(PROTEIN CARBONYL ENZYME IMMUNO-ASSAY KIT)

96 Wells

Instructions for Use

Storage

Remove box containing components G, K and L and store in freezer (-20°C)

Remove box containing components H and I and store in fridge (≈4°C)
DO NOT FREEZE

Store the remainder of the kit at room temperature

PC kits do not require to be kept under cool conditions for short term transportation. The kits are stable at room temperature for up to 6 weeks. If the kit is separated into freezer, fridge and bench as per instructions, it is stable until at least the expiry.

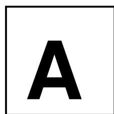
Minor changes have been made to the current batch, as highlighted in the instructions.

BIOCELL CORPORATION LTD.
19 Laureston Ave, Papatoetoe, New Zealand

www.biocellcorp.co.nz

To be used with PC Kits Batch P094

Please Note:	Crystallisation around the DNP (E) and Guanidine (F) lids is purely due to the high concentration of Guanidine. This will not affect the results of the kit.



BIOCELL PC TEST

(PROTEIN CARBONYL ENZYME IMMUNO-ASSAY KIT)

The *BIOCELL PC TEST 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 27 samples as triplicates or 41 samples as duplicates if the whole plate is used at once.

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

For research use only

Introduction

Protein carbonyls are formed by a variety of oxidative mechanisms and are sensitive indices of oxidative injury. The quantity of protein carbonyls in a protein sample can be determined by derivatising with dinitrophenylhydrazine (DNP) and measuring bound DNP colourimetrically or immunologically. The ELISA method enables carbonyls to be measured quantitatively with microgram quantities of protein.

Assay Principle

A schematic diagram of the Protein Carbonyl ELISA is shown in Figure 1. Samples containing protein are reacted with DNP; then the protein is nonspecifically adsorbed to 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. Absorbances are related to a standard curve prepared for serum albumin containing increasing proportions of hypochlorous acid-oxidized protein that has been calibrated colourimetrically.

Reagent Use and Stability

Reagents must be brought to room temperature prior to use.

PC kits do not require to be kept under cool conditions for short term transportation. The kits are stable at room temperature for up to 6 weeks. If the kit is separated into freezer, fridge and bench as per instructions, it is stable until at least the expiry.

The stability of all other reagents is as shown in Notes 1-9 on page 3.

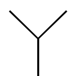
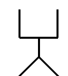
Kit Components

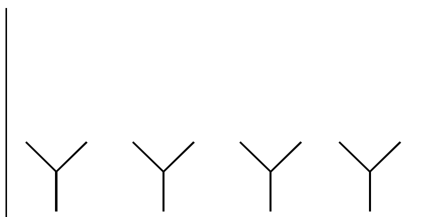
The kit components (supplied materials) are sufficient for one plate. Each kit contains reagents and components labeled as follows:

A	Instructions	G	Anti-DNP-biotin-antibody
B	ELISA 96-well-plate + plate cover	H	Streptavidin-horseradish-peroxidase
C	EIA buffer powder	I	Chromatin reagent
D	Blocking reagent	J	Stopping reagent
E	Dinitrophenylhydrazine	**K**	**Standards (6)**
F	Guanidine hydrochloride diluent	**L**	**Carbonyl control sample**

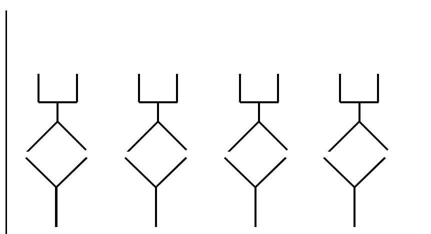
Figure 1: Schematic diagram of the Protein Carbonyl ELISA

Key:

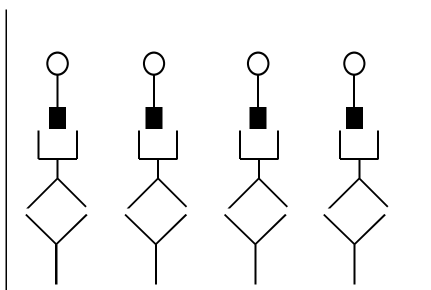
 DNP – protein  anti–DNP-biotin AB Streptavidin HRP

1. 

