

## General 4-Hydroxynonenal (4-HNE) ELISA Kit

Cat #: orb2809928 (manual)

Size: 96 tests / 48 tests

*For research use only, not for clinical diagnosis.*

### 4-HNE Introduction

4-Hydroxynonenal (4-HNE), also known as 4-hydroxy-2-nonenal, is an  $\alpha,\beta$ -unsaturated hydroxyalkenal produced by lipid peroxidation of cell membranes. As a major product of this process, it is a colorless oil and is widely present in animal tissues. Under oxidative stress conditions, the lipid peroxidation chain reaction is enhanced, leading to a significant increase in 4-HNE levels. It is currently believed that 4-HNE plays a key role in cell signal transduction and is widely involved in various biological pathways ranging from cell cycle regulation to cell adhesion.

### Detection Principle

This kit uses the enzyme-linked immunosorbent assay (ELISA) competitive method. 4-Hydroxynonenal (4-HNE) antigen is pre-coated in the wells, and then samples, standards, biotin-labeled antibodies, and HRP enzyme conjugate are added sequentially. After incubation and washing, the substrate TMB is added for color development. TMB is converted to blue in the presence of horseradish peroxidase (HRP) and then converted to the final yellow color under acidic conditions. The intensity of the color is negatively correlated with the concentration of 4-Hydroxynonenal (4-HNE) in the sample. The absorbance (OD value) is measured at 450nm using an enzyme-labeled instrument to calculate the sample concentration.

Detection Range: 0.625-40ng/mL

Sensitivity: 0.319ng/mL

Specificity: detects 4-HNE in samples with no significant cross-reactivity with its analogues.

### Product Composition

Reagents	Specifications (96T)	Specifications (48T)	Storage Conditions
Pre-coated Assay Plate	8×12	8×6	2-8°C
Standard	2 tubes	1 tube	2-8°C
Universal Diluent	2×20 mL	1×20 mL	2-8°C
Biotin-antibody (100×)	60 $\mu$ l	30 $\mu$ l	2-8°C
Streptavidin-HRP (100×)	120 $\mu$ l	60 $\mu$ l	2-8°C
Wash Buffer (20×)	2×10 mL	1×10 mL	2-8°C

TMB Substrate	10 ml	5 ml	2-8°C
Stop Solution	6 ml	3 ml	2-8°C
Plate Sealer	4 pieces	4 pieces	
Manual	1 copy	1 copy	

### Required Instruments and Reagents

1. Microplate reader (wavelength: 450nm)
2. Precision pipette with disposable tips: 0.5-10uL, 5-50uL, 20-200uL, 200-1000uL
3. 37°C incubator
4. Deionized or distilled water

### Precautions

1. Incubation is carried out in strict accordance with the specified time and temperature to ensure accurate results. All reagents must reach room temperature 20-25°C before use. Refrigerate reagents immediately after use.
2. Incorrect plate washing may result in inaccurate results. Make sure to suction out as much liquid as possible from the wells before adding the substrate. Do not allow the micropores to dry out during the incubation process.
3. Eliminate residual liquid and finger marks from the bottom of the plate, otherwise the OD value will be affected.
4. The substrate chromogenic solution should be colorless or very light in color, and the substrate solution that has turned blue cannot be used.
5. Avoid cross-contamination of reagents and specimens to avoid false results.
6. Avoid direct exposure to strong light during storage and incubation.
7. No reaction reagent should come into contact with the bleaching solvent or the strong gases emitted by the bleaching solvent. Any bleaching component will destroy the biological activity of the reaction reagent in the kit.
8. Expired products cannot be used, and components with different article numbers and batch numbers should not be mixed.
9. Recombinant proteins from sources other than the kit may not match the antibody of this kit and may not be recognized.
10. If there is a possibility of disease transmission, all samples should be managed, and samples and testing devices should be handled in accordance with the prescribed procedures.

### Sample Collection and Requirements

1. **The detection range of the kit is not equivalent to the concentration range of the analyte in the sample**, and it is recommended to estimate the concentration of the analyte in the sample through relevant literature before the experiment and determine the actual concentration of the sample through the pre-

experiment Condition. If the concentration of analyte in the sample is too high or too low, dilute or concentrate the sample appropriately.

2. If the sample to be tested is not among the samples listed in the manual, it is recommended to do a pre-test to verify the validity of the test.

