

Protein Carbonyl Assay Kit

Catalog No. 10005020

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GENERAL INFORMATION

Materials Supplied

Catalog Number	Item	Quantity
10005845	Protein Carbonyl Hydrochloric Acid	2 vials
10005846	Protein Carbonyl DNPH	2 vials
10005847	Protein Carbonyl TCA Solution	1 vial
10005848	Protein Carbonyl Guanidine Hydrochloride	1 vial
10005849	Protein Carbonyl Ethanol	3 vials
10005850	Protein Carbonyl Ethyl Acetate	3 vials
400014	96-Well Plate (Colorimetric Assay)	1 plate
400012	96-Well Cover Sheet	1 cover

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 975-3999. We cannot accept any returns without prior authorization.



WARNING: This product is for laboratory research use only; not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

Precautions

Please read these instructions carefully before beginning this assay.

For research use only. Not for human or diagnostic use.

It is recommended to take appropriate precautions when using the kit reagents (*i.e.*, lab coat, gloves, eye goggles, etc.) as some of them can be harmful.

Hydrochloric and trichloroacetic acids are corrosive and are harmful if swallowed. Contact with skin may cause burns. In case of contact with skin or eyes, rinse immediately with plenty of water for 15 minutes. Keep away from combustible materials.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

E-Mail: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed at 4°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance between 360-385 nm
2. Adjustable pipettes and a repeat pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable
4. Microcentrifuge.
5. Streptomycin sulfate for removal of nucleic acids (optional, see under **Sample Preparation**, page 11).
6. Bovine serum albumin (BSA) for determination of protein concentration (optional, see under **Calculations**, page 16).
7. Spectrophotometer for determination of nucleic acid contamination and protein concentration (optional, see under **Sample Preparation** and **Calculations**, pages 11 and 15, respectively).

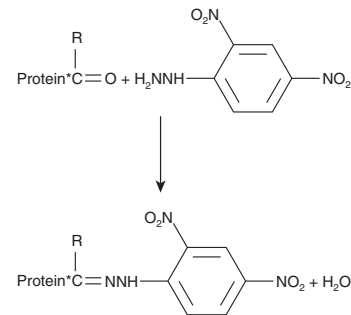
Background

Reactive oxygen species (ROS) are produced as a consequence of normal aerobic metabolism. Highly reactive free radical species can oxidize lipids, proteins, and DNA which contributes to the onset of a variety of diseases including cancer, arteriosclerosis, cardiovascular, and inflammatory diseases.¹ The most general indicator and by far the most commonly used marker of protein oxidation is protein carbonyl content.² Redox cycling cations such as Fe^{2+} or Cu^{2+} can bind to cation binding locations on proteins and with the aid of further attack by H_2O_2 or O_2 can transform side-chain amine groups on several amino acids (*i.e.*, lysine, arginine, proline, or histidine) into carbonyls. Metal-catalyzed protein oxidation is not the only mechanism by which carbonyls are introduced into proteins. Cigarette smoke and aldehydes have also been implicated in the oxidation of plasma proteins.³

Several approaches have been taken to detect and quantitate the carbonyl content in protein preparations. The most convenient procedure is the reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls. DNPH reacts with protein carbonyls, forming a Schiff base to produce the corresponding hydrazone, which can be analyzed spectrophotometrically (see Scheme 1, on page 7).⁴

About This Assay

Cayman's Protein Carbonyl Assay Kit utilizes the DNPH reaction to measure the protein carbonyl content in plasma, serum, cell lysates, or tissue homogenates in a convenient 96-well format. The amount of protein-hydrazone produced is quantified spectrophotometrically at an absorbance between 360-385 nm. The carbonyl content can then be standardized to protein concentration.



Scheme 1. Biochemistry of the Protein Carbonyl Assay

Reagent Preparation

1. Hydrochloric Acid - (Catalog No. 10005845)

The vial contains 12 M hydrochloric acid (HCl). Slowly add the contents of the vial to 40 ml of HPLC-grade water to yield 2.5 M HCl. The 2.5 M HCl is used to resuspend the DNPH. The diluted HCl is stable for at least three months at room temperature.

2. DNPH - (Catalog No. 10005846)

The vial contains 2,4-dinitrophenylhydrazine. Dissolve the contents of the vial in 10 ml of 2.5 M HCl. This is enough reagent to process 12 samples. If more samples are being treated, than resuspend the second vial. Reconstituted DNPH is stable for one week at 4°C and stored in the dark. *Do not freeze!*

3. TCA Solution - (Catalog No. 10005847)

NOTE: Wear appropriate protection when handling the TCA solution.

