Protein Carbonyl Fluorometric Assay Kit

Item No. 700490

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. For best results, remove components and store as stated below.

Item Number	Item	Size/Quantity	Storage
700491	PCF Sample Buffer (10X)	1 vial/10 ml	4°C
700492	PCF Fluorophore	2 vials	-20°C
700493	PCF TCA Solution	2 vials/20 ml	4°C
700494	PCF Guanidine Hydrochloride	1 vial/6 ml	4°C
700495	PCF Diluent (10X)	1 vial/10 ml	4°C
10011288	Half Volume 96-Well Plate (white)	1 plate	Room temperature
400012	96-Well Cover Sheet	1 cover	Room temperature

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.

Trichloroacetic acid is corrosive and is 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

Email: 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 in the **Materials Supplied** section, on 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 at an excitation wavelength between 480-490 nm, and an emission wavelength between 525-535 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. Bovine serum albumin (BSA) and Bradford reagent for the determination of protein concentration or Cayman's Protein Determination Assay Kit (Item No. 704002).
6. A supply of HPLC-grade acetone
7. Streptomycin sulfate for removal of nucleic acids (*optional*, see under **Sample Preparation**, page 7)

INTRODUCTION

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 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. This protein oxidation in plasma, as measured by an increase in plasma protein carbonyls, can be used as a measure of the increase in overall oxidative stress in the body.[3]

About This Assay

Cayman's Protein Carbonyl Fluorometric Assay Kit provides a reliable and sensitive method for determining protein carbonyl concentration in plasma, serum, cell lysate, and tissue homogenate samples. The assay relies on the 1:1 binding of a fluorophore to the protein carbonyl. Once bound, excess fluorophore is washed away. Any remaining fluorescence is directly proportional to protein carbonyl concentration.[3]

PRE-ASSAY PREPARATION

Reagent Preparation

1. PCF Sample Buffer (10X) - (Item No. 700491)

The vial contains 10 ml of 1.0 M HEPES buffer, pH 6.0. Dilute the entire contents of the vial to a total volume of 100 ml with HPLC grade water. The diluted buffer is stable for at least three months at 4°C.

2. PCF Fluorophore - (Item No. 700492)

Each vial contains a lyophilized fluorophore. Resuspend the contents of the vial with 2 ml of diluted sample buffer to yield a 200 µM solution. The resuspended fluorophore is stable for one week at -20°C or for one day at room temperature.

3. PCF TCA Solution - (Item No. 700493)

Each vial contains 20 ml of 20% trichloroacetic acid solution. The reagent is ready to use as supplied. The reagent is stable for one month at room temperature or six months at 4°C.

4. PCF Guanidine Hydrochloride - (Item No. 700494)

The vial contains 6 ml of 6.0 M Guanidine Hydrochloride. The reagent is ready to use as supplied. The reagent is stable for one month at room temperature or six months at 4°C.

5. PCF Diluent (10X) - (Item No. 700495)

The vial contains 10 ml of 1.0 M potassium phosphate, pH 7.4. Dilute the entire contents of the vial to a total volume of 100 ml with HPLC grade water. The diluted buffer is stable for at least three months at room temperature.

Sample Preparation

Removal of Nucleic Acids. Nucleic acids may erroneously contribute to a higher estimation of carbonyls. Whenever the ratio of $A_{280/260}$ 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.[4]

NOTE: Protein concentration can be determined using Cayman's Protein Determination Kit (Item No. 704002).

Plasma

Normal carbonyl levels in plasma typically range from 0.5-4.0 nmol/mg.[4]

1. Collect blood using an anticoagulant such as heparin 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 one month. Repeated freeze/thaw cycles should be avoided.
3. Plasma should be adjusted to between 5-10 mg/ml total protein concentration prior to assaying. Adjust protein concentration with diluted PCF Sample Buffer. Higher concentrations may lead to inaccurate results.

Serum

Normal carbonyl levels in serum typically range from 0.5-4.0 nmol/mg.[4]

1. Collect blood without using an anticoagulant.
2. Allow blood to clot for 30 minutes at 25°C.
3. 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 one month. Repeated freeze/thaw cycles should be avoided.
4. Serum should be adjusted to between 5-10 mg/ml total protein concentration prior to assaying. Adjust protein concentration with diluted PCF Sample Buffer. Higher concentrations may lead to inaccurate results.

Urine

Assaying urine is not recommended due to low protein concentration.

