Enzyme Immunoassay for
9(±)-Hydroxyoctadecadienoic Acid

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INTRODUCTION

Linoleic acid, the predominant polyunsaturated fatty acid (PUFA) in the human diet, can be metabolized by cyclooxygenase, lipoxygenase and P450 enzymes. The hydroxyoctadecadienoic acid (HODE) derivatives of linoleic acid, 9(R)-HODE, 9(S)-HODE and 13(S)-HODE, are the most widely distributed of the known linoleic acid metabolites. These compounds exhibit interesting biologic activities, including the regulation of platelet function, maintenance of vascular thromboresistance and transduction of the cellular responses to certain growth factors. HODE derivatives may also influence certain pathological states including psoriasis, the development of atherosclerosis and the development of cancer. This assay measures the level of total 9-HODE, which includes both 9(S)-HODE and 9(R)-HODE, in biological samples.

PRINCIPLES OF PROCEDURE

This kit is a competitive enzyme-linked immunoassay (ELISA). Briefly, the 9-HODE present in the samples or standards competes with 9(±)-HODE conjugated to horseradish peroxidase [9(±)-HODE-HRP] for binding to an antibody specific for 9(±)-HODE that is precoated on a microplate. The peroxidase activity of 9(±)-HODE-HRP results in color development when a substrate is added. The intensity of the color is proportional to the amount of 9(±)-HODE-HRP bound and is inversely proportional to the amount of unconjugated 9-HODE present in the samples or standards.

MATERIALS PROVIDED

Component	Description	Volume	Storage	Cat No
Coated Plate	96-well microplate coated with anti-9(±)-HODE antibody.	1 plate	4°C	EA80a
9(±)-HODE Standard	0.05 mg/mL 9(±)-HODE standard solution in ethanol.	20 µL	4°C	EA80b
5x Wash Buffer	Buffer used to wash the plate.	50 mL	4°C	EA80c
5x Dilution Buffer	Buffer used for diluting kit components and samples.	25 mL	4°C	EA80d
TMB Substrate	TMB substrate used for color development.	25 mL	4°C	EA80e
9(±)-HODE-HRP Conjugate	9(±)-HODE horseradish peroxidase concentrated conjugate.	320 µL	4°C	EA80f

MATERIALS NEEDED BUT NOT PROVIDED

1. Microplate reader with a 450 nm filter
2. Adjustable micropipettes (10 – 1000 µL) and tips
3. Deionized water
4. 3 N Sulfuric Acid (H_2SO_4)

EXTRACTION MATERIALS

1. Chloroform ($CHCl_3$)
2. Butylated Hydroxytoluene (BHT)
3. Triphenyl Phosphine (TPP)

4. Magnesium Chloride ($MgCl_2$)
5. Potassium Hydroxide (KOH)
6. pH 3 Water
7. Methanol (MeOH)
8. Sodium Chloride (NaCl)
9. Ethyl Acetate
10. HCl (concentrated and 1 N)
11. Nitrogen Gas (N_2)

STORAGE

1. Store the components of this kit at the temperatures specified on the labels.
2. Unopened reagents are stable until the indicated kit expiration date.
3. Desiccant bag must remain in foil pouch with unused strips. Keep pouch sealed when not in use to maintain a dry environment. Remove excess air before sealing.

WARNINGS AND PRECAUTIONS

1. Use aseptic technique when opening and dispensing reagents.
2. This kit is designed to work properly as provided and instructed. Additions, deletions or substitutions to the procedure or reagents are not recommended, as they may be detrimental to the assay.

PROCEDURAL NOTES

1. 9(±)-HODE has been reported to bind to glassware. The use of plasticware (polypropylene) or silanated glassware is therefore recommended for all procedures involving the standards, enzyme conjugate, or samples containing 9-HODE.
2. To minimize errors in absorbance measurements due to handling, wipe the exterior bottom of the microplate wells with a lint-free paper towel prior to inserting into the plate reader.

SAMPLE PREPARATION

1. Urine can be assayed after diluting with Dilution Buffer.
2. Plasma and most other mediums will need to be extracted.

EXTRACTION REAGENTS NEEDED

1. **Folch Solution + 0.005% BHT (w/v) + 0.05% TPP (w/v):** 2:1 $CHCl_3$:MeOH
2. **Folch Solution + 0.005% BHT (w/v):** 2:1 $CHCl_3$:MeOH
3. **0.43% $MgCl_2$:** 0.43% $MgCl_2$ (w/v) in deionized water
4. **MeOH + 0.005% BHT (w/v)**
5. **15% KOH:** 15% KOH (w/v) in deionized water
6. **pH 3 Water:** deionized water brought to a pH of 3 with HCl
7. **0.9% NaCl:** 0.9% NaCl (w/v) in deionized water

EXTRACTION PROTOCOL

Note: This is a suggested protocol. Varying compositions of biological fluids may alter extraction efficiency. It is therefore important to measure the 9(±)-HODE concentration of a parallel "spiked" sample (i.e. a biological sample to which a known amount of 9(±)-HODE is added prior to extraction) in order to determine extraction efficiency.

