



20 – HETE ELISA Kit

Catalog Number: 20H39-K01 (1 x 96 wells)
For Research Use Only. Not for use in diagnostic procedures.
v. 2.1 (02 NOV 23)

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INTENDED USE

The Eagle Biosciences 20-Hete ELISA Assay kit is intended for the quantitative determination of 20-hete in biological samples by enzyme linked immunoassay (ELISA). The 20-Hete ELISA Assay kit is for research use only and not to be used in diagnostic procedures

For further information about this kit, its application, or the procedures in this insert, please contact the Technical Service Team at Eagle Biosciences, Inc at www.EagleBio.com or at 866-411-8023.

ASSAY BACKGROUND

The specificity of the 20-HETE ELISA Assay kit was investigated using authentic 20-HETE and a panel of fatty acids which, based on their structure, might be anticipated to compete with 20HETE for binding to antibodies for 20-HETE. Anti-20-HETE did not cross-react with 14, 15- and 11,12DHETs, PGE2 and showed almost no cross-reactivity even with structurally extremely similar arachidonic acid (AA), linoleic acid and linolenic acid as shown in the competitive ELISA analysis. Considering the only difference between 20HETE and AA is an oxygen molecule, the specificity of the Eagle Biosciences 20-HETE ELISA is a surprise. Human essential and salt-sensitive hypertension were related to differential AA metabolism by cytochrome P450 (CYP) 4A which has AA- ω -hydroxylase (20-HETE synthesis) activity. Increased circulating insulin inhibits 20-HETE synthesis in obese hypertensive subjects. Recently, CYP4F2 genetic variants, which increased urinary 20-HETE secretion, were found to be correlated with the risk for hypertension in a Chinese population.

The 20-Hete ELISA Assay kit can be used for the determination of 20-HETE in serum, plasma, cells, and tissues following proper isolation and purification. Instructions are provided as to the proper isolation and purification in the following pages.

STORAGE AND STABILITY

The 20-Hete ELISA Assay kit will obtain optimal results if all of the components are stored at the proper temperature prior to use. Items should be stored at the designated temperatures upon receipt of the 20-Hete ELISA Assay kit. All components are stored below -20°C and should not be re-frozen and thawed more than necessary.

Item	Description	Quantity
20-HETE ELISA Plate	Solid 96-well plate coated with anti-20-HETE antibody per well	1
20-HETE Standard (2 μ L)	Stock standard at a concentration of 1 mg/mL	1
20-HETRE HRP Conjugates (12 μ L)	1000 X concentrated solution	1
Sample Dilution Stock Buffer (25 mL)	10 X solution of Tris-buffered saline with preservatives	1



HRP Buffer (15 mL)	1 X solution of Tris-buffered saline with preservatives	1
Wash Buffer Stock Solution (25 mL)	10 X solution of Tris-buffered saline with detergents and preservatives	1
TMB Substrate (22 mL)	A solution of TMB (tetra methyl benzadine)	1

MATERIALS REQUIRED BUT NOT PROVIDED

Plate reader with a 450 nm filter

- An 8-channel adjustable pipetter and an adjustable pipetter
- Storage bottles
- Costar® cluster tubes (1.2 mL) and microcentrifuge tubes
- Deionized water

PRECAUTIONS

- **Please read all instructions carefully before beginning the 20-Hete ELISA Assay kit.**
- The reagents in the 20-Hete ELISA Assay kit have been tested and formulated to perform optimally. The 20-Hete ELISA Assay kit may not perform correctly if any of the reagents are replaced or any of the procedures are modified.
- The 20-Hete ELISA Assay kit is intended for research use only and is not to be used as a diagnostic.

PROCEDURAL NOTES

- Remove all of the reagents required, including the TMB, and allow them to equilibrate to room temperature before proceeding with the assay.
- It is necessary to thoroughly mix the concentrated buffer solutions. A stir bar is contained within each buffer solution.

SAMPLE PREPARATION

There are different protocols for isolating and purifying 20-HETE depending on the medium in which it is in. Listed below are the different protocols. For optimal results follow the appropriate protocol based on the biological sample present.

20-HETE measurement in cells expressing Cytochrome P450 4A

1. Collect and homogenize and/or sonicate the cells using a solution containing a final concentration of ~0.1 mM TPP (triphenylphosphine, 0.03-0.05 mg/mL). TPP is an antioxidant, which looks like a precipitate in samples because it does not easily dissolve. Before using the stored samples containing TPP, spin samples to separate the precipitated TPP from sample solution
2. Acidify the whole homogenized cells with acetic acid to a pH of approximately 3-4. Measure using standard pH paper.



