BioCell Protein Carbonyl Assay Kit

A) Instruction manual

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To be used with Protein Carbonyl Assay Kits Batch P135

For research use only

The sensitivity of the Protein Carbonyl Assay Kit has been improved beginning with batch P132. This may result in slightly different protein carbonyl values from previous batches, particularly at the lower end of the standard curve.

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Contents

Storage

- Freezer (-20 °C): Components G, K and L.
- Fridge (4 °C): Components H and I.
- Store the remainder of the kit at room temperature.
- Reagents must be brought to room temperature prior to use.
- BioCell Protein Carbonyl Assay Kits do not need to be kept under cool conditions for short-term transport. The kits are stable at room temperature for up to 6 weeks.
- If the kit components are separated into freezer, fridge and bench as per the instructions, they are stable until at least the expiry date.

Description

The BioCell Protein Carbonyl Assay Kit is an enzyme-linked immunosorbent assay (ELISA) for the measurement of protein carbonyls in biological samples. This kit contains materials for one 96 well plate. It will measure a maximum of 25 samples in triplicate or 41 samples in duplicate.

The assay can be used to analyze biological fluids such as plasma, serum, bronchoalveolar lavage fluid and cerebrospinal fluid, as well as cell extracts and other soluble protein samples.

Introduction

Protein carbonyls are biomarkers of protein oxidation that are generated by several different mechanisms during oxidative stress. They can be formed on arginine, proline, threonine and lysine residues as a result of metal-catalyzed oxidation. Protein carbonyls can also arise from the direct reaction of proteins with reactive oxygen species (ROS), or can be introduced into proteins by covalent attachment of carbonyl-containing molecules such as reducing sugars or lipid peroxidation products.

Assay principle

A schematic diagram of the Protein Carbonyl ELISA is shown on page 4. Samples containing protein are reacted with dinitrophenylhydrazine (DNP) and then excess protein is non-specifically adsorbed to saturate an ELISA plate. Unconjugated DNP and non-protein constituents are washed away. The adsorbed protein is probed with biotinylated anti-DNP antibody followed by streptavidin-linked horseradish peroxidase. Absorbance values are related to a standard curve prepared for serum albumin containing increasing proportions of hypochlorous acid-oxidized protein that has been calibrated colorimetrically.

Kit components

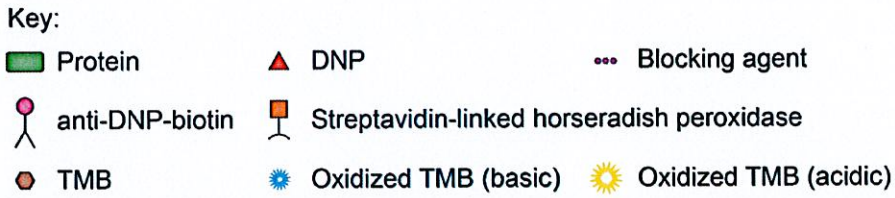
The kit components (supplied materials) are sufficient for one plate. Each kit contains reagents and components labeled as follows:

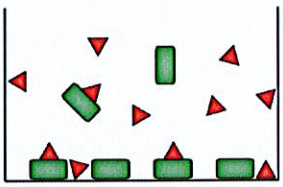
A	Instruction manual	G	Anti-DNP-biotin antibody
B	ELISA 96 well plate and plate cover	H	Streptavidin-horseradish peroxidase
C	EIA buffer powder	I	Chromogen reagent
D	Blocking reagent	J	Stopping reagent
E	Dinitrophenylhydrazine (DNP)	K	Oxidized protein standards (6)
F	Guanidine hydrochloride	L	Carbonyl control sample

Additional materials required

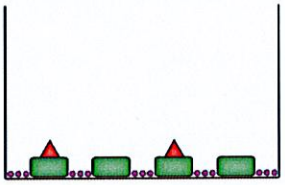
1. Pipettes with disposable plastic tips to deliver 5 µL- 1 mL.
2. Containers for reagent mixing and pipetting reservoirs.
3. The use of an automated microwell washer is recommended, otherwise use a squeeze bottle.
4. Microwell spectrophotometric reader. The reader must be able to read at 450 nm. 650 nm is useful to monitor the reaction before stopping.
5. Deionized water.
6. 1 L and 100 mL measuring cylinders.
7. 1.5 mL (Methods A-D) and 0.5 mL (Method D) tubes.
8. Trichloroacetic acid 28% (w/v) (Method D).
9. Incubator at 37 °C.

Schematic of the Protein Carbonyl ELISA

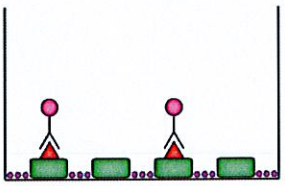


1. 

