New standard storage temperature

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> 12(S)-HETE EIA kit

Catalog # ADI-900-050

96 Well Enzyme Immunoassay Kit
For use with plasma and culture supernates

Reagents require separate storage conditions.

Check our website for additional protocols, technical notes and FAQs

For proper performance, use the insert provided with each individual kit received

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ALEXIS assay designs BIOMOL Stressgen

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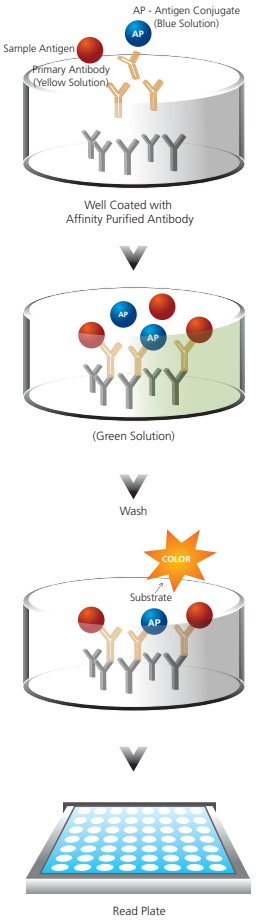
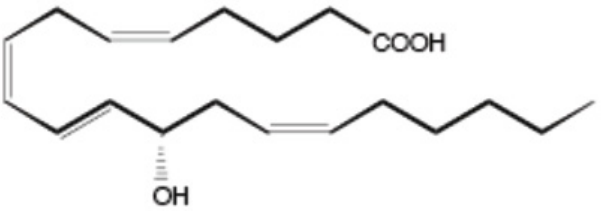
Introduction



12(S)-HETE is the stereospecific hydroxy product from the reduction of 12(S)-hydroperoxy tetraenoic eicosatetraenoic acid [12(S)-HpETE][1-2], which itself is a 12-lipoxygenase metabolite of arachidonic acid[3]. 12(S)-HETE has been shown to be chemotactic and chemokinetic for polymorphonuclear leukocytes[4] and vascular smooth cells[5]. It also acts as a second messenger in angiotensin-II induced aldosterone production[6]. Evidence also suggests that 12(S)-HETE is involved in suppressing renin production[7], stimulating insulin secretion by pancreatic tissue[8], inducing endothelial cell retraction and tumor cell adhesion[9].

12(S)-HETE



Principle

1. Standards and samples are added to wells coated with a GxR IgG antibody. A blue solution of 12(S)-HETE conjugated to alkaline phosphatase is then added, followed by a yellow solution of rabbit polyclonal antibody to 12(S)-HETE.

2. During a simultaneous incubation at room temperature the antibody binds, in a competitive manner, the 12(S)-HETE in the sample or conjugate. The plate is washed, leaving only bound 12(S)-HETE.

3. pNpp substrate solution is added. The substrate generates a yellow color when catalyzed by the alkaline phosphatase on the 12(S)-HETE conjugate.

4. Stop solution is added. The yellow color is read at 405 nm. The amount of signal is indirectly proportional to the amount of 12(S)-HETE in the sample.

Materials Supplied

1. **Assay Buffer**
   27 mL, Product No. 80-1591Tris buffered saline containing proteins and sodium azide as preservative

2. **12(S)-HETE Standard**
   0.5 mL, Product No. 80-0607
   A solution of 500,000 pg/mL 12(S)-HETE

3. **Goat anti-Rabbit IgG Microtiter Plate**
   One plate of 96 wells, Product No. 80-0060
   A clear plate of break-apart strips coated with a goat anti-rabbit polyclonal antibody

4. **12(S)-HETE Antibody**
   5 mL, Product No. 80-0611
   A yellow solution of a rabbit polyclonal antibody to 12(S)-HETE

5. **12(S)-HETE Conjugate**
   5 mL, Product No. 80-0610
   A blue solution of 12(S)-HETE conjugated to alkaline phosphatase

6. **Wash Buffer Concentrate**
   27 mL, Product No. 80-1286
   Tris buffered saline containing detergents

7. **pNpp Substrate**
   20 mL, Product No. 80-0075
   A solution of p-nitrophenyl phosphate

8. **Stop Solution**
   5 mL, Product No. 80-0247
   A solution of trisodium phosphate in water

9. **12(S)-HETE Assay Layout Sheet**
   1 each, Product No. 30-0104

10. **10. Plate Sealer**
    2 each, Product No. 30-0012

Do not mix components from different kit lots or use reagents beyond the expiration date of the kit.

