Protein Carbonyl Fluorometric Assay Kit

Item No. 701530

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TABLE OF CONTENTS

GENERAL INFORMATION
3 Materials Supplied
4 Safety Data
4 Precautions
5 If You Have Problems
5 Storage and Stability
5 Materials Needed but Not Supplied

INTRODUCTION
6 Background
7 About This Assay

PRE-ASSAY PREPARATION
8 Reagent Preparation
10 Sample Preparation

ASSAY PROTOCOL
13 Plate Set Up
14 Performing the Assay

ANALYSIS
17 Calculations
18 Performance Characteristics

RESOURCES
20 Troubleshooting
21 References
22 Plate Template
23 Notes
23 Warranty and Limitation of Remedy

GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity	Storage
701531	Protein Carbonyl Reaction Buffer	1 vial/5 ml	4°C
701532	Protein Carbonyl Assay Buffer	1 vial/60 ml	4°C
701533	Protein Carbonyl RBH	1 vial/500 µl	-20°C
700030	Protein Carbonyl Hydrochloric Acid (Fluorometric)	1 vial/1 ml	RT
701534	Protein Carbonyl Additive	1 vial/550 µl	-20°C
701535	Protein Carbonyl Wash Solution	2 vials/30 ml	-20°C
701536	Protein Carbonyl Sample Diluent (20X)	1 vial/25 ml	RT
700566	Ethanol Assay Reagent	1 vial/2 ml	RT
701537	Oxidized (High) Protein Carbonyl Control	1 vial/2 mg	-20°C
701538	Oxidized (Low) Protein Carbonyl Control	1 vial/2 mg	-20°C
400017	96-well Solid Plate (black)	1 plate	RT
400012	96-well Cover Sheet	1 ea	RT

If any of the items listed on page 3 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 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

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 in the **Materials Supplied** section (see page 3) 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 fluorescence using an excitation wavelength of 560 nm and an emission wavelength between 585-595 nm
2. Adjustable pipettes and a multichannel or repeating pipette
3. A source of ultrapure water; glass-distilled water or HPLC-grade water may not be acceptable. *NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000)*
4. Microcentrifuge
5. 100% TCA solution
6. Materials needed for Sample Preparation (see pages 10-12)

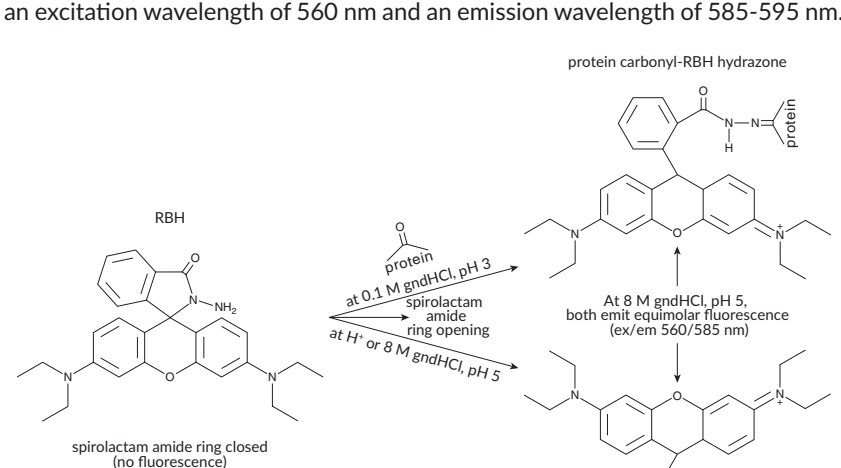
INTRODUCTION

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]

About This Assay

Cayman's Protein Carbonyl Fluorometric Assay Kit utilizes the rhodamine B hydrazide (RBH) reaction to measure the protein carbonyl content in plasma, serum, cell lysates, or tissue homogenates in a convenient 96-well format. Formation of the fluorescent protein carbonyl-RBH hydrazone is analyzed using an excitation wavelength of 560 nm and an emission wavelength of 585-595 nm.



Scheme 1. RBH Assay Mechanism.[6] Image adapted from Georgiou, C.D., Zisimopoulos, D., Argyropoulou, V., *et al.* Protein carbonyl determination by a rhodamine B hydrazide-based fluorometric assay. *Redox Biol.* **17**, 236-245 (2018).

Sold under license from Christos D. Georgiou, University of Patras.

PRE-ASSAY PREPARATION

Reagent Preparation

1. **Protein Carbonyl Reaction Buffer**

   This vial contains 5 ml of Protein Carbonyl Reaction Buffer (Item No. 701531). It is ready to use as supplied.

2. **Protein Carbonyl Assay Buffer**

   This vial contains 60 ml of Protein Carbonyl Assay Buffer (Item No. 701532). Equilibrate to room temperature and vortex to dissolve any precipitates. It is ready to use as supplied.