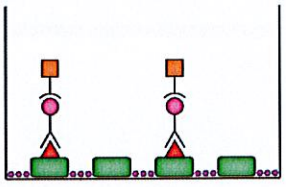
Protein samples that have been derivatized with DNP are added and directly bound to the ELISA plate, saturating all protein-binding sites.

2. 

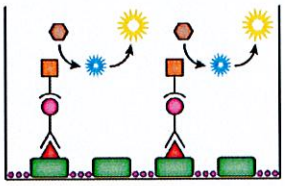
Excess protein and DNP are washed away, and unoccupied binding sites are blocked.

3. 

The adsorbed DNP-protein is probed with anti-DNP-biotin antibody.

4. 

Streptavidin-linked horseradish peroxidase is bound to the complex.

5. 

Chromogen, containing TMB and H_2O_2, is added and the horseradish peroxidase catalyses the oxidation of TMB by H_2O_2 to a blue product. The reaction is stopped by the addition of acid, which enhances the sensitivity and gives a yellow product that can be measured at 450 nm.

Reagent preparation

Note: Gloves should be worn when handling reagents.

Step 1: EIA buffer (C)

Dissolve the contents of bottle (C) in 1 L of deionized water.

Store at 4 °C.

Step 2: Blocking reagent (D)

Add 2 mL of EIA buffer to the blocking reagent container (D). Mix well and transfer to a 100 mL measuring cylinder. Add EIA buffer to a final volume of 75 mL.

Label this solution as "diluted blocking solution".

Store at 4 °C.

Step 3: Dinitrophenylhydrazine (DNP) (E)

Crystallization around the DNP (E) and Guanidine hydrochloride (F) lids may occur and is purely due to the high concentration of guanidine. This will not affect the result of the kit.

Add 1 mL of the DNP reagent (E) to 9 mL of Guanidine hydrochloride (F).

Label this solution as "diluted DNP solution".

If the "diluted DNP solution" has been stored for more than 2 weeks it is recommended that it be centrifuged at 8 000 g for 5 minutes and the supernatant used for derivatization.

Store at room temperature protected from light.

Step 4: Anti-DNP-biotin antibody (G)

Store at -20 °C until needed.

Prepare this dilution immediately before use.

Add 0.5 mL of the "diluted blocking solution" to the Anti-DNP-biotin antibody tube (G).
Mix well and make up to 20 mL with the "diluted blocking solution".

Step 5: Streptavidin-horseradish peroxidase (H)

Store at 4 °C until needed. Must NOT be frozen.

Prepare this dilution immediately before use.

Add 0.5 mL of the "diluted blocking solution" to the Streptavidin-horseradish peroxidase tube (H).
Mix well and make up to 20 mL with the "diluted blocking solution".

Step 6: Chromogen reagent (I)

Store at 4 °C until needed.

Prepare this dilution immediately before use.

Add 15 mL of the Chromogen reagent (I) to 5 mL of EIA buffer (C) and mix well.
Pour the required amount into a pipetting reservoir.
Never pipette directly from the container and do not return unused reagent to the main container as contamination of this solution may result in unintended color development.

Step 7: Stopping reagent (J)

Ready to use.

Store at room temperature.

Step 8: Oxidized protein standards (K1-6)

Store at -20 °C until needed.

Please note: The Oxidized protein standards (K1-6) and the Carbonyl control sample (L) can disperse and attach to the sides and cap of the tube. Do a short spin/pulse in the centrifuge prior to opening these colored tubes and adding deionized water.

Add 25 µL of deionized water to each of the 6 oxidized protein standards and vortex to reconstitute them. If the standards are not completely reconstituted they can be incubated for 2 hours at 37 °C, or overnight at room temperature, and then vortexed again to ensure all of the sample is dissolved.

Step 9: Carbonyl control sample (L)

Store at -20 °C until needed.

Reconstitute using the same method as for the oxidized protein standards (Step 8).

Reconstituted standards and the carbonyl control sample should be frozen at -80 °C if another test is to be performed at a later date.

Once reconstituted, the protein concentration of the standards and control is 40 mg/mL.

