# Protein Carbonyl Assay Kit

## A) Instruction Manual

Redox Innovation
University of Otago, Christchurch, New Zealand

**www.redoxinnovation.co.nz**

To be used with Protein Carbonyl Assay Kits Batch RI-PC-B1

**For research use only**

# Contents

## Storage

- Freezer (-20 °C): Components F, 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.
- Redox Innovation 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 Redox Innovation 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 analyse 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-catalysed 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 biotin hydrazide and then excess protein is non-specifically adsorbed to saturate an ELISA plate. Unconjugated biotin hydrazide and non-protein constituents are washed away. The adsorbed protein is probed with streptavidin-linked horseradish peroxidase. Absorbance values are related to a standard curve prepared for serum albumin containing increasing proportions of hypochlorous acid-oxidised protein that has been calibrated colorimetrically.

## Kit components

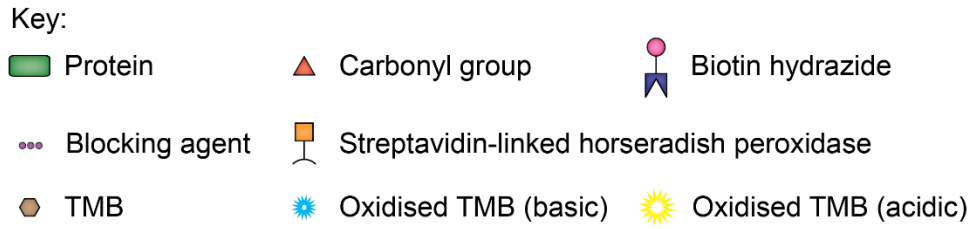
The kit components (supplied materials) are sufficient for one plate. Each kit contains reagents and components labelled as follows:

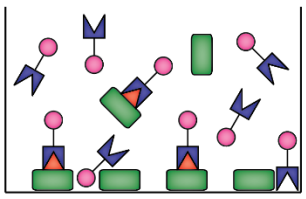
| | | | |
|---|---|---|---|
| A | Instruction manual | G | Guanidine hydrochloride |
| B | ELISA 96 well plate | H | Streptavidin-horseradish peroxidase |
| C | Plate cover | I | Chromogen reagent |
| D | EIA buffer powder | J | Stopping reagent |
| E | Blocking reagent | K | Oxidised protein standards (6) |
| F | Biotin hydrazide | L | Carbonyl control sample |

## Additional materials required

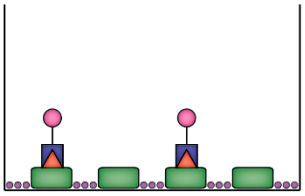
1. Pipettes with disposable plastic tips to deliver 5 µL- 1 mL.
2. Containers for reagent mixing and pipetting reservoirs.
3. The use of an automated microwell washer is recommended, otherwise use a squeeze bottle.
4. Microwell spectrophotometric reader. The reader must be able to read at 450 nm. 650 nm is useful to monitor the reaction before stopping.
5. Deionised water.
6. 1 L and 50 mL measuring cylinders.
7. Centrifuge for spinning down the contents of Biotin hydrazide (F), Oxidised protein standards (K1-6) and Carbonyl control sample (L) tubes before use.
8. 1.5 mL (Methods A-D) and 0.5 mL (Method D) tubes.
9. Trichloroacetic acid 28% (w/v) (Method D).

## Schematic of the Protein Carbonyl ELISA

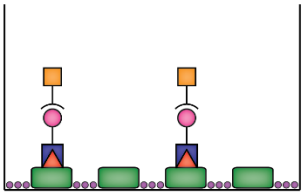



1. 

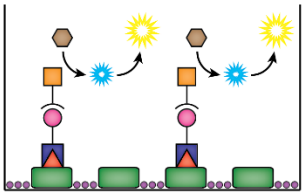
Protein samples that have been derivatised with biotin hydrazide are added and directly bound to the ELISA plate, saturating all protein-binding sites.

2. 

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

3. 

Streptavidin-linked horseradish peroxidase is bound to the biotin hydrazide-protein complex.

