ELISA PRODUCT INFORMATION & MANUAL

13-HODE

NBP2-61283

Enzyme-linked Immunosorbent Assay for quantitative detection of 13-HODE.
For research use only. Not for diagnostic or therapeutic procedures.

www.novusbio.com - P: 303.730.1950 - P: 888.506.6887 - F: 303.730.1966 - technical@novusbio.com

13(S)-HODE ELISA kit

96 Well Kit

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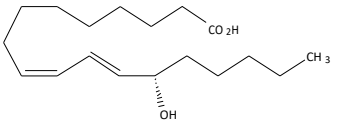
Description

The 13-(S)-HODE ELISA kit is a competitive immunoassay for the quantitative determination of free 13(S)-HODE in biological fluids. Please read the complete kit insert before performing this assay. The kit uses a polyclonal antibody to 13(S)-HODE to bind, in a competitive manner, the 13(S)-HODE in the sample or an alkaline phosphatase molecule which has 13(S)-HODE covalently attached to it. After a simultaneous incubation at room temperature the excess reagents are washed away and substrate is added. After another incubation, the enzyme reaction is stopped and the yellow color generated read on a microplate reader at 405 nm. The intensity of the bound yellow color is inversely proportional to the concentration of 13(S)-HODE in either standards or samples. The measured optical density is used to calculate the concentration of 13(S)-HODE. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

Introduction

The major human dietary poly-unsaturated fatty acid is linoleic acid, an essential fatty acid that can be metabolized to either 13-hydroxyoctadecadienoic acid via the lipoxygenase [13(S)-HODE] or P450 [13(R)-HODE] pathways or to 15-hydroxyeicosatetraenoic acid [15(S)-HETE] via the cyclo-oxygenase pathway[3,4]. Changes in the intra-cellular ratio of 13(S)-HODE to 15(S)-HETE have been linked to a number of physiological conditions dominated by cancer and cardiovascular diseases[5–7]. 13(S)-HODE helps to mediate cellular adhesion properties and excessive proliferation that is critical in the formation of tumors, metastasies and artherogenic plaques[5,9]. Continuously synthesized in non-stimulated vessel epithelia, 13(S)-HODE remains associated with the vitronectin receptor, an ubiquitous integrin adhesion molecule[8]. Cell surfaces become adhesive when HODE concentrations decline, allowing the vitronectin receptor to relocate to the cell surface.

13(S)-HODE



Precautions

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1. Some kit components contain azide, which may react with lead or copper plumbing. When disposing of reagents always flush with large volumes of water to prevent azide build-up.
2. Stop Solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in use.
3. The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} ions. The activity of the conjugate is affected by concentrations of chelators (>10 mM) such as EDTA and EGTA.
4. We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results.
5. The 13(S)-HODE Standard provided, Catalog No. 80-0996, is supplied in ethanolic buffer at a pH optimized to maintain 13(S)-HODE integrity. Care should be taken handling this material because of the known and unknown effects of eicosanoids.

Materials Supplied

1. **Goat anti-Rabbit IgG Microtiter Plate, One Plate of 96 Wells.**
   A plate using break-apart strips coated with goat antibody specific to rabbit IgG.
2. **13(S)-HODE ELISA Conjugate, 5 mL.**
   A blue solution of alkaline phosphatase conjugated with 13(S)-HODE.
3. **13(S)-HODE ELISA Antibody, 5 mL.**
   A yellow solution of a rabbit polyclonal antibody to 13(S)-HODE.
4. **Assay Buffer, 27 mL.**
   Tris buffered saline containing proteins and sodium azide as preservative.
5. **Wash Buffer Concentrate, 27 mL.**
   Tris buffered saline containing detergents.
6. **13(S)-HODE Standard, 0.5 mL.**
   A solution of 10,000 ng/mL 13(S)-HODE.
7. **pNpp Substrate, 20 mL.**
   A solution of p-nitrophenyl phosphate in buffer.
8. **Stop Solution, 5 mL.**
   A solution of trisodium phosphate in water. Keep tightly capped. Caution: **Caustic.**
9. **13(S)-HODE Assay Layout Sheet, 1 each.**
10. **Plate Sealers, 2 each.**

Storage

All components of this kit, **except the conjugate**, are stable at 4 °C until the kit's expiration date. The conjugate **must** be stored frozen at -20 °C.

Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Precision pipets for volumes between 5 µL and 1,000 µL.
3. Repeater pipets for dispensing 50 µL and 200 µL.
4. Disposable beakers for diluting buffer concentrates.
5. Graduated cylinders.
6. A microplate shaker.
7. Adsorbent paper for blotting.
8. A 37 °C Incubator.
9. Microplate reader capable of reading at 405 nm, preferably with correction between 570 and 590 nm.

