cultures to ensure that cell viability and cell numbers being analyzed are consistent. Any changes in culture conditions or viability can influence experimental results. • When using the Guava EasyCyte™ Plus instrument for flow analysis, make sure that a quality check on the instrument (e.g. calibration) is performed on a daily basis prior to use.
Staining is weak	• Some cell lines may require higher concentrations of conjugated antibodies. Try higher concentrations of antibody.
Staining is too bright	• Some cell lines may require lower concentrations of conjugated antibodies. • Reduce the DNPH incubation time.
No shift	• Cells may not have been induced. Positive and negative controls should be included for each experiment to ensure that the protocol was followed correctly and the assay is functioning properly.
False positive results (i.e. shifts in MOI when none is expected)	• Be sure to run negative control reactions (i.e. untreated). • Sub-optimal culture conditions may stress cells in culture, causing them to undergo stress in the absence of experimental induction treatment. Ensure cultures are healthy, actively growing, and have media changed regularly.

For further support, please contact Millipore's Technical services at +1(800) 437-7500

References

1. Halliwell, B. and Gutteridge, J.M.C. (1990) Role of free radicals and catalytic metal ions in human diseases: an overview. *Methods in Enzymol.* **186**:1.
2. Halliwell, B. and Gutteridge, J.M.C. (1989) Free Radicals*. Biology and Medicine, 2nd Ed.* (Oxford, Clarendon Press).
3. Stadtman, E.R. (1992) Protein oxidation and aging. *Science* **257**:1220.
4. Ching Kuang Chow, ed. (1988) *Cellular Antioxidant Defense Mechanisms* (CRC Press, FL).
5. Weizman, S.A. and Gordon, L.L. (1990) Inflammation and cancer: Role of phagocytegenerated oxidants in carcinogenesis. *Blood* **76**:655.
6. Babior, B.M. and Woodman, R.C. (1990) Chronic granulomatous disease. *Semin. Hematol.* **27**:247.
7. Cance, B., *et al.* (1979) Hydroperoxide metabolism in mammalian organs. *Physiol. Rev.* **59**:527.
8. Halliwell, B. (1994) Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *Lancet* **344**:721.
9. Miquel, J., et al., eds. (1989) *Handbook of free radicals and antioxidant in biomedicine* (CRC Press, FL).
10. Dizdaroglu, M. Chemistry of free radical damage to DNA and nucleoprotein in B. Halliwell and I. Aruomao, eds., (1993) *DNA and Free Radicals* (Chichester, Ellis Harwood), p19.
11. Ames, B.N., et al. (1991) *Oxidative Damage and Repair: Chemical, Biological and Medical Aspects*, K.J.A. Davies, ed. (Pergamon, Elmsford, NY), p181.

12. Davies, K.J.A. (1987) Protein damage and degradation by oxygen radicals. *J. Biol. Chem.* **262**:9895.
13. Schreck, R. *et al.* (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kB transcription factor and HIV-1. *EMBO J.* **10**:2247.
14. Demple, B. and Halbrook, J.H. (1983) Inducible repair of oxidative DNA damage in *Escherichia coli. Nature* **304**:466.
15. Christman, M. F., *et al.* (1985) Positive control of a regulon for defenses against oxidative stress and some heat-shock proteins in Salmonella typhimurium. *Cell* **41**:753.
16. Stadtman, E. R. (1993) Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Ann. Rev. Biochem.* **62**:797.
17. Davies, *et al.* (1987) Protein damage and degradation by oxygen radicals II. Modification of amino acids. *J. Biol. Chem.* **262**:9902.
18. Uchida, K. and Kawakishi, S. (1990) Site-specific oxidation of angiotensin I by copper(II) and L-ascorbate: conversion of histidine residues to 2-imidazolones. *Arch. Biochem. Biophys*. **283**:20.
19. Heinecke, J.W., *et al.* (1993) Dityrosine, a specific marker of oxidation, is synthesized by the myeloperoxidase-hydrogen peroxidase system of human neutrophil and macrophages. *J. Biol. Chem.* **268**:4069.
20. Farber, J.M. and Levine, R.L. (1986) Sequence of a peptide susceptible to mixed function oxidation. Probable cation binding site in glutamine synthetase. *J. Biol. Chem*. **261**:4574.
21. Climent, I., *et al.* (1989) Derivatization of r-glutamyl semialdehyde residues in oxidized proteins by fluoresceninamine. *Anal. Biochem*. **182**:226.
22. Levine, R.L. (1983) Oxidative modification of glutamine synthetase I. Interaction is due to loss of one histidine residue. *J. Biol. Chem.* **258**:11823.
23. Oliver, C.N. (1987) Inactivation of enzymes and oxidative modification of proteins by stimulated neutrophils. *Arch. Biochem. Biophys.* **253**:62.
24. Pognonec, P., *et al.* (1992) The helix-loop/leucine repeat transcription factor USF can be functionally regulated in a redox-dependent manner. *J. Biol. Chem.* **267**: 24563.
25. Rivett, A.J. (1986) Regulation of intracellular protein turnover: covalent modification as a mechanism of marking proteins for degradation. *Curr. Top. Cell Regul.* **28**:291.
26. Wolf, S.P. and Dean, R.T. (1986) Fragmentation of proteins by free radicals and its effect on their susceptibility to enzymic hydrolysis. *Biochem J.* **234**:399.
27. Davies, *et al.* (1987) Protein damage and degradation by oxygen radicals IV. Degradation of denatured protein. *J. Biol. Chem.* **262**:9914.
28. Levine, R.L., *et al.* (1981) Turnover of bacterial glutamine synthetase: Oxidative inactivation proceeds proteolysis. *Proc. Natl. Acad. Sci. USA* **78**:2120.
29. Oliver, C.N., *et al.* (1987) Age related changes in oxidized proteins. *J. Biol. Chem.* **262**:5488.
30. Smith, C.D., *et al.* (1991) Excess brain protein oxidation and enzyme dysfunction in normal aging and in Alzheimers disease. *Proc. Natl. Acad. Sci. USA.* **88**:10540.
31. Starke-Reed, P.E. and Oliver, C.N. (1989) Protein oxidation and proteolysis during aging and oxidative stress. *Arch. Biochem. Biophys.* **275**:559.
32. Berlett B. and Stadtman E. (1997) Protein Oxidation in Aging, Disease, and Oxidative Stress. *J. Biol. Chem.* 272:20313.

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