Tissue Homogenate

1. Prior to dissection, rinse the tissue with a phosphate buffered saline (PBS) solution, pH 7.4, to remove any red blood cells and clots.
2. Homogenize the tissue in 5-10 ml of cold buffer (*i.e.*, 100 mM Tris, pH 7.8) per gram of tissue.
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and store on ice.
5. If not assaying on the same day, store the sample at -20°C. The sample will be stable for at least one month when frozen.
6. Adjust the total protein concentration to between 5-10 mg/ml before assaying. Higher concentrations may lead to inaccurate results.

Cell Lysate

1. Collect cells by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, do not harvest using proteolytic enzymes; rather use a rubber policeman.
2. The cell pellet can be homogenized or sonicated in 1-2 ml of cold buffer (*i.e.*, 100 mM HEPES, pH 6.0).
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and store on ice.
5. If not assaying on the same day, freeze at -80°C. The sample will be stable for one month.
6. The sample should be adjusted to between 5-10 mg/ml total protein concentration prior to assaying. Adjust protein concentration with diluted Sample Buffer. Higher concentrations may lead to inaccurate results.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. A typical layout of Fluorophore standards and samples to be measured in duplicate is given in Figure 1 below. We suggest you record the contents of each well on the template sheet provided (see page 19).

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33
B	B	B	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
C	C	C	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
D	D	D	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
E	E	E	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
F	F	F	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
G	G	G	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
H	H	H	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40

A-H = Standards
S1-S40 = Sample Wells

Figure 1. Sample plate format

Pipetting Hints

- 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

- **This assay requires an overnight incubation at room temperature.**
- The final volume of the assay is 50 µl in all the wells.
- All reagents 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 standards and samples be assayed at least in duplicate.
- Twenty-six samples can be assayed in triplicate or forty in duplicate.
- The assay is performed at room temperature.
- Monitor fluorescence at an excitation wavelength between 480-490 nm and an emission wavelength between 525-535 nm.

Standard Preparation

***Prepare the standards on the second day of the assay**

To prepare the protein carbonyl standards, begin by transferring 10 µl of resuspended PCF Fluorophore into 990 µl of diluted PCF Diluent to yield a 2.0 µM solution. Transfer 100 µl of the 2 µM solution to 900 µl of diluted PCF diluents to yield a 200 nM standard solution. This 200 nM solution is used to prepare the standard curve. Take eight clean glass test tubes and label them A-H. Add the amount of 200 nM standard and diluted PCF Diluent to each tube as described in Table 1.

Tube	200 nM Fluorophore (µl)	Diluted PCF Diluent (µl)	Final Fluorophore Concentration (nM)
A	0	200	0
B	3	197	3
C	6	194	6
D	12	188	12
E	25	175	25
F	50	150	50
G	100	100	100
H	200	0	200

Table 1. Preparation of standards

Performing the Assay

Day 1:

1. Determine the protein concentration of each sample.
2. Adjust the protein concentration to between 5-10 mg/ml with diluted PCF Sample Buffer.
3. For each sample being assayed, transfer 50 µl of sample to a 1.5 ml microcentrifuge tube.
4. Add 50 µl of PCF Fluorophore to each sample tube.
5. Incubate the tubes at room temperature overnight, protected from light.

Day 2:

1. Add 400 µl of 20% TCA solution to each tube. Vortex and incubate on ice for 10 minutes.
2. Centrifuge the tubes in a microcentrifuge at 10,000 x g for 10 minutes.
3. Decant the supernatant and discard.
4. Add 1 ml of acetone to each tube and vortex vigorously. If the pellet does not disintegrate upon vortexing, use a small implement such as a spatula to manually break up the pellet.
5. Centrifuge the tubes in a microcentrifuge at 10,000 x g for 10 minutes.
6. Repeat the acetone wash two additional times.
7. After the final wash, decant the supernatant and allow the sample to dry for one hour (leave lids open).
8. After all of the acetone has evaporated, add 50 µl of PCF Guanidine Hydrochloride to each tube and incubate at room temperature for 10 minutes.
9. Add 450 µl of diluted PCF Diluent to each tube.
10. Vortex samples and centrifuge at 10,000 x g for 10 minutes to remove excess debris.
11. Prepare the standard curve as described in Table 1.
12. Transfer 50 µl of each standard and each sample to a corresponding well in the 96-well white plate.
13. Read the fluorescence at an excitation wavelength between 480-490 nm and an emission wavelength between 525-535 nm.

