MANUAL

RayBio® Protein Oxidation (Carbonyl Content) Assay Kit (Colorimetric)

Catalog #: MA-OxiC-C

ISO 13485

Introduction

Oxidative stress is commonly defined as a condition under which the generation of reactive oxygen species (ROS) within a cellular system exceeds the buffering capacity of endogenous antioxidant defenses. This leads to oxidative damage to lipids, DNA, and proteins, and has been linked to aging, cancer and other diseases. One of the most commonly used markers of oxidative stress is carbonylation of proteins.

RayBio® Protein Oxidation (Carbonyl Content) Assay Kit provides a simple and accurate method for quantifying protein carbonyl content in plasma, serum, cell lysates and other biological liquid samples. Carbonyl content is determined by the derivatization of protein carbonyl groups with 2,4-dinitrophenyl-hydrazine (DNPH), resulting in the formation of stable dinitrophenyl (DNP) hydrazone adducts. These adducts can be measured spectrophotometrically at 375 nm, and the absorbance is directly proportional to the carbonyl content present in the sample.

Storage

The entire kit may be stored at 2–8 °C for up to 6 months from the date of shipment. For prepared reagent storage, see table below.

Component	Size / Description	Storage After Preparation
Microplate (Item A)	One 96-well (12 strips x 8 wells) plate	RT
DNPH Solution	2x 12 ml	2–8°C dark
Acid Solution	25 ml	RT
TCA Solution	30 ml	2–8°C
Guanidine Solution	2x 15 ml	2–8°C dark
Wash Buffer I	2x 20 ml	RT
Wash Buffer II	2x 20 ml	RT

RT = room temperature

Additional Materials Required

1. Microplate reader capable of measuring absorbance at 375 nm
2. Precision pipettes to deliver 2 µl to 1 ml volumes
3. Distilled or deionized water
4. Tubes to prepare sample dilutions
5. Microcentrifuge
6. Streptomycin sulfate for removal of nucleic acids (optional)
7. Bovine serum albumin (BSA) for the determination of protein concentration (optional)
8. Spectrophotometer for the determination of nucleic acid contamination and protein concentration (optional)

Sample Tips and General Considerations

NOTE: Optimal methods of sample preparation will need to be determined by each researcher empirically based on researched literature and knowledge of the samples.

- If not using fresh samples, freeze samples as soon as possible after collection.
- Avoid multiple freeze-thaw cycles. If possible, sub-aliquot samples prior to initial storage.
- It is strongly recommended to add a protease inhibitor cocktail to cell and tissue lysate samples.
- Avoid sonication of 1 ml or less as this can quickly heat and denature proteins.
- This assay works best when samples have protein concentrations in the range of 1-10 mg/ml.
- Most samples will not need to be concentrated. If concentration is required, a spin column concentrator with a chilled centrifuge is recommended.

1. Cell lysates can be prepared as follows:

For attached cells, remove supernatant from cell culture, wash cells twice with cold 1X PBS (for suspension cells, pellet the cells by spinning down the cells at 1,000 x g for 10 minutes) making sure to remove any remaining PBS before adding lysis buffer. Solubilize the cells at $2x10^7$ cells/ml in lysis buffer containing protease inhibitors. Pipette up and down to resuspend cells and rock the lysates gently at 2–8°C for 30 minutes. Transfer extracts to microfuge tubes and centrifuge at 14,000 x g for 10 minutes.
It is recommended that sample protein concentrations should be determined using a total protein assay. Lysates should be used immediately or aliquoted and stored at -70°C. Thawed lysates should be kept on ice prior to use.

General tips for preparing lysate samples can be viewed on RayBiotech's online Resources page: https://www.raybiotech.com/tips-on-sample-preparation/

Check for nucleic acids contamination:
Nucleic acids can lead to an overestimation of carbonyl levels. Check cell lysate samples for nucleic acid contamination using the 280nm/260nm absorbance ratio. If the ratio is less than 1, incubate samples with 1% streptomycin sulfate for 15 minutes at room temperature, then centrifuge at 6000 x g for 10 min at 4°C. Collect the supernatant for protein carbonyl measurement.

2. Plasma samples:

Collect blood using an anticoagulant, such as citrate, EDTA, or heparin, and mix gently by inverting. Centrifuge at 1000 x g for 10 minutes at 4°C. Collect the plasma supernatant without disturbing the white buffy layer. Test the samples immediately or store them by freezing at -80°C.

3. Serum samples:

Collect blood in an anticoagulant-free tube. Let the blood clot at room temperature for 30 minutes. Centrifuge at 2500 x g for 20 minutes. Carefully remove the yellow serum supernatant without disturbing the white buffy layer. Test the samples immediately or store them by freezing at -80°C.

