DRG® 15-HETE (EIA-1411)

Revised 24 April 2007 **RUO in the USA**

Intended Use
The 15-HETE Enzyme Immunoassay (EIA) Kit is to be used for the quantitative determination of 15-HETE levels in biological levels. This kit is intended for Research Use Only in the United States.

Explanation of the Test
15-hydroxyeicosatetraneoic acid (15-HETE) is a hydroxy derivative of arachidonic acid by 15-lipoxygenase contained in blood platelets. It has been shown to be chemotactic and chemokinetic for polymorphonuclear leukocytes and vascular smooth muscle cells. 15-HETE acts as a second messenger in angiotensin-II induced aldosterone production. Evidence also suggests many other biological activities for this compound including stimulating insulin secretion by pancreatic tissue, inducing hyperphosphorylation of several major cytoskeletal proteins, enhancing surface expression of integrin receptors and platelet aggregation in tumor cells.

The DRG 15-HETE EIA kit is a competitive enzyme immunoassay for the determination of 15-HETE levels in biological fluids. A goat anti-rabbit antibody has been precoated onto a polystyrene 96-well plate. A rabbit polyclonal antibody to 15-HETE is bound by the antibody on the coated well. Variable amounts of 15-HETE in the samples compete with a fixed amount of alkaline phosphatase labeled 15-HETE for a limited number of sites on the anti-15-HETE antibody. The higher the concentration of the compound in the sample, the less labeled compound is bound to the specific antibody. The unbound material is removed by washing. A colored product is formed inversely proportional to the amount of unlabeled 15-HETE bound. The reaction is terminated by the addition of stop solution and the absorbance is measured at 405 nm. The absorbance is correlated with concentration through a standard curve.

Precautions for Use
1. Do not pipet solutions by mouth.
2. Reagents may contain sodium azide. Sodium azide may react with lead an dropper plumbing to form explosive metal azides. On disposal of reagents, flush with large volumes of water to prevent azide accumulation.
3. Use only the 96-well plate supplied with the kit.
4. Do not eat or smoke in areas where specimens or kit reagents are being handled.

Reagents Supplied
The 15-HETE EIA Kit contains sufficient reagents for 96 determinations including standard curves. The reagents and other components provided are:

Quality	Kit Component	Final Volume
1 vial	15-HETE Antibody, rabbit, lyophilized.	11 ml
1 vial	15-HETE Alkaline Phosphatase Conjugate, liquid	11 ml
7 vials	15-HETE Standards, lyophilized	1 ml
1 bottle	EIA Phosphate Buffer	50 ml
1 bottle	para-Nitrophenyl Phosphate Substrate 4 Tablets/bottle	---
1 bottle	para-Nitrophenyl Phosphate Diluent Concentrate, (10x)	50 ml
1 bottle	Wash Buffer Concentrate (10 x)	1 Liter

Revised 24 April 2007 **RUO in the USA**

1 bottle	Stop Reagent Concentrate (10 x)	100 ml
1 plate	96 Well Plate with Holder	---
2 sealers	Adhesive Plate Sealers	---

Materials Needed But Not Supplied

- Precision pipettes with disposable tips:
  50-200 µl adjustable multiwell pipettor or 50 µl and 100 µl multiwell pipettors; 20-200 µl and 200-1000 µl adjustable pipettor.
- Disposable reagent troughs for multiwell pipettor
- Beakers, flasks, cylinders necessary for preparation of reagents
- Constant temperature air incubator, 37+/-2^{0}C
- 96-well plate washing/aspiration device
- 96-well plate reader for measurement of absorbance at 405 nm
- Semi-log paper.
- Horizontal orbital rotator set at approximately 150 rpm for 96 well plates.

