ab133044 – 13(S)-HODE ELISA Kit

Instructions for Use

For quantitative detection of 13(S)-HODE in Tissue Culture Media, Human Serum, Saliva and Urine.

This product is for research use only and is not intended for diagnostic use.

Version 6 Last Updated 24 February 2020

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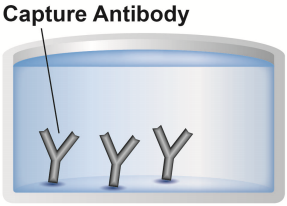
1. BACKGROUND

Abcam's 13(S)-HODE *in vitro* competitive ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the accurate quantitative measurement of 13(S)-HODE in Tissue Culture Media, Human Serum, Saliva and Urine.

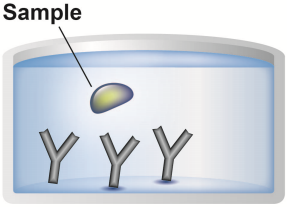
A goat anti-rabbit IgG antibody has been precoated onto 96-well plates. Standards or test samples are added to the wells, along with an alkaline phosphatase (AP) conjugated-13(S)-HODE antigen and a polyclonal rabbit antibody specific to 13(S)-HODE. After incubation the excess reagents are washed away. pNpp substrate is added and after a short incubation the enzyme reaction is stopped and the yellow color generated is read at 405 nm. The intensity of the yellow coloration is inversely proportional to the amount of 13(S)-HODE captured in the plate.

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. 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. 13(S)-HODE helps to mediate cellular adhesion properties and excessive proliferation that is critical in the formation of tumors, metastasies and artherogenic plaques. Continuously synthesized in non-stimulated vessel epithelia, 13(S)-HODE remains associated with the vitronectin receptor, a ubiquitous integrin adhesion molecule. Cell surfaces become adhesive when HODE concentrations decline, allowing the vitronectin receptor to relocate to the cell surface.

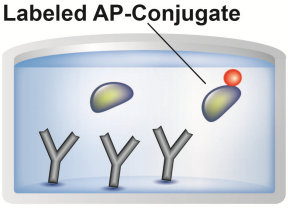
2. ASSAY SUMMARY



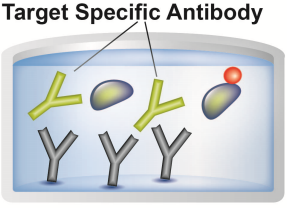
Prepare all reagents and samples as instructed.



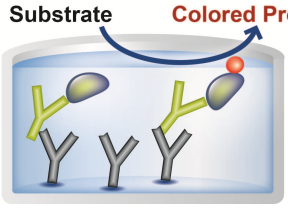
Add standards and samples to appropriate wells.



Add prepared labeled AP-conjugate to appropriate wells.



Add 13(S)-HODE antibody to appropriate wells. Incubate at room temperature.



Add pNpp substrate to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

- 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
- Stop Solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in use
- 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
- 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
- The 13(S)-HODE Standard provided, 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

4. STORAGE AND STABILITY

Store kit at +4°C immediately upon receipt, apart from the Alkaline Phosphatase Conjugate, which should be stored at -20°C. Avoid multiple freeze-thaw cycles.

Refer to list of materials supplied for storage conditions of individual components.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
Goat anti-rabbit IgG Microplate (12 x 8 wells)	96 Wells	+4ºC
13(S)-HODE Alkaline Phosphatase Conjugate	5 mL	-20ºC
13(S)-HODE Antibody	5 mL	+4ºC
13(S)-HODE Standard	500 µL	+4ºC
Assay Buffer	27 mL	+4ºC
20X Wash Buffer Concentrate	27 mL	+4ºC
pNpp Substrate	20 mL	+4ºC
Stop Solution	5 mL	+4ºC

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are required to successfully utilize this assay:

- Standard microplate reader - capable of reading at 405 nm, preferably with correction between 570 and 590 nm
- Adjustable pipettes and pipette tips. Multichannel pipettes are recommended when large sample sets are being analyzed
- Microplate Shaker
- Absorbent paper for blotting
- 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
- 0.2N hydrochloric acid
- Deionized water
- Ethanol
- Drying apparatus capable of maintaining sample at 4 º C
- Water saturated ethyl acetate (equal volumes water shaken with ethyl acetate)

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted

8. TECHNICAL HINTS

- Standards can be made up in either glass or plastic tubes
- Pre-rinse the pipette tip with the reagent, use fresh pipette tips for each sample, standard and reagent
- Pipette standards and samples to the bottom of the wells
- Add the reagents to the side of the well to avoid contamination
- 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
- 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
- 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
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions**

9. REAGENT PREPARATION

Equilibrate all reagents and samples to room temperature (18 - 25°C) prior to use.

9.1 13(S)-HODE Alkaline Phosphatase Conjugate

Allow the 13(S)-HODE Alkaline Phosphatase Conjugate to equilibrate to room temperature. Any unused conjugate should be aliquoted and re-frozen at or below -20°C.

