Product Manual

OxiSelect™ Protein Carbonyl ELISA Kit

Catalog Number

STA-310	96 assays
STA-310-5	5 x 96 assays

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures



Introduction

Protein oxidation is defined as the covalent modification of a protein induced either directly by reactive oxygen species (ROS) or indirectly by reaction with secondary by-products of oxidative stress. Oxidative modification of proteins can be induced in vitro by a wide array of pro-oxidant agents and occurs in vivo during aging and in certain disease conditions.

There are numerous types of protein oxidative modification. The most common products of protein oxidation in biological samples are the protein carbonyl derivatives of Pro, Arg, Lys, and Thr. These derivatives are chemically stable and serve as markers of oxidative stress for most types of ROS. Many of the current assays involve derivatization of the carbonyl group with dinitrophenylhydrazine (DNPH), followed by immunobloting with an anti-DNP antibody. The Protein Carbonyl ELISA was first developed by Buss and co-workers, the protein samples ($\geq$4 mg/mL) react with DNPH and then adsorb to wells of an ELISA plate before probe with anti-DNPH antibody. In their method, protein samples containing low amounts of protein must be concentrated to at least 4 mg/mL by TCA precipitation. However, TCA precipitation results a 20% loss of the total carbonyl values, and loss of protein during precipitation is also expected. In Cell Biolabs' OxiSelect™ Protein Carbonyl ELISA Kit, protein samples are first allowed to adsorb to wells of a 96-well plate and then react with DNPH. There is no need to concentrate protein in experimental and clinical samples with low amounts of protein (< 4 mg/mL) and the kit requires protein sample as little as 10 µg/mL.

The OxiSelect™ Protein Carbonyl ELISA Kit is an enzyme immunoassay developed for rapid detection and quantitation of protein carbonyls. The quantity of protein carbonyls in protein sample is determined by comparing its absorbance with that of a known reduced/oxidized BSA standard curve. Each kit provides sufficient reagents to perform up to 96 assays, including standard curve and unknown protein samples.

Assay Principle

BSA standards or protein samples (10 µg/mL) are adsorbed onto a 96-well plate for 2 hrs at 37ºC. The protein carbonyls present in the sample or standard are derivatized to DNP hydrazone and probed with an anti-DNP antibody, followed by an HRP conjugated secondary antibody. The protein carbonyl content in unknown sample is determined by comparing with a standard curve that is prepared from predetermined reduced and oxidized BSA standards.

Related Products

1. STA-304: Protein Tyrosine Nitration Control (Nitrotyrosine-BSA)
2. STA-305: OxiSelect™ Nitrotyrosine ELISA Kit
3. STA-308: OxiSelect™ Protein Carbonyl Immunoblot Kit
4. STA-309: Oxidized Protein Immunoblot Control (Carbonyl-BSA)
5. STA-318: OxiSelect™ AOPP Assay Kit
6. STA-319: AOPP-Human Serum Albumin (AOPP-HSA)
7. STA-816: OxiSelect™ N-epsilon-(Carboxymethyl) Lysine (CML) Competitive ELISA Kit
8. STA-817: OxiSelect™ Advanced Glycation End Products (AGE) Competitive ELISA Kit

Kit Components

1. 96-well Protein Binding Plate (Part No. 231001): One strip well 96-well plate.
2. Anti-DNP Antibody (1000X) (Part No. 231002): One 20 µL vial of anti-DNP Rabbit IgG.
3. Secondary Antibody, HRP Conjugate (1000X) (Part No. 231009): One 20 µL vial.
4. 25X DNPH Solution (Part No. 231010): One 500 µL amber vial.
5. 2X DNPH Diluent (Part No. 231005): One 15 mL bottle.
6. Blocking Reagent (Part No. 231006): One 20 g bottle.
7. 10X Wash Buffer (Part No. 310806): One 100 mL bottle.
8. Substrate Solution (Part No. 310807): One 12 mL amber bottle.
9. Stop Solution (Part. No. 310808): One 12 mL bottle.
10. Reduced BSA Standard (Part No. 231007): One 200 µL vial of 1 mg/mL fully reduced BSA in PBS.
11. Oxidized BSA Standard (Part No. 231008): One 200 µL vial of 1 mg/mL oxidized BSA in PBS at 7.5 nmol protein carbonyl/mg proteins. The protein carbonyl is predetermined by a spectrophotometric method as described by Reznick and Parker (See Ref. 5).

