Protein Carbonyl ELISA Kit

Enzyme Immunoassay kit for the quantification of Protein Carbonyl in purified protein, plasma, serum, cell lysate, or tissue homogenate.

Catalog number: ARG81227

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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 (Buss H, et.al. *Free Radic Biol Med*. 1997), the protein samples (at concentration >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 this Protein Carbonyl ELISA Kit, protein samples are first allowed to adsorb to wells of a 96-well plate and then react with DNPH. And the concentration of the protein samples are as little as 10 µg/mL so there is no need to concentrate protein in experimental and clinical samples with low amounts of protein (< 4 mg/mL).

PRINCIPLE OF THE ASSAY

The Protein Carbonyl ELISA Kit is an enzyme immunoassay developed for rapid detection and quantitation of protein carbonyls.

BSA standards or protein samples (10 µg/mL) are firstly adsorbed onto a 96-well plate. After wash, DNPH is adding in the well. The protein carbonyls present in the sample or standard are derivatized to DNP hydrazine. After washing away any excess DNPH, an antibody specific for DNP is added to each well and incubate. Following a washing to remove unbound substances, a secondary antibody conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After washing away any unbound antibody-enzyme reagent, a substrate solution (TMB) is added to the wells and color develops in proportion to the amount of DNP. The color development is stopped by the addition of acid and the intensity of the color is measured at a wavelength of 450nm ±2nm. 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.

MATERIALS PROVIDED & STORAGE INFORMATION

Store the unopened kit at 2-8 °C. Use the kit before expiration date.

Component	Quantity	Storage information
antibody-coated microplate	96 wells plate	4°C
Reduced BSA Standard (1 mg/ml)	200 µl	-20°C
Oxidized BSA Standard (1 mg/ml)	200 µl	-20°C
10X Wash Buffer	100 ml	4°C
1000X Anti-DNP Antibody concentrate	20 µl	4°C
1000X HRP-conjugated secondary Antibody concentrate	20 µl	4°C
25X DNPH Diluent	500 µl	4°C
2X DNPH Diluent	15 ml	4°C
Blocking Reagent Powder	20 g	4°C
TMB substrate	12ml (Ready-to-use)	4°C (Protect from light)
STOP solution	12ml (Ready-to-use)	4°C

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 450nm (optional: 620 nm as optional reference wave length)
- 1X PBS
- Ethanol
- Pipettes and pipette tips
- Multichannel micropipette reservoir
- Deionized or distilled water.

- Microplate shaker.
- Automated microplate washer (optional)

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Store Standard at -20°C and other kit components at 4°C at all times.
- Upon receipt, the Reduced and Oxidized BSA Standard should be aliquoted and stored at ≤ -20°C to avoid repeated freeze-thaw cycles.
- If crystals are observed in the 10X Wash buffer, warm to RT or 37°C until the crystals are completely dissolved.
- Ensure complete reconstitution and dilution of reagents prior to use.
- All reagents should be mixed by gentle inversion or swirling prior to use. Do not induce foaming.
- Before using the kit, spin tubes and bring down all components to the bottom of tubes.
- It is highly recommended that the standards and samples be assayed in duplicates.
- Change pipette tips between the addition of different reagent or samples.

SAMPLE COLLECTION & STORAGE INFORMATION

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Sample collection:

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Collect serum and assay immediately or aliquot and store samples at ≤ -80°C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -80°C. Avoid repeated freeze-thaw cycles.

Cell and tissue lysates:

- Since detergents (Triton X-100, NP-40, or Igepal CA-630) interfere protein coating of the plate, we suggested using lysis buffer without detergents for Cell and tissue lysates collection. However, if the detergent concentration in the 10 µg/mL protein samples is less than 0.001% may also be used.

- We suggest to homogenize the samples in PBS containing proteinase inhibitors and optional 0.005% Butylated hydroxytoluene (BHT is a generic antioxidant, prepare 5% stock of BHT in methanol before used), followed by either homogenization or sonication, centrifuge at 12000g for 10 min, and harvest the supernatant. (We recommend lysis samples by homogenization or sonication to increase lysis efficiency). Assay immediately or aliquot and store samples at ≤ -80°C. Avoid repeated freeze-thaw cycles.

- 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 (optional: precipitate protein 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 4°C.

  Note: Any DNA/RNA removal method can be used as long as no additional proteins are introduced during the procedure. Any additional proteins may contain carbonyl groups, which will influence the assay results.

Sample Preparation

1. Perform a protein assay such as Bradford or BCA on all samples to determine the protein concentration.
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 of Reduced BSA. A titration may be performed to ensure the samples fall in the range of the standard curve. For the calculation of the concentrations this dilution factor has to be taken into account.