4. 

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 (D)

Dissolve the contents of bottle (D) in 1 L of deionised water.

**Store at 4 °C**.

## Step 2: Blocking reagent (E)

Add 2 mL of EIA buffer to the blocking reagent container (E). Mix well and transfer to a 50 mL measuring cylinder. Add EIA buffer to a final volume of 50 mL.

Label this solution as “diluted blocking solution”.

**Store at 4 °C**.

## Step 3: Biotin hydrazide (F)

**Store at -20 °C protected from light until needed**.

Prepare this immediately before use.

Crystallisation around the Guanidine hydrochloride (G) lid may occur and is purely due to the high concentration of guanidine. This will not affect the result of the kit.

Please note: The Biotin hydrazide can disperse and attach to the sides and cap of the tube. Do a short spin/pulse in the centrifuge prior to opening this tube and adding Guanidine hydrochloride.

Add 0.5 mL of Guanidine hydrochloride (G) to the Biotin hydrazide tube (F).

Mix well and transfer to a 50 mL measuring cylinder. Add Guanidine hydrochloride (G) to a final volume of 20 mL.

Label this solution as “biotin hydrazide solution”.

Biotin hydrazide made up with Guanidine hydrochloride can be stored at 4 °C for up to one week if another test is to be performed at a later date. However, this may result in an extended colour development time and it is recommended to use it fresh.

## Step 4: 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”.

Streptavidin-horseradish peroxidase made up with diluted blocking solution can be stored at 4 °C for up to one week if another test is to be performed at a later date. However, this may result in an extended colour development time and it is recommended to use it fresh.

## Step 5: 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 (D) 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 colour development.**

## Step 6: Stopping reagent (J)

Ready to use.

**Store at room temperature.**

## Step 7: Oxidised protein standards (K1-6)

**Store at -20 °C until needed.**

Please note: The Oxidised 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 coloured tubes and adding deionised water.

Add 25 µL of deionised water to each of the 6 oxidised protein standards and vortex to reconstitute them.

## Step 8: Carbonyl control sample (L)

**Store at -20 °C until needed.**

Reconstitute using the same method as for the oxidised 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.

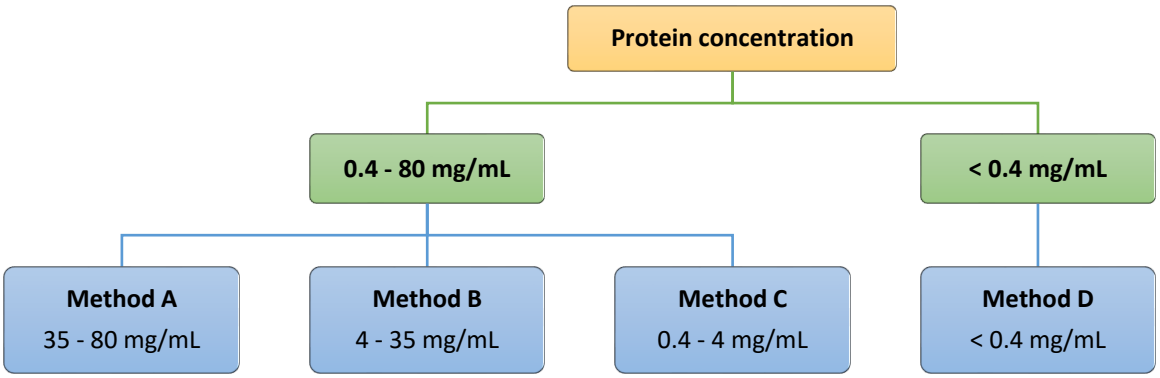
# Assay procedure

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

## Step 1: Sample derivatisation with Biotin hydrazide

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.

