# ELISA PRODUCT INFORMATION & MANUAL

# Non-species specific 12S-HETE ELISA Kit (Colorimetric)

## *NBP3-07908*

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

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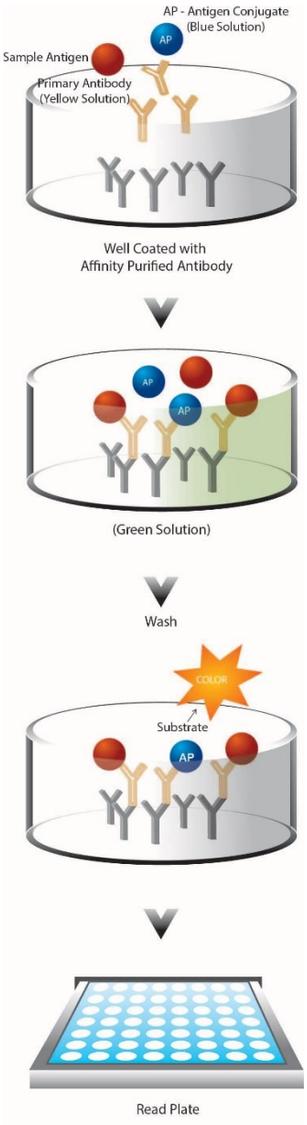
# Product Manual

## TABLE OF CONTENTS

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

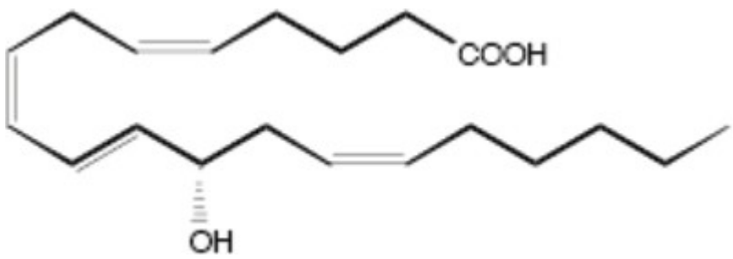

Introduction ..... 2
Principle ..... 2
Materials Supplied ..... 3
Storage ..... 4
Other Materials Needed ..... 4
Reagent Preparation ..... 5
Sample Handling ..... 6
Assay Procedure ..... 8
Calculation of Results ..... 9
Typical Results ..... 10
Typical Standard Curve ..... 10
Performance Characteristics ..... 11
Sample Recovery ..... 12
References ..... 15
Contact Information ..... 18




## 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 405nm. The amount of signal is indirectly proportional to the amount of 12(S)-HETE in the sample.

## MATERIALS SUPPLIED

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

1. **Assay Buffer Concentrate, 27ml**

   Tris buffered saline containing proteins and sodium azide as preservative

2. **12(S)-HETE Standard, 3x0.5ml**

   A solution of 500,000pg/ml 12(S)-HETE

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

3. **Goat anti-Rabbit IgG Microtiter Plate, 5 plates of 96 wells**

   A clear plate of break-apart strips coated with a goat anti-rabbit polyclonal antibody

4. **12(S)-HETE Antibody, 25ml**

   A yellow solution of a rabbit polyclonal antibody to 12(S)-HETE

5. **12(S)-HETE Conjugate, 25ml**

   A blue solution of 12(S)-HETE conjugated to alkaline phosphatase

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

6. **Wash Buffer Concentrate, 100ml**

   Tris buffered saline containing detergents

7. **pNpp Substrate, 100ml**

   A solution of p-nitrophenyl phosphate

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

8. **Stop Solution, 30ml**

   A solution of trisodium phosphate in water

9. **12(S)-HETE Assay Layout Sheet**

   1 each

10. **Plate Sealer**

    10 each

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.

Reagents require separate storage conditions.

## 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.

## OTHER MATERIALS NEEDED

1. Deionized or distilled water
2. Precision pipets for volumes between 5µl and 1,000µl
3. Repeater pipet for dispensing 50µl and 200µl
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 405nm