The standard should be handled with care due to the known and unknown effects of the antigen.

The activity of the conjugate is affected by concentrations of chelators > 10mM (such as EDTA and EGTA).

Avoid contamination by endogenous alkaline phosphatase. Do not expose reagents or supplies to bare skin.

Stop solution is caustic. Keep tightly capped.

Some components contain sodium azide, which may react with plumbing. While disposing, flush with large volumes of water to prevent build-up.

Storage

All components of this kit, except the conjugate and standard, are stable at 4°C. The conjugate and standard must be stored at or below -20°C.

Materials Needed but Not Supplied

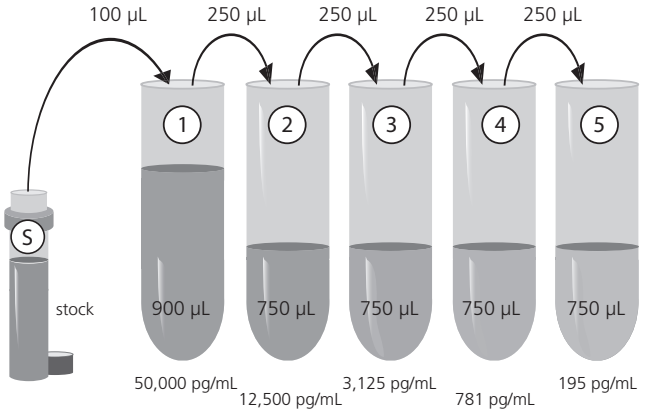
1. Deionized or distilled water
2. Precision pipets for volumes between 5 µL and 1,000 µL
3. Repeater pipet for dispensing 50 µL and 200 µL
4. Disposable beakers for diluting buffer concentrates
5. Graduated cylinders
6. Microplate shaker
7. Lint-free paper toweling for blotting
8. 37°C Incubator
9. Microplate reader capable of reading at 405 nm

Reagent Preparation

1. Wash Buffer
   Prepare the wash buffer by diluting 10 mL of the supplied Wash Buffer Concentrate with 190 mL of deionized water. This can be stored at room temperature until the kit's expiration, or for 3 months, whichever is earlier.

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.

3. Preparation of 12(S)-HETE Standard Curve



Allow the 500,000 pg/mL standard stock to warm to room temperature. Label five 12 x 75 mm tubes #1 though #5. Pipet 900 µL of the appropriate sample diluent (Assay Buffer or non-conditioned culture media, diluted at least 1:2 in Assay Buffer) into tube #1. Pipet 750 µL of the appropriate sample diluent into tubes #2 through #5. Add 100 µL of the 500,000 pg/mL standard stock into tube #1 and vortex gently. Add 250 µL of tube #1 to tube #2 and vortex gently. Add 250 µL of tube #2 to tube #3 and vortex gently. Continue this for tubes #4 through #5.

Diluted standards should be used within 1 hour of preparation. The concentration of the standards is labeled above.

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

Glass or polypropylene tubes may be used for standard preparation. Avoid polystyrene.

Sample Handling

If buffers other than those provided are used in the assay, the end-user must determine the appropriate dilution and assay validation.

This assay is suitable for measuring 12(S)-HETE in plasma and culture supernates. 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 supernates 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 supernate samples.

A minimum 1:16 dilution and 1:64 dilution is recommended for sodium heparin and EDTA plasma, respectively. These minimum dilutions are recommended to remove matrix interference of these samples in the assay (see Sample Recoveries section). 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.

Some samples normally have low levels of 12(S)-HETE present and extraction may be necessary for accurate measurement. A suitable extraction procedure is outlined below. Please refer to references 10-13 for further details of extraction protocols.