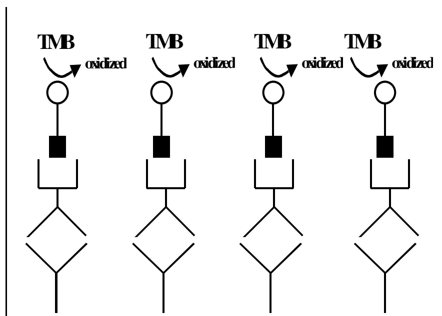
DNP reacted proteins are bound to the ELISA plate

2. 

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

3. 

Streptavidin – linked horseradish peroxidase is bound to the complex

4. 

The chromatin reagent containing peroxide is added.

The peroxide catalyses the oxidation of TMB.

The reaction is stopped by the addition of acid and the absorbance is measured for each well at 450 nm.

Materials required but not supplied

1. Single and multi -channel pipettes capable of delivering 5µl, 10µl, 25µl, 100µl, 200µl, 250µl and 1ml.
2. Disposable pipette tips.
3. Containers for reagent mixing and pipetting reservoirs.
4. The use of an automated microwell washer is recommended.
5. Microwell spectrophotometric reader. The reader must read at **450nm**.
6. Deionized water.
7. 1 L and 100 ml measuring cylinders.
8. 1.5 ml and 0.5 ml (for low protein method) eppendorf tubes.
9. Trichloroacetic acid (TCA) 28% (w/v) for low protein method.

PRE-ASSAY PREPARATION

Note: Gloves should be worn when handling reagents.
Reagents G and H can disperse and attach to the sides of the microvial in transit and become difficult to accumulate into the bottom. Proceed with new instructions as below.

1 EIA Buffer (C).
Dissolve the contents of bottle (C) in one litre of deionized water. **Store at 4°C** for up to 4 weeks.

2 Blocking reagent (D).
Add 2ml of EIA Buffer to blocking reagent container (D). Mix well and transfer to a 100ml measuring cylinder. Add EIA Buffer to a final volume of 75ml. Label this solution as “diluted blocking solution”. **Store at 4°C** for up to 4 weeks.

3 Dinitrophenylhydrazine (DNP) (E).
Add 1ml of the DNP reagent (E) to 9ml of guanidine hydrochloride (F). Label this solution as “diluted DNP solution”.
Store at room temperature. If diluted reagent has been stored for more than 2 weeks it is recommended that it be centrifuged at 8000g for 5 minutes and the supernatant used for derivatization.

4 Anti-DNP-biotin-antibody (G). **Store at -20°C until needed**.
Prepare this dilution directly before use.
Add 0.5ml of “diluted blocking solution” to the Anti-DNP-biotin-antibody microvial (G). Mix well and make up to 20ml with “diluted blocking solution”.

5 Streptavidin-horseradish-peroxidase (H). **Store at 4°C until needed**. **Must NOT be frozen.**
Prepare this dilution directly before use.
Add 0.5ml of “diluted blocking solution” to the Streptavidin-HRP microvial (H). Mix well and make up to 20ml with “diluted blocking solution”.

6 **Chromatin reagent (I).** (Ready to use) **Store at 4°C until needed**.
Contamination of this solution may result in unintended colour development.
Never pipette directly from container and do not return unused reagent to main container. Pour the required amount into a pipetting reservoir.
20ml is needed.

7 Stopping reagent (J). (Ready to use)

8 Oxidized Protein Standards (K1-6). **Store at -20°C until needed**
Add **25** µl of deionized water to each of the 6 oxidized protein samples, **vortex, stand at 37**°C for at least **30 minutes then re-vortex again to ensure ALL of the sample is dissolved**. Reconstituted standards should be frozen if another test is to be performed at a later date. **Only use the top standard K6 (red/pink) if dealing with highly oxidized samples.**

9 Carbonyl control sample (L). **Store at -20°C until needed.**
Add **25** µl of deionized water to the Carbonyl control sample, **vortex, stand at 37°C for at least 30 minutes then re-vortex again to ensure ALL of the sample is dissolved.** Reconstituted control should be frozen if another test is to be performed at a later date. The carbonyl levels should be close to the green standard (K4).

1. Sample Derivatisation with DNP

Note: Allow all reagents to equilibrate to room temperature (18-25°C) before performing the assay.

The assay is set up so that about 1 µg of derivatised 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. Two methods of sample preparation are described. The standard procedure is applicable to plasma or any sample with a protein concentration between 35g/L and 80g/L *(expected range for human plasma from healthy controls: 60-70g/L).* The low protein method, which involves concentration of the protein by precipitation with trichloroacetic acid (TCA) should be used with more dilute samples.

