Soluble Epoxide Hydrolase Activity ELISA

Catalog Number: **SH1/SH11/SH21/SH101**
Store at -20°C.
FOR RESEARCH USE ONLY

Introduction

14,15-DHET is a metabolite formed by the soluble epoxide hydrolase (sEH)-mediated metabolism of 14,15-EET which itself is generated by arachidonic acid epoxygenase activity of cytochromes P450 (CYPs) 2C and 2J. This competitive ELISA kit with an HRP detection system can been used to determine 14,15-DHET levels in biological samples and cell culture media as a measure of sEH activity. In addition, this assay can also be used to screen for inhibitors of soluble epoxide hydrolase activity. The kit is very similar to our 14,15-DHET/EET ELISA kit (DH 2) but also comes supplied with the 14,15-EET substrate so that one can measure activity or inhibition of the metabolism of EET to DHET by soluble epoxide hydrolase. The kit uses a 96-well plate format and contains enough 14,15-EET for 12 separate reaction mixtures.

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 (22 mL)	A solution of TMB (tetra methyl benzadine)	1
8	14,15-EET (90 uL)	9 micrograms in ethanol	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

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

sEH Activity Protocol for Adherent Cells:

1. Add 2.5 mL trypsin to 100 mm dish containing confluent HepG2 cells
2. Collect cells in 15 mL tube and centrifuge at 100g for 3 minutes.
3. Wash cell pellet with 3 mL of PBS and centrifuge again at 100 g for 3 minutes.
4. Resuspend cells in 5 mL growth medium (DMEM). Cell density = 300,000 cells/mL or 300 cells/uL.
5. Dilute an aliquot of the cell suspension with DMEM to 5.1 cells/uL. (i.e 20 uL cells + 1060 uL DMEM).
6. Prepare 14,15-EET solution by diluting EET with media. 15 uL EET + 90 uL DMEM. 15uL of EET = 1.5 ug. Total volume = 105 µL.
7. To 98 uL of cells add 52 uL of diluted EET. Total volume = 150 uL.
8. Incubate cell mixture for 1 hour at room temperature.
9. Dilute 3X with 1X sample dilution buffer. A 10X sample dilution buffer is supplied with the ELISA kit. (i.e. 300 uL of 1X sample dilution buffer +150 uL cell reaction mixture.
10. Centrifuge for 5 minutes at 200g to remove cells. Save supernatant for assay.
11. Add100 µL of DILUTED supernatant to well of ELISA plate. (We found that a 4X dilution usually works best, however, more or less dilution may be necessary, i.e. dilute 100 µL of sample with 300 µL of dilution buffer). Freeze remainder at minus 80 degrees C.

sEH Activity Protocol for Cytosol preparations

1. Dilute cytosol to 2.0 mg protein/mL.
2. Prepare 14,15-EET solution by diluting EET with media. 15 uL EET + 90 uL DMEM. 15uL of EET = 1.5 ug. Total volume = 105 µL.

3. To 98 uL of cytosol add 52 uL of diluted EET. Total volume = 150 uL.
4. Incubate cytosol mixture for 1 hour at room temperature.
5. Dilute 3X with 1X sample dilution buffer. A 10X sample dilution buffer is supplied with the ELISA kit. (i.e. 300 uL of 1X sample dilution buffer +150 uL cytosol reaction mixture.
6. Centrifuge for 5 minutes at 200g to particulates. Save supernatant for assay.
7. Add100 µL of DILUTED supernatant to well of ELISA plate. (We found that a 4X dilution usually works best, however, more or less dilution may be necessary, i.e. dilute 100 µL of sample with 300 µL of dilution buffer). Freeze remainder at minus 80 degrees C.

Toxicant and Drug Candidate Screening Protocol:

1. Follow steps 1 through 6 of sEH activity protocol.
2. Dilute the test compound to the appropriate concentration(s)
3. To 98 uL of cells or cytosol add 2 uL of test compound and incubate for 30 minutes with shaking at room temperature.
4. Add 52 uL of diluted EET. Total volume =152 uL.
5. Follow steps 8 through 11 (for cells) or steps 4 through 7 (for cytosol) of sEH activity protocol.