3. **Protein Carbonyl RBH**

   This vial contains 500 µl of 1 mM Protein Carbonyl RBH (Item No. 701533) in ethanol. This solution should be kept on ice and protected from light. It is ready to use as supplied.

4. **Protein Carbonyl Hydrochloric Acid**

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

   This vial contains 1 ml of Protein Carbonyl Hydrochloric Acid (Item No. 700030). It is ready to use as supplied.

5. **Protein Carbonyl Additive**

   This vial contains 550 µl of Protein Carbonyl Additive (Item No. 701534). It is ready to use as supplied.

6 **Protein Carbonyl Wash Solution**

   Each vial contains 30 ml of Protein Carbonyl Wash Solution (Item No. 701535). The wash solution should be kept at -20°C. It is ready to use as supplied.

7. **Protein Carbonyl Sample Diluent (20X)**

   This vial contains 25 ml of 1 M Protein Carbonyl Sample Diluent (20X) (Item No. 701536), which contains sodium hydroxide (NaOH). Mix the contents of the vial with 475 ml of ultrapure water to yield 50 mM NaOH Protein Carbonyl Sample Diluent (1X). The diluted NaOH sample diluent should be used for dilution of controls and samples and is stable for one month at room temperature.

8. **Ethanol Assay Reagent**

   This vial contains 2 ml of Ethanol Assay Reagent (Item No. 700566). It is ready to use as supplied.

9. **Oxidized (High) Protein Carbonyl Control**

   This vial contains 2 mg of Oxidized (High) Protein Carbonyl Control (Item No. 701537), which consists of lyophilized oxidized BSA. Reconstitute with 500 µl of ultrapure water. Mix 27 µl of this solution with 573 µl Protein Carbonyl Sample Diluent (1X) to yield 0.18 mg/ml (25 µg).

10. **Oxidized (Low) Protein Carbonyl Control**

    This vial contains 2 mg of Oxidized (Low) Protein Carbonyl Control (Item No. 701538), which consists of lyophilized oxidized BSA. Reconstitute with 500 µl of ultrapure water. Mix 27 µl of this solution with 573 µl Protein Carbonyl Sample Diluent (1X) to yield 0.18 mg/ml (25 µg). *NOTE: Controls are not used in the sample calculation. They are just indicators of the assay performance. It is not necessary to run both controls at the same time.*

Sample Preparation

This assay is recommended for testing protein carbonyls in clarified protein isolates.[6] The method of purification has to be chosen by the user based on their sample origin, experimental design and expertise. Various protein purification methods for protein carbonyl determination are discussed by Georgiou *et al.* in *Redox Biol.* **17**, 128-142 (2018).[6] This assay works best with protein concentrations in the range of 0.06-0.25 mg/ml.

Plasma

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.
3. Prepare protein isolates.

Serum

1. Collect blood without using an anticoagulant. Allow the blood to clot for 30 minutes at room temperature.
2. Centrifuge 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.
3. Prepare protein isolates.

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.
5. Prepare protein isolates.

Tissue Homogenate

1. Dissect 200-300 mg of tissue. Rinse tissue with PBS 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 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.
5. Prepare protein isolates.

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 given (see Figure 1). We suggest you 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	A	A	S1	S1	S5	S5	S9	S9	S13	S13	S17	S17
B	B	B	B1	B1	B5	B5	B9	B9	B13	B13	B17	B17
C	C	C	S2	S2	S6	S6	S10	S10	S14	S14	S18	S18
D	D	D	B2	B2	B6	B6	B10	B10	B14	B14	B18	B18
E	E	E	S3	S3	S7	S7	S11	S11	S15	S15	S19	S19
F	F	F	B3	B3	B7	B7	B11	B11	B15	B15	B19	B19
G	G	G	S4	S4	S8	S8	S12	S23	S16	S16	S20	S20
H	H	H	B4	B4	B8	B8	B12	B12	B16	B16	B20	B20

A-H = Standards
S1-S20 = Control or Sample wells
B1-B20 = Control or Sample Background wells

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- 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 100 µl in all the wells.
- All reagents except samples, Protein Carbonyl RBH, and Protein Carbonyl Wash Solution 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, controls, and standards be assayed at least in duplicate.

