

## 20-HETE Glucuronide ELISA kit

Catalog Number: **20HG1/20HG11/20HG21/20HG101**

Store at -20°C.

FOR RESEARCH USE ONLY



### 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, PGE<sub>2</sub> 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 20-HETE and AA is an oxygen molecule, the specificity of the Detroit R&D 20-HETE ELISA is a surprise.

Human essential and salt-sensitive hypertension was related to differential AA metabolism by cytochrome P<sub>450</sub> (CYP) 4A which has AA- $\omega$ -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 free 20-HETE and glucuronidated 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

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.

### 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 $\mu$ L)	Stock standard at a concentration of 1 mg/mL	1
3	20-HETE HRP Conjugates (12 $\mu$ L)	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 (24 mL)	A solution of TMB (tetra methyl benzadine)	1
8	Beta-glucuronidase	8 mg /vial	

### Additional Required Materials (Not Provided)

- Storage bottles
- Costar® cluster tubes (1.2 mL) and microcentrifuge tubes
- Deionized waterF
- 1 M Citric Acid
- 2N Sulfuric Acid

### 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.

### 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 Preparations

#### MATERIALS

1. Dissolve 8 mg of beta-glucuronidase (provided) in 8 mL of 1 M citric acid, adjust to pH 5.5.
2. 20-HETE ELISA Kit Cat# 20H1 (provided)
3. 10X Sample Dilution Buffer (provided in kit); Dilute to 1X with deionized water for use in protocol and ELISA

#### PROTOCOLS

**A. Measurement of free 20-HETE in cell lysates, urine, blood and media** There are different protocols for isolating and purifying free 20-HETE depending on the type of sample. Please follow the protocol for the type of biological sample you are using.

#### 20-HETE measurement in cells expressing Cytochrome P<sub>450</sub> 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  $\mu$ L N, N-dimethyl-formamide (DMF), to dissolve the dried-up residue for reconstitution. Add 0.5 mL of 1x Sample Dilution Buffer (provided in kit) to make a solution. Load 100  $\mu$ 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  $\mu$ L of the rest of sample plus 50  $\mu$ L of 1x Sample Dilution Buffer, and 3 wells with 10  $\mu$ L of the rest of sample plus 90  $\mu$ 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 H<sub>2</sub>O 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 or less of tissue in 4 mL of H<sub>2</sub>O, and 0.01 mg TPP.
2. Acidify the homogenate by adding 8  $\mu$ 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  $\mu$ L of 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**

1. Combine 1.8 mL of plasma (adjusted with approximately 20  $\mu$ L of acetic acid to pH 4) and 1.8 mL of ethyl acetate. Vortex thoroughly. Centrifuge at 2000 rpm for ten minutes at 22°C. Three phases should result:
  - i. Upper organic phase – ethyl acetate phase (lipoproteins)
  - ii. Interphase – proteins
  - iii. 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 Speedvac 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 H<sub>2</sub>O. Adjust the pH using 20% formic acid (132  $\mu$ 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 Speedvac, yielding the dried sample-sediment (e). Store the sediment (e) at -20°C. For ELISA assay, dissolve the dried sample-residue (e) in 20 µL N,N-dimethyl-formamide (DMF), then add 130 µL of 1x Sample Dilution Buffer.
8. For the competitive 20-HETE ELISA, the above 150 µL sample needs to be further diluted: Dilute 1:4 (e.g., 80 µL sample + 320 µL 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 µL total sample volume from 1.8 mL plasma (12-fold concentration) and then, 80 sample in 400 µL SDB (5-fold dilution). Since, the samples are concentrated 2.4-fold; to get the actual concentration, you must divide by 2.4.

Perform the ELISA for 20-HETE (according to the instructions of the manufacturer).

For free 20-HETE in cell lysates, extract the lysates using ethyl acetate as described below, dry and dissolve in 20 µL DMF and then dilute the dissolved sample with 1X Sample Dilution Buffer to 0.5 mL and apply sample to ELISA plate (100 µL/well). For free 20-HETE in media and urine, dilute media with Sample Dilution Buffer and apply to ELISA (100 µL/well). A 4X dilution is recommended.