The vial contains a 2 g/ml solution of trichloroacetic acid (TCA). Slowly add 12 ml of the TCA solution to 108 ml of HPLC-grade water. This dilution results in a 20% TCA solution. Transfer 40 ml of the 20% TCA solution to another vessel and add 40 ml of HPLC-grade water. This results in a 10% TCA solution. Both solutions are stable at room temperature for at least one week.

4. Guanidine Hydrochloride - (Catalog No. 10005848)

The vial contains a solution of guanidine hydrochloride. It is ready to use as supplied.

5. Ethanol - (Catalog No. 10005849)

Each vial contains 30 ml of ethanol. It is ready to use as supplied.

6. Ethyl Acetate - (Catalog No. 10005850)

Each vial contains 30 ml of ethyl acetate. Mix the contents of one vial (30 ml) with 30 ml of ethanol (Catalog No. 10005849) for a 1:1 mixture of ethanol:ethyl acetate. This is enough reagent to process nine samples. If more samples are being treated, mix together additional ethanol and ethyl acetate vials.

Sample Preparation

This assay works best when samples have protein concentrations in the range of 1-10 mg/ml.

Plasma

Typically human plasma has a protein carbonyl content of 0.5-4.0 nmol/mg.⁵

1. Collect blood using an anticoagulant such as heparin, EDTA, or citrate.
2. Centrifuge the blood at 700-1,000 x g for 10 minutes at 4°C. Pipette off the top yellow plasma layer without disturbing the white buffy layer. Store plasma on ice until assaying or freeze at -80°C. The plasma sample will be stable for at least one month.

Serum

Typically human serum has a protein carbonyl content of 0.1-1.0 nmol/mg.⁶

1. Collect blood without using an anticoagulant such as heparin, EDTA, or citrate. Allow blood to clot for 30 minutes at room temperature.
2. Centrifuge the blood at 2,000 x g for 15 minutes at 4°C. Pipette off the top yellow serum layer without disturbing the white buffy layer. Store serum on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for at least one month.

Urine

Assaying urine is not recommended due to low protein concentration.

Cell Lysate

1. Collect cells by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C).
2. The cell pellet can be homogenized or sonicated on ice in 1-2 ml of cold buffer (*i.e.*, 50 mM MES or phosphate, pH 6.7, containing 1 mM EDTA).
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and store on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for at least one month.
5. Check the supernatant absorbance at 280 nm and 260 nm to determine if there are contaminating nucleic acids present in the sample. Use the homogenization buffer as a blank. If the ratio 280/260 is less than 1, a further step to remove nucleic acids with 1% streptomycin sulfate is needed.

Tissue Homogenate

1. Dissect 200-300 mg of tissue. Rinse tissue with a phosphate buffered saline solution to remove any red blood cells or clots. Samples containing significant amounts of heme, especially hemoglobin will interfere with the assay.
2. Homogenize the tissue in 1-2 ml of cold buffer (*i.e.*, 50 mM MES or phosphate, pH 6.7, containing 1 mM EDTA).
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and store on ice. If not assaying the same day, freeze at -80°C. The sample will be stable for at least one month.
5. Check the supernatant absorbance at 280 nm and 260 nm to determine if there are contaminating nucleic acids present in the sample. Use the homogenization buffer as a blank. If the ratio 280/260 is less than 1, a further step to remove nucleic acids with 1% streptomycin sulfate is needed.

Removal of Nucleic Acids. Nucleic acids may erroneously contribute to a higher estimation of carbonyls. Whenever the ratio of 280/260 nm is less than 1, samples should be incubated with streptomycin sulfate at a final concentration of 1% in the sample (A 10% streptomycin sulfate stock solution should be made in 50 mM potassium phosphate, pH 7.2). Incubate the samples at room temperature for 15 minutes and then centrifuged at 6,000 x g for 10 minutes at 4°C. Use the supernatant for determining protein carbonyl content.⁵

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of samples to be measured in duplicate is given (see Figure 1). We suggest you record the contents of each well on the template sheet provided (see page 23).