Triplicate analyses of each sample are recommended (27 samples per whole plate).

A: Standard Procedure (samples with 35-80 g/L protein)

Heparinised and EDTA plasma or serum from humans, rats or mice (but not rabbits) can be used in the assay. ***Plasma samples should be centrifuged before use.***

- Set up and label the required 1.5 ml reaction tubes.
  (eg. If doing triplicates: 32 tubes for **6 Standards, 1 Carbonyl control**, and 25 samples)
- Add 200 µl "diluted DNP-solution" to each tube.
- Add 5 µl of each sample, standard or control to the appropriate tube.
- Mix and incubate for 45 min. To ensure that the incubation time is similar for all samples, start the timer after the first sample has been added and perform the later dilution into EIA-buffer at approximately the same speed as when adding the sample to the "diluted DNP-solution".

- Prepare complimentary set of 1.5 ml tubes for samples, standards and controls.
- Add 1 ml EIA Buffer into each tube.
- Add 5 µl of each DNP-treated sample to the appropriate tube and mix well.

The samples are now ready for ELISA Procedure (3).

B: Low Protein Procedure

Appropriate for cell extracts or biological fluids such as cerebrospinal fluid and bronchoalveolar lavage fluid.

- Determine total protein concentration of samples. If these are within 35-80 g/L, use procedure A. For concentrations below this range, proceed as follows.
- Initially, make a 10-fold dilution of the standards and quality controls by diluting 5 µl with 45 µl EIA Buffer.
- Transfer a volume containing 20 µg protein from each sample to a labeled 0.5 ml tube and make all samples up to an equal volume with deionised water.
- Add 0.8 volumes of ice cold 28% (w/v) TCA (not provided), mix and leave on ice for 10 minutes.
- Centrifuge at 10000 rpm for 3 min and carefully aspirate the supernatant from the inner side of each tube **without disturbing the pellet.**
- Add 5 µl EIA Buffer plus 15 µl "diluted DNP-solution" to each sample tube. Vortex thoroughly.
- Take 5 µl of each diluted standard and control and add 15 µl "diluted DNP-solution".
- Leave at room temperature for 45 min. To ensure that the incubation time is similar for all samples, start the timer after the first sample has been added and perform the later dilution into EIA-buffer at approximately the same speed as when adding the sample to the "diluted DNP-solution".
- Prepare complimentary set of 1.5 ml tubes for samples, standards and controls.
- Add 1 ml EIA Buffer into each tube.
- Add 5 µl of each DNP-treated sample to the appropriate tube and mix well.

The samples are now ready for ELISA Procedure (3).

2. Plate Set Up

- Create a plate map indicating location of samples, and standards. We recommend the samples and standards should be analyzed in triplicate (preferred) or duplicate.

3. ELISA Procedure

- Add 200 µl of each sample (in EIA-buffer) into each of the assigned ELISA-plate wells. Cover the plate with sealing tape (can be cut to size if required).
- Leave plate at 4°C overnight (preferred procedure) or for 2 hours at 37°C.
- Wash plate with EIA Buffer (5 x approx. 300 µl per well).
- Add 250 µl of "diluted blocking solution" per well and incubate for 30 min at room temperature.
- Wash plate with EIA Buffer (5 x approx. 300 µl per well).
- Add 200 µl of "diluted anti-DNP-biotin-antibody" per well and incubate for 1 hour at 37°C.
- Wash plate with EIA Buffer as above.
- Add 200 µl of "diluted streptavidin-HRP" per well and incubate 1 hour at room temperature.
- Wash plate with EIA Buffer as above.

4. Colour Development and Measurement

- Add 200 µl of Chromatin reagent (I) per well and let colour develop until highest standard becomes dark blue (A_{650} 0.7-1.0). This typically takes 20-30 min at 37°C or 40-60 min at RT. Start the timer when the Chromatin reagent is being added with the multi-channel pipette to the first row or column and keeps the time intervals between pipetting rows or columns constant. Positive wells will turn blue and can be measured at 650nm. However, stopping the reaction with an acid solution is preferred. This will result in a colour change to yellow, increasing absorbances about threefold.

