ab133034 – 12(S)-HETE ELISA Kit

Instructions for Use

For quantitative detection of 12(S)-HETE in cell culture supernatants and plasma (heparin, EDTA).

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

Version 5a Last Updated 22 March 2023

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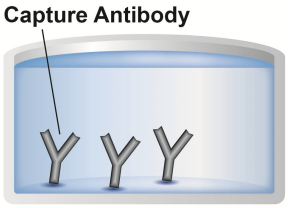
1. BACKGROUND

Abcam's 12(S)-HETE *in vitro* competitive ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the accurate quantitative measurement of 12(S)-HETE in cell culture supernatants and plasma (heparin, EDTA).

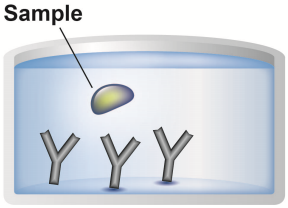
A goat anti-rabbit IgG antibody has been precoated onto 96-well plates. Standards or test samples are added to the wells, along with an alkaline phosphatase (AP) conjugated-12(S)-HETE antigen and a polyclonal rabbit antibody specific to 12(S)-HETE. After incubation the excess reagents are washed away. pNpp substrate is added and after a short incubation the alkaline phosphatase 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 12(S)-HETE captured in the plate.

12(S)-HETE is the stereospecific hydroxy product from the reduction of 12(S)-hydroperoxy tetraenoic eicosatetraenoic acid (12(S)-HpETE), which itself is a 12-lipoxygenase metabolite of arachidonic acid. 12(S)-HETE has been shown to be chemotactic and chemokinetic for polymorphonuclear leukocytes and vascular smooth cells. It also acts as a second messenger in angiotensin-II induced aldosterone production. Evidence also suggests that 12(S)-HETE is involved in suppressing renin production, stimulating insulin secretion by pancreatic tissue, inducing endothelial cell retraction and tumor cell adhesion.

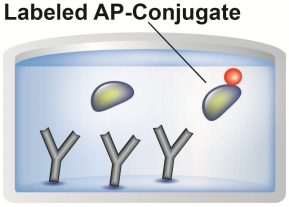
2. ASSAY SUMMARY



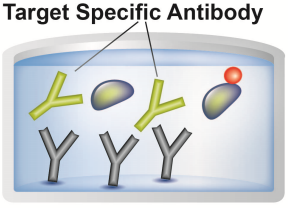
Prepare all reagents and samples as instructed.



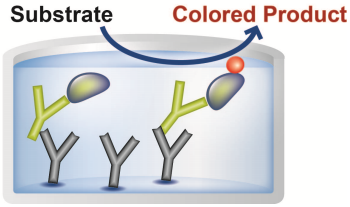
Add standards and samples to appropriate wells.



Add prepared labeled AP-conjugate to appropriate wells.



Add 12(S)-HETE 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

- ## STORAGE AND STABILITY

Store kit at +4°C immediately upon receipt, apart from the 12(S)-HETE Alkaline Phosphatase Conjugate and 12(S)-HETE Standard, which should be stored at -20°C. Avoid multiple freeze-thaw cycles.

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

4. MATERIALS SUPPLIED

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

5. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Standard microplate reader - capable of reading at 405 nm, preferably with correction between 570 and 590 nm.
- Automated plate washer (optional)
- Adjustable pipettes and pipette tips. Multichannel pipettes are recommended when large sample sets are being analyzed
- Eppendorf tubes
- Microplate Shaker
- Absorbent paper for blotting
- 200 mg C_{18} Reverse Phase Extraction Columns (only required for extraction of samples containing low levels of 12(S)-HETE)
- 2M hydrochloric acid (only required for extraction of samples containing low levels of 12(S)-HETE)
- Deionized water
- Ethanol
- Hexane
- Ethyl Acetate

6. 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

7. 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**

8. REAGENT PREPARATION

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

9.1 **12(S)-HETE Alkaline Phosphatase Conjugate**

Allow the 12(S)-HETE Alkaline Phosphatase Conjugate to equilibrate to room temperature. Any unused conjugate should be aliquoted and re-frozen at or below -20 °C.

9.2 **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 Assay Buffer. The dilution should be used within 3 hours of preparation. This 1:10 dilution is intended for use in the Total Activity wells ONLY.

9.3 **1X Wash Buffer**

Prepare the 1X Wash Buffer by diluting 10 mL of the 20X Wash Buffer Concentrate in 190 mL of deionized water. Mix thoroughly and gently.

9. 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 For:

10.1.1 **Serum/Plasma** samples dilute the 12(S)-HETE standard with Assay Buffer.