ANALYSIS

Calculations

1. Calculate the average fluorescence for each standard and sample. Subtract the average fluorescence of standard A from itself and all other standards and samples. This is the adjusted relative fluorescence unit (RFU).
2. Plot the adjusted RFU of the standards as a function of the final concentration of fluorophore from Table 1. See Figure 2 for a typical standard curve.

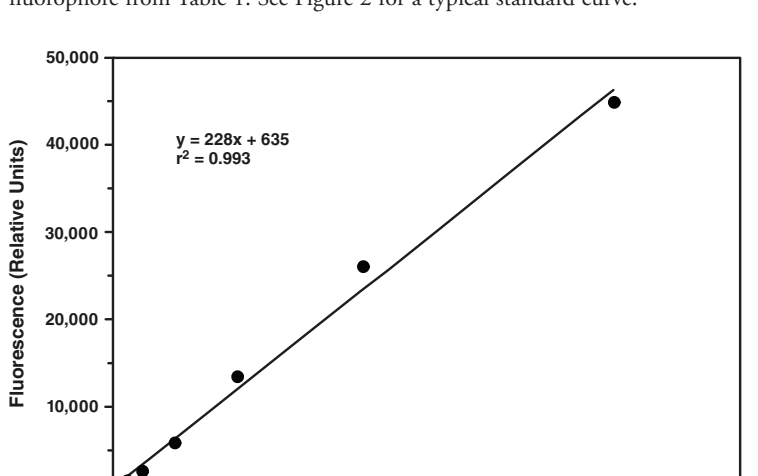


Figure 2. Typical standard curve

3. Calculate the carbonyl concentration of the samples using the equation obtained from the linear regression of the standard curve, substituting adjusted RFU values for each sample.

$$\text{Protein carbonyl (nM)} = \left[\frac{\text{adjusted sample RFU - (y-intercept)}}{\text{slope}}\right] \times 10^{*}$$

*Correction factor accounts for dilution of the sample in the microcentrifuge tube

4. To calculate the total nmol/mg of protein carbonyl in the original sample, use the following equation.

$$\text{Carbonyl content (nmol/mg)} = \left[\frac{\text{carbonyl (nM)}}{\text{protein (mg/ml) x 1,000}^{*}}\right] \times \text{dilution}^{*}$$

*Correction Factor to go from nM to nmol/ml and dilution factor used to dilute the sample to 5-10 mg/ml.

Performance Characteristics

Precision:

When a series of eight plasma carbonyl measurements were performed on the same day under the same experimental conditions, the intra-assay coefficient of variation was 5.2%. When a series of eight plasma carbonyl measurements were performed on eight different days under the same experimental conditions, the inter-assay coefficient of variation was 4.6%.

Interferences

The following reagents were tested for interference in the assay.

	Reagent	Will Interfere (Yes or No)
Buffers:	Tris	No
	HEPES	No
	MES	No
	Phosphate	No
Detergents:	Polysorbate 20 (1%)	No
	Triton X-100 (1%)	No
Chelators:	EDTA (1 mM)	No
	EGTA (1 mM)	Yes
Protease Inhibitors/ Enzymes:	Trypsin (10 µg/ml)	No
	PMSF (200 µM)	No
	Leupeptin (10 µg/ml)	No
	Antipain (100 µg/ml)	No
	Chymostatin (10 µg/ml)	No
Solvents:	Ethanol (5%)	No
	Methanol (5%)	Yes
	DMSO (5%)	No
Others:	Glycerol (10%)	No

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
Sample values are very low	A. The sample is too dilute B. Too much pellet was lost during the washing phase	A. Make sure the sample protein concentration is between 5-10 mg/ml and re-assay the sample B. Run the assay again, centrifuge pellet longer and decant acetone carefully
Sample values are too high	The sample is too concentrated	Make sure the sample protein concentration is between 5-10 mg/ml and re-assay the sample

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. Mohanty, J., Bhamidipaty, S., Evans, M.K., *et al.* A fluorimetric semi-microplate format assay of protein carbonyls in blood plasma. *Anal. Biochem.* **400(2)**, 289-294 (2010)
4. Reznick, A.Z. and Packer, L. Oxidative damage to proteins: Spectrophotometric method for carbonyl assay. *Methods Enzymol.* **233**, 357-363 (1994).

