

Soluble Epoxide Hydrolase Activity ELISA

Catalog Number: **SH1/SH11/SH21/SH101**

Store at -20°C.

FOR RESEARCH USE ONLY

Version 111814



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 epoxigenase activity of cytochromes P450 (CYPs) 2C and 2J. This competitive ELISA kit with an HRP detection system can be 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 (24 mL)	A solution of TMB (tetra methyl benzadine)	1
8	14,15-EET (90 uL)	100 µM stock solution	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

Recommended sEH Activity & Inhibitor screening Protocol for Adherent Cells:

- 1) Cells are detached from the growing surface by trypsinization.
- 2) Cells are washed once with PBS and resuspended in 10 mL media . Optimal cell density is approximately 180,000 cells/mL.
- 3) Cells are transferred to a 96-well clear bottom assay plate (900 cells in 50ul/well) and are allowed to attach to the plate for 12-18 hours in an humidified incubator under 37C.
- 4) Wells are washed with PBS and 88ul of media (serum free) are added to each well. If screening for an inhibitor of soluble epoxide hydrolase, dilute the test compound to the appropriate concentration(s) and add to the well (recommended final volume of 11ul) and incubate for 30 minutes. Better results may be observed with 2 hours of incubation.
- 5) Prepare the 14,15-EET solution by diluting the 100 µM stock EET solution (supplied with the kit) with media to a concentration of 10 µM. Add 11ul of diluted EET to each well (final concentration of 1 µM EET).
- 6) Incubate cell mixture for 1 hour at room temperature.
- 7) Transfer 100ul of media to the 14,15 DHET ELISA plate. (We found that no dilution works best).

sEH Activity Protocol for Cytosol preparations (recommended protocol based on liver cytosol; adjust as necessary if using cytosol prepared from other tissue)

1. Mix 400µl of 1X sample dilution buffer and 50 µl cytosol (final protein concentration should be between 4 and 200ug/mL) in a microtube
2. If screening for inhibitors of soluble epoxide hydrolase activity, dilute the test compound(s) to the appropriate concentration (100X inhibitor stock solutions).

Add 5 μ L of inhibitor to to 395 μ L of 1X sample dilution buffer and then add 50 μ L cytosol. Incubate for 30 minutes with shaking at room temperature. Better results may be observed with 2 hours of incubation.

3. For all reactions, add 50 μ L of 10 μ M EET (final concentration 1 μ M).
4. Incubate at 37°C for 30 minutes
5. Add 100 μ L to each well of the 14,15 DHET Elisa plate and follow direction for how to use the ELISA kit

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.

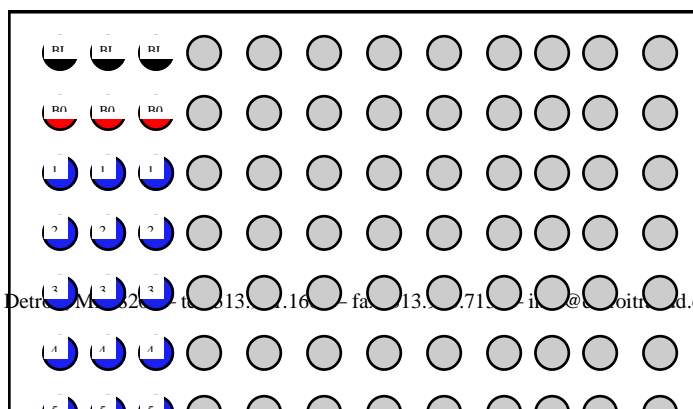
HRP Conjugate: Dilute 1 vial of the 14,15-DHET-HRP conjugate (0.012 mL) with 12.00 mL of 1 X HRP buffer. One vial makes enough conjugate for one plate. The conjugate must be used the same day and should not be stored for later use.

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 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 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-formanamide (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 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  =S₁—S₆  =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 microliters of 2 N sulfuric acid to all of the wells.

Step 11: Read the plate at 450 nm.

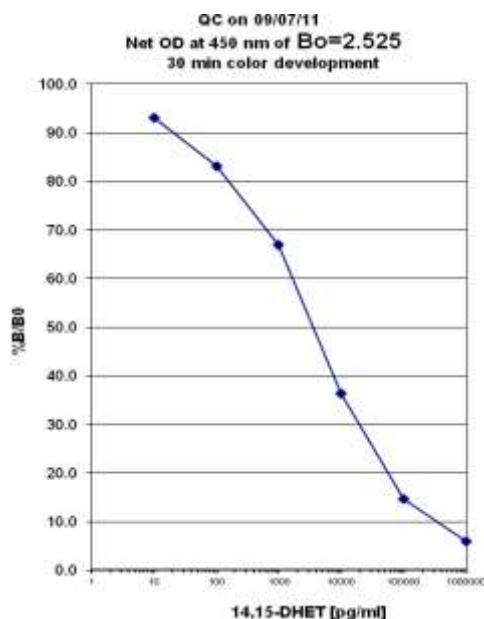
Calculating the Results

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_0 wells. This is your maximum binding.
3. Calculate the $\%B/B_0$ 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 $\%B/B_0$ versus the concentration of 14,15-DHET from the standards using semi-log paper.
5. Calculate the $\%B/B_0$ 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_0 wells = 2.525

Standard	Concentration	O.D.	%B/ B_0
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 eicosanoids.

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

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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