Sample Handling

The ELISA Kit is compatible with extracted 13(S)-HODE samples reconstituted in Assay Buffer. Samples in the majority of Tissue Culture Media, including those containing fetal bovine serum, can also be read in the assay, provided the standards have been diluted into the tissue culture media instead of Assay Buffer. There will be a small change in binding associated with running the standards and samples in media. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions. Users should only use standard curves generated in media or buffer to calculate concentrations of 13(S)-HODE in the appropriate matrix. Most samples should be extracted before running in this assay. A suitable extraction procedure is outlined below. **All extraction steps should be carried out on ice**. Polypropylene and silanated glassware should be used whenever possible to limit loss of 13(S)-HODE. For users wishing to analyze whole saliva samples, it is recommended that the sample be passed through a 0.45 µm filter prior to dilution and use in the assay

Materials Needed

1. Lysis or Homogenization Buffer. A typical cell lysis buffer is 10 mM Tris-HCl, pH 7.4, 400 mM NaCl, 1 mM EDTA, 1.0% SDS. Tissue samples can be homogenized in phosphate buffered saline (PBS), pH 7.4.
2. 0.2N hydrochloric acid, deionized water, water saturated ethyl acetate (equal volumes water shaken with ethyl acetate) and ethanol.
3. Drying apparatus capable of maintaining sample at 4 °C during drying.

Procedure

Cell Culture Samples

1. Wash cells twice with ice cold PBS.
2. Pipet ice cold lysis buffer onto cells and suspend quickly by scraping and pipetting into and out of pipet tip. An aliquot can be saved for protein determination.
3. Centrifuge sample at 4 °C for 30 seconds at 2100 x g. Transfer the supernatant to a fresh tube.
4. Slowly acidify the sample to pH 3.5-4.0 with 0.2N HCl, using small volumes (5-10 µL) each addition. Should need about 25-30 µL acid per each 200 µL sample volume.
5. Add a 3-fold excess of saturated ethyl acetate to the sample and shake to mix. Centrifuge at 4 °C for 5 minutes at 82 x g to separate the phases.
6. Collect the upper layer (organic phase) into a fresh tube. Be careful to avoid contamination with the materials at the phase, interface and aqueous layer.
7. Repeat steps 5 and 6 for a total of three extractions, collecting the organic layers into a single tube.
8. Dry samples completely, maintaining cold temperature throughout the process. Samples can be stored at -70 °C in desiccation.
9. Dissolve dried sample in 25 µL ethanol. Finish sample dilution with Assay Buffer so that the sample alcohol content is ≤20%.

Tissue Samples

1. Frozen tissue samples should be homogenized on ice in cold PBS, pH 7.4. Homogenize for one minute on low speed. An aliquot can be reserved for protein determination.
2. Continue the extraction as with cell culture samples, starting at step 3.

Procedural Notes

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4 °C in the sealed bag provided. The wells should be used in the frame provided.
8. **Care must be taken to minimize contamination by endogenous alkaline phosphatase.** Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands.
9. **Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results.**

Reagent Preparation

1. 13(S)-HODE Standard

Allow the 10,000 ng/mL 13(S)-HODE standard solution to warm to room temperature. Label five 12 x 75 mm glass tubes #1 through #5. Pipet 1 mL of standard diluent (Assay Buffer or Tissue Culture Media) into tube #1. Pipet 750 µL of standard diluent into tubes #2 through #5. Remove 100 µL of diluent from tube #1. Add 100 µL of the 10,000 ng/mL standard to tube #1. Vortex thoroughly. Add 250 µL of tube #1 to tube #2 and vortex thoroughly. Add 250 µL of tube #2 to tube #3 and vortex. Continue this for tubes #4 and #5.

The concentration of 13(S)-HODE in tubes #1 through #5 will be 1,000, 250, 62.5, 15.6 and 3.9 ng/mL respectively. See 13(S)-HODE Assay Layout Sheet for dilution details. Diluted standards should be used within 60 minutes of preparation.

2. 13(S)-HODE Conjugate

Allow the conjugate to warm to room temperature. Any unused conjugate should be aliquoted and re-frozen at or below -20 °C. Avoid repeated freeze thaws of the aliquots.

3. Conjugate 1:10 Dilution for Total Activity Measurement

Prepare the Conjugate 1:10 Dilution by diluting 50 µL of the supplied Conjugate with 450 µL of standard diluent. The dilution should be used within 3 hours of preparation. **This 1:10 dilution is intended for use in the Total Activity wells ONLY**.