9.2 1X Wash Buffer

Prepare the 1X Wash Buffer by diluting 5 mL of the 20X Wash Buffer Concentrate in 95 mL of deionized water. Mix thoroughly and gently.

9.3 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**

10. STANDARD PREPARATIONS

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use. Diluted standards should be used within 60 minutes of preparation.

10.1 Allow the reconstituted 10,000 ng/mL 13(S)-HODE **Stock Standard** solution to equilibrate to room temperature. The standard solution should be stored at -20°C. Avoid repeated freeze-thaw cycles.

10.2 Label five tubes with numbers #1 – #5.

10.3 Add 900 µL of appropriate diluent (Assay Buffer or Tissue Culture Media) to tube #1

10.4 Add 750 µL appropriate diluent to tubes #2 through #7.

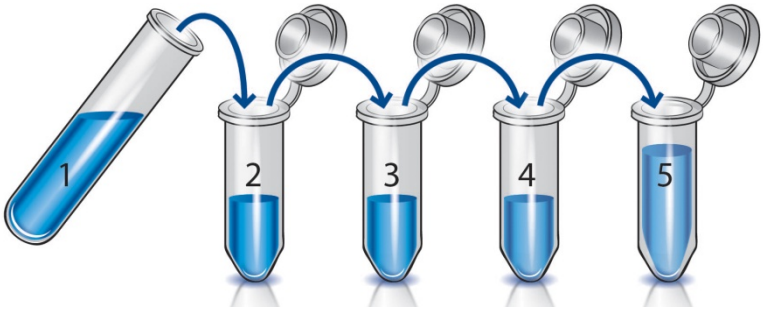
10.5 Prepare a 1,000 ng/mL **Standard 1** by transferring 100 µL of the 10,000 ng/mL Stock Standard tube 1. Vortex thoroughly.

10.6 Prepare **Standard 2** by transferring 250 µL from Standard 1 to tube #2. Vortex thoroughly.

10.7 Prepare **Standard 3** by transferring 250 µL from Standard 2 to tube 3. Vortex thoroughly.

10.8 Using the table below as a guide, repeat for tubes #4 and #5.

Standard	Sample to Dilute	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (ng/mL)	Final Conc. (ng/mL)
1	Standard	100	900	10,000	1,000
2	Standard 1	250	750	1,000	250
3	Standard 2	250	750	250	62.5
4	Standard 3	250	750	62.5	15.6
5	Standard 4	250	750	15.6	3.9



11. SAMPLE COLLECTION AND STORAGE

- The 13(S)-HODE kit is compatible with 13(S)-HODE samples in a wide range of matrices after dilution in Assay Buffer. However, the end user must verify that the recommended dilutions are appropriate for their samples
- 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
- For tissue, urine and plasma samples, prostaglandin synthetase inhibitors such as indomethacin or meclofenamic acid at concentrations up to 10 µg/mL should be added to either the tissue homogenate or urine and plasma samples. Some samples normally have very low levels of 13(S)-HODE present and extraction may be necessary for accurate measurement. A suitable extraction procedure is outlined below:

11.1 Cell Culture Supernatants

11.1.1 Wash cells twice with ice cold PBS.

11.1.2 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.

11.1.3 Centrifuge sample at 4 °C for 30 seconds at 2,100 x g. Transfer the supernatant to a fresh tube.

11.1.4 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.

11.1.5 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.

11.1.6 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.

11.1.7 Repeat steps 5 and 6 for a total of three extractions, collecting the organic layers into a single tube.

11.1.8 Dry samples completely, maintaining cold temperature throughout the process. Samples can be stored at -70 °C in desiccation.

11.1.9 Dissolve dried sample in 25 µL ethanol. Finish sample dilution with Assay Buffer so that the sample alcohol content is ≤20%.

11.2 Tissue Samples

11.2.1 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.

11.2.2 Continue the extraction as with cell culture samples, starting at step 11.1.3.

12. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents
- Unused well strips should be returned to the plate packet and stored at 4°C
- For each assay performed, a minimum of 2 wells must be used as blanks, omitting primary antibody from well additions
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates)
- Well effects have not been observed with this assay

	1	2	3	4
A	B_s	Std 1	Std 5	
B	B_s	Std 1	Std 5	
C	TA	Std 2	Sample 1	
D	TA	Std 2	Sample 1	
E	NSB	Std 3	Sample 2	
F	NSB	Std 3	Sample 2	
G	B_0	Std 4	etc	
H	B_0	Std 4	etc	

Plate layout shows controls, blanks and standards required for each assay. Use additional strips of wells to assay all your samples.

Key:

B_s = Blank; contains substrate only.

TA = Total Activity; contains conjugate (5 µL) and substrate.

NSB = Non-specific binding; contains standard diluent, assay buffer, conjugate and substrate.

B_0 = 0 pg/mL standard; contains standard diluent, conjugate, antibody and substrate

13. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use**
- **It is recommended to assay all standards, controls and samples in duplicate**

13.1 Add 100 µL appropriate diluent* into the NSB (non-specific binding) wells. (*Use the same diluent used to prepare standards in section 10, either Assay Buffer or Tissue Culture Media).

