Enzyme Immunoassay for
15-epi-Lipoxin A_4
Product No. EA 46

For Research Use Only

Please read all instructions carefully before beginning this assay
Store lyophilized conjugate at –20ºC or less.

Store all other kit components at 4ºC at all times.

DESCRIPTION

15-epi-Lipoxin A_4 is an aspirin-triggered eicosanoid believed to be involved in the positive attributes of aspirin therapy for heart, cancer, and human immunodefiency virus patients. During inflammation, neutrophils are activated. 15-epi-LXA_4, when administered in vivo, inhibits neutrophil activation and dampens inflammation (Takano, 1997). 15-epi-LXA_4 is naturally formed in the body via this pathway:

Arachidonic Acid (AA) -> 15(R)-HETE -> 15-epi-LXA_4 Aspirin is thought to be involved in the acetylation of prostaglandin G/H synthase, which triggers the conversion of AA to 15(R)-HETE.

The development of this assay, which is both sensitive and selective for 15-epi-LXA_4, will be essential to researchers studying anti-inflammatory medications.

PRINCIPLE OF ASSAY

This is an ELISA (Enzyme-Linked Immunosorbent Assay) for the quantitative analysis of 15-epi-Lipoxin A_4 levels in biological fluid. This test kit operates on the basis of competition between the enzyme conjugate and 15-epi-Lipoxin A_4 in the sample for a limited number of binding sites.

First, the sample or standard solution is added to the microplate. Next, the diluted enzyme conjugate is added and the mixture is shaken and incubated at room temperature for one hour. During the incubation, competition for binding sites is taking place. The plate is then washed, removing all the unbound material. The bound enzyme conjugate is detected by the addition of substrate which generates an optimal color after 30 minutes. Quantitative test results may be obtained by measuring and comparing the absorbance reading of the wells of the samples against the standards with a microplate reader at 650 nm. The extent of color development is inversely proportional to the amount of 15-epi-Lipoxin A_4 in the sample or standard. For example, the absence of 15-epi-Lipoxin A_4 in the sample will result in a bright blue color, whereas the presence of 15-epi-Lipoxin A_4 will result in decreased or no color development.

MATERIALS PROVIDED

1. **EIA BUFFER:** 30 mL. Provided to dilute enzyme conjugate and 15-epi-Lipoxin A_4 standards.
2. **WASH BUFFER 10x:** 20 mL. Dilute10-fold with deionized water. Diluted wash buffer is used to wash all unbound enzyme conjugate, samples and standards from the wells after the one hour incubation.
3. **SUBSTRATE:** 20 mL. Stabilized 3,3', 5,5' Tetramethylbenzidine (TMB) plus Hydrogen Peroxide (H_2O_2) in a single bottle. It is used to develop the color in the wells after the wash step. LIGHT SENSITIVE. Keep substrate refrigerated.
4. **EXTRACTION BUFFER 5x:** 30 mL. Dilute 5-fold with deionized water. This is used for diluting extracted and non-extracted samples.
5. **15-EPI-LIPOXIN A_4 ENZYME CONJUGATE:** Two vials of lyophilized 15-epi-LXA_4 horseradish peroxidase conjugate. Reconstitution with 75 µL of deionized water results in a 50:1 concentrate. Blue capped vials.
6. **15-EPI-LIPOXIN A_4 STANDARD:** 50 µL. 15-epi-Lipoxin A_4 standard at the concentration of 1 µg/mL. Green capped vial.
7. **15-EPI-LIPOXIN A_4 ANTIBODY COATED PLATE:** A 96 well Costar® microplate with anti-15-epi-LXA_4 rabbit antibody precoated on each well. The plate is ready to use as is. **DO NOT WASH!**

MATERIALS NEEDED BUT NOT PROVIDED

1. 300 mL deionized water for diluting wash buffer, extraction buffer and conjugate.
2. Precision pipettes that range from 10 µL-1000 µL and disposable tips.

NOTE: If all or several strips are to be used at one time, it is suggested that a multichannel pipette be used.

3. Clean test tubes used to dilute the standards and conjugate.
4. Graduated cylinders to dilute and mix wash buffer and extraction buffer.
5. Microplate reader with 650 nm filter.
6. Plastic film or plate cover to cover plate during incubation.