3. **Serum:** Whole blood specimens collected in serum separators are stored at room temperature for 2 hours or 4°C overnight, then centrifuge at 1000×g for 20 minutes, and the supernatant can be taken or stored at -20°C or -80°C, but repeated freeze-thaw should be avoided.

4. **Plasma:** Collect specimens with EDTA or heparin as anticoagulants and centrifuge the specimen at 1000×g at 2-8°C for 15 minutes within 30 minutes of collection, take the supernatant for detection, or store the supernatant at -20°C or -80°C, but avoid repeated freeze-thaw cycles.

5. **Tissue homogenate:** Rinse the tissue with pre-chilled PBS (0.01M, pH=7.4) to remove residual blood (lysed red blood cells in the homogenate can affect the measurement), and mince the tissue after weighing. The minced tissue is compared to the corresponding volume of PBS (generally according to the weight-to-volume ratio of 1:9, for example, 1g of tissue sample corresponds to 9mL of PBS, the specific volume can be adjusted appropriately according to the needs of the experiment, and a record should be made. It is recommended to add protease inhibitors to PBS) to a glass homogenizer and grind well on ice. To further lyse the histiocytes, the homogenate can be sonicated, or repeatedly freeze-thaw. Finally, the homogenate was centrifuged at 5000×g for 5~10 minutes, and the supernatant was taken for detection.

6. **Cell culture supernatant:** Centrifuge at 1000×g for 20 minutes to detect, or store the supernatant at -20°C or -80°C, but avoid repeated freeze-thaw cycles.

7. **Cell lysate:** For adherent cells, gently wash with pre-cooled PBS, then digest with trypsin, and collect the cells by centrifuging at 1000×g for 5 minutes; suspended cells can be collected directly by centrifugation. Wash the collected cells with pre-cooled PBS 3 times and resuspend 150-200µL PBS for every 1×10<sup>6</sup> cells (it is recommended to add a protease inhibitor in the PBS; if the content is very low, you can appropriately reduce the volume of PBS) and lyse the cells by repeated freeze-thaw cycles or sonication. Centrifuge the extracted solution at 1500×g for 10 minutes at 2-8°C and collect the supernatant for detection.

8. **Other biological specimens:** Centrifuge at 1000×g for 20 minutes, and take the supernatant for detection.

9. **Appearance of the sample:** The sample should be clear and transparent, and the suspended solids should be centrifuged and removed.

10. **Sample preservation:** If the sample is tested within 1 week after collection, it can be stored at 4°C, if it cannot be tested in time, please divide it into one-time use and freeze it at -20°C (test within 1 month), or -80°C (testing within 6 months), avoid repeated freezing and thawing, hemolysis of the specimen will affect the final test result, so hemolyzed specimens should not be tested for this test.

## Sample Dilution Protocol

**Please estimate the concentration range of the samples in advance. If your test sample needs to be diluted, refer to the dilution protocols below.**

**Dilution 100 times:** One-step dilution. Take 5µL of the sample and add it to 495µL of universal diluent to make a 100-fold dilution;

**Dilution 1000 times:** Two-step dilution. Take 5 $\mu$ L of the sample and add it to 95 $\mu$ L of universal diluent to make a 20-fold dilution, then take 5 $\mu$ L of the 20-fold diluted sample and add it to 245 $\mu$ L of universal diluent to make a 50-fold dilution, resulting in a total dilution of 1000 times;

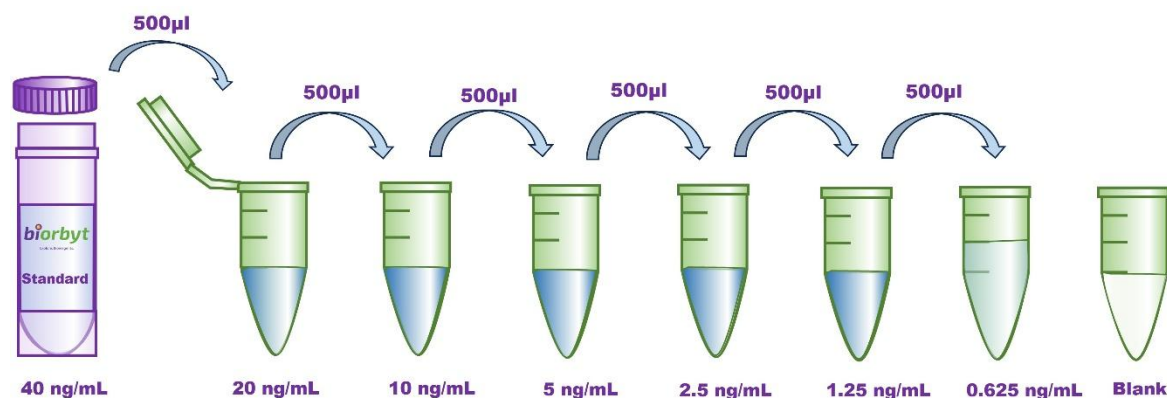
**Dilution 100000 times:** Three-step dilution. Take 5 $\mu$ L of the sample and add it to 195 $\mu$ L of universal diluent to make a 40-fold dilution, then take 5 $\mu$ L of the 40-fold diluted sample and add it to 245 $\mu$ L of universal diluent to make a 50-fold dilution, and finally take 5 $\mu$ L of the 2000-fold diluted sample and add it to 245 $\mu$ L of universal diluent to make a 50-fold dilution, resulting in a total dilution of 100000 times;