10.1.2 **Tissue culture media** samples dilute the 12(S)-HETE standard with tissue culture media diluted 1:2 with assay buffer.

10.2 Allow the 500,000 pg/mL Gastrin 1 Stock Standard solution to equilibrate to room temperature. The standard solution should be stored at -20°C. Avoid repeated freeze-thaw cycles.

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

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

10.5 Add 750 µL appropriate diluent to tubes #2-#5

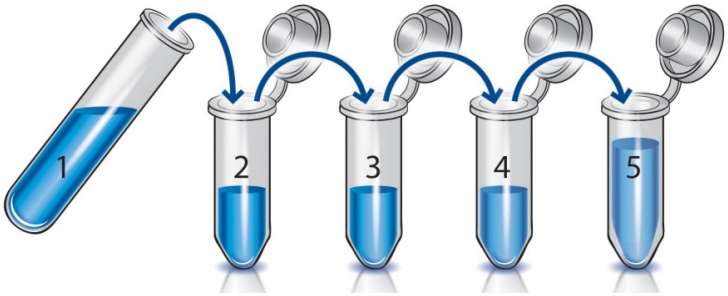
10.6 Prepare a 50,000 pg/mL **Standard 1** by adding 100 µL of the 500,000 pg/mL Stock Standard to tube 1. Vortex thoroughly.

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

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

10.9 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. (pg/mL)	Final Conc. (pg/mL)
1	Stock	100	900	500,000	50,000
2	Standard 1	250	750	50,000	12,500
3	Standard 2	250	750	12,500	3,125
4	Standard 3	250	750	3,125	781
5	Standard 4	250	750	781	195



10. SAMPLE COLLECTION AND STORAGE

- This assay is suitable for measuring 12(S)-HETE in plasma and culture supernatants. Prior to assay, frozen samples should be slowly brought to 4˚C and centrifuged, if necessary, to isolate residual debris. Samples containing rabbit IgG may interfere with the assay.
- Culture supernatants may be run directly in the assay provided the same non-conditioned media is used as the standard diluent. It is recommended that culture media be diluted a minimum of 1:2 in the assay buffer, for both standard diluent and sample, prior to use in the assay. There may be a small change in binding associated with culture supernatant samples.
- A minimum 1:16 dilution and 1:64 dilutions are recommended for sodium heparin and EDTA plasma, respectively. These minimum dilutions are recommended to remove matrix interference of these samples in the assay. Samples outside of the standard range may require further dilution with the assay buffer or extraction. The optimal dilution for any sample must be determined by the investigator.

11.1 Some samples normally have very low levels of 12(S)-HETE present and extraction may be necessary for accurate measurement. A suitable extraction procedure is outlined below:

11.1.1. Acidify the sample by addition of 2M HCl to pH of 3.5. Approximately 50 µL of HCl will be needed per mL of plasma. Allow to sit at 4°C for 15 minutes. Centrifuge samples in a microcentrifuge for 2 minutes to remove any precipitate.

11.1.2. Prepare the C18 reverse phase column by washing with 10 mL of 100% ethanol followed by 10 mL of deionized water.

11.1.3. Apply the sample under a slight positive pressure to obtain a flow rate of about 500 µL/minute. Wash the column with 10 mL of water, followed by 10 mL of 15%

ethanol, and finally 10 mL hexane. Elute the sample from the column by addition of 10 mL ethyl acetate.

11.1.4. If analysis is to be carried out immediately, evaporate samples under a stream of nitrogen. Add at least 250 µL of Assay Buffer to dried samples. Vortex well, then allow to sit for five minutes at room temperature. Repeat twice more.

11.1.5. If analysis is to be delayed, store samples as the eluted ethyl acetate solutions at -80°C until the immunoassay is to be run. Evaporate the organic solvent under a stream of nitrogen prior to running assay and reconstitute as above.

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 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. Contents of each well can be recorded on the template sheet included in the Resources section

	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**
- **Refer to the recommended plate layout in Section 12 before proceeding with the assay**

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 50 µL of Assay Buffer into the NSB wells.

13.4 Add 100 µL of prepared standards and 100 µL diluted samples to appropriate wells

13.5 Add 50 µL of 12(S)-HETE Alkaline Phosphatase Conjugate (blue) into NSB, B_0, standard and sample wells, i.e. not the Total Activity (TA) and B_s wells.

13.6 Add 50 µL of 12(S)-HETE antibody (yellow) into B_0, standard and sample wells, i.e. not B_s, TA and NSB wells

Note: Every well used 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 12(S)-HETE Alkaline Phosphatase Conjugate 1:10 dilution to the TA wells.

