Protein Carbonyl Colorimetric Assay Kit

Item No. 10005020

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity
401137	Hydrochloric Acid	1 vial/50 ml
10005846	Protein Carbonyl DNPH	2 vials/20 mg
10005848	Protein Carbonyl Guanidine Hydrochloride	1 vial/50 ml
10005849	Protein Carbonyl Ethanol	3 vials/30 ml
10005850	Protein Carbonyl Ethyl Acetate	3 vials/30 ml
400014	96-Well Solid Plate (Colorimetric Assay)	1 plate

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

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 acid is corrosive and 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

Email: techserv@caymanchem.com

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 repeating 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 **Sample Preparation**, page 10)
6. Bovine serum albumin (BSA) for determination of protein concentration (optional, see **Calculations**, page 16)
7. Spectrophotometer for determination of nucleic acid contamination and protein concentration (optional, see **Sample Preparation** and **Calculations**, pages 10 and 16, respectively)
8. 10% and 20% TCA solutions
9. Materials needed for Sample Preparation (see pages 10-12)

INTRODUCTION

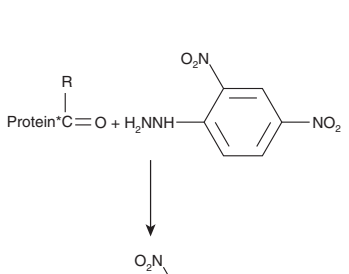
Background

Protein carbonylation, a post-translational modification producing protein-carbonyl adducts, occurs during oxidative stress.[1] Carbonyl groups are generated at a variety of amino acids, particularly lysine, arginine, proline, or threonine residues, by radical and nonradical reactive oxygen species (ROS). The modifications are chemically and metabolically stable, making them useful targets for the detection of oxidative damage. Protein carbonylation can also be catalyzed by redox cycling cations such as Fe^{2+} or Cu^{2+} in conjunction with non-radical ROS, toxicants such as cigarette smoke, or by secondary reactions with aldehydes produced during lipid peroxidation.[1-3] Protein carbonylation is elevated in plasma, urine, bronchoalveolar lavage fluid (BALF), or tissues in a variety of disease states, including acute respiratory distress syndrome (ARDS), Alzheimer's disease, chronic renal failure, sepsis, and diabetes.[4,5]

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).[6]

About This Assay

Cayman's Protein Carbonyl Colorimetric 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 the protein concentration.



Scheme 1. Biochemistry of the protein carbonyl assay

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Hydrochloric Acid - (Item No. 401137)**

   NOTE: Wear appropriate protection when handling the hydrochloric acid solution.

   The vial contains 2.4 M hydrochloric acid (HCl), which is used to resuspend the DNPH.

2. **DNPH - (Item No. 10005846)**

   The vial contains DNPH. Dissolve the contents of the vial in 10 ml of 2.4 M HCl. This will be a sufficient volume of reagent to process 12 samples. If more samples are being assayed, resuspend the second vial. The reconstituted DNPH will be stable for one week when stored at 4°C in the dark. *Do not freeze!*

3. **Guanidine Hydrochloride - (Item No. 10005848)**

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

4. **Ethanol - (Item No. 10005849)**

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

5. **Ethyl Acetate - (Item No. 10005850)**

   Each vial contains 30 ml of ethyl acetate. Mix the contents of one vial with 30 ml of ethanol for a 1:1 mixture of ethanol:ethyl acetate. This is enough reagent to process nine samples. If more samples are being assayed, 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. It is recommended to use Cayman's Protein Determination (BCA) Kit (Item No. 701780), Protein Determination Kit (Item No. 704002), or a similar protein determination assay to measure the total protein concentration.

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. 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 280:260 ratio is less than 1, a further step to remove nucleic acids with 1% streptomycin sulfate is needed. (See **Removal of Nucleic Acids** on page 12.)

Tissue Homogenate

1. Dissect 200-300 mg of tissue. Rinse tissue with a phosphate-buffered saline 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 buffer, 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 280:260 ratio is less than 1, a further step to remove nucleic acids with 1% streptomycin sulfate is needed. (See **Removal of Nucleic Acids** below.)