For each dilution step, the volume taken should be no less than 3 $\mu$ L, and the dilution factor should not exceed 100 times. Each dilution step must be mixed thoroughly to avoid foaming.

## Reagent Preparation

1. Remove the kit from the refrigerator 10 minutes in advance and equilibrate to room temperature.
2. **Standard gradient working solution:** Add 1mL of universal dilution to the lyophilized standard, let it stand for 15 minutes, let it completely dissolve, and then mix gently (at a concentration of 40 ng/mL), then dilute at the following concentrations: 40 ng/mL, 20 ng/mL, 10 ng/mL, 5 ng/mL, 2.5 ng/mL, 1.25 ng/mL, 0.625 ng/mL, 0 ng/mL.

Doubling dilution method: take 7 EP tubes, add 500 $\mu$ L of general dilution solution to each tube, and draw 500 $\mu$ L from 40ng/mL standard working solution to the first EP tube to mix well to prepare 20ng/mL standard working solution, and then pipette and mix well according to this step. The last tube is used directly as a blank well, and there is no need to draw liquid from the penultimate tube, as shown in the figure below.



3. **Preparation of biotinylated antibody detection solution:** 15 minutes before use, centrifuge the concentrated biotinylated antibody at 1000 $\times$ g for 1 minute, and dilute the 100 $\times$  concentrated biotinylated antibody into a 1 $\times$  working concentration (10  $\mu$ L concentrated + 990  $\mu$ L universal diluent) in a universal diluent, and use it on the same day.
4. **Preparation of enzyme conjugate working solution:** Centrifuge 100 $\times$  concentrated streptavidin-HRP at 1000 $\times$ g for 1 minute before use, and dilute 100 $\times$  concentrated streptavidin-HRP to 1 $\times$  working concentration in a universal diluent (10  $\mu$ L concentrated + 990  $\mu$ L universal diluent) for use on the same day.
5. **Preparation of 1 $\times$  washing solution:** take 10ml of 20 $\times$  washing solution to 190ml of distilled water (the concentrated washing solution taken out of the refrigerator may have crystallization, which is a normal phenomenon, it can be placed at room temperature, shake evenly, and then configure after the crystallization is completely dissolved).

## Operation Steps

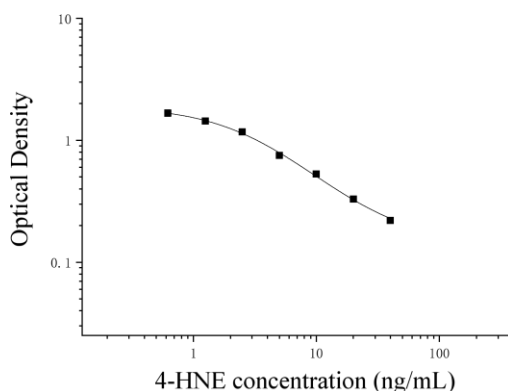
1. Remove the desired slats from the foil pouch after 10 minutes of room temperature equilibration, and place the remaining slats back at 4°C with a ziplock bag.
2. Loading: Add 50 µl of samples or standards of different concentrations to the corresponding wells, and add 50 µL of universal diluent to the blank wells, immediately add 50µL of Biotin-antibody working solution to each well, incubate for 1 h at 37°C after covering with the plate sealer. (Suggestion: Samples to be tested are diluted by a minimum of 1-fold with a universal diluent before being added to the microplate for testing to reduce the impact of matrix effects on the test results, and the sample concentration should be multiplied by the corresponding dilution factor when calculating the sample concentration. All samples and standards to be tested are recommended to be double-well).
3. Wash the plate: Discard the liquid, add 300µL of 1x wash solution to each well, let it stand for 1 minute, shake off the wash solution, pat dry on absorbent paper, and repeat the wash 3 times (you can also use a plate washer to wash the plate).
4. Add streptavidin-HRP working solution: Add 100µL of streptavidin-HRP working solution to each well, cover the plate and incubate at 37°C for 30 minutes.
5. Wash the plate: Discard the liquid and wash the plate 5 times according to the washing method in step 3.
6. Add TMB Substrate: 90 µL of TMB Substrate was added to each well, covered with a plate sealer, and incubated at 37°C for 15 minutes in the dark.
7. Add stop solution: Remove the microplate, add 50 µL of stop solution directly to each well, and immediately measure the OD value of each well at a wavelength of 450 nm.

