RayBio® 4-Hydroxynonenal (4-HNE) Competitive ELISA kit

Catalog #: EIA-4-HNE

User Manual
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RayBiotech
Empowering your proteomics

ISO 13485 Certified

3607 Parkway Lane, Suite 100
Norcross, GA 30092
Tel: 770-729-2992, Fax:770-206-2393
Web: www.RayBiotech.com, Email: info@raybiotech.com

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Please read the entire manual carefully before starting your experiment

I. Introduction

4-Hydroxynonenal (4-HNE), a prominent lipid peroxidation product, is a highly reactive aldehyde generated during the oxidative degradation of polyunsaturated fatty acids in cell membranes. This molecule has garnered significant attention due to its profound influence on a wide range of biological and pathological processes. It exerts its effects by forming covalent adducts with proteins, nucleic acids, and lipids, thereby altering their structure and function.

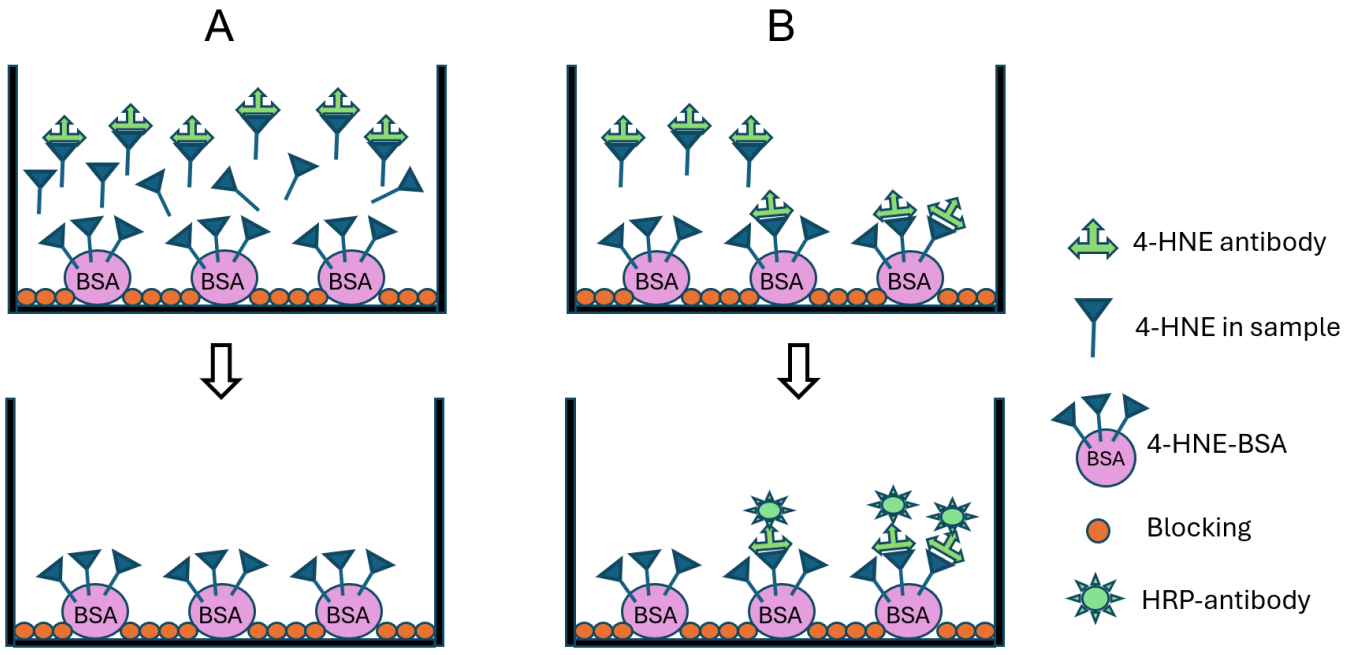
Extensive research demonstrates that 4-HNE is a critical mediator in the pathogenesis of various diseases, including cancer, neurodegenerative disorders, cardiovascular diseases, and metabolic syndromes. Moreover, 4-HNE plays a pivotal role in modulating key cellular signaling pathways, such as oxidative stress response, inflammation, and apoptosis. These interactions highlight its dual nature as both a biomarker and a modulator of disease development and progression.