## REAGENT PREPARATION

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

### 1. Wash Buffer

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

### 2. Assay Buffer

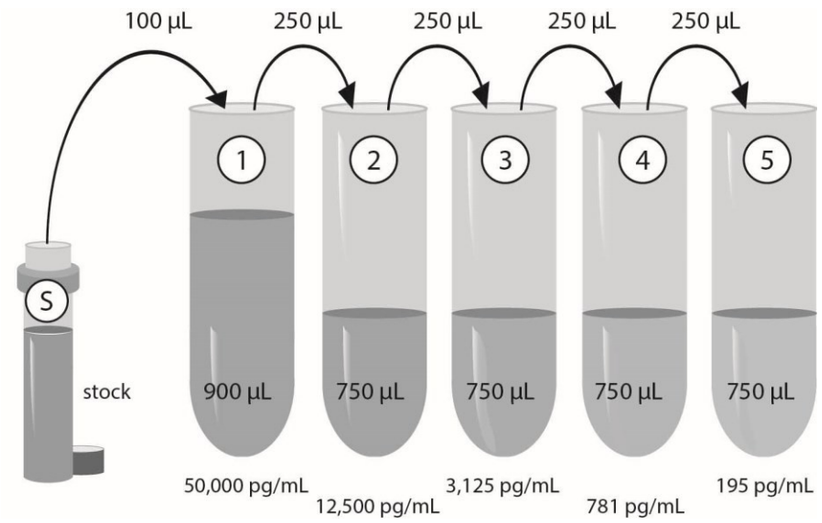
Glass or polypropylene tubes may be used for standard preparation. Avoid polystyrene.

Just before use, prepare the assay buffer by diluting 10 mL of the supplied Assay Buffer Concentrate with 90 mL of deionized water. Discard unused buffer or add up to 0.09% sodium azide (w/v) for storage.

### 3. Conjugate 1:10 Dilution for Total Activity Measurement

Prepare the Conjugate 1:10 Dilution by diluting 50µl of the supplied Conjugate with 450µl 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.

### 4. Preparation of 12(S)-HETE Standard Curve




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

## 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. 200mg C18 Reverse Phase Extraction Columns.

### Extraction Procedure

1. Acidify the sample by addition of 2M HCl to pH of 3.5. Approximately 50µl 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 10ml of 100% ethanol followed by 10ml of deionized water.

3. Apply the sample under a slight positive pressure to obtain a flow rate of about 0.5ml/minute. Wash the column with 10ml of water, followed by 10ml of 15% ethanol, and finally 10ml hexane. Elute the sample from the column by addition of 10ml ethyl acetate.
4. If analysis is to be carried out immediately, evaporate samples under a stream of nitrogen. Add at least 250µl 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

**Bring all reagents to room temperature for at least 30 minutes prior to opening. All standards and samples should be run in duplicate.**

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

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 re-seal the bag. Store unused wells at 4°C.

All standards and samples should be run in duplicate.

2. Pipet 100µl of standard diluent (Assay Buffer or 1:2 diluted culture media) into the NSB and the Bo (0pg/ml) wells.
3. Pipet 50µl of Assay Buffer into the NSB wells.
4. Pipet 100µl of Standards #1 through #5 in to the appropriate wells.

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

5. Pipet 100µl of the Samples into the appropriate wells.
6. Pipet 50µl of blue conjugate into each well except the Blank and TA wells.
7. Pipet 50µl 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. Seal the plate. Incubate for 2 hours on a plate shaker (~500 rpm) at room temperature.

Pipet the reagent to the side of the wells to avoid possible contamination.

9. Empty the contents of the wells and wash by adding 400µl 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.
10. Pipet 5µl of the blue conjugate 1:10 dilution (see Step 2, Reagent Preparation, on page 5) to the TA wells.
11. Add 200µl of the substrate solution into each well.

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

12. Incubate at 37°C for 3 hours without shaking.
13. Pipet 50µl of stop solution into each well.
14. After blanking the plate reader against the substrate blank, read optical density at 405nm. If plate reader is not capable of adjusting for the blank, manually subtract the mean OD of the substrate blank from all readings.