Materials Not Supplied

1. Protein samples such as purified protein, plasma, serum, cell lysate, or tissue homogenate
2. 1X PBS
3. Ethanol
4. 10 µL to 1000 µL adjustable single channel micropipettes with disposable tips
5. 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
6. Multichannel micropipette reservoir
7. Microplate reader capable of reading at 450 nm (620 nm as optional reference wave length)

Storage

Upon receipt, aliquot and store both the Reduced and Oxidized BSA Standards at -20ºC to avoid multiple freeze/thaw cycles. Store all other components at 4ºC.

Preparation of Reagents

- 1X Wash Buffer: Dilute the 10X Wash Buffer Concentrate to 1X with deionized water. Stir to homogeneity.
- 1X DNPH Diluent: Dilute the 2X DNPH Diluent to 1X with deionized water. Mix well.
- Blocking Solution: Weigh out 5 g of Blocking Reagent, dissolve in 100 mL of 1X PBS, and store at 4ºC for up to one week.

- DNPH Working Solution: Based on the number of tests, FRESHLY prepare appropriate amount of DNPH Working Solution by diluting the 25X DNPH Solution to 1X in 1X DNPH Diluent. For example: for 20 assays, transfer 80 µL of 25X DNPH Solution to a tube containing 1.92 mL of 1X DNPH Diluent, mix well and use it IMMEDIATELY.
- Anti-DNP Antibody and Secondary Antibody: Immediately before use dilute the Anti-DNPH antibody 1:1000 and Secondary Antibody 1:1000 with 1X Blocking Solution. Do not store diluted solutions.

Preparation of Protein Carbonyl BSA Standards

1. Freshly Prepare 10 µg/mL of reduced or oxidized BSA by diluting the 1 mg/mL BSA standards in 1X PBS. Example: Add 20 µL to 1.98 mL of 1X PBS.
2. Prepare a series of carbonyl BSA standards by mixing the oxidized BSA and reduced BSA in the proper ratios according to Table 1.

Standard Tubes	10 µg/mL Oxidized BSA (µL)	10 µg/mL Reduced BSA (µL)	[Protein Carbonyl] (nmol/mg)
1	400	0	7.5
2	320	80	6.0
3	240	160	4.5
4	160	240	3.0
5	80	320	1.5
6	40	360	0.75
7	20	380	0.375
8	0	400	0

Table 1. Preparation of Protein Carbonyl BSA Standard Curve

Preparation of Samples

1. Perform a protein assay such as Bradford or BCA on all samples to determine the protein concentration.

   Notes for cell and tissue lysates:
   - *Lysates should not be prepared in lysis buffer containing Triton X-100, NP-40, or Igepal CA-630 because these detergents interfere with protein coating of the plate unless the detergent concentration in the 10 µg/mL protein samples is no more than 0.001%. We recommend lysis by homogenization or sonication.*
   - *A high concentration of nucleic acid in cell or tissue lysates can erroneously contribute to higher estimation of carbonyl content. To remove nucleic acid, we recommend one of the following procedures:*
     1. *Pretreat lysate with nuclease, followed by ammonium sulfate precipitation of high percentage saturation.*
     2. *Add streptomycin sulfate or PEI to a final concentration of 1% and 0.5% respectively, incubate 30 minutes at room temperature and remove the nuclei acid precipitates by centrifuging at 6000 g for 10 minutes at 4ºC.*

2. Dilute each protein sample to 10 µg/mL in 1X PBS prior to use in the assay.

   Note: Samples with high concentrations of protein carbonyl content may be further diluted 5-10 fold in 10 µg/mL Reduced BSA. A titration may be performed to ensure the samples fall in the range of the standard curve.

