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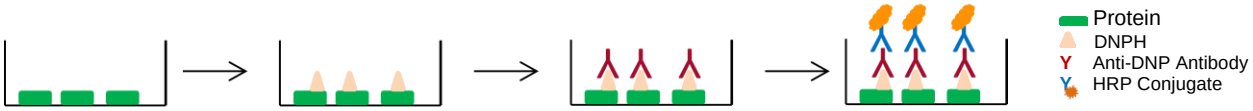
Protein Carbonyl ELISA Kit

06/21

(Catalog # E4910-100, 100 assays; Store at -20 °C)

I. Introduction:

Protein Carbonyl (PC) groups such as aldehydes and ketones are added on the protein side chains of proline, arginine, lysine, and threonine by the free radical-mediated oxidation of proteins either directly by reactive oxygen species (ROS) or indirectly by secondary by-products of oxygen species. Oxidative damage to proteins can affect their functions as receptors, enzyme activity, and transport and can lead to aggregation, crosslinking or fragmentation. PC content has been shown to be elevated in pathologies including cancer, inflammation, neurodegenerative disorders etc. PC moieties are chemically stable and are abundant. Thus, they are used as a biomarker of oxidative stress and a measure of protein oxidation. **BioVision's Protein Carbonyl ELISA Kit** is an enzyme-linked immunosorbent assay kit designed for the quantitative measurement of protein carbonyls in biological samples including serum, cell lysates, tissue lysates etc. In this kit, protein samples, as little as 10 µg/ml are coated to the 96-well strip plate, the protein carbonyl group of which then reacts with 2,4-Dinitrophenylhydrazine (DNPH). The protein-conjugated DNPH is then detected using a specific Anti-DNPH antibody followed by HRP conjugated secondary antibody. Subsequently, a TMB substrate is added to visualize the HRP enzymatic reaction thereby generating a blue colored product, which turns yellow once the stop solution is added. The intensity of the yellow color is proportional to the amount of protein carbonyl in samples. The quantity of protein carbonyls in samples is determined by comparing their absorbance with a known reduced/oxidized BSA Standard. The assay is accurate, sensitive and can detect as low as 0.35 nmol/mg protein carbonyl in samples.



II. Application:

- Determination of Protein Carbonyl amounts in biological samples

III. Sample Types:

- Biological fluids: Serum, etc.
- Cell Lysates: Hela cells, etc.
- Tissue Homogenates: Mouse liver, etc.

IV. Kit Contents:

Components	E4910-100	Cap Code	Part Number
96-Well Strip Plate	8 x 12 Strips	--	E4910-100-1
DNPH Solution	120 µl	Amber	E4910-100-2
DNPH Diluent	200 µl	White	E4910-100-3
Blocking Buffer	25 ml	WM	E4910-100-4
Wash Buffer (10X)	50 ml	WM	E4910-100-5
Anti-DNP Antibody	10 µl	Yellow	E4910-100-6
HRP Conjugate Stock	25 µl	Blue	E4910-100-7
Antibody Diluent	50 ml	NM	E4910-100-8
Reduced BSA Standard (1 mg/ml)	120 µl	Green	E4910-100-9
Oxidized BSA Standard (1 mg/ml)	120 µl	Orange	E4910-100-10
TMB Substrate	20 ml	Amber	E4910-100-11
Stop Solution	20 ml	NM	E4910-100-12
Plate Sealing Film	2	--	E4910-100-13

V. User Supplied Reagents and Equipment:

- Multiwell microplate Spectrophotometer capable of measuring absorbance at 650 nm and 450 nm
- Adjustable pipettes, pipette tips and reagent reservoir. Multichannel pipettes are recommended
- Phosphate Buffered Saline (PBS)
- dH_2O
- Ethanol (Pure, ≥ 99.9%)
- Syringe and Syringe Filter (0.22 µm)
- Absorbent paper

VI. Storage Conditions and Reagent Preparation:

Store the kit at -20 °C, protected from light. Briefly centrifuge all small vials prior to opening. Read the entire protocol before performing the experiment.

