20-HETE Glucuronide Test Kit

For measuring free and glucuronidated 20-HETE

Catalog Number: **20HG1**

Introduction

This competitive ELISA kit is for determination of free and glucuronidated 20-HETE (also known as 20-OH-AA) levels in biological samples. The specificity of the 20-HETE ELISA was investigated using authentic 20-HETE and a panel of fatty acids which, based on their structure, might be anticipated to compete with 20-HETE for binding to antibodies for 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 (AA), linoleic acid and linolenic acid. Human essential and salt-sensitive hypertensions 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.

This kit can be used for the determination of glucuronidated 20-HETE as provided in the following pages. High levels of the glucuronidated form of 20-HETE have been found in human urine.

This competitive ELISA kit, based on competition between 20-HETE epitope and the 20-HETE-HRP conjugate for a limited number of binding sites available from the anti-20-HETE antibody, which is coated to the wells of the 96 well ELISA plate. The conjugate concentration is held as a constant in each well, while the concentration of the 20-HETE is variable, based on the concentration of the sample or standard. Thus, the amount of the 20-HETE conjugate which is able to bind to each of the wells is inversely proportional to the concentration of 20-HETE in the standard or sample. The amount of the conjugate which is bound to each well is then determined by the amount of color obtained, when TMB is added. The TMB reacts with the HRP available in the well. With the addition of sulfuric acid, the blue colored product is converted into a yellow-colored product, which can be read on a plate reader at 450 nm.

Storage and Stability

This 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 this kit.

All components are stored below -20°C and should not be re-frozen and thawed more than necessary.

Precautions

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

Materials Provided

Part Number	Item	Description	Quantity
1	20-HETE ELISA Plate	Solid 96-well plate coated with anti-20-HETE antibody in each well	1
2	20-HETE Standard (2 µL)	Stock standard at a concentration of 1 mg/mL	1
3	20-HETE-HRP Conjugates (12 µL)	1000 X concentrated solution	1
4	Sample Dilution Buffer (25 mL)	10 X solution of Tris-buffered saline with preservatives	1
5	HRP Buffer (15 mL)	1 X solution of Tris-buffered saline with preservatives	1
6	Wash Buffer Solution (25 mL)	10 X solution of Tris-buffered saline with detergents and preservatives	1
7	TMB Substrate (22 mL)	A solution of TMB (tetra methyl benzadine)	1
8	Beta-Glucuronidase enzyme	8 mg solid	1

Additional Required Materials (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
- 2 N Sulfuric Acid

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.

Sample Preparations

20-HETE measurement in <u>urine.</u>

1. Extraction using ethyl acetate is not necessary to measure <u>free</u> 20-HETE and the net glucuronidated 20-HETE. It is recommended that the urine sample be diluted 4-fold with 1X sample dilution buffer.
2. To measure net glucuronidated level of 20-HETE, it is necessary to treat the urine sample with the enzyme beta-glucuronidase (see protocol below).
3. When calculating the concentration of 20-HETE consider any dilution factors used.

<u>Free and Glucuronidated 20-HETE Measurement (no extraction method)</u>

Materials

1. Dissolve 8 mg of β-glucuronidase (provided) in 8 mL of 1 M citric acid, adjust to pH 5.5. (400 U/mL).

Protocol

A. Measurement of free 20-HETE: Dilute urine 4-fold with 1X Sample Dilution Buffer and apply to ELISA plate (100 µL/well). A 4X dilution is recommended although other dilution factors may be tried, too.

B. Measurement of glucuronidated 20-HETE

This method is for determining the level of glucuronidated 20-HETE in urine after digestion of the molecule with glucuronidase. Subtract the level of the molecule in the sample without glucuronidase from the levels in the sample with glucuronidase after complete digestion (usually 3 hrs) to obtain the level of glucuronidated molecule. By then adding the values obtained in parts A and B, one has a measure of total 20-HETE in the urine sample.