**B. Measurement of glucuronidated 20-HETE in cell lysates (Based on  $5 \times 10^4$  cells/mL reaction mixture)**

**(i) Extraction**

1. Collect cells from reaction mixture by centrifuging at 300 x g.
2. Suspend cell pellet in 1.0 mL media, vortex vigorously, centrifuge at 300 x g, remove media leaving about 300 µL of media behind. Vortex vigorously.
3. For beta-glucuronidase digestion, bring up to 1.0 mL with 1X Sample Dilution Buffer and pipet 0.5 mL into two tubes.

**ii) Glucuronidase digestion**

**1 Add 200 µL of the beta-glucuronidase solution, pH 5.5, to one tube (see MATERIALS), final pH 5.5.**

**2 Add 200 µL of the 1 M citric acid solution to the second tube (no enzyme).**

**3 Incubate both tubes at 37°C for 3 hours.**

**4. If performing the ELISA immediately then go onto step (iii). If not, freeze immediately.**

4. Add 1.0 mL ethyl acetate to pellet and vortex vigorously.
5. Centrifuge at high speed to separate aqueous and organic layers. (If performing multiple extractions with ethyl acetate, combine the ethyl acetate fractions and proceed to next step).
6. Remove ethyl acetate layer and dry using a speed vac or under a gentle stream of nitrogen or argon.
7. Re-suspend dried extract in 20 µL ethanol, dissolve

**(iii) ELISA**

1. Follow instructions for ELISA kit. Samples may need to be diluted prior to adding to the ELISA plate. A 4X dilution is recommended.
2. To calculate the amount of glucuronidated 20-HETE, subtract the value of the zero time point from the 3-hour time point. Please note that the zero time point does not equal free 20-HETE, it is simply a starting value that must be subtracted from the 3 hour time point. To measure free 20-HETE, follow protocol in A.

**C. Measurement of glucuronidated 20-HETE in Media and Urine (Note—Extraction is not necessary).**

**(i) Beta-Glucuronidase digestion**

1. Pipet 0.5 mL of extracellular media or urine into two tubes.
2. Add 125 µL of the beta-glucuronidase enzyme to each tube (see MATERIALS), final pH 5.5.
3. Immediately flash-freeze one of the tubes. This is the zero time point.
4. Incubate the other tube at 37°C for 3 hours. This is the 3 hour time point.

**(ii) ELISA**

1. Follow instructions for ELISA kit. Samples may need to be diluted prior to adding to the ELISA plate. A 4X dilution is recommended.

2. To calculate the amount of glucuronidated 20-HETE, subtract the value of the zero time point from the 3 hour time point. Please note that the zero time point does not equal free 20-HETE, it is simply a starting value that must be subtracted from the 3 hour time point. To measure free 20-HETE, follow protocol in A.

## ELISA 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.

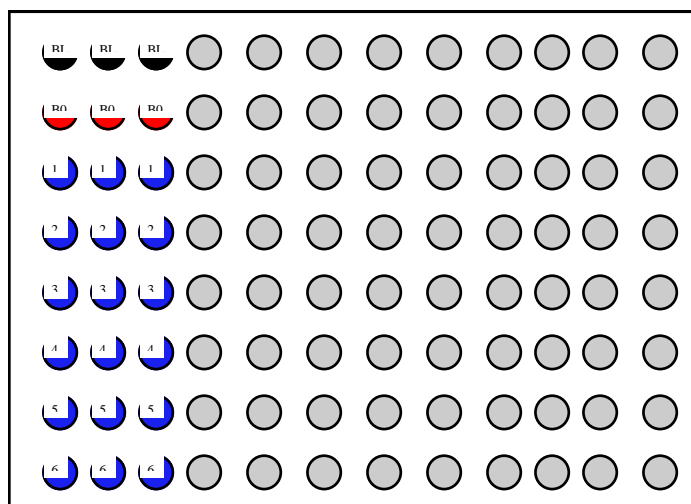
**HRP Conjugate:** Dilute 1 vial of the 20-HETE-HRP conjugate (0.012 mL) with 12.00 mL of 1 X HRP buffer. One vial makes enough conjugate for one plate. The conjugate must be used the same day and should not be stored for later use.

