14,15-DHET Human Urine Test Kit

For measuring free and glucuronidated 14,15-DHET

Catalog Number: **DH3, DH13, DH23, DH103**

V. 10232012

Introduction

This competitive ELISA kit is for determination of free and glucuronidated 14,15-DHET levels in urine. The 14,15-DHET is a representative metabolite of soluble epoxide hydrolase-mediated metabolism of EETs, which are generated by arachidonic acid epoxygenase activity of cytochromes P450. 14,15-DHET level exhibited strong positive correlation with hypertension in rat and human and brain injury and stroke in rodents. Human urine and blood 14,15-DHET levels were measured using the 14,15-DHET ELISA kit.

High levels of the glucuronidated form of 14,15-DHET have been found in human urine but not in urine collected from rodents. This kit can be used for the determination of free and glucuronidated 14,15-DHET in human urine following proper isolation and purification as provided in the following pages

This competitive ELISA kit, based on competition between the14,15-DHET epitope and the 14,15-DHET-HRP conjugate for a limited number of binding sites available from the anti-14,15-DHET 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 14,15-DHET is variable, based on the concentration of the sample or standard. Thus the amount of the 14,15-DHET conjugate which is able to bind to each of the wells is inversely proportional to the concentration of 14,15-DHET 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	14,15-DHET ELISA Plate	Solid 96-well plate coated with anti-14,15-DHET antibody in each well	1
2	14,15-DHET Standard (2 µL)	Stock standard at a concentration of 1 mg/mL	1
3	14,15-DHET-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 (24 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
- 2N 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. A stir bar is contained within each buffer solution.

Sample Preparations

Materials

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

Protocols

I. Measurement of A) free and B)glucuronidated 14,15-DHET in sample by extraction with ethyl acetate.

A. **Measurement of free 14,15-DHET:** Extract 4 mL of urine with ethyl acetate using the **Extraction Protocol** described below.

B. Measurement of free and 14,15-DHET glucuronide

This method is for determining the level of glucuronidated 14,15-DHET in urine after digestion of the molecule with glucuronidase. Collect the first sample as soon as the β-glucuronidase is added to a reaction mixture (0 hour digestion) and then a second sample at a time when digestion of the glucuronic acid moiety of the molecule is completed. Subtract the level of the molecule in the first sample at 0 hour from the levels in the second sample after complete digestion (usually 3 hrs) to obtain the level of glucuronidated molecule.

β-Glucuronidase digestion

1. To 4 mL of urine add 1 mL of the β-glucuronidase solution, pH 5.5, to each tube (pH < 6.0).
2. Immediately transfer 2 mL of urine to a clean tube and flash-freeze. This is the zero time point.
3. Incubate the remaining 2 mL at 37°C for 3 hours. This is the 3-hour time point.
4. **Extract**
5.

Extraction protocol

a) Combine an equal amount of urine sample from steps 2 and 3 above (adjusted with approximately 20 µL of acetic acid to pH 4) and ethyl acetate. Vortex thoroughly. Centrifuge at 2000 rpm for ten minutes at 22°C. Three phases should result:
b) Upper organic phase – ethyl acetate phase (lipoproteins)
c) Interphase – proteins
d) Lower phase – aqueous phase
e) Collect the upper organic phase (a) and set aside.
f) 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.
g) 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).
h) Saponification (to cleave fatty acid from glycerol backbone): Dissolve the dried residues (b) in 2 mL of 20% KOH solution (for preparation see 14,15-DHET measurement in cells). Vortex thoroughly and incubate for 1 h at 50°C. This will yield an aqueous solution (c).
i) Dilute 2 mL of the aqueous solution (c) with 3 mL of H_2O. Adjust the pH using 20% formic acid (132 µL) 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 containing saponified lipids.
j) Dry the pooled ethyl acetate upper phase (d) and dry in a Speedvac, yielding the dried sample-sediment (e). Store the sediment (e) at -20°C. For ELISA assay, dissolve the sediment (e) in 20 µL of DMF or ethanol, then add 130 µL of 1X Sample Dilution Buffer.
k) For the competitive 14,15-DHET 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 final concentration, consider all dilution factors.
l) Perform the ELISA for 14,15-DHET according to the instructions of the manufacturer

II. Measurement of A) Free and B) Glucuronidated 14,15-DHET without extraction

A. Measurement of free 14,15-DHET: Dilute 1 mL 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 14,15-DHET

This method is for determining the level of glucuronidated 14,15-DHET in urine after digestion of the molecule with glucuronidase. Collect the first sample as soon as the β-glucuronidase is added to a reaction mixture (0 hour digestion) and then a second sample at a time when digestion of the glucuronic

acid moiety of the molecule is completed. Subtract the level of the molecule in the first sample at 0 hour from the levels in the second sample after complete digestion (usually 3 hrs) to obtain the level of glucuronidated molecule.