β-Glucuronidase digestion

1. Dilute 1 mL of urine 4-fold with 1X Sample Dilution Buffer.
2. Divide into two 2 mL aliquots.
3. To one 2 mL aliquot, add 0.5 mL of the β-glucuronidase solution, pH 5.5 (pH < 6.0).
4. To the other 2 mL aliquot of urine, add 0.5 mL of 1 M citric acid buffer, pH 5.5.
5. Incubate both aliquots at 37°C for 3 hours.
6. Follow the instructions for the ELISA kit (see below). Make sure that all dilution factors are accounted for in calculating the amounts of free and glucuronidated 20-HETE.
7. To calculate the amount of glucuronidated 20-HETE, subtract no-enzyme value from the with enzyme value.
8. To calculate total amount of 20-HETE in the sample, add the value obtained in Step 7 to the amount of free 20-HETE in Part A.

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 kit.

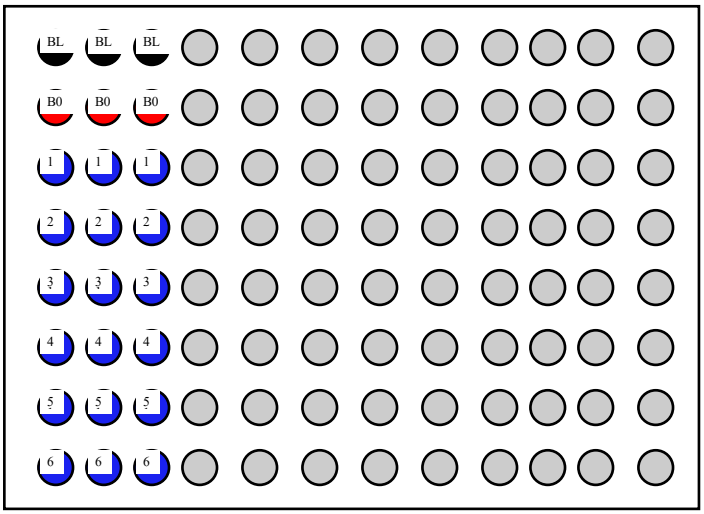
HRP Conjugate: Dilute 1 vial of the 20-HETE-HRP conjugate (0.012 mL) with 12.00 mL of 1 X HRP buffer. One vial makes enough conjugate for one plate. The conjugate must be used the same day and should not be stored for later use.

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 for Standards 1 to 5. Spin down the enclosed 20-HETE standard vial (2 µL, 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 the 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 of N, N-dimethyl-formanmide (DMF, 10 µL to 20 µL) and vortex well. Before ELISA assay, add 100 µL 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 ELISA test.

Performing the Assay

Plate Setup: Each plate must contain a minimum of three blank wells (B_L), three maximum binding wells (B_O), and a six point standard curve (S_1-S_6). Each sample should be assayed in triplicate. A suggested plate format is shown below:



Standard Dilutions Table =B_L =B_O =S_1—S_6 =Samples

Standards	Final Concentration (pg/mL)	Add Sample Dilution Buffer (mL)	Serial Dilutions Procedure
No. 6	1,000,000	1.998	2 µL 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

Step 1: Load 200 microliters of Sample Dilution Buffer into the blank (B_L) wells and 100 microliters of Sample Dilution Buffer into the maximum binding (B_O) wells.

Step 2: Load 100 microliters of each of the standards into the appropriate wells.

Step 3: Load 100 microliters of each of the samples into the appropriate wells.

Step 4: Load 100 microliters of the diluted 20-HETE-HRP conjugate in the B_O wells, the standard wells, and the sample wells. Do NOT add HRP conjugate into the B_L wells.

Step 5: Incubate the plate at room temperature for two hours.

Step 6: Wash the plate three times with 400 microliters of the diluted Wash Buffer per well.

Step 7: After the last of the three wash cycles pat the plate dry onto some paper toweling.

Step 8: Add 200 microliters of the TMB substrate to all of the wells (including B_L wells).

Step 9: Incubate the plate at room temperature for 15-30 minutes.

Step 10: Add 50 micoliters of 2 N sulfuric acid to all of the wells.

Step 11: Read the plate at 450 nm.

Calculating the 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 B_O wells. This is your maximum binding.
3. Calculate the %B/B_O for Standard 1 by averaging the corrected absorbance of the two S_1 wells, divide the average by the maximum binding, then multiply by 100. Repeat this formula for the remaining standards.
4. Plot the %B/B_O versus the concentration of 20-HETE from the standards using semi-log paper.
5. Calculate the %B/B_O 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.

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.

Warranty

Detroit R&D, Inc., makes no warranty of any kind expressed, or implied, including, but not limited to the warranties of fitness for a particular purpose and merchantability.

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