OPTIONAL MATERIALS

7. 1 N HCl.
8. Microplate shaker.

If performing an extraction on samples, the following will be required:

9. 1 N HCl
10. Ethanol
11. C_{18} Sep-Pak® light column (Waters® Corporation)
12. Petroleum ether
13. Methanol
14. Nitrogen gas
15. Centrifuge

WARNINGS AND PRECAUTIONS

1. **DO NOT** use components beyond expiration date.
2. **DO NOT** mix any reagents or components of this kit with any reagents or components of any other kit. This kit is designed to work properly as provided.
3. **DO NOT** pipette reagents by mouth.
4. Always pour substrate out of the bottle into a clean test tube. **DO NOT** pipette out of the bottle. An unclean tip could contaminate the substrate.
5. All specimens should be considered potentially infectious. Exercise proper handling precautions.
6. **DO NOT** smoke, eat or drink in areas where specimens or reagents are being handled.
7. Use aseptic technique when opening and removing reagents from vials and bottles.
8. Keep plate covered except when adding reagents, washing or reading.
9. Kit components should be refrigerated at all times when not in use; lyophilized conjugate, frozen.
10. Ensure that the conjugate is completely reconstituted before use. Each vial, when reconstituted, provides sufficient reagent to perform 64 assays (8 strips). If more than 64 assays are to be run, reconstitute both vials and pool the reconstituted conjugate.

PROCEDURAL NOTES

1. It is not necessary to allow reagents to warm to room temperature before use.
2. Desiccant bag must remain in foil pouch with unused strips. Keep pouch sealed when not in use to maintain a dry environment. Seal with a heat sealer. If a heat sealer is not available, thoroughly close the open end with tape. Remove excess air before sealing.
3. Always use clean pipette tips for the buffer, enzyme conjugate, standards and samples.
4. Before pipetting a reagent, rinse the pipette tip three times with that reagent (i.e. fill the tip with the desired amount of reagent and dispense back into the same vial - repeat 2 times). Now the tip is properly rinsed and ready to dispense the reagent into your well or test tube.
5. When pipetting into the wells, DO NOT allow the pipette tip to touch the inside of the well, or any of the reagents already in the well. This can result in cross contamination.
6. Standards and samples should be assayed in duplicate.
7. To quantitate, always run a standard curve in duplicate when testing samples. If testing a sample that is not extracted, dilute standards in the same type of medium being tested. This medium should be known to be negative.
8. Gently mix specimens and reagents before use. Avoid vigorous agitation.
9. Before opening the lyophilized conjugate vial, examine the vial to ensure that lyophilized material has not been trapped in the cap. If material is in the cap, gently tap the upright vial to dislodge the trapped material.
10. To reconstitute the lyophilized conjugate, add 75 µL of deionized water to a vial. Rehydrate the conjugate by gently rotating the vial. Do not vortex or shake the contents. Avoid excess foaming. After the solid material has gone into solution, allow the conjugate to incubate at least 15 minutes before dilution. Write the date of reconstitution on the label. Concentrated, reconstituted conjugate has a shelf life of at least two weeks when stored at 4ºC.
11. The enzyme conjugate is most stable in its concentrated form. Dilute only the volume necessary for the amount of strips currently being used.
12. Before taking an absorbance reading, wipe the outside bottom of the wells with a lint-free wiper to remove dust and fingerprints.

SAMPLE PREPARATION

Usually, urine and tissue culture supernatant can be assayed directly by diluting them with the diluted extraction buffer. Dilute specimens may require extraction in order to concentrate 15-epi-LXA_4. Plasma and most other mediums will need to be extracted.

EXTRACTION OF 15-EPI-LIPOXIN A_4

1. For 1 mL plasma or urine: acidify to pH 3.5 with 1 N HCl. 1 mL of plasma requires about 150 µL of 1 N HCl.
   For tissue: Homogenize the tissue in ethanol (5 mL/g) and centrifuge to obtain supernatant. Dilute 1 mL of the supernatant with 5 mL of water and acidify to pH 3.5 with 1 N HCl.
2. Precondition the C_{18} Sep-Pak® light column (Waters® Corporation) by washing the column with 2 mL of ethanol followed by 2 mL of water.
3. Apply the above sample to the column and adjust the flow rate to 1 mL per minute. Reducing the flow rate to 0.5 mL per minute may increase extraction efficiencies. Some samples may clog the column. These samples may be diluted 1:5 in water to improve the flow rate.
4. Wash the column with 1 mL of water followed by 1 mL of petroleum ether.
5. Elute 15-epi-LXA_4 with 2 mL of methyl formate.
6. Evaporate methyl formate with a stream of Nitrogen gas.
7. Dissolve the residue in an appropriate volume of diluted extraction buffer and assay for 15-epi-Lipoxin A_4 content.