The RayBio® 4-HNE ELISA Kit is a competitive enzyme immunoassay developed for rapid detection and quantitation of A-to-I in RNA samples collected from various species including humans, rats or mice. The quantity of 4-HNE is determined by a known 4-HNE standard curve. This kit has a high sensitivity of approximately 0.363 ng/mL. Each kit provides sufficient reagents to perform up to 96 assays, including standard curve and unknown samples.

II. General Description

The RayBio® 4-HNE Enzyme Immunoassay (EIA) Kit is an *in vitro* quantitative assay for detecting 4-HNE based on the competitive enzyme immunoassay principle. In this assay, the samples and standards are added to a microplate pre-coated with 4-HNE. In the plate wells, the endogenous 4-HNE or the standards compete with the pre-coated 4-HNE for binding to the anti-4-HNE antibody. After a wash step, any bound 4-HNE antibody then interacts with horseradish peroxidase (HRP)-secondary antibody, which catalyzes a color development reaction. The intensity of the colorimetric signal is directly proportional to the amount of bound 4-HNE antibody and inversely proportional to the amount of endogenous 4-HNE in the standard or samples. A standard curve of known concentration of 4-HNE can be established and the concentration of 4-HNE in the samples can be calculated accordingly.

III. How It Works



A. High level of endogenous 4-HNE in sample. Anti-4-HNE is bound by excess free 4-HNE and washed off the plate. **B.** Low to moderate level of endogenous 4-HNE RNA editing in sample. Anti-4-HNE binds to coated 4-HNE due to low competition and is then detected by HRP-conjugated secondary antibody.

IV. Storage

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

V. Reagents

Component	**Size / Description**	**Storage / Stability <u>After Preparation</u>**
4-HNE Microplate	96 wells (12 strips x 8 wells) coated with 4-HNE.	1 month at 4°C*
Assay Diluent B (5X)	15 mL concentrated buffer. Diluent for standards, samples, and antibodies.	1 month at 4°C
Standard 4-HNE	2 vials. 1 vial is enough to run each standard in triplicate.	1 week at 4°C
Positive Control	2 vials of Lyophilized Positive Control. 1 vial is enough to run in triplicate.	1 month at 4°C
Anti-4-HNE Antibody (1000X)	10 µL concentrated anti-4-HNE Antibody.	1 month at 20°C
HRP-conjugated secondary antibody (5000X)	5 µL concentrated antibody.	Do not store and reuse
Wash Buffer (20X)	25 mL of 20X concentrated solution.	1 month at 4°C
TMB One-Step Substrate Reagent	12 mL of 3,3,5,5'-tetramethylbenzidine (TMB) in buffer solution.	1 month at 4°C
Stop Solution	8 mL of 0.2 M sulfuric acid.	12 months at 4°C

*Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

VI. Additional Materials Required

1. Microplate reader capable of measuring absorbance at 450 nm
2. Precision pipettes to deliver 2 µL to 1 mL volumes
3. Adjustable 1-25 mL pipettes for reagent preparation
4. 100 mL and 1-liter graduated cylinders
5. Absorbent paper
6. Distilled or deionized water
7. GraphPad Prism or SigmaPlot software (or other software which can perform four-parameter logistic regression models)
8. Tubes to prepare standard or sample dilutions
9. Orbital shaker
10. Aluminum foil
11. Plastic wrap

VII. Reagent Preparation

Keep kit reagents on ice during reagent preparation steps.

A. Preparation of Plate and Anti-4-HNE Antibody

1. Equilibrate plate to room temperature before opening the sealed pouch.
2. Label removable 8-well strips as appropriate for your experiment.
3. 5X Assay Diluent B should be diluted 5-fold with deionized or distilled water.
4. Briefly centrifuge the anti- 4-HNE antibody vial and dilute 1000-fold with 1X Assay Diluent B for working solution.