Assay Protocol

1. Prepare unknown samples according to the Preparation of Samples section above. Each 10 µg/mL protein sample and BSA Standard should be assayed in duplicate or triplicate.
2. Add 100 µL of 10 µg/mL protein samples or reduced/oxidized BSA standards to the 96-well Protein Binding Plate. Incubate at 37ºC for at least 2 hours or 4ºC overnight.
3. Wash wells 3 times with 250 µL 1X PBS per well. After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess wash solution.
4. Add 100 µL of the DNPH Working Solution and incubate for 45 minutes at room temperature in the dark.
5. Wash wells with 250 µL of 1X PBS/Ethanol (1:1, v/v) with incubation on an orbital shaker for 5 minutes. Repeat washing a total of 5 times, aspirating between each. After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess wash solution. Wash 2 times with 250 µL of 1X PBS.
6. Add 200 µL of Blocking Solution per well and incubate for 1-2 hours at room temperature on an orbital shaker.
7. Wash 3 times with 250 µL of 1X Wash Buffer with thorough aspiration between each wash. After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess 1X Wash Buffer.
8. Add 100 µL of the diluted anti-DNP antibody to all wells and incubate for 1 hour at room temperature on an orbital shaker. Wash the strip wells 3 times according to step 7 above.
9. Add 100 µL of the diluted HRP conjugated secondary antibody to all wells and incubate for 1 hour at room temperature on an orbital shaker. Wash the strip wells 5 times according to step 7 above.
10. Warm Substrate Solution to room temperature. Add 100 µL of Substrate Solution to each well, including the blank wells. Incubate at room temperature on an orbital shaker. Actual incubation time may vary from 2-30 minutes.

Note: Watch plate carefully; if color changes rapidly, the reaction may need to be stopped sooner to prevent saturation.

11. Stop the enzyme reaction by adding 100 µL of Stop Solution to each well. Results should be read immediately (color will fade over time).
12. Read absorbance of each well on a plate reader using 450 nm as the primary wave length. Using the fully reduced BSA standard as absorbance blank.

Example of Results

The following figures demonstrate typical Protein Carbonyl ELISA results. One should use the data below for reference only. This data should not be used to interpret actual results.

