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14,15-DHET

ELISA KIT

Detroit R&D, Inc.

Rev. date: 8/15/2007

US Patents 6,440,682 and 6,534,282

コスモ・バイオ株式会社

Trouble Shooting

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 tempera ture.
- The lab is to 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 OD obtained from same sample

- Redo assay using an 8-channel pipetman making sure that all 8 channels are equal volume while loading.

CONTINUED FROM PAGE 13.

Production of 14,15-DHET ELISA:
A goat was immunized with 14,15-DHET conjugated to KLH via carboxyl group of the 14,15-DHET. Specificity of the 14,15-DHET IgG was assayed with authentic eicosanoids structurally similar to 14,15-DHET (see Table 1).

Quantitation of 14,15-EET level using 14,15-DHET ELISA:
The 14,15-DHET ELISA can be used for measurement of 14,15-EET levels after chemical oxidation of the 14,15-EET to 14,15-DHET. 14,15-EET+14,15-DHET levels have to be obtained. Then, to obtain 14,15-EET levels, 14,15-DHET levels have to be subtracted from 14,15-EET+14,15-DHET levels after measuring 14,15-DHET levels. 14,15-DHET standard has to go through the same chemical oxidation procedure to compensate loss or acid effect. The 14,15-DHET antibody recognizes the immunoreactive epitope unique to 14,15-DHET.

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 et al. Computational characterization of a series of eicosanoids. Lett. Drug Design & Discovery 2, 239, 2005.

[3] Sinal et al. Targeted disruption of soluble epoxide hydrolase reveals a role in blood pressure regulation. J. Biol. Chem. 275, 40504, 2000.

[4] Yu et al. Soluble epoxide hydrolase regulates hydrolysis of vasoactive epoxyeicosatrienoic acids. Circulation Research 87, 992, 2000.

[5] Makita et al. Cytochrome P450, the arachidonic acid cascade, and hypertension: new vistas for an old enzyme system. FASEB J. 10, 1456, 1996, and references therein.

[6] Spiecker et al. Risk of coronary artery disease associated with polymorphism of the cytochrome p450 epoxygenase CYP2J2. Circulation 110, 2132, 2004.

[7] 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.

[8] Wang, et al. Cytochrome P-450 Epoxygenase Promotes Human Cancer Metastasis Cancer Res. 67, 6665-6667, July 15, 2007.

[9] 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.

[10] Alkayed, et al. Polymorphisms in the Human Soluble Epoxide Hydrolase Gene EPHX2 Linked to Neuronal Survival after Ischemic Injury. The Journal of Neuroscience, 27(17): 464-4649, April 25, 2007.

* Please contact us for urine or plasma extraction procedure or human study.

Table of Contents

Kit Contents

Part Number	Item	Description	Quantity
1	14,15-DHET ELISA Plate	Solid 96-well plate coated with anti-14,15-DHET antibody per 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 Stock Buffer (25 ml)	A 10 X solution of Tris-buffered saline with preservatives	1
5	HRP Dilution Buffer (30 ml)	A concentrated solution of Tris-buffered saline with preservatives	1
6	Wash Buffer Stock Solution (50 ml)	A 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

Notes:

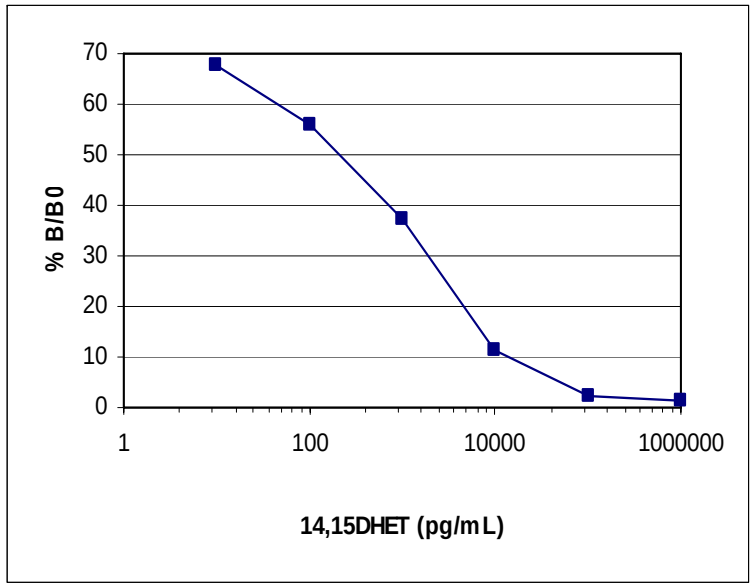
Table 1. Specificity of anti-14,15-DHET IgG .
The specificity of the 14,15-DHET ELISA was investigated using authentic 14,15-DHET and a panel of eicosanoids.