Reagent Preparation

Reagent	PREPARATION
Microplate (Item A)	No preparation
Acid Solution	
DNPH Solution	Warm to room temperature before use
Guanidine Solution	
20% TCA Solution	Slowly add 20 ml TCA solution into 30 ml dH2O. Keep it on ice.
10% TCA Solution	Slowly add 10 ml TCA solution into 40 ml dH2O. Keep it on ice.
Wash Buffer	Mix 40 ml each of Wash Buffer I and Wash Buffer II in a 1:1 ratio. Wash Buffer should be prepared fresh right before the experiment.

Assay Procedure

Plate Set Up

A representative plate layout is given below. We recommend measuring all samples in duplicate. (S1 – S24: Sample 1 – 24. C1 – C24: Control Samples 1 – 24)

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	C1	C1	S9	S9	C9	C9	S17	S17	C17	C17
B	S2	S2	C2	C2	S10	S10	C10	C10	S18	S18	C18	C18
C	S3	S3	C3	C3	S11	S11	C11	C11	S19	S19	C19	C19
D	S4	S4	C4	C4	S12	S12	C12	C12	S20	S20	C20	C20
E	S5	S5	C5	C5	S13	S13	C13	C13	S21	S21	C21	C21
F	S6	S6	C6	C6	S14	S14	C14	C14	S22	S22	C22	C22
G	S7	S7	C7	C7	S15	S15	C15	C15	S23	S23	C23	C23
H	S8	S8	C8	C8	S16	S16	C16	C16	S24	S24	C24	C24

Assay Protocol

This assay works best when samples have protein concentrations in the range of 1-10mg/ml.

1. For each sample, prepare two 2 ml tubes, one for sample (S#) and the other for control (C#). Add 200 µl of sample to both tubes.
2. Add 800 µl DNPH solution to the sample tube and add 800 µl Acid Solution to the control tube.
3. Incubate all the tubes in the dark at room temperature for 1 hour. Vortex each tube briefly every 15 minutes during the incubation.
4. Add 1 ml 20% TCA (See “Reagent Preparation”) to each tube and vortex. Incubate for 5 minutes on ice.
5. Centrifuge tubes at 10,000 x g for 10 minutes at 4°C in a microcentrifuge.
6. Discard the supernatant and resuspend the pellet in 1 ml 10% TCA (See “Reagent Preparation”). Incubate for 5 minutes on ice.
7. Centrifuge tubes at 10,000 x g for 10 minutes at 4°C in a microcentrifuge.
8. Discard the supernatant and resuspend the pellet in 500 µl Wash Buffer (See “Reagent Preparation”). Vortex thoroughly and centrifuge the tubes at 10,000 x g for 10 minutes at 4°C in a microcentrifuge.
9. Repeat step 8 two more times.
10. After the final wash, resuspend the protein pellet in 500 µl of Guanidine Solution and incubate at 37°C for 10 minutes with gentle mixing. Centrifuge the tubes at 10,000 x g for 10 minutes at 4°C in a microcentrifuge to remove any precipitation.
11. Transfer 220 µl supernatant from the sample tube to two wells of the 96-well plate.
12. Transfer 220 µl supernatant from the control tube to two wells of the 96-well plate.
13. Measure the absorbance at a wavelength of 375 nm using a plate reader.

Determination of protein content of pellets (optional)

Proteins may be lost during washing steps. The BCA assay can be used to accurately determine protein levels in the final reconstituted sample from the sample tube. The protein levels obtained can then be used to normalize the measured carbonyl content (see Calculation of Results below).

Note: For samples containing over 1 mg of protein, we recommend diluting at least 10-fold before performing the BCA assay. The Bradford protein assay is unsuitable for this purpose due to interference from guanidine.

Assay Procedure Summary

1. Prepare all reagents and samples as instructed.
2. Incubate samples with DNPH in the dark at RT for one hour.
3. Use 20% TCA to precipitate the proteins.
4. Use 10% TCA to precipitate the proteins.
5. Use wash buffer to wash the pellet 3 times.
6. Resuspend the protein pellet in guanidine solution.
7. Transfer 220 µl supernatant to 96-well plate.
8. Read absorbance at 375 nm on a microplate reader.
9. Determine protein concentration by BCA assay. (Optional)

Calculation of Results

Calculate the mean absorbance for each set of duplicate samples and controls and subtract the average absorbance of the control from the average absorbance of sample. This is the Corrected Absorbance (CA).

Determine the concentration of the carbonyls by inserting the corrected absorbance into the following equation:

Protein Carbonyl (nmol/ml) = [(CA) / (*0.011µM^{-1})] (500µl / 200µl)

*The actual extinction coefficient for dinitrophenylhydrazine is 22,000M^{-1}cm^{-1}(0.022uM^{-1}cm^{-1}). This value has been adjusted for the pathlength of the solution in the well.

If protein concentration is measured, carbonyl content can be adjusted as follows:

Carbonyl content (nmol/mg) = (Carbonyl nmol/ml) / (protein mg/ml)

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