Freeing Esterified 9-HODE

From Plasma or Other Fluids:

1. Add 20 mL of Folch Solution + 0.005% BHT + 0.05% TPP to a 50 mL conical tube and place on ice.
2. Add 1 mL of plasma or other fluid.
3. Shake or vortex well for 1 minute.
4. Add 10 mL of ice cold 0.43% $MgCl_2$ and shake or vortex well for 1 minute.
5. Centrifuge for 3 minutes at 2500 x *g* and 4°C.
6. Discard the top layer and transfer the bottom organic layer to a new 50 mL tube, being careful not to transfer any protein layer that may be present.
7. Evaporate the organic layer under N_2.
8. Add 0.5-2 mL of MeOH + 0.005% BHT (depending on the amount of lipid present) and an equal volume of 15% KOH, swirling after each addition.
9. Incubate the sample at 37°C for 30 minutes.
10. Adjust to pH 3 with 1 N HCl using approximately 2.5 times the volume of 15% KOH that was added.
11. Dilute with pH 3 Water so that the volume of MeOH added is ≤5% of the total volume. The sample is now ready for liquid phase extraction as described below.

From Tissue Samples:

1. Add 20 mL of Folch Solution + 0.005% BHT to a 40 mL flat bottom tube and place on ice.
2. Weigh 0.5 to 1 gram of tissue and add to tube on ice.
3. Shake or vortex well for 1 minute.
4. Homogenize with a blade homogenizer or sonicator for 30 seconds.
5. Allow to stand under N_2 in a sealed tube for one hour at room temperature, vortexing occasionally.
6. Add 4 mL of 0.9% NaCl.
7. Vortex vigorously and centrifuge for 3 minutes at 2500 x *g* and 4°C.
8. Discard the upper layer and transfer the lower phase to a new 50 mL conical tube, carefully avoiding the protein layer.
9. Evaporate under N_2.
10. Add 2-4 mL of MeOH + 0.005% BHT and an equal volume of 15% KOH, swirling after each addition.
11. Incubate at 37°C for 30 minutes.
12. Adjust to pH 3 with 1 N HCl using approximately 2.5 times the volume of 15% KOH that was added.
13. Dilute to 40–80 mL with pH 3 Water so the MeOH is ≤5% of the total volume. The sample is now ready for liquid phase extraction as described below.

Extraction from Plasma, Serum or Tissue Culture Medium

1. Acidify to pH 3 with concentrated HCl.
2. Extract with 3x the sample volume of water saturated Ethyl Acetate. Centrifuge at a low speed or allow to stand until phases separate.
3. Remove the organic (upper) phase and transfer it to a new container, being careful not to contaminate it with the aqueous phase.
4. Repeat steps 2 and 3, combining the organic phases with that from the first extraction.
5. Evaporate completely under N_2 or in a centrifugal evaporator.
6. Bring samples up in 25 µL Methanol, then add 975 µL Dilution Buffer. (If solubility is a problem, it may be necessary to increase the pH.)

NOTE: This ELISA assay is sensitive to differences in pH among samples and/or standards. Hence, it is critical to ensure that all samples and standards are adjusted to the same pH prior to running the assay.

REAGENT PREPARATION

1. **5x Dilution Buffer:** Add 25 mL of 5x Dilution Buffer to 100 mL of deionized water.
2. **5x Wash Buffer:** Add 50 mL of 5x Wash Buffer to 200 mL of deionized water.
3. **9(±)-HODE-HRP Conjugate:** Add 300 μL of Conjugate to 11.70 mL Dilution Buffer.

STANDARD CURVE PREPARATION

The 9(±)-HODE Standard is provided as a 0.05 mg/mL stock solution in ethanol. Make a 1000 ng/mL working standard stock solution by adding 980 μL of Dilution Buffer directly to the vial containing 20 μL of 9(±)-HODE Standard. Use the table on the following page to construct an eight-point standard curve.