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

Continued on Page 14.

Typical Results



The data shown here is an example of typical results obtained using the Detroit R & D 14,15-DHET 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.

Blank wells = 0.070
B_0 wells = 0.869

Standard	Concentration	O.D.	% B/B_0
No. 1	10 pg/ml	0.540	67.60
No. 2	100 pg/ml	0.445	55.70
No. 3	1,000 pg/ml	0.300	37.50
No. 4	10,000 pg/ml	0.094	11.80
No. 5	100,000 pg/ml	0. 020	2 .50
No. 6	1000,000 pg/ml	0. 011	1.40

PRECAUTIONS

- Please read these instructions carefully before beginning this 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

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

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 at –20 °C and should not be re-frozen and thawed any more than is necessary.

Additional Items Required

- Plate reader with a 450 nm filter
- A 8-channel adjustable pipetter and An adjustable pipetter
- Storage bottles
- Costar® cluster tubes (1.2 mL) and Microcentrifuge tubes
- Deionized water

Background Information

The level of 14,15-DHET or 14,15-DHET epitope has been shown to exhibit correlation with hypertension in rodents[1,2,3] 14,15-DHET is a representative metabolite of cytosolic epoxide hydrolase-mediated metabolism of EET's, which are generated by arachidonic acid epoxygenase activity of cytochrome P450's[4] .

This is a competitive ELISA kit, based on competition between 14,15-DHET epitope and 14,15-DHET-HRP conjugate for 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 in the conjugate forming a blue color in the well, which will be more or less intense based upon the amount of 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. For additional information see NOTE on Page 13 and 14.

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.

Calculations

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 obtain 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 users manual for 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 S1 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.

Assay Preparations

- **Note:** Remove all of the reagents required, including the TMB, and allow them to equilibrate to room temperature before proceeding. It is necessary to thoroughly mix the concentrated buffer . A stir bar is contained within each buffer solution.

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 Conctrate (50 ml) with 450 ml of deionized water to yield a final volume of 500 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 11.88 ml of HRP dilution buffer to yield a final volume of 12 ml. 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 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.

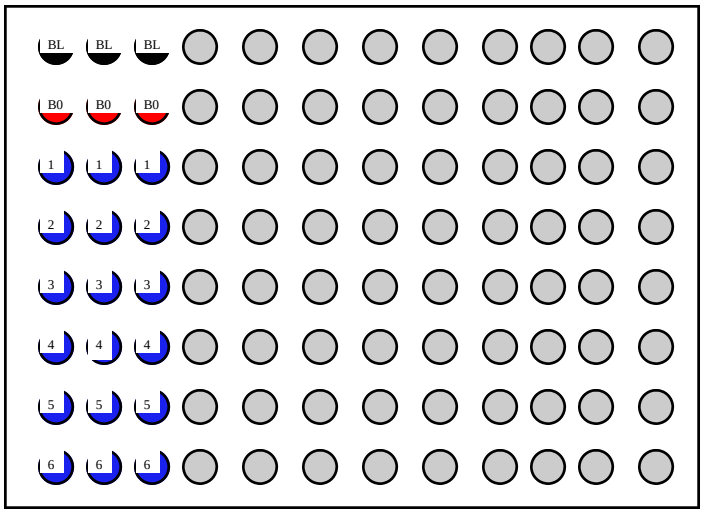
SAMPLES

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 or N, N-dimethyl-formamide (DMF, 10 uL to 20uL) and vortex well. Before ELISA assay, add 100 ul of 1 X Sample Dilution Buffer to make stock sample solution ready for quantitation 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 (BL), three maximum binding wells (B_0), and a six point standard curve (S1-S6). Each sample should be assayed in triplicate. A suggested plate format is shown as follows:



Standards Dilution Table

Standards	Final Concentration (pg/mL)	Add Sample Dilution Buffer (mL)	Serial Dilutions Procedure
• No.6	1,000,000	1.998	2uL of stock soln.
• No.5	100,000	0.9	Add 0.1mL of No.6
• No.4	10,000	0.9	Add 0.1mL of No.5
• No.3	1,000	0.9	Add 0.1mL of No.4
• No.2	100	0.9	Add 0.1mL of No.3
• No.1	10	0.9	Add 0.1mL of No.2

Step 1) Load 100 microliters of Sample Dilution Buffer into the maximum binding (B_0) wells and the blank (BL) wells.

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

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

Step 4) Load 100 microliters of the diluted 14,15-DHET -HRP conjugate into the B_0 wells, the standard wells, and the sample wells. Do NOT add HRP conjugate into blank (BL) wells.

Step 5) Incubate the plate at room temperature for 2 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 BL (blank wells).

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

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Measurement of 14, 15-DHET in mouse tissue (Liver or Heart)

Tissue homogenization

(1 g tissue +4 ml H_2O+0.1 mg triphenylphosphine. For liver sample, 1 g liver tissue +4ml H_2O+ 0.1 mg triphenylphosphine)

↓

Acidify homogenate by acetic acid
(Add 8 µl acetic acid to each homogenate)

↓

Extraction three times by ethyl acetate
(Get 6-8 ml clean organic solution)

↓

Dry up organic substance with argon or nitrogen gas
(It takes about 1 hr to dry up the organic solvent)

↓

Dissolve dried extraction with DMF or ethanol
(Add 10 ~ 20 µl of DMF or ethanol to reconstitute the dry-up extraction)

↓

Dilute further with sample dilution buffer
{add 0.5 ml (it can be more or less) sample dilution buffer and centrifuge diluted sample at 10000 rpm for 5 min at RT, then take the supernatant for ELISA measuring}

↓

ELISA for 14, 15-DHET
(According to the instruction of manufacturer)

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Protocol #1:

EET Formation Activity Measurement using DHET ELISA Kit Cat. # **DH2**, **DH12**, **DH22**, **DH5**, **DH15** and **DH25**

It is well know that arachidonic acid (AA) will be converted to EET by P450 arachidonic acid epoxygenase (AA epoxygenase) and EET will be converted to DHET by soluble epoxide hydrolase (sEH) *in vivo*. Cytochrome P450 2J2 (CYP2J2) is a predominant human AA epoxygenase that produces all four EET. In human carcinoma cells, rAAV-mediated overexpression of CYP2J2 resulted in marked increase in 14,15-DHET level in cell plasma, whereas rAAV-anti2J2-mediated silence of CYP2J2 expression significantly decreased its production (1). Our EET/DHET kit can be used to measure EET level in the cultured cells which express sEH (1).

1. Collect and homogenize and/or sonicate the cells using a solution containing a final concentration of 0.1mM TPP (triphenyphosphine). TPP is an antioxidant, which looks like precipitate in samples because it does not easily dissolve. Before using the stored samples with TPP, spin to separate the TPP from the samples.

2. Acidify the whole homogenized cells with acetic acid to make pH 3~4 using PH standard paper (Be cautious of the PH change by adding 1μl acetic acid each time).

3. Extraction with ethyl acetate. Add the equal volume of ethyl acetate to the homogenized cells, and vortex very well. Take the upper organic phase into a clean 50 ml conical tube after centrifuge spin-down. Then add another equal volume ethyl acetate to the homogenized cells to start the second-time extraction. Three times of extraction are strongly recommended.

4. Evaporate pooled ethyl acetate extraction until all dry-up under the Argon gas.

5. Add 10 μl to 20 μl ethanol or N, N-dimethyl-formamide (DMF) to dissolve the dry-up extraction for reconstitution. Add 0.5 mL sample dilution buffer (provided in the kit) to make a solution. Load 100 μl in each well in triplicates on the ELISA plate. (Note: We recommended measuring a different dilution of sample in attempt to fit the results to the

standard curve. e.g., Add 3 wells by 50 μl of the rest of sample and 50 μl sample dilution buffer and 3 wells of 10 μl of the rest of sample and 90 μl sample dilution buffer.)