3. Extraction with ethyl acetate. Add an equal volume of ethyl acetate to the homogenized cells and vortex very well. Place the upper organic phase into a fresh clean tube after centrifugation. Then add another equal volume of ethyl acetate to the homogenized cells to start the second-time extraction. It is strongly recommended that extraction is performed three times.
4. Evaporate the pooled ethyl acetate from the extractions until all has dried up under argon or nitrogen gas.
5. **Saponification if needed (see below)**
6. Add 20 μ L ethanol, or N, N-dimethyl-formamide (DMF), to dissolve the dried-up residue for reconstitution. Add 0.5 mL of 1x Sample Dilution Buffer (provided in the 20-HETE ELISA Assay kit) to make a solution. Load 100 μ L in each well, in triplicates, on the ELISA plate. (Note: We recommend measuring a different dilution of sample in attempt to fit the results to the standard curve. e.g., load 3 wells with 50 μ L of the rest of sample plus 50 μ L of 1x Sample Dilution Buffer, and 3 wells with 10 μ L of the rest of sample plus 90 μ L of 1x Sample Dilution Buffer.)
7. Perform the ELISA for 20-HETE (according to the instructions of the manufacturer).

Saponification (to cleave fatty acid from glycerol backbone):

1. Dissolve dried fatty acids (obtained from 3X ethyl acetate extractions) in 2 mL of 20% KOH solution (make working solution: 1 mL of 2 M KOH + 4 mL methanol so that the final conc. of KOH = 0.4 N).
2. Vortex and incubate for 1 h at 50°C.
3. Add 1.5X H₂O to the solution and adjust pH with 20% formic acid to pH ~5.
4. Re-extract the solution with ethyl acetate (1 part aqueous solution + 1 part ethyl acetate) and dry.

20-HETE measurement in tissues

1. Homogenize 1 g of tissue, 4 mL of H₂O, and 0.01 mg TPP.
2. Acidify the homogenate by adding 8 μ L of acetic acid to each homogenate.
3. Extract with an equal amount of ethyl acetate, vortex thoroughly, spin down, and collect the organic phase. Repeat this extraction twice more and combine all of the organic phases.
4. Dry the organic phase with argon or nitrogen gas.
5. Saponification if needed (see 20-HETE measurement in cells)
6. Dissolve the dried residue from above step # 4 with N,N-dimethyl-formamide (DMF). (Add approximately 20 μ L of ethanol or DMF to reconstitute the dried-up residue.)
7. Dilute further with 1x Sample Dilution Buffer: Add approximately 0.5 mL of 1x Sample Dilution Buffer and centrifuge at 10,000 rpm for five minutes at room temperature. The supernatant will be used for ELISA.
8. Perform the ELISA for 20-HETE (according to the instructions of the manufacturer).

20-HETE measurement in plasma or serum

Below is a protocol for measuring 20-HETE in plasma or serum using a starting volume of 1.0 mL of fluid. Starting volumes below 1.0 mL can also be used (i.g., for mouse studies) for measuring 20-HETE by making the appropriate proportional adjustments to all volumes and dilutions. Please contact our technical team at info@eaglebio.com for any assistance.