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




### Method A 35-80 mg/mL

1. Determine the number of 1.5 mL tubes that are needed for derivatisation. 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 "Biotin hydrazide solution" (Tube set A).
3. Add 5 µL of each standard, control or sample to the appropriate labelled 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 derivatisation. 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 "Biotin hydrazide 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 "Biotin hydrazide solution" tube.
5. Add a volume of sample containing 200-300 µg protein to the appropriate "Biotin hydrazide solution" tube and make all samples up to an equal volume with deionised 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 derivatisation. 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 "Biotin hydrazide 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 "Biotin hydrazide solution" tube.
5. Add a volume of sample containing 20-30 µg protein to the appropriate "Biotin hydrazide solution" tube and make all samples up to an equal volume with deionised 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 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 labelled tubes for derivatisation (Tube set A).
4. For each sample transfer a volume containing 20 µg of protein to a labelled 0.5 mL tube (Tube set A) and make all samples up to an equal volume with deionised 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 "Biotin hydrazide 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 analyse the standards, carbonyl control and samples in triplicate (25 samples per plate).

## Step 3: ELISA procedure

### Derivatised protein adsorption to wells

Add 200 µL of each derivatised 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).

### Detection

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: Colour development and measurement

Add 200 µL of the diluted Chromogen reagent (I) per well. A blue colour 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 0.25 at 650 nm.

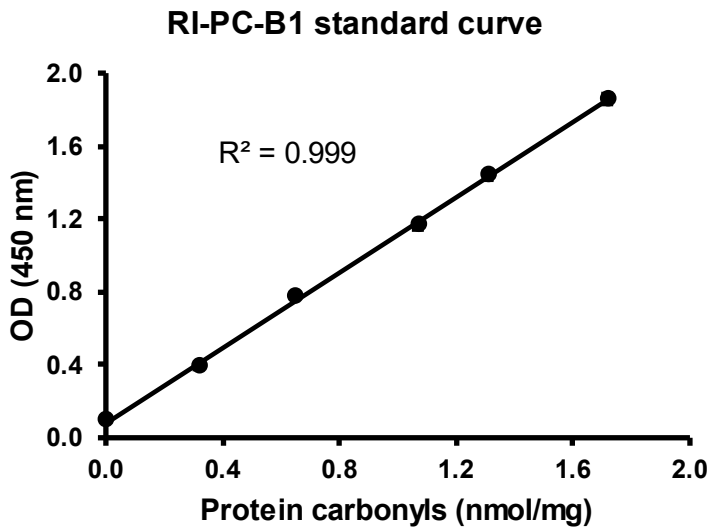
Stop the reaction with 100 µL of Stopping reagent (J) per well. Shake the plate gently to mix reagents. This will change the colour to an intense yellow, increasing the absorbance values about threefold.

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 colour | Value (nmol/mg) |
|---|---|---|
| K1 | White/clear | 0.00 |
| K2 | Purple | 0.32 |
| K3 | Orange | 0.65 |
| K4 | Green | 1.07 |
| K5 | Blue | 1.31 |
| K6 | Red/pink | 1.72 |

**Colour development: 10 minutes**
**Carbonyl control sample (L): 0.87 ± 0.05 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 (< 0.30 nmol/mg) can have a higher inter-assay variation of about 12% because they are closer to the low end of the standard curve.

See https://www.redoxinnovation.nz/ for Frequently Asked Questions.

## References

Buss, H., T.P. Chan, K.B. Sluis, N.M. Domigan, and C.C. Winterbourn, *Protein carbonyl measurement by a sensitive ELISA method.* Free Radic Biol Med, 1997. **23**(3): p. 361-6.

Winterbourn, C.C. and I.H. Buss, *Protein carbonyl measurement by enzyme-linked immunosorbent assay.* Methods Enzymol, 1999. **300**: p. 106-11.

Buss, I.H. and C.C. Winterbourn, *Protein carbonyl measurement by ELISA.* Methods Mol Biol, 2002. **186**: p. 123-8.

## Recent citations

A complete list of citations is available at https://www.redoxinnovation.co.nz.

## Manufacturer Contact / Technical Support

Redox Innovation
University of Otago
Christchurch
New Zealand

Website: www.redoxinnovation.co.nz
Email: info@redoxinnovation.co.nz

## Plate map

**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 |
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| A | | | | | | | | | | | | |
| B | | | | | | | | | | | | |
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