MANUAL

RayBio® Oxidation (Carbonyl) Detection Kit For Western Blots

Catalog #: MA-OxiC-W

RayBiotech
Empowering your proteomics

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.

The RayBio® Oxidation (Carbonyl) Detection Kit utilizes the 'biotin probes' method to biotin label carbonyl groups of oxidized proteins. In this method, the biotin probes first react with carbonyl groups of oxidized proteins to form unstable Schiff bases, which are then further reduced to more stable amines. The biotinylated proteins can then be detected by western blotting. Furthermore, the oxidation sites can be mapped using mass spectrometry after selectively isolating the biotinylated proteins/peptides using avidin resin.

Storage

The entire kit may be stored at -20°C for up to 6 months from the date of shipment. For extended storage, it is recommended to store at -80°C. Avoid repeated freeze-thaw cycles. For prepared reagent storage, see table below.

Component	Size / Description	Storage	Storage After Preparation
Oxidation Buffer A	12 ml	RT	RT
Oxidation Buffer B	3 ml	RT	RT
Oxidation Labeling Reagent	1 vial	-20 °C	-20 °C
Oxidation Stabilizing Reagent	1 vial	-20 °C	-20 °C
1000X HRP-Streptavidin	1 vial (50 µl)	-20 °C	2–8 °C (up to 3 months)

RT = room temperature

Additional Materials Required

1. 20% (v/v) trichloroacetic acid (TCA) solution, ice-cold
2. 1:1 (v/v) ethanol:ethyl acetate
3. Desalting column or dialysis membrane
4. 1.5 mL microcentrifuge tubes
5. 15 mL tubes (polypropylene)
6. 10 mL graduated cylinders (X2)
7. Benchtop centrifuge and microcentrifuge (4°C)
8. Distilled or deionized water
9. Precision pipettes to deliver 2 µl to 1 ml volumes
10. Adjustable 1-25 ml pipettes for reagent preparation

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.
- Most samples will not need to be concentrated. If concentration is required, a spin column concentrator with a chilled centrifuge is recommended.

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 1000x g for 10 min) 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 aliquot 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/

Reagent Preparation

NOTE:

- Thaw frozen reagents to room temperature immediately before use. If buffers contain visible crystals, warm to room temperature, and mix gently until dissolved.
- This kit contains enough reagent to label 40 samples containing 100-200 µg of total protein each.

COMPONENT	PREPARATION
Oxidation Buffer A	No preparation
Oxidation Buffer B	No preparation
Oxidation Labeling Reagent	No preparation
Oxidation Stabilizing Reagent	Add 31 µl Oxidation Stabilizing Reagent into 5ml Oxidation Buffer A to make the diluted Stabilizing Reagent
1000X HRP-Streptavidin	Recommended dilution: 1000-fold for western blot
20% (v/v) trichloroacetic acid (TCA) (not included)	1 part trichloroacetic acid (TCA) mixed with 4 parts dH_2O, pre-chilled (4°C or on ice)
1:1 (v/v) ethanol:ethyl acetate (not included)	1 part ethanol mixed with 1 part ethyl acetate

Assay Procedure

1. Prepare 100 µl of sample with a total protein concentration in the range of 1-2 mg/mL. If the sample contains Tris or glycine, remove it using a desalting column or dialysis bag against Oxidation buffer A.
2. Add 12 µl of Oxidation Labeling Reagent to each sample and gently mix.
3. Incubate the samples at room temperature for 2 hours with gentle shaking.
4. Transfer the reaction to ice and incubate for 15 min.
5. Add 112 µl of diluted Stabilizing Reagent (see reagent preparation) to each sample.
6. Incubate the samples at room temperature for 1 hour.
7. Quick spin. Add 224 µl of ice-cold 20% TCA solution to each sample and incubate on ice for 15 mins.
8. Centrifuge the samples at 10,000 × g for 5 min at 4ºC.
9. Carefully discard supernatants, then add 250 µl ice-cold 20% TCA and briefly vortex.
10. Centrifuge the samples at 10,000 × g for 5 min at 4ºC.
11. Carefully discard supernatants, then add 250 µl of 1:1 (v/v) ethanol:ethyl acetate and briefly vortex.
12. Repeat step 10 and 11 at least twice.
13. Centrifuge the samples at 10,000 × g for 5 min, at 4ºC and carefully discard supernatants.
14. Allow the pellets to dry at room temperature for about 15 min to allow complete solvent evaporation. Do not over-dry pellet, or it may not be dissolved properly.
15. Reconstitute each protein pellet in 20-40 µl Oxidation Buffer B. Or directly reconstitute in SDS sample loading buffer. Vortex and/or incubate overnight at 4ºC would help to dissolve the pellet.
16. The sample is now ready for analysis by western blot. HRP-Streptavidin concentrate provided in this kit may be used for this application. The labeled sample can be stored at -20°C for future analysis.

Notes:

1. For western blot, sample loading amount should be determined empirically, recommend a serial dilution in an initial experiment.
2. For western blot, blocking reagent containing dry milk may cause high background signal.
3. The 1000X HRP-Streptavidin may be used at 1:1000 dilution. Develop the membrane(s) with ECL reagents or other peroxidase compatible substrate.

Assay Procedure Summary

1. Prepare reagents and samples.
2. Label samples with Oxidation Labeling Reagent.
3. Add Oxidation Stabilizing Reagent to samples.
4. Precipitate proteins with ice-cold 20% TCA.
5. Wash protein pellet twice with 1:1 (v/v) ethanol:ethyl acetate.
6. Resuspend protein pellets in Oxidation Buffer B.

Troubleshooting Guide

Problem	Possible Cause	Solution
Oxidation signal not detected	Labeling or stabilizing reagent were not added	Add labeling reagent followed by stabilizing reagent
	Incomplete removal of Labeling and stabilizing reagent	Add more washes after TCA precipitation
	Oxidation levels were too low	Add positive and negative control for further analysis
High background in Western Blot	Detection reagents were excessively used	Increase dilution factor of detection reagent
	Insufficient/incorrect membrane blocking or washing	Use 2% BSA in PBS as blocking buffer and wash more times

This product is for research use only.