- **DNPH Solution and DNPH Diluent:** Store the DNPH Solution at -20 °C, protected from light. DNPH Diluent can be kept at 4 °C or at room temperature (RT). **Note: DNPH Diluent contains high concentration of Sodium Hydroxide. Wear gloves and follow safe laboratory practices when handling.**
- **Wash Buffer (10X):** Warm to RT prior to use. If crystals are present, mix gently until the crystals are completely dissolved. Prepare 100 ml of 1X Wash Buffer by diluting 10 ml of Wash Buffer (10X) with 90 ml dH_2O. The 1X Wash Buffer is stable for one month at 4 °C.

- **Anti-DNP Antibody and HRP Conjugate Stock:** Store at -20 °C, protected from light. Keep on ice while in use.
- **Reduced BSA Standard** and **Oxidized BSA Standard:** Divide into aliquots and store at -20 °C. Avoid frequent freeze-thaw cycles. Keep on ice while in use.
- **Blocking Buffer, Antibody Diluent, TMB Substrate** and **Stop Solution:** Provided as a ready-to-use solution. Bring to RT 30 min prior to use. When not in use, reseal the bottle immediately and store at 4 °C, protected from light.

VII. Protein Carbonyl ELISA Protocol:

1. **Sample Preparation: For biological fluids:** Collect the samples and centrifuge at 1,000-2,000 x *g* for 15 min at 4 °C. Collect the supernatants and keep the samples on ice. **For cells or tissues:** Rapidly Homogenize the pelleted cells (~1 x 10^6) or tissue (~10-50 mg) in 200~500 µl ice-cold PBS on ice for 15 min. Centrifuge the sample at 12,000 x *g* for 15 min at 4 °C and collect the supernatant. The supernatant can be stored at -20 °C for up to one week prior to the assay. For all samples, estimate the protein concentration using BCA protein assay kit (BioVision Cat# K813-2500) or any preferred method. Dilute the samples to 10 µg/ml using ice-cold PBS and keep the diluted samples on ice.

   Notes:

   i. Prepare diluted samples (10 µg/ml) as needed, immediately prior to use. If sample stock concentration is < 10 µg/ml, homogenize samples in lower volume of PBS.
   ii. Detergents such as Triton X-100, Tween, CHAPS, Brij 35 etc. affect the assay. These detergents will interfere with the protein coating on the 96-well plate.
   iii. Sonication may be used for lysing the samples.

2. **BSA Standard Preparation:**

   a. In clean microfuge tube, dilute the Reduced BSA Standard (1 mg/ml) at 1:100 ratio with PBS to prepare (10 µg/ml) Reduced BSA Standard. i.e. mix 12 µl of Reduced BSA Standard with 1188 µl PBS.

   b. In another clean microfuge tube, dilute the Oxidized BSA Standard (1 mg/ml) at 1:100 ratio with PBS to prepare (10 µg/ml) Oxidized BSA Standard. i.e. mix 12 µl of Oxidized BSA Standard with 1188 µl PBS.

   c. Mix diluted Reduced BSA Standard with diluted Oxidized BSA Standard to prepare the Protein Carbonyl Standards as shown below. Suggested Standards are 8, 5.6, 3.2, 1.6, 0.8 and 0 nmol/mg respectively.

Standards	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**
Diluted Reduced BSA (10 µg/ml)	0 µl	90 µl	180 µl	240 µl	270 µl	300 µl
Diluted Oxidized BSA (10 µg/ml)	300 µl	210 µl	120 µl	60 µl	30 µl	0 µl
Mix Well						
Protein Carbonyl (nmol/mg)	**8**	**5.6**	**3.2**	**1.6**	**0.8**	**0**

3. **Assay Plate Preparation:**

   a. Add 100 µl of Sample (10 µg/ml) to wells labeled as **Sample** and **Sample Control** and 100 µl of **Standards** to Standard wells that you intend to coat. Seal the plate and incubate the sealed plate overnight at 4 °C, protected from light. **Note: i.** A Standard Curve must be run with each assay. **ii.** Coating Solution should be prepared fresh, directly before use.

   b. Following overnight incubation, aspirate the Coating Solution and add 250 µl of PBS to each well on an orbital shaker and incubate for 5 min. Aspirate the PBS from each well and pat it dry against clean absorbent paper. Repeat this rinse step once more (for a total of 2 rinses). After the last wash, remove any remaining PBS by aspirating or decanting. Clap the plate on clean absorbent papers.