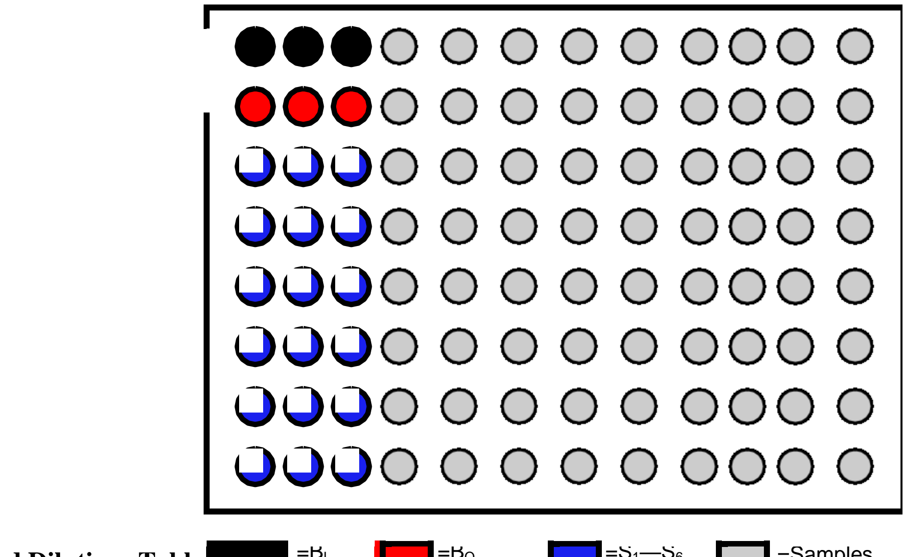
Assay Preparations

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 toserially 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

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.

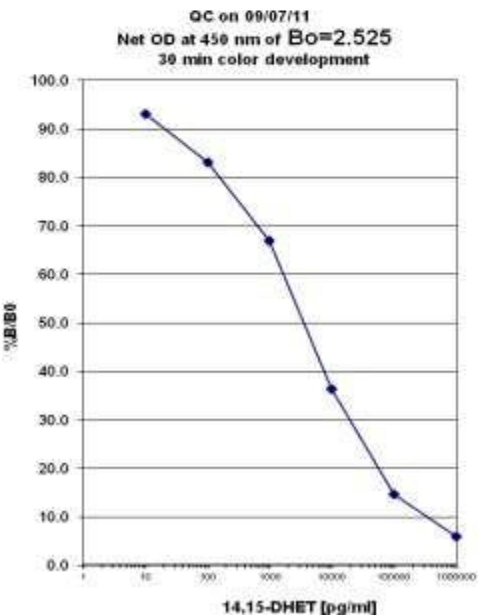
Calculating the Results

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.

References

1. Kim et al. Two divisional US Patents: 6,440,682 and 6,534,282, issued on 8/27/2002 & 3/18/2003, respectively. (http://patft.uspto.gov/netahtml/srchnum.htm)
2. Garson, Kim et al. Computational characterization of a series of eicosanoids. Lett. Drug Design & Discovery 2, 322, 2005.
3. Spiecker et al. Risk of coronary artery disease associated with polymorphism of the cytochrome p450 epoxygenase CYP2J2. Circulation 110, 2132, 2004.
4. Wang et al. Cytochrome P450 2J2 promotes the neoplastic phenotype of carcinoma cells and is up-regulated in human tumors. Cancer Res. 65, 4707, 2005.
5. Alkayed et al. Polymorphisms in the Human Soluble Epoxide Hydrolase Gene EPHX2 Linked to Neuronal Survival after Ischemic Injury. J. Neurosci. 27(17): 464-4649, April 25, 2007.
6. Wang et al. Cytochrome P-450 Epoxygenase Promotes Human Cancer Metastasis. Cancer Res. 67, 6665-6667, July 15, 2007.
7. Zeldin et al. Cytochrome P-450 epoxygenases protect endothelial cells from apoptosis induced by tumor necrosis factor-a via MAPK and PI3K/Akt signaling pathways. Am J. Physiol Heart Circ Physiol 293: H142-H151, 2007.
8. Alkayed et al. Soluble Epoxide Hydrolase Gene Deletion Is Protective Against Experimental Cerebral Ischemia. Stroke: 39: 2073-2078, 2008.
9. Wang et al. Selective Inhibitors of CYP2J2 Related to Terfenadine Exhibit Activity Strongly against Human Cancers *in vitro* and *in vivo*. JPET, 152017, 2009.
10. Chen et al. Synergystic effect of cytochrome P450 epoxygenase CYP2J2*7 polymorphism with smoking on the onset of premature myocardial infarction. Atherosclerosis, 195, 199-206, 2007.
11. Alkayed et al. Role of Soluble Epoxide Hydrolase in the Sex-Specific Vascular Response to Cerebral Ischemia. J. of Cerebral Blood Flow & Metabolism: 29, 1475-1481, 2009.

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.

Detroit R&D, Inc.

Metro Center For High Technology Bldg. (MCHT)
2727 Second Ave. Suite 4113
Detroit, MI 48201

Phone: 313.961.1606
Fax: 313.963.7130
E-mail: info@detroitrandd.com
www.DetroitRandD.com