## 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

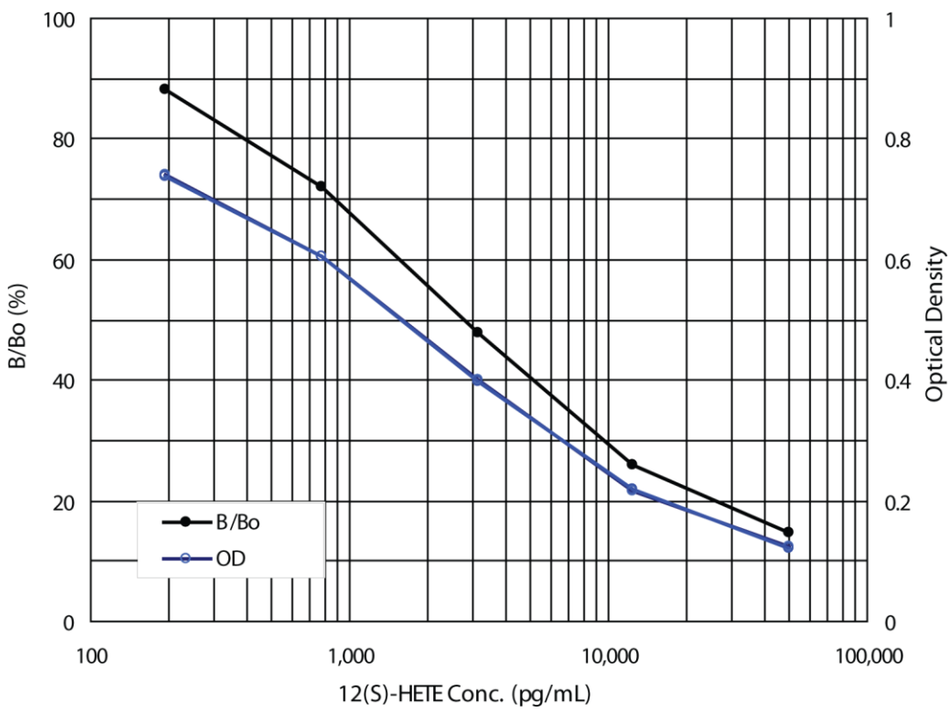
3. Plot the Percent Bound (B/Bo) versus concentration of 12(S)-HETE for the standards. Approximate a straight line through the points. The concentration of the unknowns can be determined by interpolation.

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

## TYPICAL RESULTS

| Sample | Average Net OD | 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** |

## TYPICAL STANDARD CURVE



## PERFORMANCE CHARACTERISTICS

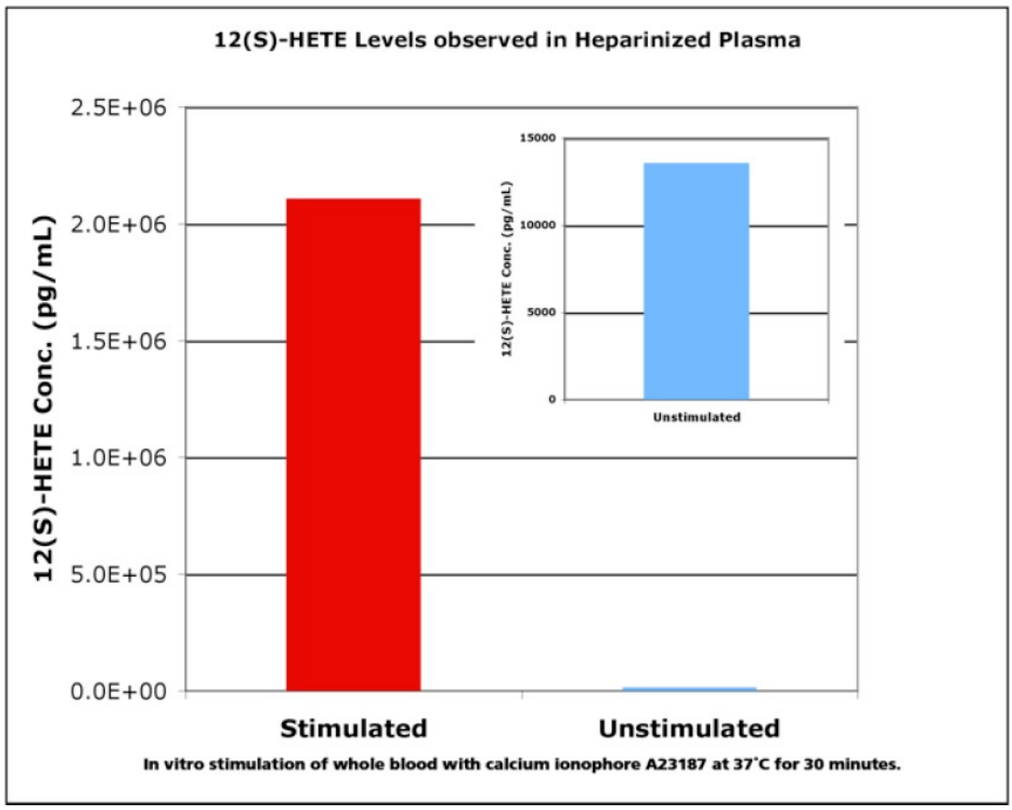
### Specificity

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 $B_2$ | 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 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 Dilution |
|---|---|---|
| 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 Heparinized 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 | --- | |

## Sensitivity

Sensitivity was calculated as the ratio of the mean OD plus 2 standard deviations of 16 replicates of the 0pg/ml standard to the mean of 16 replicates of the lowest standard, multiplied by the concentration of that standard (195pg/ml). This value was determined to be 146.3pg/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 | --- | 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% |

## 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

## NOTES

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