4. Wash Buffer

Prepare the Wash Buffer by diluting 5 mL of the supplied concentrate with 95 mL of de-ionized water. This can be stored at room temperature until the kit expiration date, or for 3 months, whichever is earlier.

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4 °C.

2. Pipet 100 µL of standard diluent (Assay Buffer or Tissue Culture Media) into the NSB and the Bo (0 pg/mL Standard) wells.

3. Pipet 100 µL of Standards #1 through #5 into the appropriate wells.

4. Pipet 100 µL of the Samples into the appropriate wells.

5. Pipet 50 µL of Assay Buffer into the NSB wells.

6. Pipet 50 µL of blue Conjugate into each well, except the Total Activity (TA) and Blank wells.

7. Pipet 50 µL of yellow Antibody into each well, except the Blank, TA and NSB wells.

NOTE: Every well used should be **Green** in color except the NSB wells which should be **Blue**. The Blank and TA wells are empty at this point and have no color.

8. Incubate the plate at room temperature on a plate shaker for 2 hours at ~500 rpm. The plate may be covered with the plate sealer provided, if so desired.

9. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 2 more times for a total of **3 washes.**

10. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.

11. Add 5 µL of the blue Conjugate 1:10 Dilution (**see Step 3, Reagent Preparation, on page 5**) to the TA wells.

12. Add 200 µL of the pNpp Substrate solution to every well. Incubate at 37 °C for 2 hours without shaking. The plate should be covered with the provided plate sealer.

13. Add 50 µL of Stop Solution to every well. This stops the reaction and the plate should be read immediately.

14. Blank the plate reader against the Blank wells, read the optical density at 405 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the Blank wells, manually subtract the mean optical density of the Blank wells from all readings.

Calculation of Results

Several options are available for the calculation of the concentration of 13(S)-HODE in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of 13(S)-HODE can be calculated as follows:

1. Calculate the average net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:

   Average Net OD = Average Bound OD - Average NSB OD

2. Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula:

   $$\text{Percent Bound} = \frac{\text{Net OD}}{\text{Net Bo OD}} \times 100$$

3. Plot Percent Bound versus Concentration of 13(S)-HODE for the standards. Approximate a straight line through the points. The concentration of 13(S)-HODE in the unknowns can be determined by interpolation.

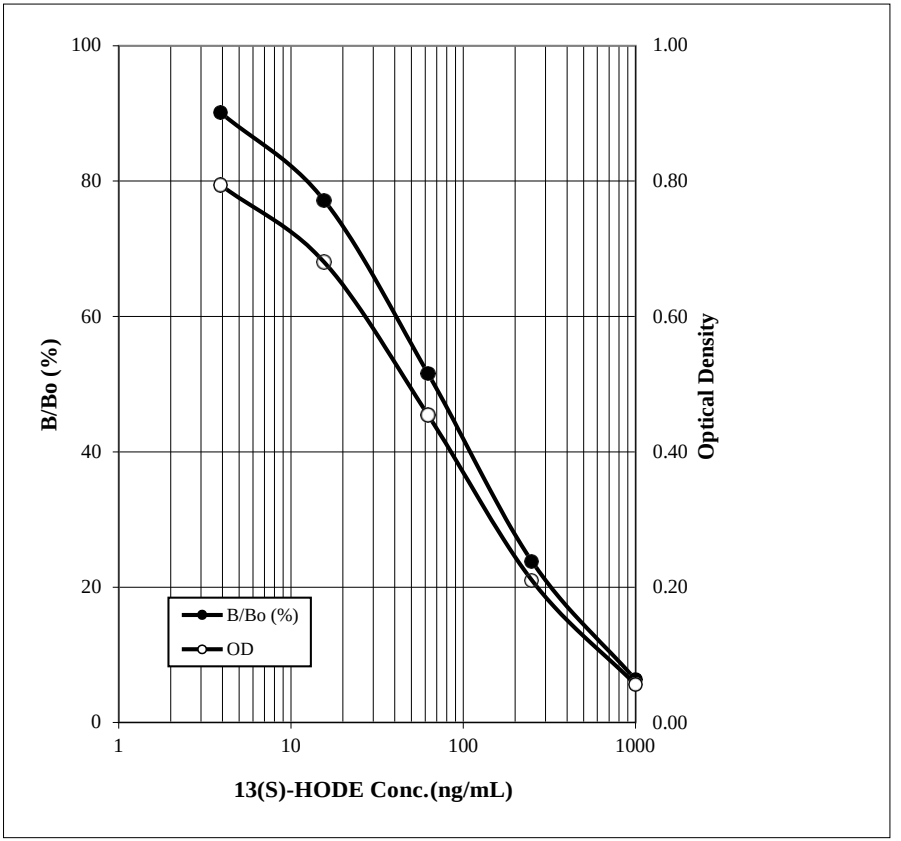
Typical Results

The results shown below are for illustration only and **should not** be used to calculate results.