REAGENT PREPARATION

- 1X Wash buffer: Dilute 10X Wash buffer into distilled water to yield 1X Wash buffer, mix well. Storage at 2-8°C.
- 1X DNPH Diluent: Dilute 2X DNPH Diluent into distilled water to yield 1X DNPH Diluent, mix well. Storage at 2-8°C.
- 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 with 1X DNPH Diluent. (E.g.: for 20 assays, add 100 µL of 25X DNPH Solution to a tube containing 2.4 mL of 1X DNPH Diluent, mix well and use it IMMEDIATELY.
- 1X Blocking Reagent: Weigh out 5 g of Blocking Reagent Powder, dissolve in 100 mL of 1X PBS. The 1X Blocking Reagent can be stored at 4°C for up to one week.
- 1X Anti-DNP Antibody working solution: Dilute the antibody immediately before use; dilute the 1000X Anti-DNP Antibody concentrate into 1X Blocking reagent to yield 1X Anti-DNP Antibody working solution. (E.g.: 10 µl of the Anti-DNP Antibody concentrate (1000X) + 9.990ml of 1X Blocking reagent) Do not store diluted solutions.
- 1X HRP-conjugated secondary Antibody working solution: Dilute the antibody immediately before use; dilute the 1000X HRP-conjugated secondary Antibody concentrate into 1X Blocking reagent to yield 1X HRP-conjugated secondary Antibody working solution. (E.g.: 10 µl of the HRP-conjugated secondary Antibody concentrate (1000X) + 9.990 ml of 1X Blocking reagent) Do not store diluted solutions.

- 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. (E.g.: Add 20 µL to 1.98 mL of 1X PBS)
2. Prepare a series dilution of Protein Carbonyl BSA Standards by mixing the oxidized BSA and reduced BSA as according to the suggested concentration table below:

Standard No	10 µg/mL Oxidized BSA (µL)	10 µg/mL Reduced BSA (µL)	[Protein Carbonyl] (nmol/mg)
S1	400	0	7.5
S2	320	80	6.0
S3	240	160	4.5
S4	160	240	3.0
S5	80	320	1.5
S6	40	360	0.75
S7	20	380	0.375
S0	0	400	0

ASSAY PROCEDURE

1. Prepare unknown samples according to the Samples Preparation 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. Cover the plate and incubate the plate at **37°C for at least 2 hours** or **4°C overnight.**
3. Aspirate each well and wash, repeating the process 2 times for a total **3 washes**. Wash by filling each well with **250 µl of 1× PBS** using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels.
4. Add **100 µl** of the **DNPH Working Solution** and incubate for **45 minutes at room temperature** in the dark.
5. Aspirate each well and wash, repeating the process 4 times for a total **5 washes**. Wash by filling each well with **250 µl of 1X PBS/Ethanol (1:1, v/v)**. Incubate the plate on an orbital shaker for 5 minutes at each wash step. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels.
6. Aspirate each well and wash, repeating the process 1 times for a total **2 washes**. Wash by filling each well with **250 µl of 1X PBS**. Incubate the plate on an orbital shaker for 5 minutes at each wash step. Complete removal of liquid at each is essential to good performance. After the last

wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels.

7. Add **200 µl** of the **1X Blocking reagent** to each well, and incubate for **1-2 hour at RT** on a microplate shaker.
8. Aspirate each well and wash, repeating the process 2 times for a total **3 washes**. Wash by filling each well with **250 µl of 1× Wash Buffer** using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating, decanting or blotting against clean paper towels.
9. Add **100 µl** of the 1:1000 **diluted 1X anti-DNP antibody working solution** to each well, cover the plate and incubate for **1 hour at RT** on a microplate shaker.
10. Aspirate each well and **wash** as step 8.
11. Add **100 µl** of the 1:1000 **diluted 1X HRP-conjugated secondary antibody working solution** to all wells, cover the plate and incubate for **1 hour at RT** on a microplate shaker.
12. **Warm TMB substrate solution to RT** before next wash step.
13. Aspirate each well and wash as step 8, but wash for a total **5 washes** at this step. Proceed immediately to the next step.
14. Add **100 µl** of **TMB substrate solution** into each well. Incubate for **2-30 mins at RT** on microplate shaker. Avoid exposure to light.
    Note: Watch plate carefully; if color changes rapidly, the reaction may need to be stopped sooner to prevent saturation.
15. Add **100 µl of Stop Solution** to each well and shake lightly to ensure

homogeneous mixing.

16. Read the OD with a microplate reader at 450nm immediately. Using the fully reduced BSA standard as absorbance blank.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards, controls and samples.

2. Using linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.

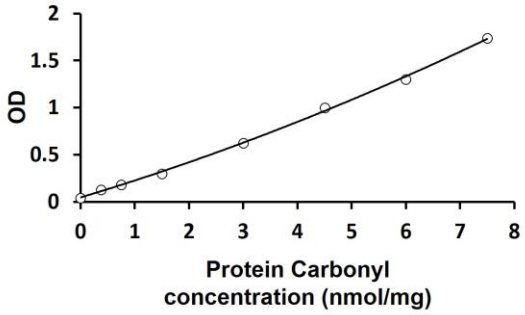
3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.

4. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.

5. If the samples have been diluted, the concentration read from the standard curve must be further converted by the appropriate dilution factor according to the sample preparation procedure as described above.

EXAMPLE OF TYPICAL STANDARD CURVE

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.



QUALITY ASSURANCE

Sensitivity

1 ng/ml

Assay Range

1.56 - 100 ng/ml