Materials Needed

1. A sample with a known concentration of 12(S)-HETE to allow extraction efficiency to be accurately determined.
2. 2M hydrochloric acid, deionized water, ethanol, hexane and ethyl acetate.
3. 200 mg C18 Reverse Phase Extraction Columns.

Extraction Procedure

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.
2. Prepare the C18 reverse phase column by washing with 10 mL of 100% ethanol followed by 10 mL of deionized water.
3. Apply the sample under a slight positive pressure to obtain a flow rate of about 0.5 mL/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.
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.
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.

Assay Procedure

Refer to the Assay Layout Sheet to determine the number of wells to be used. Remove the wells not needed for the assay and return them, with the desiccant, to the mylar bag and seal. Store unused wells at 4°C.

1. Pipet 100 µL of standard diluent (Assay Buffer or 1:2 diluted culture media) into the NSB and the Bo (0 pg/mL) wells.
2. Pipet 50 µL of Assay Buffer into the NSB wells.
3. Pipet 100 µL of Standards #1 through #5 in to the appropriate wells.
4. Pipet 100 µL of the Samples into the appropriate wells.
5. Pipet 50 µL of blue conjugate into each well except the Blank and TA wells.
6. 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.
7. Seal the plate. Incubate for 2 hours on a plate shaker (~500 rpm) at room temperature.
8. Empty the contents of the wells and wash by adding 400 µL of wash buffer to every well. Repeat 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.
9. Pipet 5 µL of the blue conjugate 1:10 dilution (see Step 2, Reagent Preparation, on page 5) to the TA wells.
10. Add 200 µL of the substrate solution into each well.
11. Incubate at 37°C for 3 hours without shaking.
12. Pipet 50 µL stop solution into each well.
13. After blanking the plate reader against the substrate blank, read optical density at 405 nm. If plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

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

All standards and samples should be run in duplicate.

Pre-rinse each pipet tip with reagent. Use fresh pipet tips for each sample, standard, and reagent.

Pipet the reagents to the sides of the wells to avoid possible contamination.

Prior to the addition of substrate, ensure there is no residual wash buffer in the wells. Remaining wash buffer may cause variation in assay results.

Calculation of Results

Make sure to multiply sample concentrations by the dilution factor used during sample preparation.

Several options are available for the calculation of the concentration of 12(S)-HETE 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 concentrations can be calculated as follows:

1. Calculate the average Net OD for each standard and sample by subtracting the average NSB OD from the average OD for each standard and sample.

   Average Net OD = Average 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:

   Percent Bound = Net OD ÷ Net Bo OD x 100

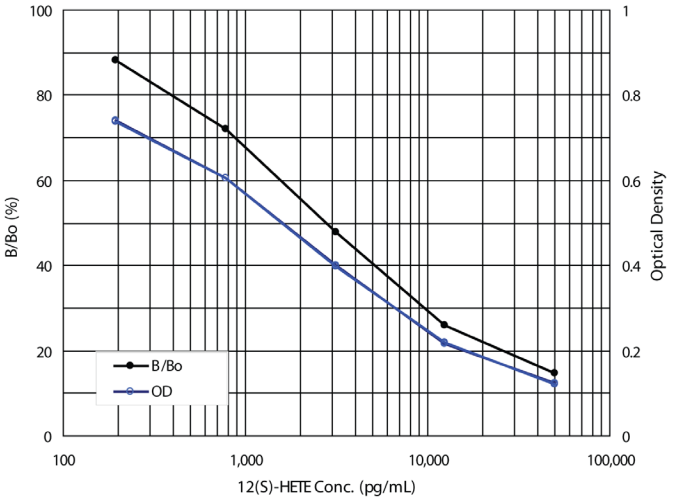
3. Using logit-log paper, plot the Percent Bound (B/Bo) versus concentration of 12(S)-HETE for the standards. Approximate a straight line through the points. The concentration of the unknowns can be determined by interpolation.

Samples with concentrations outside of the standard curve range will need to be re-analyzed using a different dilution.