Related Products

Acetylation Stimulating Protein (human) EIA Kit - Item No. 10008491
Antioxidant Assay Kit - Item No. 709001
Catalase Assay Kit - Item No. 707002
Glutathione Assay Kit - Item No. 703002
Glutathione Peroxide Assay Kit - Item No. 703102
Glutathione Reductase Assay Kit - Item No. 703202
Glutathione S-Transferase Assay Kit - Item No. 703302
8-hydroxy-2-deoxy Guanosine EIA Kit - Item No. 589320
Hydrogen Peroxide Assay Kit - Item No. 706011
8-Isoprostane EIA Kit - Item No. 516351
Lipid Hydroperoxide Assay Kit - Item No. 705002
$iPF_{2\alpha}$-VI EIA Kit - Item No. 516301
Protein Carbonyl Assay Kit - Item No. 10005020
Protein Determination Kit - Item No. 704002
Superoxide Dismutase Assay Kit - Item No. 706002
TBARS Assay Kit - Item No. 10009055
Thioredoxin Reductase Assay Kit - Item No. 10007892
Xanthine Oxidase Assay Kit - Item No. 10010895

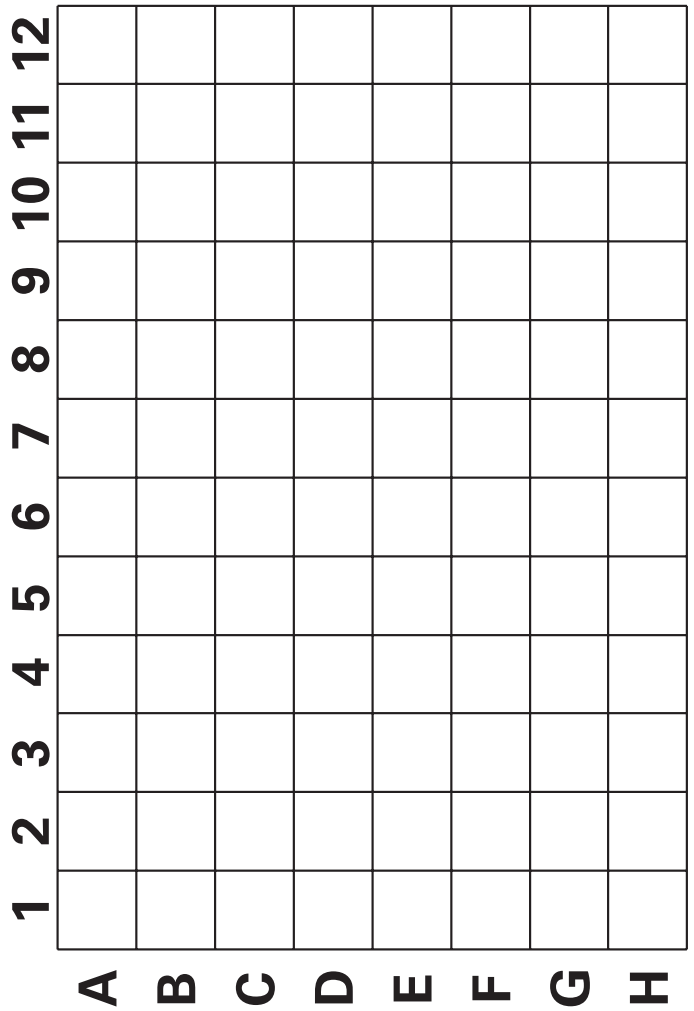
Warranty and Limitation of Remedy

Cayman Chemical Company makes **no warranty or guarantee** of any kind, whether written or oral, expressed or implied, including without limitation, any warranty of fitness for a particular purpose, suitability and merchantability, which extends beyond the description of the chemicals hereof. Cayman **warrants only** to the original customer that the material will meet our specifications at the time of delivery. Cayman will carry out its delivery obligations with due care and skill. Thus, in no event will Cayman have **any obligation or liability**, whether in tort (including negligence) or in contract, for any direct, indirect, incidental or consequential damages, even if Cayman is informed about their possible existence. This limitation of liability does not apply in the case of intentional acts or negligence of Cayman, its directors or its employees.

Buyer's **exclusive remedy** and Cayman's sole liability hereunder shall be limited to a refund of the purchase price, or at Cayman's option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to Cayman within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

For further details, please refer to our Warranty and Limitation of Remedy located on our website and in our catalog.



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