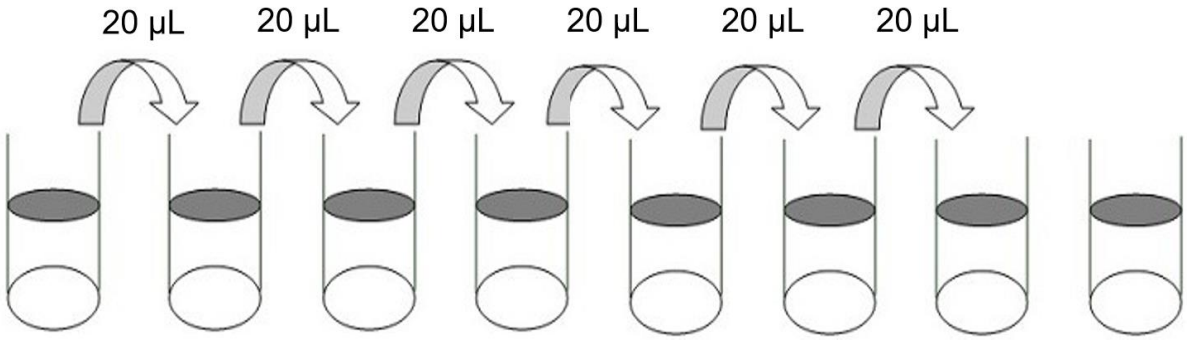
B. Preparation of Standards

5. Label 8 microtubes with the following concentrations: S1 (100,000 ng/mL), S2 (10,000 ng/mL), S3 (1,000 ng/mL), S4 (100 ng/mL), S5 (10 ng/mL), S6 (1 ng/mL), S7 (0.1 ng/mL) and S8 (0 ng/mL). Pipette 180 µL of 1X Assay Diluent B into S2-S8.
6. Briefly centrifuge the vial of 4-HNE Standard. Reconstitute with 200 µL of 1X Assay

Diluent B. Mix thoroughly. This solution serves as S1.

7. To make the S2 (10,000 ng/mL) standard, pipette 20 µL of the S1 standard into the tube labeled S2 containing 180 µL of 1X Assay Diluent B. Mix thoroughly.

8. Repeat this step with successive concentration, preparing a dilution series as shown in the illustration below. Each time, add 20 µL of the prior concentration until the S7 is reached. Mix each tube thoroughly before the next transfer. 1X Assay Diluent B serves as the blank (S8, 0 ng/mL).



	S1	S2	S3	S4	S5	S6	S7	S8
Diluent Volume	200 µL	180 µL	180 µL	180 µL	180 µL	180 µL	180 µL	180 µL
Conc.	100,000 ng/mL	10,000 ng/mL	1,000 ng/mL	100 ng/mL	10 ng/mL	1 ng/mL	0.1 ng/mL	0 ng/mL

C. Positive Control Preparation

9. Briefly centrifuge the Positive Control vial and reconstitute with 200 µL of 1X Assay Diluent B.

Positive Control is a mouse serum sample that serves to verify that the kit components are functioning. The resulting OD is not used to calculate final concentrations of samples. If no positive competition is observed please contact RayBiotech Technical Support. The Positive Control may be diluted further if desired.

D. Sample Preparation

10. Serum and plasma samples from humans, mice and rat can be detected. Use immediately or aliquot and store at -20ºC until use. Avoid repeated freeze-thaws. Dilute your sample with the 1X Assay Diluent B at 2-4-fold.

I. Urine: Urine should be aseptically collected from the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge samples at 5000 g for 10 minutes, or filter through 0.45 µm filter, prior to use in the assay.

II. Serum: Avoid hemolyzed and lipemic blood samples. Collect blood in a tube with no anticoagulant. Allow the blood to clot at room temperature for 30 minutes. Centrifuge at 5000 x g for 10 minutes at room temperature. Remove the yellow serum supernatant without disturbing the white buffy layer.

III. Plasma: Avoid hemolyzed and lipemic blood samples. Collect blood with EDTA, heparin or citrate and centrifuge at 5000 g for 10 minutes at room temperature. Remove the plasma layer and avoid disturbing the white buffy layer.

Recommended Dilution: 2-fold for serum and plasma.

If you have any questions regarding the recommended dilutions, you may contact technical support at 770-729-2992 or techsupport@raybiotech.com.

E. Preparation of Wash Buffer and HRP

11. If Wash Buffer contains visible crystals, warm them to room temperature and mix gently until dissolved.
12. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to yield 400 mL of 1X Wash Buffer.
13. Briefly centrifuge the HRP-Secondary Antibody vial before use. Dilute the HRP-Secondary Antibody concentrate 5000-fold with 1X Assay Diluent B.

VIII. Assay Procedure

1. Remove the appropriate number of microtiter wells of 4-HNE coated microplate from foil pouch and place them into the well holder. Return any unused wells to the foil pouch with desiccant pack, reseal along entire edge, and store at -20ºC.

2. Keep kit reagents on ice during reagent preparation steps. It is recommended that all standards and samples be run at least in duplicate.

3. Add 50 µL of each standard (S1-S8), Positive Control and sample to appropriate wells. Incubate at room temperature for 10 minutes on an orbital shaker.