4. **Assay Plate Layout and Reagent Preparation:**
   Notes: i. Intensive washing is essential step for accurate results, we recommend setting the orbital shaker speed to 800 rpm or higher in washing steps to achieve best results. This applies to all the following wash steps. **ii.** DNPH is light sensitive. Thus, protect the plate from light during the entire process by wrapping the plate with an aluminum foil.

   a. Prepare **DNPH working solution** immediately before use by adding 20 µl of DNPH Solution and 32 µl DNPH Diluent to 10 ml dH_2O and mix well. We recommend filtering the DNPH working solution using a 0.22 µm Syringe filter before use. Add **100 µl DNPH working solution** to each well containing **Sample**, **Sample Control** and **Standard**s. Incubate the plate at RT for 45 min with gentle orbital shaking (~400 rpm), protected from light. **Note:** Do not store or reuse the DNPH working solution.

   b. Prepare the **1X DNPH Wash Buffer** immediately before use by mixing equal volume of pure Ethanol and 1X Wash Buffer **(1:1, v/v)**. For example, mix 20 ml Ethanol with 20 ml of 1X Wash Buffer to prepare 40 ml 1X DNPH Washing Buffer. Aspirate the DNPH working solution and wash each well by filling with 250 µl of **1X DNPH Wash Buffer** on an orbital shaker (≥ 800 rpm) and incubating for 5 min. Repeat the washing step for a total of 5 times. After the last wash, remove any remaining DNPH Wash Buffer by aspirating or decanting. Clap the plate on absorbent filter papers.

   c. Add 250 µl **1X Wash Buffer** to each well on an orbital shaker (≥ 800 rpm) and incubate for 5 min. Repeat the wash step for a total of 2 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Clap the plate on absorbent filter papers.

5. Add **250 µl Blocking Buffer** to each well and incubate the plate at RT for 1.5~2 hr with gentle orbital shaking (~400 rpm), protected from light. Aspirate the Blocking Buffer and rinse wells twice with 250 µl of 1X Wash Buffer on an orbital shaker (≥ 800 rpm) and incubate for 5 min. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Clap the plate on absorbent filter papers or other absorbent materials.

6. **Antibody Reaction:**

   Note: Prepare the **Antibody working solution** fresh. Do not store or reuse the Antibody working solution.

   a. Prepare a 1000 fold dilution of the Anti-DNP Antibody. i.e. mix 1 µl of Anti-DNP Antibody with 999 µl Antibody Diluent. Further perform a 60 fold dilution of the diluted Anti-DNP Antibody by mixing 50 µl of diluted Anti-DNP Antibody with 2950 µl Antibody Diluent to prepare the **Anti-DNP Antibody working solution**. Add 100 µl of Anti-DNP Antibody working solution to each well containing Sample and Standards. **Add 100 µl of Antibody Diluent to the Sample Control** well. Incubate the plate for 1 hr at RT with gentle orbital shaking, protected from light. **Note:** Do not add Anti-DNP Antibody working solution to the Sample Control well.

b. Aspirate the Anti-DNP Antibody working solution or the Antibody Diluent and add 250 µl of 1X Wash Buffer to each well. Place the plate on an orbital shaker (≥ 800 rpm) and incubate for 5 min. Aspirate the Wash Buffer from each well and pat it dry against clean absorbent paper. Repeat the wash step a total of 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Clap the plate on absorbent filter papers.

c. Prepare a 1000 fold dilution of HRP Conjugate Stock by mixing 1 µl HRP Conjugate Stock with 999 µl Antibody Diluent. Further perform a 100 fold dilution of the diluted HRP Conjugate (i.e. mix 30 µl of diluted HRP Conjugate with 2970 µl Antibody Diluent) to prepare the **HRP Conjugate working solution**. Add **100 µl of HRP Conjugate working solution** to each well containing Sample, Sample Control and Standards. Incubate the plate for 1 hr at RT with gentle orbital shaking, protected from light.

d. Aspirate the **HRP Conjugate working solution** and add 250 µl of 1X Wash Buffer to each well. Place the plate on an orbital shaker (≥ 800 rpm) and incubate for 5 min. Aspirate the Wash Buffer from each well and pat it dry against clean absorbent paper. Repeat this wash step for 5 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Clap the plate on absorbent filter papers.