Typical Results

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

Sample	Average OD	Net	Percent Bound	12(S)-HETE (pg/mL)
Blank (mean)	(0.094)		---	---
TA	0.554		---	---
NSB	0.000		0%	---
Bo	0.839		100%	**0**
S1	0.123		14.6%	**50,000**
S2	0.217		25.8%	**12,500**
S3	0.401		47.8%	**3,125**
S4	0.605		72.1%	**781**
S5	0.740		88.3%	**195**
Unknown 1	0.692		82.5%	**359**
Unknown 2	0.387		46.1%	**3393**



Performance Characteristics

For detailed cross-reactivity protocol, see our website.

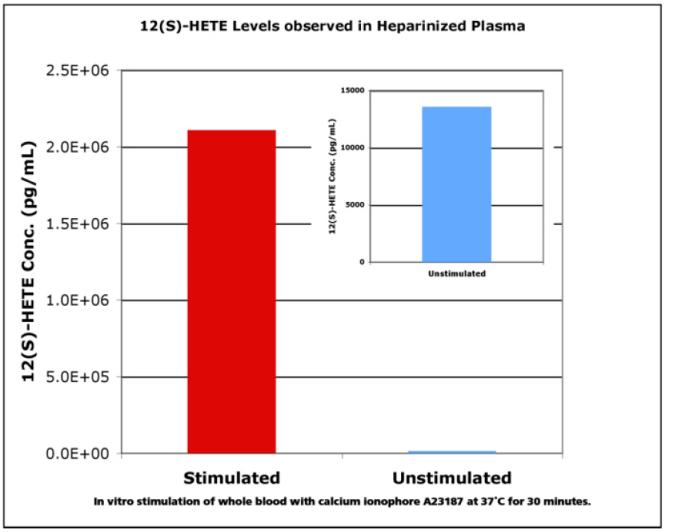
Specificity

The cross reactivities for a number of related compounds were determined by diluting cross reactants in the assay buffer at several concentrations. These samples were then measured in the assay.

Compound	Cross Reactivity
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%

Whole Blood Stimulation Experiment

This experiment was adapted from a protocol outline in reference #14. Calcium ionophore A23187 was added to whole blood to a final concentration of 50 µM. The blood was then incubated for 30 minutes at 37°C to stimulate 12(S)-HETE production by white blood cells and platelets. After centrifugation, plasma was collected. Results for treated and non-treated plasma are provided in the following graph.



Sample Recovery

12(S)-HETE concentrations were measured in a variety of different samples including human plasma and culture media. Due to the presence of binding proteins to 12(S)-HETE, spiking 12(S)-HETE into plasma is not recommended. For samples in culture media, ensure that the standards have been diluted into the same media (refer to page 6). 12(S)-HETE was spiked into diluted culture media to determine recovery and recommended dilution.

Sample	% Recovery	Recommended
Culture Media	94	1:2
Sodium heparin plasma	104	≥1:16
EDTA plasma	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	433472	111	104
	1:32	428272	109	
	1:64	385664	99	
	1:128	372608	95	
	1:256	391168	---	
EDTA Plasma	1:64	433472	91	97
	1:128	428272	103	
	1:256	385664	---	

Sensitivity

Sensitivity 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). This value was determined to be 146.3 pg/mL.

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	---	9518	---
1:2	4759	4895	103%
1:4	2380	2500	105 %
1:8	1190	1216	102%
1:16	595	606	102%

Precision

Intra-assay precision was determined by assaying 16 replicates of three buffer controls containing 12(S)-HETE in a single assay.

pg/mL	%CV
342	5.2
1,153	10.1
4,762	15.5

Inter-assay precision was determined by measuring buffer controls of varying 12(S)-HETE concentrations in multiple assays over several days.

pg/mL	%CV
224	4.1
1,127	9.1
5,294	20.8

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Notes

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MSDS (Material Safety Data Sheet) available online

Use of Product

This product contains research chemicals. As such, they should be used and handled only by or under the supervision of technically qualified individuals. This product is not intended for diagnostic or human use.

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