1. Combine 1.0 mL of plasma (adjusted with approximately 20 μ L of acetic acid to pH 4) and 1.0 mL of ethyl acetate. Vortex thoroughly. Centrifuge at 2000 rpm for ten minutes at 22°C. Three phases should result:
 - a) Upper organic phase – ethyl acetate phase (lipoproteins)
 - b) Interphase – proteins
 - c) Lower phase – aqueous phase
2. Collect the upper organic phase (a) and set aside.
3. Discard the interphase. Transfer the lower phase with a glass pipette to a new tube, and repeat the ethyl acetate extraction step 2 more times.
4. Evaporation of pooled organic phase: There should be approximately 5-6 mL of the ethyl acetate phase (a). Dry the pooled organic phase in a Speed vac to get the extracted sediment (b).
5. Saponification (to cleave fatty acid from glycerol backbone): Dissolve the dried-up residues (b) in 2 mL of 20% KOH solution (for preparation see 20-HETE in cells). Vortex thoroughly and incubate for 1 h at 50°C. This will yield an aqueous solution (c).
6. Dilute 2 mL of the aqueous solution (c) with 3 mL of H₂O. Adjust the pH using 20% formic acid (132 μ L) to pH ~5.5. Add ethyl acetate (1 part aqueous solution (c) + 1 part ethyl acetate), vortex thoroughly, and centrifuge at 2000 rpm for ten minutes at 22°C. Repeat the procedure twice more using an equal volume of ethyl acetate per sample. Collect the upper phase with saponified lipids.
7. Dry the pooled ethyl acetate upper phase (d) in a Speed vac, yielding the dried sample sediment (e). Store the sediment (e) at -20°C. For ELISA assay, dissolve the dried sample residue (e) in 20 μ L of N,N-dimethyl-formamide (DMF), then add 130 μ L of 1x Sample Dilution Buffer.
8. . For the competitive 20-HETE ELISA, the above 150 μ L sample needs to be further diluted: Dilute 1:4 (e.g., 80 μ L sample + 320 μ L 1x Sample Dilution Buffer). Check the final pH (should be pH 7.4). When calculating the concentration, consider the dilution factor. In this case, 150 μ L total sample volume from 1.8 mL plasma (12-fold concentration) and then, 80 sample in 400 μ L SDB (5-fold dilution). Since, the samples are concentrated 2.4 fold; to get the actual concentration, you must divide by 2.4.
9. Perform the ELISA for 20-HETE (according to the instructions of the manufacturer).

20-HETE measurement in urine

1. Extraction using ethyl acetate is not necessary. It is recommended that the urine sample be diluted 4- to 10-fold with 1X sample dilution buffer and 100 μ L of sample added directly to the ELISA plate well.
2. When calculating the concentration, consider the dilution factor.

ASSAY PREPARATIONS

The solid 96-well plate and TMB solution are provided ready to use. The preparations of other assay reagents are detailed below.

Wash Buffer: Mix the solution with a stir bar, applying low heat until a clear colorless solution is obtained. Dilute the entire contents of the Wash Buffer Concentrate (25 mL) with 225 mL of deionized water to yield a final volume of 250 mL of 1 X Wash Buffer. This can then be refrigerated for the entire life of the 20-Hete ELISA Assay kit.



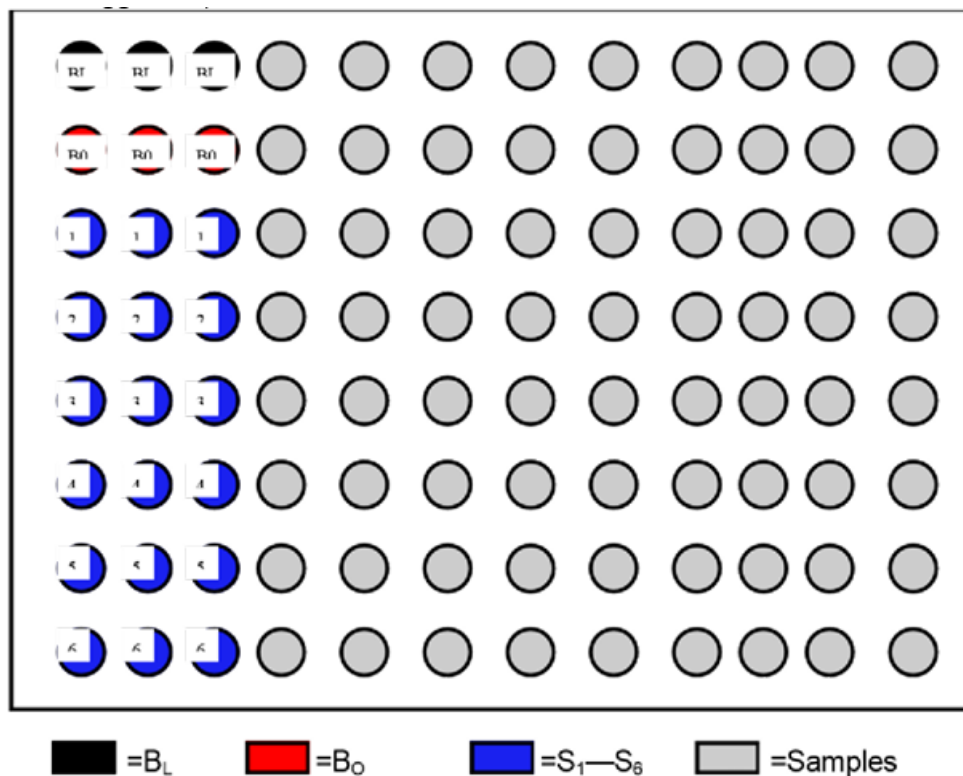
HRP Conjugate: Dilute 1 vial of the 20-HETE-HRP conjugate (0.012 mL) with 12.00 mL of 1 X HRP buffer. One vial makes enough conjugate for one plate. The conjugate must be used the same day and should not be stored for later use.