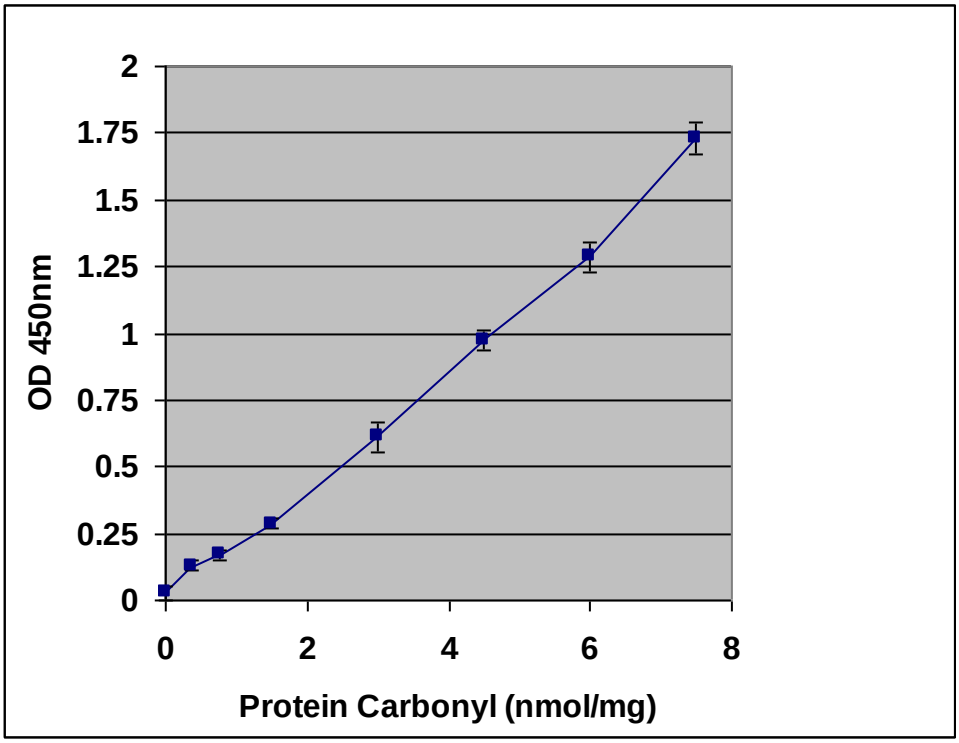


Figure 1: Protein Carbonyl ELISA Standard Curve

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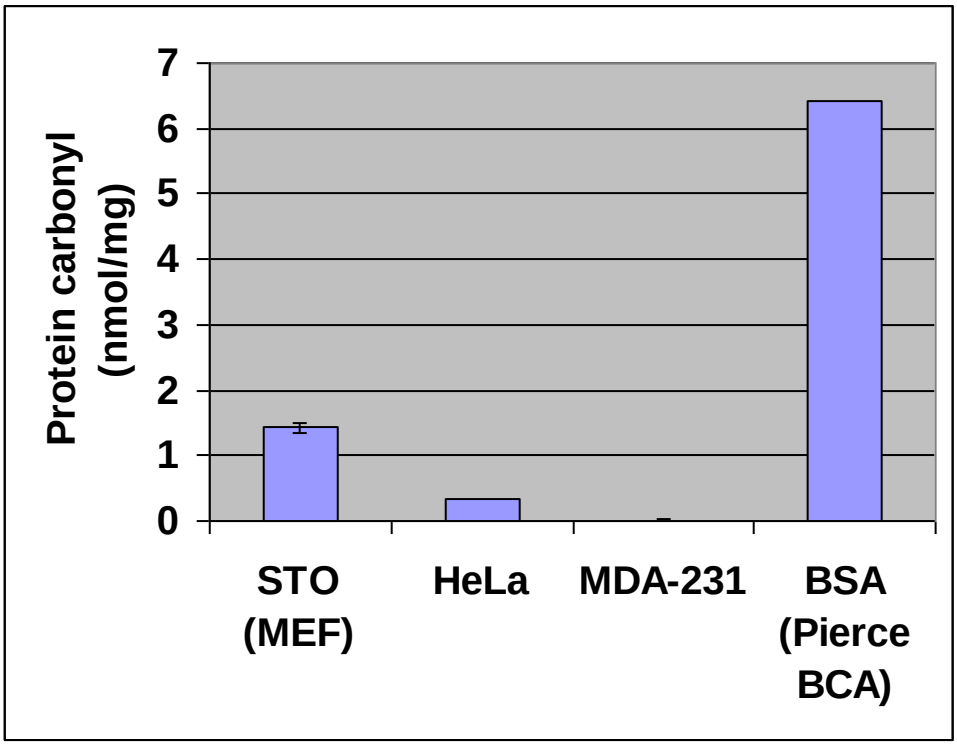


Figure 2: Amount of Protein Carbonyl Content for Cell Lysate and BSA Standard. STO (MEF), HeLa and MDA-231 cells were sonicated in 25mM HEPES, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 2% Glycerol. Cell Lysates and BSA Standard from Pierce BCA Protein Assay were diluted to 10 µg/mL with 1X PBS and coated onto a 96-well Protein Binding Plate. The protein carbonyl levels were determined as described in the Assay Protocol.

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