Reagent Preparation

1. **15-HETE Antibody**
   The antibody is supplied lyophilized in a phosphate buffer containing bovine serum albumin (BSA) and sodium azide. Reconstitute with 11 ml of the EIA Phosphate Buffer provided. The solution can be stored at 2-8^{0}C for up to 3 months.
2. **15-HETE Alkaline Phosphatase Conjugate**
   The conjugate is supplied ready to use in 0.01M Phosphate Buffer containing 0.9% sodium chloride, 0.1% BSA, 0.1% magnesium chloride and 0.1% sodium azide at pH 7.4. The conjugate may be stored at 2-8^{0}C until the expiration date. DO NOT FREEZE. Protect from prolonged exposure to light.
3. **15-HETE Standards**
   The standards are supplied lyophilized in a phosphate buffer containing BSA and sodium azide. Reconstitute with 1 ml each of EIA Phosphate Buffer provided. Mix well. Standard concentrations of 0, 50, 100, 250, 1000, 2500 and 5000 pg/ml are supplied. Use only plastic or siliconized glass pipettes for transfer. After reconstitution, standards are stable for 3 months when stored at -20^{0}C. Avoid repeated freeze/thaw cycles.
4. **EIA Phosphate Buffer**
   This buffer is supplied ready to use and contains 0.01M phosphate, 0.9% sodium chloride, 0.1% magnesium chloride, 0.1% bovine serum albumin (BSA) and 0.1% sodium azide at pH 7.4. Buffer is stable until the expiration date when stored at 2-8^{0}C.
5. **para-Nitrophenyl Phosphate (pNPP) Substrate**
   Each of the four pNPP tablets supplied contains 25 mg of pNPP. To prepare substrate, allow tablets to reach room temperature and dissolve 1 tablet per 12.5 ml of pNPP Substrate Diluent. **Do not prepare in advance of the assay**. Prepare only the amount of substrate needed approximately 10 minutes prior to the end of incubation with the conjugate according to the table below:

Number of Tests	#of pNPP tablets	Substrate Diluent
Assaying 1-30 tests	1	12.5 ml
Assaying 30-60 tests	2	15.0 ml
Assaying 60-96 tests	3	37.5 ml

pNPP tablets should be stored at a temperature of 2-8^{0}C and are stable until the expiration date on the bottle when stored properly. pNPP is sensitive to light

and moisture. AVOID CONTACT WITH EYES, SKIN AND CLOTHING. WASH IMMEDIATELY AFTER HANDLING.

6. **para-Nitrophenyl Phosphate Diluent Concentrate**
   The diluent is supplied as a 10x concentrate. Dilute the concentrate to a final volume of 50 ml with distilled water. **Adjust the diluted diluent to Ph 10.0 if necessary**. The diluted buffer contains 0.05M sodium bicarbonate, magnesium chloride and sodium azide. The diluent is stable until the expiration date on bottle when stored at 2-8^{0}C.
7. **Wash Buffer Concentrate**
   The wash buffer is supplied as a 10x concentrate. The diluted buffer contains tris buffered saline and surfactant. Prepare Wash Buffer for use in the assay by adding 100 ml of the Wash Buffer Concentrate to 900 ml of deionized water. Mix well. Wash Buffer is stable for 6 months when stored at 2-8^{0}C.
8. **Stop Reagent Concentrate**
   The concentrate is supplied as 2N sodium hydroxide. To prepare 0.2N sodium hydroxide required as the stop reagent in the assay, dilute the concentrate to a final volume of 100 ml with deionized water. Mix well. The concentrate and diluted solution are stable until the expiration date on the bottle when stored at 2-8^{0}C.
9. **96-Well Plate with Holder**
   The plate is supplied ready to use. It has been precoated with goat anti-rabbit antibody. Use only the number of well-strips necessary to perform the assay. Unused strips should be removed from holder and stored in foil pouch provided. They are stable until the expiration date when stored at 2-8^{0}C.

Sample Handling
Blood should be collected in siliconized glass or plastic tubes containing EDTA and a biosynthesis inhibitor and processed immediately. Extraction of samples is recommended prior to assay. If assays are not performed immediately, the plasma should be stored at -20^{0}C or below and assays carried out as soon as possible.

Extraction of Eicosanoids
A wide variety of general and specified methods have been reported. The following method is adapted from Powell and utilizes an ODS-silica cartridge:

1. Sample volumes should be 1-5 ml of plasma or tissue homogenate or 10 ml of urine*. A control should also be prepared to determine extraction efficiency by adding approximately 7,000 dpm of the appropriate tritiated eicosanoid to a sample and allowing it to equilibrate at 4^{0}C for 10 minutes.
2. Add ethanol to tissue homogenates, subcellular fractions and cell supernatants to achieve a final concentration of 15% ethanol. Centrifuge at 375 x g for 10 minutes at 4^{0}C. Retain supernatant for testing.
3. Wash the ODS-silica cartridge with 10 ml of deionized water followed by 10 ml of ethanol and another 5 ml of deionized water.
4. Acidify the biological sample to pH 3.0 with 3% formic acid and apply to ODS-silica cartridge.
5. Wash the cartridge with: 5 ml deionized water followed by 5 ml 15% ethanol followed by 5 ml petroleum ether.
6. Elute the eicosanoid from the cartridge with 10 ml of freshly distilled methyl formate or redistilled ethyl acetate.
7. Evaporate the organic phase under nitrogen.
8. Reconstitute the dried residue in kit buffer for assay.