## Result Judgment

1. Calculate the average OD value of the standard and sample replicate wells and subtract the OD value of the blank well as the correction value. Using the concentration as the abscissa and the OD value as the ordinate, the standard curve of the four-parameter logistic function is drawn on a double-logarithmic coordinate paper (the value of the blank group is removed when plotting).
2. If the OD value of the sample is higher than the upper limit of the standard curve, it should be diluted appropriately and remeasured and multiplied by the corresponding dilution factor when calculating the sample concentration.

The following data and standard curves are for reference only, and experimenters need to establish standard curves according to their own experiments.

<b>Concentration (ng/mL)</b>	40	20	10	5	2.5	1.25	0.625	0
<b>OD value</b>	0.22	0.33	0.53	0.75	1.17	1.44	1.67	2.21



Note: This figure is for reference only, and the specimen content should be calculated using the standard curve drawn for the same test standard.

### Kit Performance

1. Repeatability: The coefficient of variation within the plate is less than 10%, and the coefficient of variation between plates is less than 10%.
2. Recovery: Recovery was calculated by adding 3 different concentration levels of 4-HNE to selected healthy serum and plasma.

Sample type	Range	Average recovery
Serum (n=8)	87-108	96
Plasma (n=8)	91-111	101

3. Linear dilution: Linearity was assessed by adding high concentrations of 4-HNE to 4 selected healthy serum, plasma, and cell culture supernatants, respectively, and diluting within the standard curve kinetics.

Dilution range	Recovery (%)	Serum	Plasma
1: 2	Range (%)	87-113	86-106
	Average recovery (%)	101	98
1: 4	Range (%)	88-112	83-114
	Average recovery (%)	100	104

### Troubleshooting

#### High background/non-specific staining

Description of results	Possible reason	Recommendations and precautions

<p>After stopping the reaction, the entire plate shows a uniform yellow or light color, or the standard curve is linear but the background signal is excessively high.</p>	<p>Yellowing of the entire plate may be caused by incorrect addition of reagents.</p>	<p>Before starting the experiment, verify the components and lot numbers of all reagents to ensure they belong to the same kit. Reagents from different kits or different lot numbers must not be mixed.</p>
	<p>The ELISA plate was not washed sufficiently.</p>	<p>Ensure that the same volume of Wash Solution is added to each microwell during the washing process. After washing, firmly tap the ELISA plate on absorbent paper to remove any residual buffer.</p>
	<p>Incubation time was too long.</p>	<p>Strictly follow the procedures outlined in the manual.</p>
	<p>Streptavidin-HRP contaminated the pipette tip or TMB container, or the positive control contaminated the pre-coated microplate.</p>	<p>Replace pipette tips when dispensing different reagents. Use separate reagent containers when preparing different reagent components, and always use a pipette during handling.</p>
	<p>The concentration of Biotinylated Antibody or Streptavidin-HRP was too high.</p>	<p>Verify that concentration calculations are correct, or perform further dilution if necessary.</p>
	<p>The substrate was exposed to light or contaminated prior to use.</p>	<p>Store reagents in the dark at all times prior to substrate addition.</p>
	<p>Color development time was too long.</p>	<p>Strictly follow the procedures outlined in the manual.</p>
	<p>An incorrect filter was used when reading the absorbance value.</p>	<p>When TMB is used as the substrate, measure absorbance at 450 nm.</p>