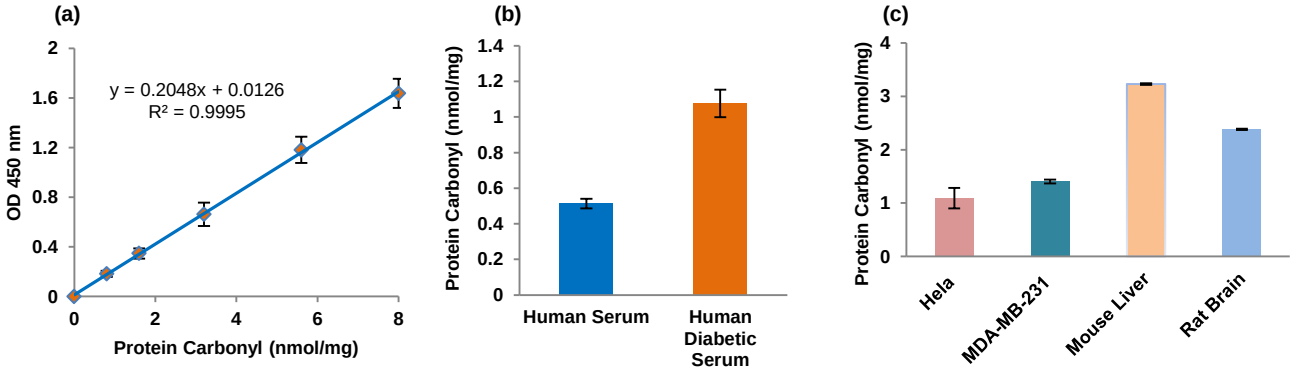
7. **Measurement:** Remove any residual Wash Buffer from the wells and add 100 µl TMB Substrate to all wells. Measure the absorbance at 650 nm for 2-30 min at RT and monitor the color development. Add 100 µl Stop Buffer into each well to stop the reaction.

Notes:

i. The incubation time following addition of the TMB substrate must be optimized to avoid over development of the color. Recommended absorbance (OD 650 nm) for BSA Standard S1 well is 0.6-0.8. After adding the Stop Solution, mix well and read the plate immediately.

ii. The OD value at 450 nm will be roughly twice the OD value at 650 nm.

8. **Calculation:** Subtract the **Standard S6** (0 nmol/mg) reading from all the Standards and Sample readings. Plot the Protein Carbonyl Standard Curve as the relative $OD_{450\ nm}$ of each Standard (Y) vs. the respective protein carbonyl amounts (nmol/mg) of the Standards (X). **If the Sample Control reading is higher than the Standard S6 reading, subtract the Sample Control readings from the Sample readings instead.** Apply the corrected Sample readings to the Protein Carbonyl Standard Curve to get nmol/mg of Protein Carbonyl amounts in samples.



Figures: (a) Protein Carbonyl Standard Curve. This Standard Curve is for demonstration only. *A Standard Curve must be run with each assay.* **(b)** Estimated Protein Carbonyl amounts (nmol/mg) in healthy human serum (10 µg/ml) and in human diabetic serum (10 µg/ml), respectively. **(c)** Estimated Protein Carbonyl amounts (nmol/mg) in Hela cell, MDA-MB-231 cell, mouse liver and rat brain lysates. All cells or tissue samples were prepared in PBS and coated on plate at a concentration of 10 µg/ml. Data presented are mean ± SD of triplicates or greater wells. Assays were performed following the kit protocol.

VII. Related Products:

Protein Carbonyl Content Assay Kit (K830)
Advanced Glycation End Products (AGEs) Assay Kit (K929)
BCA Protein Assay Kit II (K813)
Protein Carbonyl Content Assay Kit (Fluorometric) (K563)
AGE-BSA (2221)
Dounce Tissue Homogenizer (1998)

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