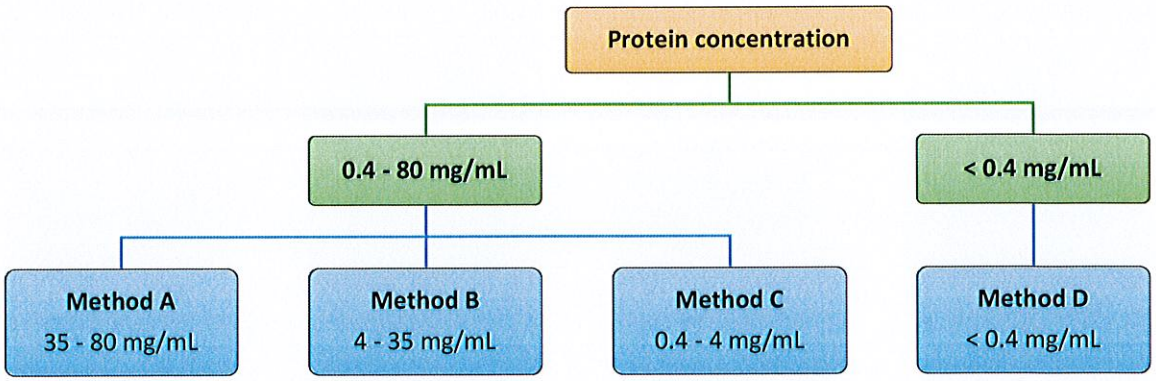
Assay procedure

Allow all reagents to equilibrate to room temperature before performing the assay.

Step 1: Sample derivatization with DNP

The assay is set up so that about 1 µg of derivatized protein is applied to each well of the ELISA plate. This is sufficient to saturate the well with protein, so some variation in the amount of protein applied to the plate will not affect the response.

After determining the protein concentration range of the samples, use the flow diagram below to choose which derivatization method to use.



Method A 35-80 mg/mL

1. Determine the number of 1.5 mL tubes that are needed for derivatization. One tube is needed for each standard (6) and carbonyl control (1), as well as one tube for each sample.
2. Label tubes and add 200 µL of "diluted DNP solution" (Tube set A).
3. Add 5 µL of each standard, control or sample to the appropriate labeled tube.
4. Mix and incubate for 45 minutes at room temperature (18-25 °C).
5. Label a second set of tubes and add 1 mL of EIA buffer to each (Tube set B).
6. Take 5 µL from each "Tube A" and add to the corresponding "Tube B" and mix well.

Method B 4-35 mg/mL

1. Determine the number of 1.5 mL tubes that are needed for derivatization. One tube is needed for each standard (6) and carbonyl control (1), as well as one tube for each sample.
2. Label tubes and add 200 µL of "diluted DNP solution" (Tube set A).
3. Dilute the standards and control 1/10 in EIA buffer for a protein concentration of 4 mg/mL.
4. Add 50 µL of each standard or control to the appropriate "diluted DNP solution" tube.
5. Add a volume of sample containing 200-300 µg protein to the appropriate "diluted DNP solution" tube and make all samples up to an equal volume with deionized water.
6. Mix and incubate for 45 minutes at room temperature (18-25 °C).
7. Label a second set of tubes and add 1 mL of EIA buffer to each (Tube set B).
8. Take 5 µL from each "Tube A" and add to the corresponding "Tube B" and mix well.

Method C 0.4-4mg/mL

1. Determine the number of 1.5 mL tubes that are needed for derivatization. One tube is needed for each standard (6) and carbonyl control (1), as well as one tube for each sample.
2. Label tubes and add 200 µL of "diluted DNP solution" (Tube set A).
3. Dilute the standards and control 1/100 in EIA buffer for a protein concentration of 0.4 mg/mL.
4. Add 50 µL of each standard or control to the appropriate "diluted DNP solution" tube.
5. Add a volume of sample containing 20-30 µg protein to the appropriate "diluted DNP solution" tube and make all samples up to an equal volume with deionized water.
6. Label a second set of tubes and add 1 mL of EIA buffer to each (Tube set B).
7. Take 50 µL from each "Tube A" and add to the corresponding "Tube B" and mix well.

Method D < 0.4 mg/mL

1. Label 2 sets of 0.5 mL tubes for each standard (6) and carbonyl control (1).
2. Into 1 set of tubes dilute each standard and control by taking 5 µL and adding 45 µL of EIA buffer.
3. Take 5 µL of each diluted standard and control and transfer to the second set of labeled tubes for derivatization (Tube set A).
4. For each sample transfer a volume containing 20 µg of protein to a labeled 0.5 mL tube (Tube set A) and make all samples up to an equal volume with deionized water.
5. Add 0.8 volumes of ice cold 28% (w/v) trichloroacetic acid (not provided) to each sample tube, mix and leave on ice for 10 minutes.
6. Centrifuge at 10 000 rpm for 3 minutes and carefully aspirate the supernatant from the inner side of each tube without disturbing the pellet.
7. Add 5 µL of EIA buffer to each sample tube.
8. Add 15 µL of "diluted DNP solution" to each sample, diluted standard and diluted control tube (Tube set A).
9. Mix and incubate for 45 minutes at room temperature (18-25 °C).
10. Label a second set of tubes and add 1 mL of EIA buffer to each (Tube set B).
11. Take 5 µL from each "Tube A" and add to the corresponding "Tube B" and mix well.