**NO color plates**

Description of results	Possible reason	Recommendations and precautions
<p>After the color development step, all wells of the ELISA plate are colorless, and the positive control is not clearly detectable. s</p>	<p>Components from different reagent sets were mixed.</p>	<p>Carefully read reagent labels when preparing or using them.</p>
	<p>During plate washing or sample/enzyme addition, the enzyme label was contaminated or inactivated, resulting in loss of its ability to catalyze color development.</p>	<p>Confirm that the container holding the ELISA plate does not contain enzyme inhibitors (such as NaN<sub>3</sub>), and ensure that the container used to prepare the Wash Solution has been thoroughly cleaned.</p>
	<p>A reagent or procedural step was omitted.</p>	<p>Review the manual carefully and strictly follow the operating procedures.</p>

**Light color**

Description of results	Possible reason	Recommendations and precautions
The Standard appears normal, but the sample color is weak.	The sample contains $\text{NaN}_3$ preservative, which inhibits the enzyme reaction.	Samples must not contain $\text{NaN}_3$ .
	The sample being tested may not contain strongly positive material, so the result may be normal.	If there is any doubt about the results, repeat the assay.
The visual result appears normal, but the microplate reader values are low.	An incorrect filter was used for absorbance measurement.	When TMB is used as the substrate, absorbance should be measured at 450 nm.
All wells, including Standards and Samples, show weak color development.	Insufficient incubation time.	Ensure accurate timing using a timer.
	Inadequate color development.	Typically 15–30 minutes.
	Excessive washing, or the dilution ratio of the concentrated Wash Buffer does not meet requirements.	Minimize the impact of washing by diluting the concentrated Wash Buffer and setting the washing time according to the manual. Accurately record the number of washes and the volume used.
	Poor quality distilled water.	The prepared Wash Buffer should be tested to confirm that the pH is neutral.
	During plate washing or sample addition, the enzyme label was contaminated or inactivated, resulting in loss of catalytic activity.	Confirm that the container holding the ELISA plate does not contain enzyme inhibitors (such as $\text{NaN}_3$ ), that the container used to prepare the Wash Buffer has been thoroughly cleaned, and that the purified water used meets the required standards and is not contaminated.
	The kit has expired or was improperly stored.	Use the kit within its expiration date and store it according to the conditions recommended in the manual to avoid contamination.
	Reagents and samples were not equilibrated to room temperature before use.	Allow all reagents and samples to equilibrate at room temperature for approximately 30 minutes before use.
	Insufficient pipetting volume, overly rapid dispensing, excessive liquid remaining on the inner wall of the tip, or unclean tip walls.	Calibrate the pipette properly. Ensure tips are compatible and fit securely, pipette at an appropriate speed, and fully dispense the liquid. Tips should have clean inner walls and be used only once
Poor repeatability.	Incubation temperature was not properly controlled.	Maintain a constant incubation temperature and avoid localized temperature extremes.
	Excess liquid remained on the inner wall of the wells during	When adding liquids, dispense along the lower inner wall of the wells without

	liquid addition.	touching the bottom.
	Reuse of consumables.	Replace pipette tips when drawing different reagents, and use separate containers when preparing different reagent components.
	The bottom of the microwell is scratched or contaminated.	Handle the plate carefully. Avoid touching the well bottoms and clean the bottom of the microplate to remove dirt or fingerprints.
		Perform technical replicates of the same sample three times, ensuring that at least two values are comparable.
	Cross-contamination during sample addition.	Minimize the risk of cross-contamination during sample addition.
The color development across the plate is uneven and irregular.	Cross-contamination during manual plate washing.	When washing plates manually, discard the first three washes immediately, then allow soaking during subsequent washes to reduce cross-contamination.
	Cross-contamination during plate tapping.	Use appropriate absorbent paper when tapping the plate. Avoid introducing foreign material into the wells, and avoid tapping in the same position repeatedly to reduce cross-contamination.

Description of results	Possible reason	Recommendations and precautions
The color development of the plate is uneven and irregular	The liquid dispensing head of the plate washer is clogged, leading to improper liquid dispensing or excessive residual liquid after aspiration, which results in uneven and irregular color development across the plate.	Unclog the liquid dispensing head to ensure that each well is properly filled with wash solution during plate washing and that minimal residual liquid remains after aspiration.
	Incomplete centrifugation of the sample, resulting in coagulation within the reaction wells or interference from sediment or residual cellular components.	Serum and plasma samples should be fully centrifuged at 3000 rpm for more than 6 minutes.
	The sample was stored for an excessively long period, leading to contamination.	Samples should be kept fresh or stored at low temperatures to prevent contamination.
	Incorrect preparation of the Wash Solution or direct use of the concentrated Wash Solution.	Prepare all reagents strictly according to the manual.