Sample	Mean OD (-Blank)	Average Net OD	Percent Bound	**13(S)-HODE (ng/mL)**
Blank OD	(0.096)			
TA	0.172	0.167		
NSB	0.005	0.000	0.00%	
Bo	0.886	0.881	100%	**0**
S1	0.061	0.056	6.3%	**1,000**
S2	0.215	0.210	23.8%	**250**
S3	0.459	0.454	51.5%	**62.5**
S4	0.685	0.680	77.1%	**15.6**
S5	0.799	0.794	90.1%	**3.9**
Unknown 1	0.715	0.710	80.5%	**11.8**
Unknown 2	0.508	0.503	57.1%	**48.2**

Typical Standard Curve

A typical standard curve is shown below. This curve **must not** be used to calculate 13(S)-HODE concentrations; each user must run a standard curve for each assay.



Typical Quality Control Parameters

Total Activity Added	=	0.172 x 100 = 17.2
%NSB	=	0.3%
%Bo/TA	=	51.3%
Quality of Fit	=	1.0000 (Calculated from 4-parameter logistic curve fit)
20% Intercept	=	313 ng/mL
50% Intercept	=	68 ng/mL
80% Intercept	=	12 ng/mL

Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols[10].

Sensitivity

Sensitivity was calculated by determining the average optical density bound for sixteen (16) wells run as Bo, and comparing to the average optical density for sixteen (16) wells run with Standard #5. The detection limit was determined as the concentration of 13(S)-HODE measured at two (2) standard deviations from the zero along the standard curve.

Average Optical Density for the Bo = 0.961 ± 0.024 (2.5%)
Average Optical Density for Standard #5 = 0.844 ± 0.038 (4.5%)

Delta Optical Density (0-3.9 ng/mL)
0.961 - 0.844 = 0.117

2 SD's of the Zero Standard = 2 x 0.024 = 0.048

Sensitivity = $\frac{0.048}{0.117}$ x 3.9 ng/mL = **1.6 ng/mL**

Linearity

A sample containing 200 ng/mL 13(S)-HODE was diluted 5 times 1:2 in the kit Assay Buffer and measured in the assay. The data was plotted graphically as actual 13(S)-HODE concentration versus measured 13(S)-HODE concentration.

The line obtained had a slope of 1.016 and a correlation coefficient of 0.997.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of 13(S)-HODE and running these samples multiple times (n=16) in the same assay. Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of 13(S)-HODE in multiple assays (n=8).

The precision numbers listed below represent the percent coefficient of variation for the concentrations of 13(S)-HODE determined in these assays as calculated by a 4 parameter logistic curve fitting program.

	13(S)-HODE (ng/mL)	Intra-assay %CV	Inter-assay %CV
Low	23	7.3	
Medium	64	6.8	
High	105	6.4	
Low	20		11.3%
Medium	62		5.4%
High	99		6.1%

Cross Reactivities

The cross reactivities for a number of related eicosanoid compounds was determined by dissolving the cross reactant (purity checked by N.M.R. and other analytical methods) in Assay Buffer at concentrations from 50,000 to 5 ng/mL. These samples were then measured in the 13(S)-HODE assay, and the measured 13(S)-HODE concentration at 50% B/Bo calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

Compound	Cross Reactivity
13(S)-HODE	100%
13(R)-HODE	2.00%
13-KODE	0.48%
Linoleic Acid	0.31%
γ-Linolenic Acid	0.28%
9(S)-HODE	0.13%
12(R)-HETE	0.07%
15(S)-HETE	0.03%
12(S)-HETE	<0.01%
Arachidonic Acid	<0.01%
DGLA	<0.01%
5(S)-HETE	<0.01%
PGE_1	<0.01%

Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard preparation.

13(S)-HODE concentrations were measured in a variety of different samples including Tissue Culture Media and human biological matrices. This assay has not been validated for the use with human plasma. The end user must validate this sample type. 13(S)-HODE was spiked into samples which were diluted with the appropriate diluent. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
Tissue Culture Media	101.6	1:2
Human Serum	89.3	1:50
Human Saliva	107	1:8
Human Urine	115	1:8

* See Sample Handling instructions on page 4 for details.

References

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