Step 2: Plate set up

Create a plate map (back cover) indicating the location of the standards and samples. It is recommended to analyze the standards, carbonyl control and samples in triplicate (25 samples per plate).

Step 3: ELISA procedure

Derivatized protein adsorption to wells

Add 200 µL of each derivatized standard, carbonyl control or sample from "Tube B" into each of the assigned ELISA plate wells.
Cover the plate with sealing tape.
Leave the plate at 4 °C overnight (preferred procedure) or for 2 hours at 37 °C.
Wash the plate with EIA buffer (5 x 200 µL per well).

Blocking

Add 250 µL of the "diluted blocking solution" per well and incubate for 30 minutes at room temperature.
Wash the plate with EIA buffer (5 x 200 µL per well).

Primary antibody

Add 200 µL of the "diluted anti-DNP-biotin antibody" per well and incubate for 1 hour at 37 °C.
Wash the plate with EIA buffer (5 x 200 µL per well).

Secondary antibody

Add 200 µL of the "diluted streptavidin-horseradish peroxidase" per well and incubate for 1 hour at room temperature.
Wash the plate with EIA buffer (5 x 200 µL per well).

Step 4: Color development and measurement

Add 200 µL of the diluted Chromogen reagent (I) per well. A blue color will develop over approximately 5-20 minutes at room temperature.

Start the timer when the Chromogen reagent is being added with the multi-channel pipette to the first row or column and keep the time intervals between pipetting rows or columns constant.

The reaction can be followed at 650 nm. The reaction should continue until the highest standard K6 reaches an OD of 0.3 at 650 nm.

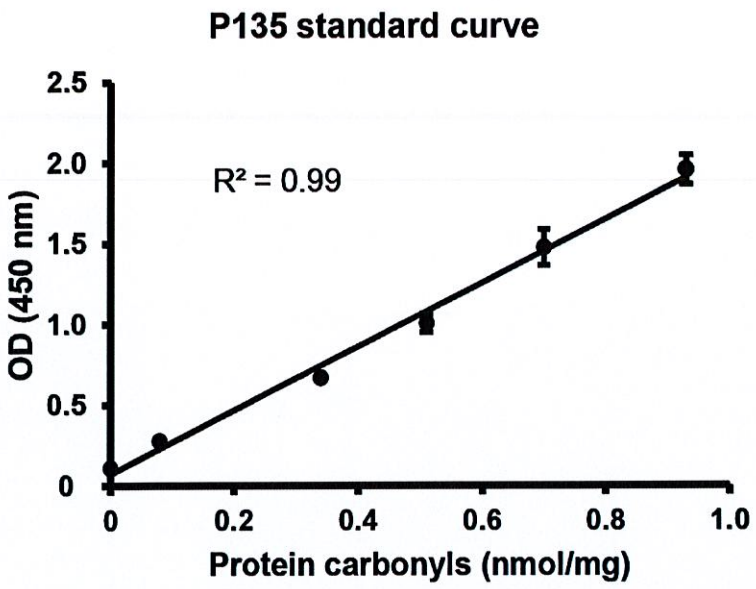
Stop the reaction with 100 µL of Stopping reagent (J) per well. Shake the plate gently to mix reagents. This will change the color to an intense yellow, increasing the absorbance values about threefold.

Read the absorbance values at 450 nm immediately after stopping the reaction.

Step 5: Analysis of results

Construct a linear or best fit regression standard curve by plotting the nmol/mg protein carbonyl concentration of the standards against their absorbance values. An R^2 value of close to 1 should be obtained (see example on the following page).

Batch specific details for standard curve



Std ID	Tube color	Value (nmol/mg)
K1	White/clear	0.00
K2	Purple	0.08
K3	Orange	0.34
K4	Green	0.51
K5	Blue	0.70
K6	Red/pink	0.93

Color development: 4 minutes
Carbonyl control sample (L): 0.46 ± 0.04 nmol/mg

Calculate the carbonyl content of the samples (nmol/mg protein) from the standard curve.

The intra- and inter-assay variation (for assays performed on the same or on different days) of samples with high carbonyls is expected to be around 5%. Samples with low carbonyls (< 0.10 nmol/mg) can have a higher inter-assay variation of about 15% because they are closer to the low end of the standard curve.

See https://biocellcorp.co.nz/diagnostics/ for Frequently Asked Questions.

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A complete list of citations is available at https://biocellcorp.co.nz/diagnostics/.

Notes

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Plate map

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