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 centrifuge at 6,000 x g for 10 minutes at 4°C. Use the supernatant for determining protein carbonyl content.[7]

ASSAY PROTOCOL

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 provided (see Figure 1). It is suggested to record the contents of each well on the template sheet provided (see page 22).

	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 a multichannel 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 2.4 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 five 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 five 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 the 1:1 ethanol:ethyl acetate mixture. Manually suspend the pellet with a 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 leftover 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:

Protein Carbonyl (nmol/ml) = [(CA)/(*0.011 μM^{-1})](500 µl/200 µl)

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

Determination of protein content in pellets (optional)

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

1. Transfer 100 µl of the sample control (C#) from one of the wells to a 1 ml quartz cuvette and add 900 µl of guanidine hydrochloride. This is a 1:10 dilution. If further dilutions are needed, dilute with guanidine hydrochloride.
2. Prepare BSA standards (not included) in guanidine chloride in a range of concentrations of 0.25-2.0 mg/ml.
3. Determine the absorbance at 280 nm of each diluted sample control (C#) and each BSA standard using a spectrophotometer.
4. Plot the absorbance *versus* concentration for the BSA standards to obtain a standard curve. Calculate concentration of each sample control using their absorbance and the equation of the BSA standard curve as shown below.

Protein concentration (mg/ml) = [(A_{280} - y-intercept)/slope] x *2.5 x 10 (dilution factor)
*2.5 is the correction factor that adjusts the results back to the original sample volume.

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

Alternatively, Cayman's Protein Determination (BCA) (Item No. 701780), Protein Determination Kit (Item No. 704002), or a similar assay can be used to measure the total protein concentration.

Performance Characteristics

Precision:

When 19 high- and 19 low-oxidized control measurements were performed on the same day, the intra-assay coefficients of variation were 8 and 12%, respectively. When seven high- and seven low-oxidized control measurements were performed on four different days under the same experimental conditions, the inter-assay coefficients of variation were 12 and 18%, respectively.

Sensitivity:

The limit of detection (LOD) of this assay is 0.74 nmol/ml.*

** Determined using human albumin*

RESOURCES

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:	Polysorbate 20 (≤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
	BSA	Yes
	DTT (<0.5%)	No

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of replicates	A. Poor pipetting/ technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
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

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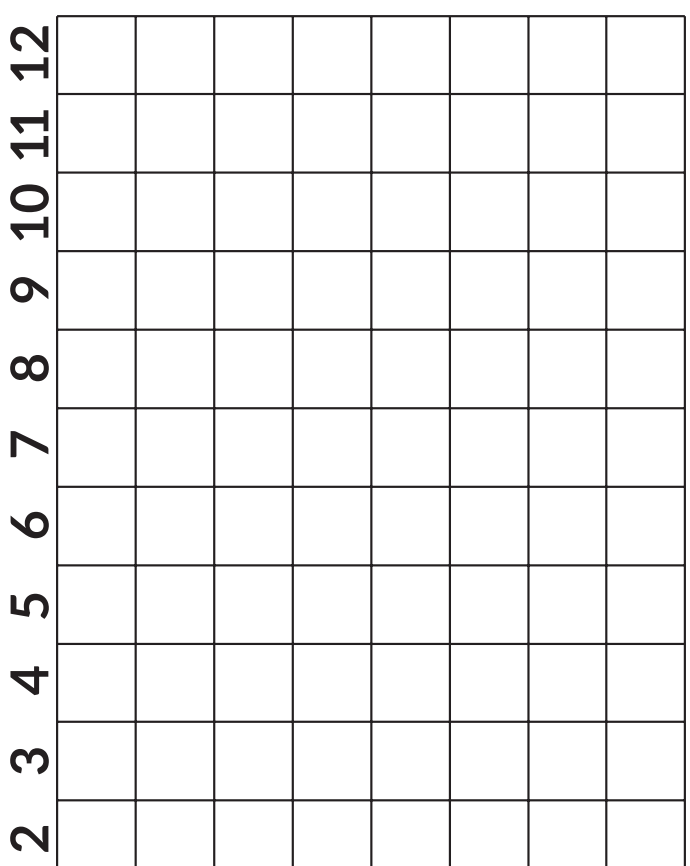

NOTES

Warranty and Limitation of Remedy

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