Table 2: Standard Curve Preparation

Standard	9(±)-HODE Conc. (ng/mL)	Vol. of Dilution Buffer (μL)	Transfer Vol. (μL)	Transfer Source	Final Vol. (μL)
S_7	500	400	400	Stock	600
S_6	100	800	200	S_7	900
S_5	10	900	100	S_6	600
S_4	5	400	400	S_5	600
S_3	1	800	200	S_4	600
S_2	0.5	400	400	S_3	600
S_1	0.1	800	200	S_2	1000
B_0	0	1000	-	-	600

ASSAY PROCEDURE

1. Add 100 μL of Standards or Samples to the corresponding wells on the microplate in duplicate. See **Scheme I** for a sample plate layout.
2. Add 100 μL of diluted 9(±)-HODE-HRP Conjugate to each well. Incubate at room temperature for two hours.
3. Wash the plate three times with 300 μL of diluted Wash Buffer per well.
4. Add 200 μL of TMB Substrate to each well. Incubate at room temperature for 45-60 minutes.
5. Add 50 μL of 3 N H_2SO_4 to each well to stop the reaction.
6. Read the plate at 450 nm.
   NOTE: If accounting for substrate background, use 2 wells as blanks (BLK) with only 150 μL TMB Substrate in the wells. Subtract the average of these absorbance values from the absorbance values of the wells being assayed.

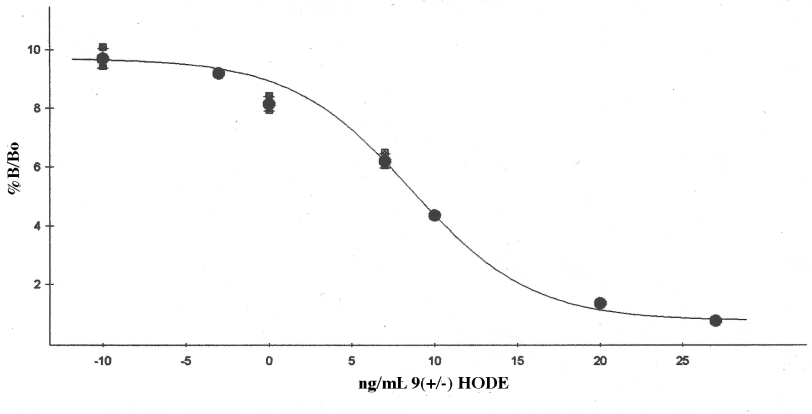
Scheme I: Sample Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	B_0	B_0	U_1	U_1	U_9	U_9	U_{17}	U_{17}	U_{25}	U_{25}	U_{33}	U_{33}
B	S_1	S_1	U_2	U_2	U_{10}	U_{10}	U_{18}	U_{18}	U_{26}	U_{26}	U_{34}	U_{34}
C	S_2	S_2	U_3	U_3	U_{11}	U_{11}	U_{19}	U_{19}	U_{27}	U_{27}	U_{35}	U_{35}
D	S_3	S_3	U_4	U_4	U_{12}	U_{12}	U_{20}	U_{20}	U_{28}	U_{28}	U_{36}	U_{36}
E	S_4	S_4	U_5	U_5	U_{13}	U_{13}	U_{21}	U_{21}	U_{29}	U_{29}	U_{37}	U_{37}
F	S_5	S_5	U_6	U_6	U_{14}	U_{14}	U_{22}	U_{22}	U_{30}	U_{30}	U_{38}	U_{38}
G	S_6	S_6	U_7	U_7	U_{15}	U_{15}	U_{23}	U_{23}	U_{31}	U_{31}	U_{39}	U_{39}
H	S_7	S_7	U_8	U_8	U_{16}	U_{16}	U_{24}	U_{24}	U_{32}	U_{32}	BLK	BLK

CALCULATIONS

1. Average the blank absorbance values and subtract the average from each well.
2. Average standard replicates (S_1 through S_7) and divide by the average of the B_0 values and multiply by 100 to obtain the $\%B_0$ value.
3. Graph the $\%B_0$ on the y-axis (linear) vs. the standard concentration on the x-axis (logarithmic) to obtain a standard curve (see **Figure 1** for a typical standard curve).
4. Average the replicates of each unknown and divide by the average B_0 values and multiply by 100 to obtain the $\%B/B_0$, then determine the corresponding concentration using the standard curve.

Figure 1: Typical Standard Curve



CROSS REACTIVITY

9(±)-HODE	100.0%	9-oxo-Octadecadienoic Acid	1.2%
9(S)-HODE	100.0%	13-oxo-Octadecadienoic Acid	2.4%
9(R)-HODE	100.0%	11(S)-HETE	0.0%
13(S)-Hydroxyoctadecadienoic Acid	1.2%	15(S)-HETE	0.0%
13(R)-Hydroxyoctadecadienoic Acid	1.2%	Linoleic Acid	0.0%

REFERENCES

1. Spindler, S.A., Clark, K.S., Callewaert, D.M., Reddy, R.G.; (1996) *Biochem. Biophys. Res. Comm.* **218**:187-191

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