	1	2	3	4	5	6	7	8	9	10	11	12
A	(S1)	(S1)	(S5)	(S5)	(S9)	(S9)	(S13)	(S13)	(S17)	(S17)	(S21)	(S21)
B	(C1)	(C1)	(C5)	(C5)	(C9)	(C9)	(C13)	(C13)	(C17)	(C17)	(C21)	(C21)
C	(S2)	(S2)	(S6)	(S6)	(S10)	(S10)	(S14)	(S14)	(S18)	(S18)	(S22)	(S22)
D	(C2)	(C2)	(C6)	(C6)	(C10)	(C10)	(C14)	(C14)	(C18)	(C18)	(C22)	(C22)
E	(S3)	(S3)	(S7)	(S7)	(S11)	(S11)	(S15)	(S15)	(S19)	(S19)	(S23)	(S23)
F	(C3)	(C3)	(C7)	(C7)	(C11)	(C11)	(C15)	(C15)	(C19)	(C19)	(C23)	(C23)
G	(S4)	(S4)	(S8)	(S8)	(S12)	(S12)	(S16)	(S16)	(S20)	(S20)	(S24)	(S24)
H	(C4)	(C4)	(C8)	(C8)	(C12)	(C12)	(C16)	(C16)	(C20)	(C20)	(C24)	(C24)

S1-S24 - Samples 1-24

C1-C24 - Control Samples 1-24

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that an adjustable pipette be used to deliver reagents to the wells.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 220 µl in all the wells.
- All reagents except samples must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the samples be assayed in duplicate.
- Monitor the absorbance at 360-385 nm using a plate reader.

Performing the Assay

Follow the procedure below for each sample to be analyzed:

1. Transfer 200 μl of sample to two 2 ml plastic tubes. One tube will be the sample tube (S#) and the other will be the control tube (C#).
2. Add 800 μl of DNPH to the sample tube and add 800 μl of 2.5 M HCl to the control tube.
3. Incubate both tubes (S# & C#) in the dark at room temperature for one hour. Vortex each tube briefly every 15 minutes during the incubation.
4. Add 1 ml of 20% TCA to each tube and vortex. Place tubes on ice and incubate for 5 minutes.
5. Centrifuge tubes at 10,000 x g for 10 minutes at 4°C in a microcentrifuge.
6. Discard the supernatant and resuspend the pellet in 1 ml of 10% TCA. Place tubes on ice and let sit for 5 minutes.
7. Centrifuge tubes at 10,000 x g for 10 minutes at 4°C in a microcentrifuge.
8. Discard the supernatant and resuspend the pellet in 1 ml of (1:1) ethanol/ethyl acetate mixture. Manually suspend pellet with spatula, vortex thoroughly, and centrifuge tubes at 10,000 x g for 10 minutes at 4°C in a microcentrifuge.
9. Repeat step 8 two more times.
10. After the final wash, resuspend the protein pellets in 500 μl of guanidine hydrochloride by vortexing.
11. Centrifuge tubes at 10,000 x g for 10 minutes at 4°C in a microcentrifuge to remove any left over debris.
12. Transfer 220 μl of supernatant from the sample (S#) tube to two wells of the 96-well plate.
13. Transfer 220 μl of supernatant from the control (C#) tube to two wells of the 96-well plate.
14. Measure the absorbance at a wavelength between 360-385 nm using a plate reader.

ANALYSIS

Calculations

1. Calculate the average absorbance of each sample and control.
2. Subtract the average absorbance of the controls from the average absorbance of the samples. This is the Corrected absorbance (CA).
3. Determine the concentration of the carbonyls by inserting the corrected absorbance into the following equation:

$$\text{Protein Carbonyl (nmol/ml)} = [(CA)/(*0.011 \mu\text{M}^{-1})](500 \mu\text{l}/200 \mu\text{l})$$

*The actual extinction coefficient for dinitrophenylhydrazine at 370 nm is 22,000 $\text{M}^{-1}\text{cm}^{-1}$ (0.022 $\mu\text{M}^{-1}\text{cm}^{-1}$). This value has been adjusted for the pathlength of the solution in the well.

Determination of protein content of pellets (optional)

Proteins are lost during the washing steps so protein levels are determined on the final pellet after the washes.

1. Transfer 100 μ l of the sample control (C#) from one of the wells to a 1 ml quartz cuvette and then add 900 μ l of guanidine hydrochloride. This is a 1:10 dilution. If further dilutions are needed, dilute with guanidine hydrochloride.
2. Determine the absorbance at 280 nm of each diluted sample control (C#) using a spectrophotometer.
3. The amount of protein is calculated from a bovine serum albumin (BSA) standard curve (0.25-2.0 mg/ml) dissolved in guanidine hydrochloride and read at 280 nm.