13.10 Add 200 µL of the pNpp Substrate solution to every well. Incubate at 37ºC for 3 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 blank 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

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

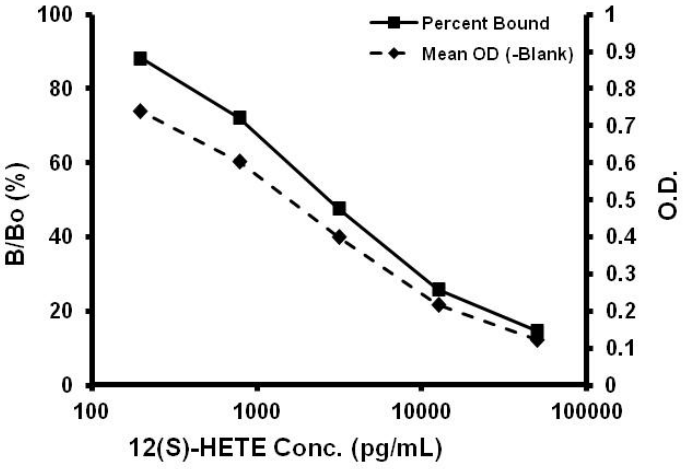
14.3 Plot the Percent Bound (B/B_0) and the net OD versus concentration of 12(S)-HETE for the standards. The concentration of 12(S)-HETE in the unknowns can be determined by interpolation of net OD values.

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	Average OD (-B_s)	Percent Bond	12(S)-HETE (pg/mL)
B_s	(0.094)		
TA	0.554		
NSB	0	0	
Standard 1	0.123	14.6	50,000
Standard 2	0.217	25.8	12,500
Standard 3	0.401	47.8	3,125
Standard 4	0.605	72.1	781
Standard 5	0.740	88.3	195
B_0	0.839	100	0
Unknown 1	0.692	82.5	359
Unknown 2	0.387	46.1	3393

16. TYPICAL SAMPLE VALUES

SENSITIVITY –

The sensitivity, minimum detectable dose of 12(S)-HETE using this Abcam ELISA kit was found to be 146.3 pg/mL. This was calculated as the ratio of the mean OD plus 2 standard deviations of 16 replicates of the 0 pg/mL standard to the mean of 16 replicates of the lowest standard, multiplied by the concentration of that standard (195 pg/mL).

PRECISION –

Intra-Assay

	12(S)-HETE (pg/mL)	%CV
Low	342	5.2
Medium	1,153	10.1
High	4,762	15.5

Inter-Assay

	12(S)-HETE (pg/mL)	%CV
Low	224	4.1
Medium	1,127	9.1
High	5,294	20.8

LINEARITY –

A buffer sample containing 12(S)-HETE was serially diluted 1:2 in assay buffer and measured in the assay. The results are shown in the table below.

Dilution	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
Neat	-	9,518	-
1:2	4,759	4,895	103
1:4	2,380	2,500	105
1:8	1,190	1,216	102
1:16	595	606	102

SAMPLE RECOVERY –

Recovery was determined by 12(S)-HETE into tissue culture media. Mean recoveries are as follows:

Sample Type	Average % Recovery	Recommended Dilution
Tissue Culture Media	94	1:2
Plasma (Heparin)	104	≥1:16
Plasma (EDTA)	97	≥1:64

PARALLELISM -

Plasma recovery and recommended dilutions were determined using plasma samples with 12(S)-HETE levels that read within the range of the assay.

	Dilution	Observed Corrected (pg/mL)	Recovery (%)	Average % Recovery
Na Heperinized Plasma	1:16	433,472	111	104
	1:32	428,272	109	
	1:64	385,664	99	
	1:128	372,608	95	
	1:256	391,168	-	
EDTA Plasma	1:64	433,472	91	97
	1:128	428,272	103	
	1:256	385,664	-	

17. ASSAY SPECIFICITY

This kit detects both endogenous and recombinant 12(S)-HETE.

CROSS REACTIVITY –

The cross reaction of the antibody calculated at 50% is:

12(S)-HETE	100 %
12(R)-HETE	2.5 %
15-HETE	0.3 %
5(S)-HETE	0.2 %
8,15-diHETE	0.1 %
5,15-diHETE	0.1 %
PGE_2	0.1 %
PGF_2 alpha	0.1 %
PGD_2	0.1 %
6-keto-PGF_1 alpha	0.1 %
Thromboxane B2	0.1 %
Arachidonic Acid	0.1 %
Leukotriene B_4	0.1 %
Leukotriene C_4	0.1 %
Leukotriene D_4	0.1 %
Leukotriene E_4	0.1 %
8-HETE	< 0.1 %
9-HETE	< 0.1 %
11-HETE	< 0.1 %

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

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)