**As true circulating amounts of eicosanoids seldom exceed a few pg/ml of plasma, it is advisable to extract at least 5 ml of plasma and 10 ml of urine per test.*

Assay Procedure

Note: For convenience, an optional one-day procedure is presented here as an alternative to the regular procedure shown below. The first two incubations are 2 hours each and should be performed at room temperature on a horizontal orbital rotator set at 150± rpm. The substrate incubation should be performed at 37^0 C, no shaking.

Bring all reagents to room temperature.

1. Mix all reagents thoroughly without foaming before use.
2. Determine the number of strips required to test the desired number of samples plus 16 wells needed for running substrate blanks, buffer blanks and standards. Remove extra strips from holder and store at 2-8^{0}C in the bag provided. If more than one assay will be performed with this kit, the ***standards must be frozen at -20^{0}C***. *Do not freeze and thaw the standards more than three times.*
3. **Leaving the blank wells empty**, pipette 100 µl of standard, buffer (for buffer blanks) or sample, in duplicate, into the antibody precoated wells according to the following scheme and diagram.. **Gently agitate plate by tapping the edge of the holder for at least 15 seconds to thoroughly mix contents of each well.**

Wells	Samples	Wells	Samples
1A, 1B	Substrate Blank	2C, 2D	15-HETE Standard 5 (1000 pg/ml)
1C, 1D	15-HETE Standard 1 (0 pg/ml)	2E, 2F	15-HETE Standard 6 (2500 pg/ml)
1E, 1F	15-HETE Standard 2 (50 pg/ml)	2G, 2H	15-HETE Standard 7 (5000 pg/ml)
1G, 1F	15-HETE Standard 3 (100 pg/ml)	3A-12H	Other Samples
2A, 2B	15-HETE Standard 4 (250 pg/ml)		

4. Leaving the substrate blank wells empty (1A, 1B), pipette 100 µl of 15-HETE Antibody to all other wells and tap gently to mix.
5. Cover the wells with a plate sealer and incubate overnight at 2-8^{0}C. (*Optional: 2 hours at room temperature on a horizontal orbital rotator set at approximately 150 ± rpm.)*
6. **Leaving the substrate blank wells empty 1A, 1B)**, add 100 µl of 15-HETE Alkaline Phosphatase Conjugate to all other wells and tap gently to mix.
7. Cover the wells with a plate sealer and incubate at 2-8^{0}C.for 3 hours. (*Optional: room temperature for 2 hours on a horizontal orbital rotator set at approximately 150 ± rpm*

Approximately 10 minutes prior to the end of incubation with the conjugate, prepare pNPP Substrate Solution by dissolving the appropriate number of pNPP tablets in the Substrate Diluent. Refer to the Reagent Preparation section for dilution instructions.

8. Remove and save sealer. Aspirate solution from all wells. Wash wells 3 times with approximately 400 µl of Wash Buffer per well with thorough aspiration between washes. Invert and blot plate on paper towel after final wash.
9. Pipette 3000 µl of pNPP substrate solution into all wells. Incubate covered for 2 hours at 37±2^{0}C. *(Optional: Incubate covered for 2 hours at 37±2^{0}C, no shaking).*
10. Pipette 50 µl Stop Solution into all wells. Tap plate gently to mix.

11. Read absorbance of wells at 405 nm versus substrate blank. The absorbance should be read as soon as possible after the completion of the assay, but may read up to 2 hours after addition of Stop Solution when wells are kept protected from light at room temperature.

Note: It is important that Stop Solution be added to wells prior to reading at 405 nm. Addition of Stop Solution causes an increase in absorbance of the chromagen and a shift in absorption spectrum.

Calculations

Standard Curve

1. Record the absorbance at 405 nm for each standard well.
2. Average the duplicate values and record the averages.
3. Plot the absorbance (vertical axis) versus the 15-HETE concentration in picograms/ml (horizontal axis) of the standards using a linear scale for absorbance and a log scale for concentration.
4. Draw the best fitting curve.