Protein concentration (mg/ml) = $[(A_{280} - y\text{-intercept})/\text{slope}] \times 2.5 \times 10$ (dilution factor)

*2.5 is the correction factor that adjusts the results back to the original sample.

Carbonyl content (nmol/mg) = (Carbonyl nmol/ml)/(protein mg/ml)

Performance Characteristics

Precision:

When a series of thirty-six human serum samples were assayed on the same day, the intra-assay coefficient of variation was 4.7% (n = 4). When a series of thirty-six human serum samples were assayed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 8.5% (n = 4).

Assay Range:

This assay works best when samples have protein concentrations in the range of 1-10 mg/ml.

Interferences

The following reagents were tested for interference in the assay.

	Reagent	Will Interfere (Yes or No)
Buffers:	Tris	No
	Borate	No
	HEPES	No
	Phosphate	No
Detergents:	Tween 20 ($\leq 1\%$)	No
	Triton X-100	Yes
Protease Inhibitors/ Chelators:	Antipain (≤ 0.1 mg/ml)	No
	PMSF (≤ 1 mM)	No
	Leupeptin (≤ 1 mg/ml)	No
	Pepstatin (≤ 0.1 mg/ml)	No
	Chymostatin (≤ 1 mg/ml)	No
	EGTA (≤ 1 mM)	No
	EDTA (≤ 1 mM)	No
Others:	Glycerol	Yes
	Bovine serum albumin	Yes
	DTT ($< 0.5\%$)	No

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Carefully tap the side of the plate with your finger to remove bubbles B. Be careful not to splash the contents of the wells
No pellet is formed upon centrifugation	Either the TCA solution was not added or the protein concentration was not 1-10 mg/ml	See Sample Preparation for guidelines
The final pellet does not totally dissolve in the guanidine hydrochloride solution		It is normal to see some cellular debris. The final centrifugation step will remove any residual debris
The control (C#) absorbance was higher than the sample (S#) absorbance	The control sample was not washed thoroughly with the ethanol:ethyl acetate solution	
Sample or control wells are cloudy	The control or sample was not washed thoroughly with the ethanol:ethyl acetate solution	
No protein carbonyls were detected in the sample	The DNPH was not added to the sample or not incubated long enough with the sample. – or – The protein concentration of the sample was not high enough	See Sample Preparation for guidelines

References

1. Halliwell, B. Oxidative stress, nutrition and health. Experimental strategies for optimization of nutritional antioxidant intake in humans. *Free Radic. Res.* **25**, 57-74 (1996).
2. Stadtman, E.R. and Oliver, C.N. Metal-catalyzed oxidation of proteins. Physiological consequences. *J. Biol. Chem.* **266**(40), 2005-2008 (1991).
3. Reznick, A.Z., Cross, C.E., Hu, M.-L., *et al.* Modification of plasma proteins by cigarette smoke as measured by protein carbonyl formation. *Biochem. J.* **286**, 607-611 (1992).
4. Levine, R.L., Williams, J.A., Stadtman, E.R., *et al.* Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol.* **233**, 346-357 (1994).
5. Reznick, A.Z. and Packer, L. Oxidative damage to proteins: Spectrophotometric method for carbonyl assay. *Methods Enzymol.* **233**, 357-363 (1994).
6. Zusterzeel, P.L.M., Mulder, T.P.J., Peters, W.H.M., *et al.* Plasma protein carbonyls in nonpregnant, healthy pregnant and preeclamptic women. *Free Radic. Res.* **33**, 471-476 (2000).

Related Products

Acetylation Stimulating Protein (human) EIA Kit - Cat. No. 10008491
Antioxidant Assay Kit - Cat. No. 709001
Catalase Assay Kit - Cat. No. 707002
Glutathione Assay Kit - Cat. No. 703002
Glutathione Peroxide Assay Kit - Cat. No. 703102
Glutathione Reductase Assay Kit - Cat. No. 703202
Glutathione S-Transferase Assay Kit - Cat. No. 703302
Hydrogen Peroxide Assay Kit - Cat. No. 706011
8-Isoprostane EIA Kit - Cat. No. 516351
Lipid Hydroperoxide Assay Kit - Cat. No. 705002
iPF_{2α}-VI EIA Kit - Cat. No. 516301
Superoxide Dismutase Assay Kit - Cat. No. 706002
TBARS Assay Kit - Cat. No. 10009055
Thioredoxin Reductase Assay Kit - Cat. No. 10007892
Xanthine Oxidase Assay Kit - Cat. No. 10010895

NOTES

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