4. Add 50 µL of the diluted Anti-4-HNE Antibody to each well. Cover wells and incubate at room temperature for 1 hour on an orbital shaker.

5. Discard the solution and wash wells 5 times with 1X Wash Solution Buffer (200-300 µL each). Washing may be done with a multichannel pipette or an automated plate washer. Complete removal of liquid at each step is essential to good assay performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 100 µL of prepared HRP-Secondary antibody solution (see Reagent Preparation Step 13) to each well. Incubate for 1 hour at room temperature with gentle shaking.

7. Discard the solution and wash 5 times as directed in Step 5.

8. Add 100 µL of TMB One-Step Substrate Reagent to each well. Incubate for 2-10 minutes at room temperature in the dark with gentle shaking (1-2 cycles/sec).

9. Add 50 µL of Stop Solution to each well. Read at 450 nm immediately. Results should be read immediately (color will fade over time).

Note: Reliable standard curves are obtained when OD values do not exceed 2.5 units for the blanks (S8).

IX. Assay Procedure Summary

1. Prepare all reagents, samples and standards as instructed.

2. Add 50 µL standards or samples to appropriate well. Incubate for 10 minutes at room temperature.

3. Add 50 µL anti-4-HNE to appropriate well. Incubate 1 hour at room temperature. And wash 5 times.

4. Add 100 µL prepared HRP-Secondary antibody solution. Incubate 1 hour at room temperature. And wash 5 times.

5. Add 100 µL TMB One-Step Substrate Reagent to each well. Incubate 2-10 minutes at room temperature.

6. Add 50 µL Stop Solution to each well. Read at 450 nm immediately.

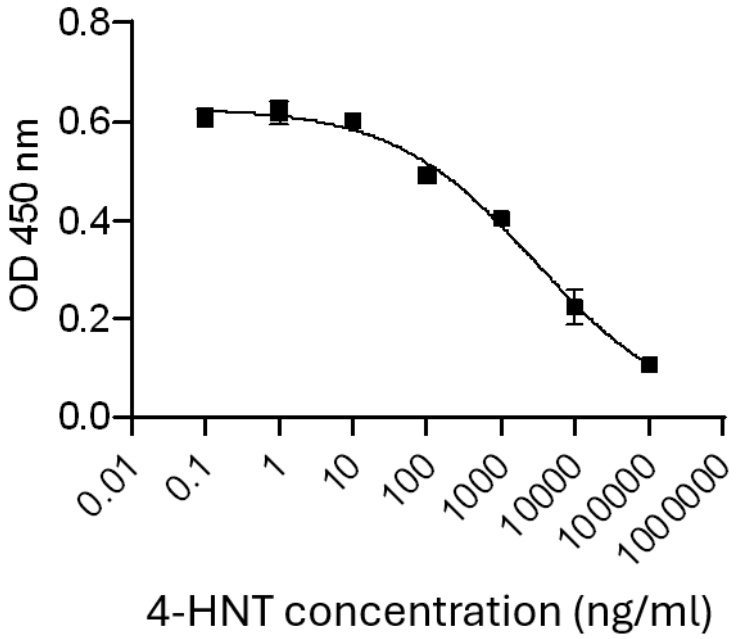
X. Calculation of Results

Calculate the mean absorbance for each set of duplicate stands, controls, and samples and subtract the blank optical density. Plot the standard curve using GraphPad Prism or Sigma Plot software (or other software which can perform four-parameter logistic regression models). If the samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

The measurement range is 0.363 – 10000 ng/mL. Any sample reading lower than the highest standard should be diluted with dilution buffer in higher dilution and re-assayed. Dilution factors need to be taken into consideration in calculating the 4-HNE concentration.

A. Typical Data

These standard curves are for demonstration only. A standard curve must be run with each assay.



B. Sensitivity

The limit of detection (LOT, Blank-3SD) is 0.363 ng/mL.