Samples

1. Record the absorbance at 405 nm for each sample well.
2. Average the duplicate value and record the averages.
3. Locate the average absorbance value which corresponds to each sample on the vertical axis and follow a horizontal line intersecting the standard curve. At the point of intersection, read the

15-HETE concentration (pg/ml) from the horizontal axis.

Typical Data for the 15-HETE EIA

Well	Sample	Concentration	A405	Average A405
1A	Substrate Blank		0	0
1B	Substrate Blank		0	0
1C	15-HETE Standard 1	0 pg/ml	1.824	1.802
1D	15-HETE Standard 1	0 pg/ml	1.780	
1E	15-HETE Standard 2	50 pg/ml	1.310	1.297
1F	15-HETE Standard 2	50 pg/ml	1.284	
1G	15-HETE Standard 3	100 pg/ml	1.141	1.142
1H	15-HETE Standard 3	100 pg/ml	1.142	
2A	15-HETE Standard 4	250 pg/ml	0.837	0.828
2B	15-HETE Standard 4	250 pg/ml	0.819	
2C	15-HETE Standard 5	1000 pg/ml	0.582	0.582
2D	15-HETE Standard 5	1000 pg/ml	0.582	
2E	15-HETE Standard 6	2500 pg/ml	0.440	0.429
2F	15-HETE Standard 6	2500 pg/ml	0.418	
2G	15-HETE Standard 7	5000 pg/ml	0.295	0.295
2H	15-HETE Standard 7	5000 pg/ml	0.295	

Do not use this data to construct a standard curve and calculate sample values. A standard curve must be generated at the time of assay for calculation of sample values.

Revised 24 April 2007 **RUO in the USA**

Typical Data for the 15-HETE EIA (Optional Procedure)

Well	Sample	Concentration	A405	Average A450
1A	Substrate Blank		0	0
1B	Substrate Blank		0	0
1C	15-HETE Standard 1	0 pg/ml	2.132	2.131
1D	15-HETE Standard 1	0 pg/ml	2.130	
1E	15-HETE Standard 2	50 pg/ml	1.822	1.816
1F	15-HETE Standard 2	50 pg/ml	1.811	
1G	15-HETE Standard 3	100 pg/ml	1.498	1.489
1H	15-HETE Standard 3	100 pg/ml	1.480	
2A	15-HETE Standard 4	250 pg/ml	1.292	1.290
2B	15-HETE Standard 4	250 pg/ml	1.288	
2C	15-HETE Standard 5	1000 pg/ml	1.045	1.018
2D	15-HETE Standard 5	1000 pg/ml	0.991	
2E	15-HETE Standard 6	2500 pg/ml	0.848	0.815
2F	15-HETE Standard 6	2500 pg/ml	0.782	
2G	15-HETE Standard 7	5000 pg/ml	0.706	0.704
2H	15-HETE Standard 7	5000 pg/ml	0.702	

Do not use this data to construct a standard curve and calculate sample values. A standard curve must be generated at the time of assay for calculation of sample values.

Specific Performance Characteristics

Cross Reactivity at 50% B/B_0

15-HETE	100.0%
5-HETE	0.1%
12-HETE	0.5%
5, 15-di-HETE	1.0%
8, 15-di-HETE	1.0%
Arachidonic Acid	0.1%
Prostaglandin D_2	0.1%
Prostaglandin E_S	0.1%
Prostaglandin F_{2a}	0.1%
Prostaglandin B_2	0.1%

Sensitivity

The detection limit of the DRG 15-HETE EIA Kit is calculated to be 1.24. pg/ml. (*Optional Procedure: 3.55 pg/ml)*

Precision

Intra-assay precision was determined from the mean of 10 replicates per sample.

Sample Pool	Mean (pg.ml)	Standard Deviation (pg/ml)	Coefficient of Variation (%)
A	459	26.9	5.9
B	1083	67.0	6.0
C	2484	147	5.9

Inter-assay precision was determined from the mean of the average of duplicate samples for 10 different runs.

Sample	Mean	Standard Deviation	Coefficient of Pool	(pg.ml)	(pg/ml)	Variation (%)
A	359	18.9	5.3			
B	810	56.7	7.3			
C	1638	126	7.6			

Recovery Studies

Spiked samples were prepared by adding 15-HETE to the assay buffer. Recovery was calculated as: (Calculated 15-HETE ÷ Observed 15-HETE x 100.