Performing the Assay

Follow the procedure below for each sample to be analyzed:

1. Transfer 140 µl of control or diluted sample to two microcentrifuge tubes. One tube will be the control/sample tube (S#) and the other will be the control/sample background tube (B#).
2. Add 50 µl of Protein Carbonyl Reaction Buffer to each tube.
3. Add 5 µl of Protein Carbonyl Hydrochloric Acid to each tube.
4. Add 5 µl of Protein Carbonyl RBH to each sample/control tube and 5 µl of Ethanol Assay Reagent to each sample/control background tube.
5. Vortex tubes and incubate protected from light for 1 hour at 37°C.
6. Add 5 µl of Protein Carbonyl Additive to each tube and vortex. Incubate for 10 minutes at room temperature.
7. Add 25 µl of 100% TCA solution to each tube and vortex. Incubate for 15 minutes on ice.
8. Centrifuge tubes at 16,000 x g for 5 minutes at 4°C to precipitate proteins.
9. Carefully remove the supernatant and discard. Wash the protein pellet with 500 µl cold Protein Carbonyl Wash Solution. Vortex vigorously and centrifuge at 16,000 x g for 5 minutes at 4°C. *NOTE: When vortexing with the Protein Carbonyl Wash Solution, part of the protein pellet may spread as a thin layer on the internal wall of the centrifuge tube and may not be visible if the pellet is small.*
10. Repeat step 9.
11. Carefully remove the supernatant and discard. Leave the tubes open at room temperature for 30 minutes to dry the protein pellets.
12. Add 300 µl of Protein Carbonyl Assay Buffer to each tube and vortex vigorously to dissolve the pellets. Break up the protein pellet with a spatula if necessary.
13. Centrifuge tubes at 16,000 x g for 5 minutes at room temperature to precipitate DNA as a clear pellet. Carefully collect supernatant.
14. Prepare the RBH standards according to Figure 2: Label eight microcentrifuge tubes A-H. Aliquot 990 µl of Protein Carbonyl Assay Buffer into tube A and 600 µl into tubes B-H. Transfer 10 µl of the 1 mM Protein Carbonyl RBH to tube A and mix thoroughly. Serially dilute the standard by removing 200 µl from tube A and placing it in tube B; mix thoroughly. Next, remove 200 µl from tube B and place it in to tube C; mix thoroughly. Repeat this process for tubes D-G. Tube H contains Protein Carbonyl Assay Buffer only.
15. Pipette 100 µl of each standard, control, and sample per well in the designated wells on the plate (see Figure 1. Sample Plate Format on page 13).
16. Measure the fluorescence using an excitation wavelength of 560 nm and an emission wavelength between 585-595 nm.

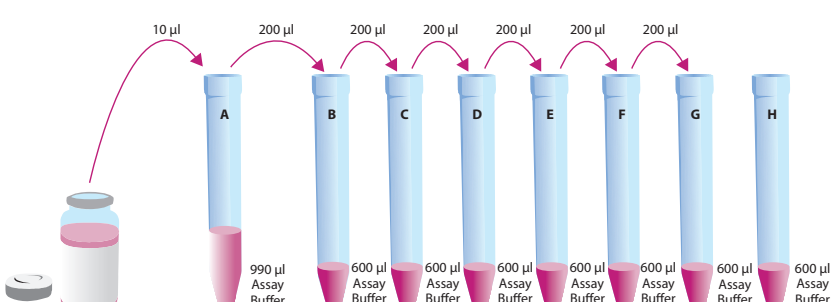


Figure 2. Preparation of RBH standards

ANALYSIS

Calculations

1. Calculate the average fluorescence of each standard, control, and sample.
2. Subtract the average fluorescence of Standard H from itself and all other standards, controls, and samples. This is the corrected fluorescence. *NOTE: If the sample background fluorescence is higher than the average fluorescence of the standard H, subtract the average sample background fluorescence (instead of the standard H) from the average sample fluorescence.*
3. Plot the corrected fluorescence of the standards as a function of the final concentration of RBH from Figure 3. See Figure 3 for a typical standard curve.
4. Calculate the RBH concentration of the controls and samples using the equation obtained from the linear regression of the standard curve, substituting corrected fluorescence values for each sample.

$$\text{RBH (nM)} = \left[\frac{\text{(Sample corrected fluorescence) - (y - intercept fluorescence)}}{\text{Slope (Fluorescence/nM)}}\right] \text{x Sample dilution}$$

5. Protein is commonly lost during the wash steps. Therefore, protein concentrations are determined using the resuspended protein prepared after the wash steps. Determine protein concentration using the Protein Determination (BCA) Kit (Item No. 701780) or a similar protein determination assay. The standard for protein determination should be prepared in Protein Carbonyl Assay buffer.
6. Calculate the concentration of the carbonyls per mg of protein by inserting the RBH concentration into the following equation:

$$\text{Protein Carbonyl (nmol/mg)} = \frac{\text{RBH (nM)}}{\text{1,000 (ml/L)}} \text{ / Protein concentration (mg/ml)}$$