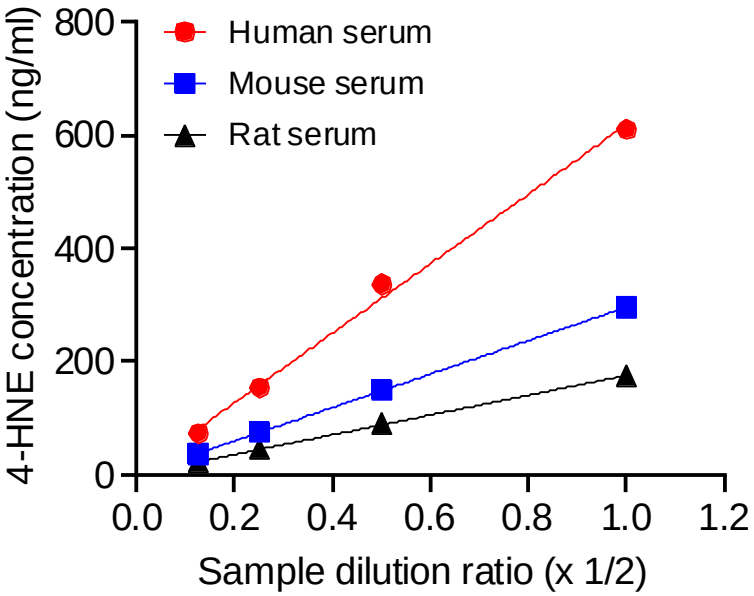
C. Precision

Intra-assay (Within-Run) CV% < 5%.

Inter-assay (Run-to-Run) CV% < 5%.

D. Linearity

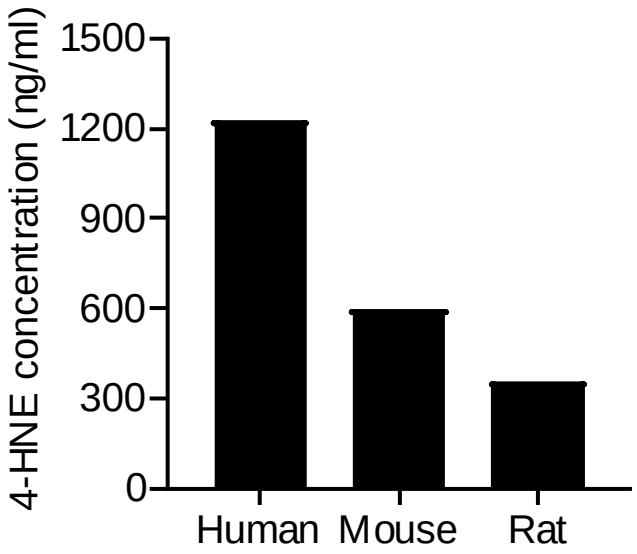
Human serum, plasma and urine samples were serially diluted from 2-16-fold (n=3). The recovery ranges from 97.98 to 110.36%.



Sample	Dilution	Observed (ng/mL)	Expected (ng/mL)	Recovery (%)
Human serum	1/2	612.626	612.626	
	1/4	338.061	306.313	110.365
	1/8	156.058	153.157	101.895
	1/16	75.034	76.578	97.984
Mouse serum	1/2	297.854	297.854	
	1/4	152.793	148.927	102.596
	1/8	76.314	74.463	102.485
	1/16	38.910	37.232	104.508
Rat serum	1/2	176.779	176.779	
	1/4	93.027	88.390	105.246
	1/8	46.664	44.195	105.588
	1/16	23.018	22.097	104.165

E. Example of Test Results

The concentration of 4-HNE in serum from human, mouse and rat (n=3/group).



XIV. Troubleshooting Guide

Problem	Cause	Solution
Poor standard curve	○ Inaccurate pipetting ○ Improper standard dilution	○ Check pipettes. ○ Briefly centrifuge and dissolve the powder thoroughly by gently mixing
Low signal	○ Improper preparation of standard and/or biotinylated antibody ○ Too brief incubation times Inadequate reagent volumes or improper dilution	○ Briefly spin down vials before opening. Dissolve the powder thoroughly. ○ Ensure sufficient incubation time; assay procedure step 2 may be done overnight. ○ Check pipettes and ensure correct preparation.
Large CV	○ Inaccurate pipetting Air bubbles in wells	○ Check pipettes. ○ Remove bubbles in wells
High background	○ Plate is insufficiently washed. ○ Contaminated wash buffer	○ Review the manual for proper wash. If using a plate washer, ensure that all ports are unobstructed. ○ Make fresh wash buffer
Low sensitivity	○ Improper storage of the ELISA kit ○ Stop solution	○ Follow storage recommendations in sections IV and V. Keep substrate solution protected from light. ○ Add stop solution to each well before reading plate

RayBio® ELISA Kits

Over 4,500 ELISA kits available, visit www.RayBiotech.com/ELISA-Kits for details.

This product is for research use only.