Sample Type	Average Recovery %
Buffer Diluent	98.9

Dilution Linearity Studies

Three assay buffer pools were spiked with 15-HETE. These pools were diluted such that they produced at least four values within the standard curve. Results were as follows:

Sample Type	Average Recovery %
Serum Pool	98.0

Material Safety Data

The physical and chemical properties of the reagents contained in this kit have not been tested as a whole.

It is strongly recommended that laboratory employees wear lab coats, gloves and safety glasses when handling the reagents provided with this kit. Reagents do not contain ingredients which have been determined to be health hazards and which comprise greater than 1% of the mixture or which could be released from the mixture in concentrations which would exceed an OSHA permissible exposure limit or ACGIH TLV or could present a health hazard to employees during normal use following good laboratory practices.

Hazardous Ingredients

Less than 1% sodium azide is used as a preservative. This information is provided for the sake of completeness. Sodium azide has a TLV in air of 0.3m^3 as NaN_3 and 0.1 ppm as HN_3 (hydrazoic acid). It may cause skin and eye irritation and may be fatal if inhaled, swallowed or absorbed through the skin. Acute health effects include nausea, headache and vomiting. In case of contact, immediately flush eyes or skin with copious amounts of water for at least 15 minutes while removing contaminated clothing. If inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen and call a physician. Fight fire with dry chemical powder. Emits toxic fumes under fire conditions. Avoid contact with acids and metals. Azide reacts with many heavy metals such as lead, copper, mercury, silver, and gold to form explosive compounds. Copper and lead azides are more sensitive than nitrogylcerine.

Incompatible with chromyl chloride, hydrazine, bromine, carbon disulfide, dimethyl sulfate, dibromomalonitrile. If spilled, absorb while wearing suitable protective clothing. Dispose of in accordance with EPA regulations.

Standards are chemically pure, biologically active. Standards are supplied lyophilized. Acute effects of overexposure: dizziness, hypotension, nausea and irritation. Contact a physician. Harmful if swallowed, inhaled or absorbed through the skin. Avoid contact with eyes, skin or clothing. Exercise Caution: Chronic effects of exposure unknown.

Contains Sodium Hydroxide. Corrosive. Wear safety glasses, gloves and lab coat. Harmful if swallowed, inhaled or in contact with skin or eyes.

Contains p-Nitrophenyl Phosphate Tablets. The toxicological properties of this material have not been thoroughly investigated. Dust from tablets may be irritating to the eyes and mucous membranes. Wear chemical resistant gloves and lab coat. Wash after handling.

Physical and Chemical Data

Components are stable in closed containers under normal temperatures and pressures. Sodium azide may react with lead or copper plumbing to form explosive metal azides. If drain disposed, flush with a large volume of water to prevent accumulation.

Fire and Explosion Data

Components are non-combustible. Negligible fire hazard when exposed to heat or flame. Fire fighting media: dry chemical extinguisher.

Health Hazards

No effects of overexposure have been documented. Individual components may cause skin irritation or be harmful if swallowed. Avoid contact with skin and eyes. FIRST AID: If swallowed, give water or milk to dilute (if conscious) and induce vomiting. In case of contact with eyes, flush with copious amounts of water for at least 15 minutes, assure adequate flushing by separating the eyelids with fingers. Call a physician. In case of skin contact wash with soap or mild detergent and large amounts of water.

Reactivity Data

Components are stable in closed containers under normal temperatures and pressures

Handling and Storage Instructions

Safety glasses and gloves should be worn to prevent skin and eye contact. Wear protective clothing such as lab coats to prevent contact. Store as directed in package insert.

Disclaimer

This information is believed to be correct but does not purport to be all inclusive and shall be used only as a guide. DRG International, Inc. shall not be held liable for any damage resulting from handling or from contact with the above product. See catalog for additional terms and conditions of sale.

References

1. Bailey, J.M., Bryant, R. W., Whiting, J. and Salata, K., Characterization on 11-HETE and 15-HETE, together with prostacyclin, as major products of the cyclooxygenase pathway in cultured rat aorta smooth muscle cells. J. Lipid Res. 24:1419 (1983).

2. Benedetto, C., McDonald-Gibson, R.G., Nigam, S. and Slater, T.F. (eds), Prostaglandins and Related Substances, IRL Press, Washington, D.C., p.20 (1987).

3. Bryant, R.W., and Hwang, D.H., Development of a radioimmunoassay for 15-HETE and its application to 15-HETE production by reticulocytes. Prostaglandins 16:375 (1983).