6. ELISA for 14, 15-DHET (According to the instruction of manufacturer)

Reference:

(1) Cancer Res. 2005; 65:4707-15
(2) Circulation 2004; 110:2132
(3) *Letters in Drug Design & Discovery 2005; 2:239*, etc.

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Protocol #2:

Free EET + DHET Formation Activity Measurement using DHET ELISA Kit Cat. # DH2, DH12, DH22, DH5, DH15 and DH25 after the Change of EET to DHET by Acidic Hydrolysis

1. Biological samples have to be collected in TPP (triphenyphosphine) with a final concentration of 0.1mM. TPP is an antioxidant, which looks like precipitate in samples because it does not easily dissolve. Before using the stored samples with TPP, spin to separate the TPP from samples.

2. Acidify the samples with acetic acid to pH 3~4. After acidification, the samples are extracted three times with ethyl acetate. For each extraction, add equal volume of ethyl acetate to the sample, vortex thoroughly and spin down and collect the organic phase. After extracting three times for the same sample, pool the organic phase (ethyl acetate) together and evaporate under the argon.

3. After dissolving the extracted eicosanoids in a minimal amount of ethanol, add 20 µL acetic acid to make pH 3~ 4. In the acidic condition, EET hydrolyzed to DHET. The reaction usually takes 12 hr at 45ºC or 18 hr (overnight) at room temperature. The reaction vial has to be flushed with Argon and keep under Argon blanket (Argon blank is like a pouch to keep the Argon gas flow during the hydrolysis). If Argon blank is not available at your place, you can add clean powdered dry ice to get rid of residual oxygen.

4. After the reaction, the samples are extracted three times with ethyl acetate. For each extraction, add 100 µl of ethyl acetate to the sample, vortex well and spin down and collect the organic phase. After three times of extraction, pool all the organic phase (ethyl acetate) together and evaporate under the argon.

5. Dissolve the dried-up eicosanoids with a minimal amount of ethanol or N, N-dimethyl-formamide (DMF, 10 µL to 20 µL) and vortex thoroughly. Before the ELISA assay, add 100 µl of 10X TBS pH 7.5, which is called sample dilution buffer and is provided in our kit to make stock sample solution for quantitation with ELISA. Check the pH with pH paper. The stock sample solution can be diluted in a proper range of concentration for ELISA test.

6. Use the14,15 -DHET ELISA kit to measure DHET, which includes DHET converted from EET. At same time, measure the DHET level without hydrolysis of EET in the same sample, and subtract it from the EET + DHET level. Then you will obtain EET level.

Reference:

(1) Cancer Res. 2005; 65:4707-15
(2) Circulation 2004; 110:2132
(3) *Letters in Drug Design & Discovery 2005; 2:239*, etc.

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Protocol #3:

Free and Esterified EET + DHET Formation Activity Measurement using DHET ELISA Kit Cat. # DH2, DH12, DH22, DH5, DH15 and DH25 after the Cleavage of Esterified Eicosanoids by Alkaline Hydrolysis, Followed by Change of EET to DHET by Acidic Hydrolysis

1. Biological samples have to be collected in TPP (triphenyphosphine) with a final concentration of 0.1mM. TPP is an antioxidant, which looks like precipitate in samples because it does not easily dissolve. Before using the stored samples with TPP, spin to separate the TPP from the samples.

2. Acidify the samples with acetic acid to pH 3~4. After acidification, the samples are extracted three times with ethyl acetate. For each extraction, add equal volume of ethyl acetate to the sample, vortex thoroughly and spin down and collect the organic phase. After extracting three times for the same sample, pool the organic phase (ethyl acetate) together and evaporate under the argon.

3. Prepare 20% KOH solution from 1 ml 2M KOH and 4 ml Methanol (final concentration KOH = 0.4 N). To cleave the esterified eicosanoids, 2 mL of 20% KOH was added and mixed very well. The mixture was incubated at 50 ºC for 1 hour.