Performance Characteristics

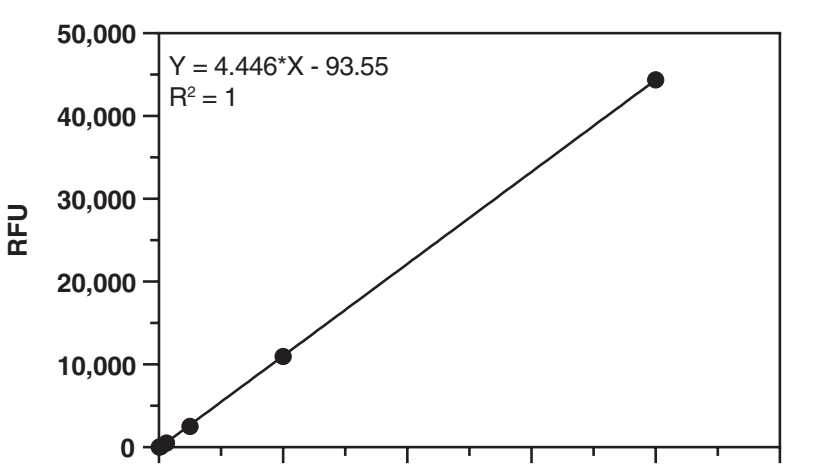


Figure 3. Typical standard curve

Precision:

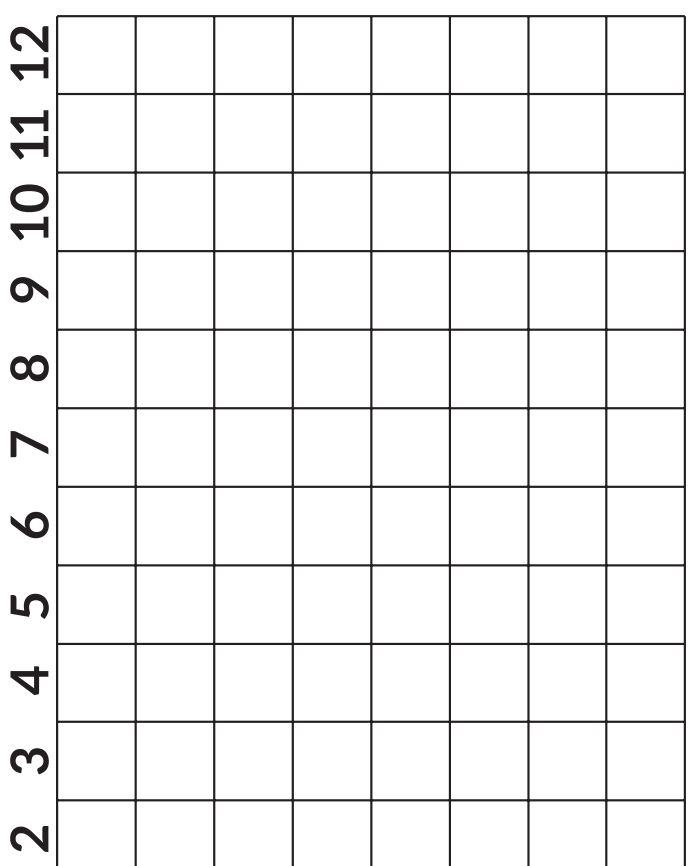
When a series of six high- and six low-oxidized controls were assayed for protein carbonyl concentration on the same day, the intra-assay coefficients of variation were 8.8 and 8.1%, respectively. When a series of high- and low-oxidized controls were assayed on three different days under the same experimental conditions, the inter-assay coefficients of variation were 9.9 and 7.2%, respectively.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	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 Protein Carbonyl TCA solution was not added or the protein concentration was too low	See **Sample Preparation** for guidelines
The final pellet does not totally dissolve in the Protein Carbonyl Assay Buffer		It is normal to see some cellular debris; the final centrifugation step will remove any residual debris
The background fluorescence was higher than the sample fluorescence	The protein concentration of the sample was too low	
Sample or control wells are cloudy	The sample or background was not washed thoroughly with the wash solution	
No protein carbonyls were detected in the sample	The Protein Carbonyl RBH 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. Dalle-Donne, I., Giustarini, D., Colombo, R., *et al.* Protein carbonylation in human diseases. *Trends Mol. Med.* **9(4)**, 169-176 (2003).
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(Pt 2)**, 607-611 (1992).
4. Dalle-Donne, I., Rossi, R., Giustarini, D., *et al.* Protein carbonyl groups as biomarkers of oxidative stress. *Clin. Chim. Acta* **329(1-2)**, 23-38 (2003).
5. Lenz, A-G., Jorens, P.G., Meyer, B. *et al.* Oxidatively modified proteins in bronchoalveolar lavage fluid of patients with ARDS and patients at-risk for ARDS. *Eur. Respir. J.* **13(1)**, 169-174 (1999).
6. Georgiou, C.D., Zisimopoulos, D., Argyropoulou, V., *et al.* Protein carbonyl determination by a rhodamine B hydrazide-based fluorometric assay. *Redox Biol.* **17**, 236-245 (2018).



NOTES

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