**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 1 to 5. Spin down the enclosed 20-HETE standard vial (2  $\mu$ 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 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-formamide (DMF, 10  $\mu$ L to 20  $\mu$ L) and vortex well. Before ELISA assay, add 100  $\mu$ 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 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:



 =B<sub>L</sub>    
  =B<sub>O</sub>    
  =S<sub>1</sub>—S<sub>6</sub>    
  =Samples

### Standard Dilutions Table

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

**Step 1:** Load 200 microliters of Sample Dilution Buffer into the blank (B<sub>L</sub>) wells and 100 microliters of Sample Dilution Buffer into the maximum binding (B<sub>O</sub>) 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<sub>O</sub> wells, the standard wells, and the sample wells. Do NOT add HRP conjugate into the B<sub>L</sub> 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<sub>L</sub> wells).

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

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

**Step 11:** Read the plate at 450 nm.

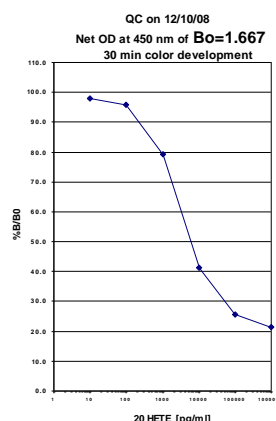
### Calculating the Results

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

## Typical Results



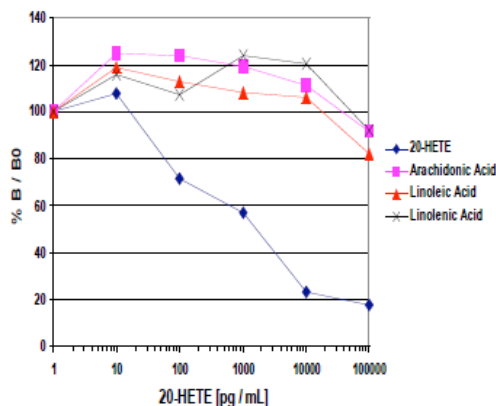
The data shown here is an example of typical results obtained using the Detroit R & D 20-HETE ELISA kit. These results are only a guideline, and should not be used to determine values from your samples. The user must run their own standard curve every time.

B<sub>L</sub> wells = 0.063  
B<sub>O</sub> wells = 1.667

Standard	Concentration	O.D.	%B/B <sub>O</sub>
No. 1	10 pg/mL	1.630	97.8
No. 2	100 pg/mL	1.597	95.8
No. 3	1,000 pg/mL	1.319	79.1
No. 4	10,000 pg/mL	0.687	41.2
No. 5	100,000 pg/mL	0.428	25.7
No. 6	1,000,000 pg/mL	0.356	21.3

## Specificity of anti-20-HETE

Anti-20-HETE did not cross-react with 14,15- and 11,12-DHETs, PGE<sub>2</sub> 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.

## References

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3. Dolegowska, B., Blogowski, W., Domanski, L.. Is it possible to predict the early post-transplant allograft function using 20-HETE measurements? A preliminary report. *Transpl Int.* 2009 22:546-553.
4. Liu et al. Overexpression of cytochrome P450 4F2 in mice increases 20-hydroxyeicosatetraenoic acid production and arterial blood pressure. *Kidney International* 75, 1288-1296, 2009.
5. Wang et al. Selective Inhibitors of CYP2J2 Related to Terfenadine Exhibit Activity Strongly against Human Cancers *in vitro* and *in vivo*. *JPET*, 152017, 2009.



## Warranty

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