4. Acidify the reaction solution with formic acid to pH 5. After acidification, the samples are extracted three times with ethyl acetate. For each extraction, add equal volume of ethyl acetate to the sample, vortex thoroughly and spin down and collect the organic phase. After three times of extraction, pool all the organic phase (ethyl acetate) together and evaporate under the argon.

5. After dissolving the extracted eicosanoids in a minimal amount of ethanol, add 20 µL acetic acid to make about pH 3 ~ 4. In the acidic condition, EET is hydrolyzed to DHET. The reaction usually takes 12 hr at 45 ºC or 18 hr (overnight) at room temperature. The reaction vial has to be flushed with Argon and keep under Argon blanket (Argon blank is like a pouch to keep the Argon gas flow during the hydrolysis). If an Argon blank is not available at your place, you can add clean powdered dry ice to get rid of the residual oxygen.

6. After reaction, the samples are extracted three times with ethyl acetate. For each extraction, add 100 µl of ethyl acetate to the sample, vortex thoroughly and spin down and

collect the organic phase. After three times of extraction, pool all the organic phase (ethyl acetate) together and evaporate under the argon.

7. Dissolve dried-up eicosanoids with a minimal amount of ethanol or N, N-dimethyl-formamide (DMF, 10 µL to 20 µL) and vortex well. Before the ELISA assay, add 100 µl of 10X TBS pH 7.5, which is called sample dilution buffer and is provided in our kit to make stock sample solution for quantitation with ELISA. Check the pH with pH paper. The stock sample solution can be diluted in a proper range of concentration for ELISA test.

8. Use the 14,15 -DHET ELISA kit to measure DHET, which includes DHET converted from EET. At same time, measure the DHET level without hydrolysis of EET in the same sample, and subtract it from the EET + DHET level. Then you will obtain EET level.

Reference:

(1) Cancer Res. 2005; 65:4707-15
(2) Circulation 2004; 110:2132
(3) *Letters in Drug Design & Discovery 2005; 2:239*, etc.

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Measuement of 14,15-DHET in plasma or serum

DHET extraction protocol

Step 1: Purification
1.8mL plasma adjusted with approximately 20µL acetic acid to pH 4 + 1.8mL ethyl acetate
Vortex
Centrifugation: t=10 min., T: 22°C, 2000rpm
Phase separation: upper phase = Ethyl acetate phase (lipoproteins) = organic phase
Interphase = proteins
Lower phase = aqueous phase
Collect upper phase (a)
2X: add equal volume ethyl acetate to lower phase after transferring lower phase with glass pipette to new tube, discard interphase (proteins), centrifuge and collect again upper phase

Step 2: Evaporation
Approximately 4mL ethyl acetate phase (a)
Dry in Speedvac = sediment (b)

Step 3: Saponification: (to cleave fatty acid from glycerol backbone)
20% KOH Solution: 1mL of 2M KOH + 4mL Methanol (MeOH) (final concentration KOH = 0.4N)
Sediment (b) + 2 mL 20% KOH
Vortex
Incubate: t = 1 h, T = 50° C
= aqueous solution (c)

Step 4: Re-extraction
2mL aqueous solution (c) + 3 mL H_2O
Adjust pH with 20% formic acid to pH 7.4 (132µL)
Add 2mL ethyl acetate
(1 part aqueous solution (c) + 1 part ethyl acetate)
Vortex
Centrifuge: t = 10 min., T = 22°C, 2000 rpm
Repeat procedure with ethyl acetate 2X (equal volume ethyl acetate per sample)
Collect upper phase (with saponified lipids)
Check pH of upper phase (should be 7.4)

Step 5: Evaporation
6mL ethyl acetate phase, upper phase (d)
Dry in speedvac = sediment (e)
Store sediment (e) at -20° C
For ELISA assay, dissolve sediment in 20µL ethanol, then add 130µL ELISA-sample buffer.
Check pH (should be 7.4 after adding ethanol)

Step 6: Competitive 14, 15-DHET – ELISA
150µL Probe: a) Dilution 1:5, (i.e., 80µL sample + 320µL ELISA-sample buffer
b) For calculating concentration, consider dilution factor (in this case 1.875, i.e., 80µL sample from the 150µL total sample volume)