Standards: Label 5 microtubes as Standard 1 through Standard 5. Dilute the entire contents of Sample Dilution Stock buffer (25 mL) with 225 mL deionized water to yield a final volume of 250 mL of 1 X Sample Dilution Buffer. Add 0.9 mL of the Sample Dilution Buffer to the microtubes 1 to 5. Spin down the enclosed 20-HETE standard vial (2 μ L, filled with inert gas) and add 1.998 mL of Sample Dilution Buffer to obtain 2 mL of solution. Label this Standard 6. Add 0.1 mL of Standard 6 to the microtube labeled Standard 5 and mix thoroughly. Next, add 0.1 mL of Standard 5 into the microtube labeled Standard 4 and mix thoroughly. Continue to serially dilute the standards using 1:10 dilutions for the remaining standards.

Samples: Samples can be directly diluted into the 1 X Sample Dilution Buffer if it is in solution. For extracted and dried samples, it is recommended to dissolve the dried-up samples with a minimal amount of ethanol or N, N-dimethyl-formamide (DMF, 10 μ L to 20 μ L) and vortex well. Before ELISA assay, add 100 μ L of 1 X Sample Dilution Buffer to make the stock sample solution ready for quantification with ELISA. The stock sample solution can be further diluted to a proper range of concentration for the 20-HETE ELISA Assay test.

PERFORMING THE ASSAY

Plate Setup: Each plate must contain a minimum of three blank wells (BL), three maximum binding wells (BO), and a six point standard curve (S1-S6). Each sample should be assayed in triplicate. A suggested plate format is shown below:





Standard Dilutions Table

Standards	Final Concentration (pg/mL)	Add Sample Dilution Buffer (mL)	Serial Dilutions Procedure
No. 6	1,000,000	1.998	2 μ L of stock solution.
No. 5	100,000	0.9	Add 0.1 mL of No. 6
No. 4	10,000	0.9	Add 0.1 mL of No. 5
No. 3	1,000	0.9	Add 0.1 mL of No. 4
No. 2	100	0.9	Add 0.1 mL of No. 3
No. 1	10	0.9	Add 0.1 mL of No. 2

ASSAY PROCEDURE

1. Load 200 microliters of Sample Dilution Buffer into the blank (BL) wells and 100 microliters of Sample Dilution Buffer into the maximum binding (BO) wells.
2. Load 100 microliters of each of the standards into the appropriate wells.
3. Load 100 microliters of each of the samples into the appropriate wells.
4. Load 100 microliters of the diluted 20-HETE-HRP conjugate in the BO wells, the standard wells, and the sample wells. Do NOT add HRP conjugate into the BL wells.
5. Incubate the plate at room temperature for two hours.
6. Wash the plate three times with 400 microliters of the diluted Wash Buffer per well.
7. After the last of the three wash cycles pat the plate dry onto some paper toweling.
8. Add 200 microliters of the TMB substrate to all of the wells (including BL wells).
9. Incubate the plate at room temperature for 15-30 minutes.
10. Add 50 microliters of 2 N sulfuric acid to all of the wells.
11. Read the plate at 450 nm.

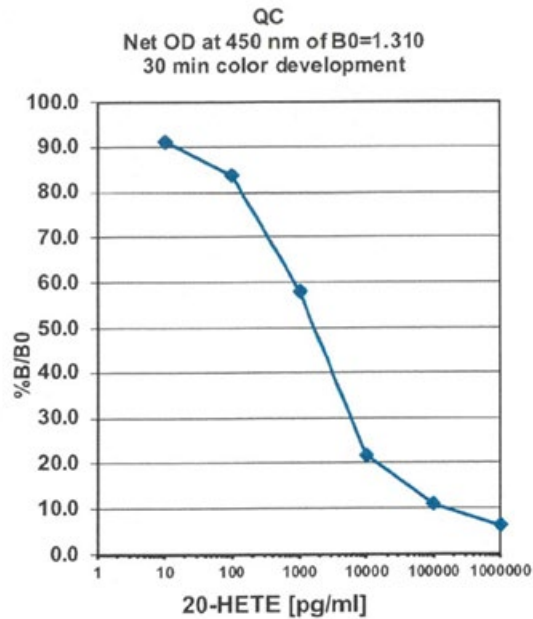
CALCULATION OF RESULTS

Most plate readers provide data reduction software that can be used to plot the standard curve and determine the sample concentrations. If your plate reader does not have this option, then a data reduction program can be used (4 parameter of log-log curve fit). If you do not have these options, the results can be obtained manually as follows:

1. Average the absorbance readings from the blanks and subtract that value from each well of the plate to obtain the corrected readings. (Note: Some plate readers do this automatically. Consult the user manual of your plate reader.)
2. Average the corrected absorbance readings from the BO wells. This is your maximum binding.
3. Calculate the %B/BO for Standard 1 by averaging the corrected absorbance of the two S1 wells, divide the average by the maximum binding, then multiply by 100. Repeat this formula for the remaining standards.



4. Plot the %B/BO versus the concentration of 20-HETE from the standards using semi-log paper.
5. Calculate the %B/BO for the samples and determine the concentrations, utilizing the standard curve.
6. Multiply the concentrations obtained for each of the samples by their corresponding dilution factor.



The data shown here is an example of typical results obtained using the 20-HETE ELISA kit. These results are only a guideline, and should not be used to determine values from your samples. The user must run their own standard curve every time.

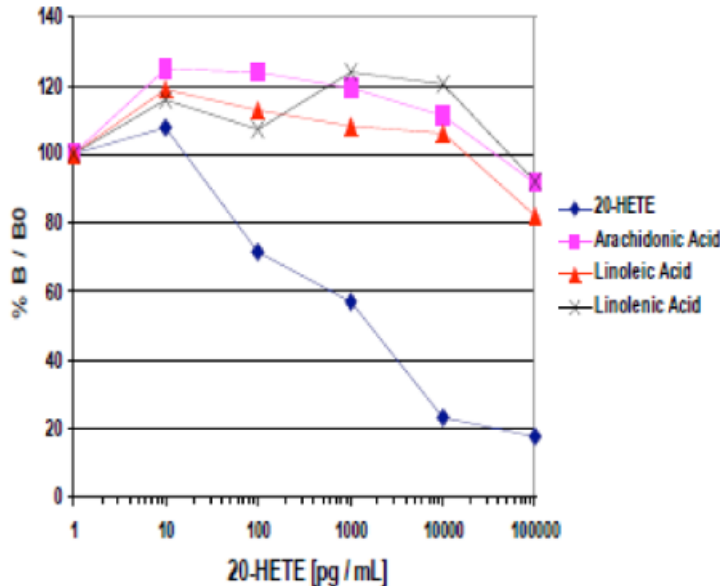
BL wells = 0.063
BO wells = 1.667

Standard	Concentration	O.D.	%B/B ₀
No. 1	10 pg/mL	1.630	91.2
No. 2	100 pg/mL	1.597	83.6
No. 3	1,000 pg/mL	1.319	58.0
No. 4	10,000 pg/mL	0.687	21.7
No. 5	100,000 pg/mL	0.428	10.9
No. 6	1,000,000 pg/mL	0.356	6.1



SPECIFICITY OF ANTI-20-HETE

Anti-20-HETE did not cross-react with 14,15- and 11,12-DHETs, PGE2 and showed almost no cross-reactivity even with structurally extremely similar arachidonic acid, linoleic acid and linolenic acid as shown in the competitive ELISA analysis (shown below).



TROUBLESHOOTING

No color present in standard wells.

- The HRP conjugate was not added. Redo the assay and add the conjugate at the proper step.
- The HRP conjugate was not incubated for the proper time. Redo the assay and incubate for the proper time.

No color in any wells, including the TA wells.

- The TMB substrate was not added. Add substrate.
- The TMB substrate was not incubated for the proper time. Continue incubation until desired color is reached.

The color is faint.

- One or all of the incubation times were cut short. Redo the assay with the proper incubation times.
- The TMB substrate was not warmed up to room temperature. Redo the assay making sure all reagents are at room temperature.
- The lab is too cold. Be sure the lab temperature is between 21-27°C and redo the assay.

The background color is very high.

- The TMB substrate has been contaminated. Redo the assay with a fresh bottle of substrate.



Scattered O.D. obtained from the sample.

- Redo assay using an 8-channel pipetman making sure that 8 channels are equal volume while loading.

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