13.2 Add 100 µL appropriate diluent (Assay Buffer or tissue culture media) into the B_0 (0 pg/mL standard) wells

13.3 Add 100 µL of prepared standards and 100 µL diluted samples to the appropriate wells.

13.4 Add 50 µL of Assay Buffer into the NSB wells.

13.5 Add 50 µL of 13(S)-HODE Alkaline Phosphatase Conjugate (blue) into NSB, B_0, standard and sample wells, i.e. not the Total Activity (TA) and B_s well.

13.6 Add 50 µL of 13(S)-HODE Antibody (yellow) into B_0, standard and sample wells, i.e. not B_s, TA and NSB wells *Note*: Every well should be green in color except the NSB wells which should be blue. The B_s and TA wells are empty at this point and have no color.

13.7 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.

13.8 Empty the contents of the wells and wash by adding 400 µL of 1X Wash Buffer to every well. Repeat the wash 2 more times for a total of 3 washes. 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.

13.9 Add 5 µL of the 13(S)-HODE Alkaline Phosphatase Conjugate 1:10 dilution (see step 9.2 Reagent Preparation section) to the TA wells.

13.10 Add 200 µL of the pNpp Substrate solution to every well. Incubate at 37°C for 2 hours without shaking.

13.11 Add 50 µL Stop Solution into each well. The plate should be read immediately.

13.12 Blank the plate reader against the B_s wells, read the O.D. absorbance 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.

14. CALCULATIONS

14.1 Calculate the average net absorbance measurement (Average Net OD) for each standard and sample by subtracting the average NSB absorbance measurement from the average absorbance measurement (Average OD) for each standard and sample.

Average Net OD = Average Bound OD - Average NSB OD

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

$$\text{Percent Bound} = \frac{\text{Average Net OD}}{\text{Average Net } B_0 \text{ OD}} \times 100$$

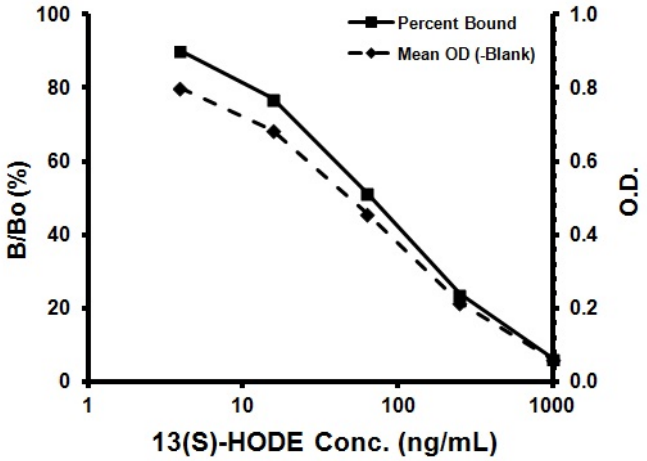
14.3 Plot the Percent Bound (B/B_0) and the net OD versus concentration of 13(S)-HODE for the standards. The concentration of 13(S)-HODE in the unknowns can be determined by interpolation of net OD values.

A four parameter algorithm (4PL) provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4 parameter logistic). Interpolate protein concentrations for unknown samples from the standard curve plotted.

Samples producing signals greater than that of the highest standard should be further diluted and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.

15. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.



Sample	Mean OD ($-B_s$)	% Bound	13(s)HODE ng/mL
B_s	(0.096)	-	-
TA	0.172	-	-
NSB	0.005	0.0	-
Standard 1	0.061	6.3	1,000
Standard 2	0.215	23.8	250
Standard 3	0.459	51.5	62.5
Standard 4	0.685	77.1	15.6
Standard 5	0.799	90.1	3.9
B_0	0.886	100	0
Unknown1	0.715	80.5	11.8
Unknown 2	0.508	57.1	48.2

16. TYPICAL SAMPLE VALUES

SENSITIVITY –

Sensitivity was calculated by determining the average optical density bound for 16 wells run as B_O, and comparing to the average optical density for 16 wells run with Standard 5. The detection limit was determined as the concentration of 13(S)-HODE measured at 2 standard deviations from the zero along the standard curve determined to be 1.6 ng/mL.

SAMPLE RECOVERY –

Recovery was determined by 13(S)-HODE into tissue culture media, Human saliva, serum, and urine. Mean recoveries are as follows:

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

LINEARITY OF DILUTION –

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.

Intra-Assay

	13(S)-HODE (ng/mL)	%CV
Low	23	7.3
Medium	64	6.8
High	105	6.4

Inter-Assay

	13(S)-HODE (ng/mL)	%CV
Low	20	11.3
Medium	62	5.4
High	99	6.1

17.ASSAY SPECIFICITY

CROSS REACTIVITY –

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/B_0 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

18. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standards dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Samples give higher value than the highest standard	Starting sample concentration is too high	Dilute the specimens and repeat the assay
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the kit	Store the all components as directed

19.NOTES

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