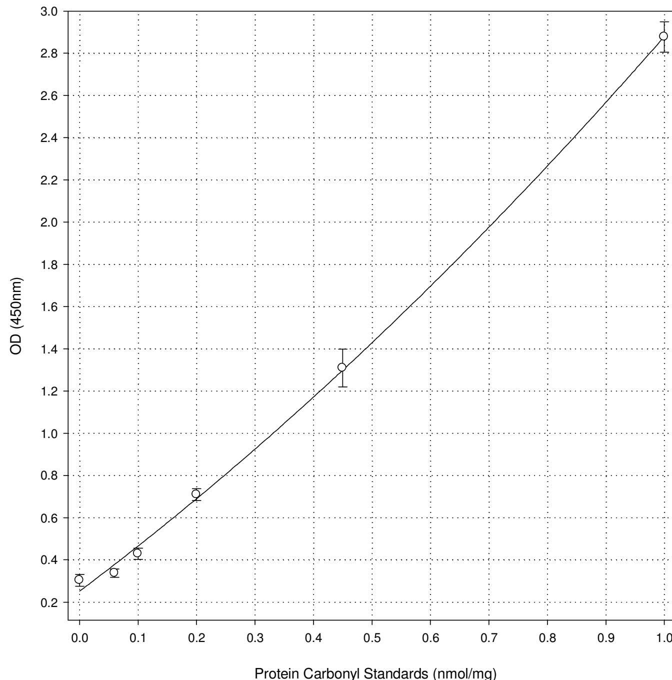
- Stop reaction with 100 µl of Stopping Reagent (J) per well, ensuring that all wells are exposed to the Chromatin reagent for the same amount of time. Add the Stopping Reagent to the rows or columns of the plate by keeping to the same time intervals used with the Chromatin reagent. Shake plate gently to mix reagents. Read absorbances at **450** nm directly after stopping reaction.

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 absorbances. An r^2 value of close to 1 should be obtained. (See example on following page)

Batch Specific Details for Standard Curve – PCKIT P094

Incubation 30min @ 37°C



Std ID	Tube Colour	Value (nmol/mg)
K1	White / Clear	0.00
K2	Purple	0.06
K3	Orange	0.10
K4	Green	0.20
K5	Blue	0.45
K6	Red / pink **(optional)**	1.00

Graphs: Sample of the Actual Standard Curves obtained from the PC Kit P094 standards

- Calculate the carbonyl content of your samples (nmol/mg protein) from your standard curve by using the regression factors obtained from your standard curve (A = intercept with y-axis, B = slope). Many plate readers can be programmed to construct a best fit standard curve and calculate sample concentrations from it. Alternatively, this can be performed manually using graph paper.

The control provided with the kit have should protein carbonyl levels similar to K4. Including this control in the assay is optional; it is intended to provide reassurance that the assay detects protein carbonyls in a sample when analysed by the customer.

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 (<0.100nmol/mg) can have a higher inter-assay variation of about 15%, because they are closer to the low end of the standard curve.

The protein carbonyl content of plasma from most healthy human adults, as determined by ELISA, is below 0.1nmol/mg. It is elevated in some disease states (please refer to the published literature for details).

References

Buss, H., Chan, T.P., Sluis, K.B., Domigan, N.M. and Winterbourn, C.C. *Protein Carbonyl Measurement By A Sensitive ELISA Method*. Free Rad. Biol. Med. (1997) Vol 23, pp 361-366.

Winterbourn, Christine C. and Buss, I. Hendrikje. *Protein Carbonyl Measurement by Enzyme-linked Immunosorbent Assay.* Methods in Enzymology, (1999) Vol 300, pp 106-111.

Buss IH. Darlow BA. Winterbourn CC. *Elevated protein carbonyls and lipid peroxidation products correlating with myeloperoxidase in tracheal aspirates from premature infants*. Pediatric Research. (2000) Vol 47, pp 640-645.

Inder T. Mocatta T. Darlow B. Spencer C. Volpe JJ. Winterbourn C. *Elevated free radical products in the cerebrospinal fluid of VLBW infants with cerebral white matter injury.* Pediatric Research (2002) Vol 52, pp 213-8.

Manufacturer Contact / Technical Support

BIOCELL CORPORATION LTD
19 Laureston Avenue,
Papatoetoe,
New Zealand.

Phone (+64)-9-2700755
Fax : (+64)-9-2700751
Web: www.biocellcorp.co.nz
Email: jan.lauder@biocellcorp.co.nz

SAMPLES: **DATE:**

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

SAMPLES: **DATE:**

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												