β-Glucuronidase digestion

6. Dilute 1 mL of urine 4-fold with 1X Sample Dilution Buffer
7. To 4 mL of urine add 1 mL of the β-glucuronidase solution, pH 5.5, to each tube (pH < 6.0).
8. Immediately transfer 2 mL of urine to a clean tube and flash-freeze. This is the zero time point.
9. Incubate the remaining 2 mL at 37°C for 3 hours. This is the 3-hour time point.
10. Follow the instructions for the ELISA kit (see below).
11. To calculate the amount of glucuronidated 14,15-DHET, subtract the 0 time value from the 3 hr value.

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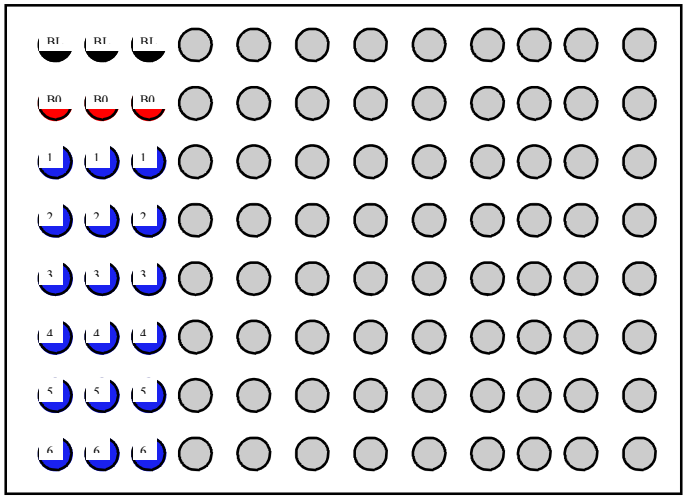
Assay Preparations

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 14,15-DHET-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 14,15-DHET 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 14,15-DHET-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.

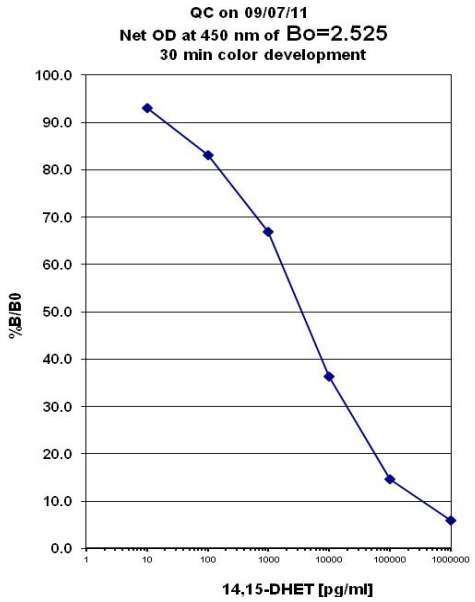
Calculating the Results

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 14,15-DHET 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.

Typical Results



The data shown here is an example of typical results obtained using the Detroit R & D 14,15-DHET 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_L wells = 0.059
B_O wells = 2.525

Standard	Concentration	O.D.	%B/B_O
No. 1	10 pg/mL	2.349	93.0
No. 2	100 pg/mL	2.099	83.1
No. 3	1,000 pg/mL	1.691	67.0
No. 4	10,000 pg/mL	0.916	36.3
No. 5	100,000 pg/mL	0.372	14.7
No. 6	1,000,000 pg/mL	0.148	5.8

Specificity of anti-14,15-DHET IgG

The specificity of the 14,15-DHET ELISA was investigated using authentic 14,15-DHET and a panel of eiconsanoids.

14,15-DHET	100.00 %
8,9-DHET	3.30 %
11,12-DHET	3.30 %
14,15-EET	1.5 %*
15(s) HETE	1.00 %
8,9-EET	0.40 %
5(s)15(s)DiHETE	0.20 %
11,12-EET	0.05 %
Arachidonic Acid	0.05 %
5,6-DHET	0.02 %
5,6-EET	0.02 %
Thromboxane B_2	0.02 %
PGE_2	<0.01 %
PGF_{2a}	<0.01 %
6-keto-PGF_{1a}	<0.01 %

*Recent experiment showed 0.3% cross-reactivity.

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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Warranty

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