Note: Extraction buffer must be diluted 5 fold with deionized water before use. Any precipitant present must be brought into solution before dilution.

TEST PROCEDURES

1. Prepare standards as follows:

STANDARD	PREPARATION
A	stock solution 1 µg/mL (this is provided)
B	take 10 µL of A, add to 490 µL of EIA buffer and mix = 20 ng/mL
C	take 200 µL of B, add to 1.8 mL of EIA buffer and mix = 2 ng/mL
D	take 200 µL of C, add to 1.8 mL of EIA buffer and mix = 0.2 ng/mL

Continue standard preparation following Scheme I.

Scheme I

Standard	ng/mL	EIA buffer (µL added)	C standard µL	D Standard µL
S_0	0	as is	-	-
S_1	0.02	900	-	100
S_2	0.05	750	-	250
S_3	0.1	500	-	500
S_4	0.2	-	-	as is
S_5	0.5	750	250	-
S_6	1	500	500	-
S_7	2	-	as is	-

2. Determine the number of wells to be used.

NOTE: Allow for extra wells when calculating amount of conjugate to dilute to allow for loss during pipetting (i.e. 4 extra wells if using a single pipette; 10 extra wells if using a multichannel pipette).

3. Dilute the reconstituted 15-epi-Lipoxin A_4 enzyme conjugate. Add 1 µL of conjugate into 50 µL total volume of EIA buffer for each well assayed. For the entire plate, add 110 µL of the 15-epi-Lipoxin A_4 enzyme conjugate into 5.5 mL total volume of EIA buffer. Mix the solution thoroughly.

   NOTE: If more concentrated conjugate is needed than is contained in the first conjugate vial, reconstitute and use the second vial. Do not use the separate contents of both vials in the same assay as some assay variability may result. If the contents of both vials are required for an assay, pool the concentrated conjugates. Use the expiration date of the oldest reconstituted vial for the pool. Alternatively, prepare the necessary volumes of diluted conjugate and pool before using in the assay.

4. Add 50 µL of standards (S) or unknown (U) (some samples may require diluting) to the appropriate wells in duplicate.

   See Scheme II for suggested template design.

5. Add 50 µL of the diluted enzyme conjugate to each well. Use 8-channel pipette or 12-channel pipette for rapid addition.
6. Mix by shaking plate gently. A microplate shaker may be used.
7. Cover plate with plastic film or plate cover and incubate at room temperature for one hour. **Note:** Keep plate away from drafts and temperature fluctuations.
8. Dilute concentrated wash buffer with deionized water (i.e. 20 mL of wash buffer plus 180 mL of deionized water). Mix thoroughly.
9. After incubation, dump out the contents of the plate. Tap out contents thoroughly on a clean lint-free towel.
10. Wash each well with 300 µL of the diluted wash buffer. Repeat for a total of three washings. An automated plate washer can be used.
11. Add 150 µL of substrate to each well. Use multichannel pipette for best results. Mix by shaking plate gently.
12. Incubate at room temperature for 30 minutes.
13. Gently shake plate before taking a reading to insure uniform color throughout each well.

14. Plate is read in a microplate reader at 650 nm. If a dual wavelength is used, set W_1 at 650 nm and W_2 at 490 nm.
15. If accounting for substrate background, use 2 to 8 wells as blanks with only substrate in the wells (150 µL/well). Subtract the average of these absorbance values from the absorbance values of the wells being assayed.

NOTE: Some microplate readers can be programmed to do these subtractions automatically when reading the plate. Consult your instrument manual.

OPTIONAL TEST PROCEDURES

16. Add 50-100 µL of 1 N HCl to each well to stop enzyme reaction.
17. Read plate at 450 nm, if 1 N HCl solution was used.
18. Plot the standard curve and estimate the concentrations of the samples from the curve. See "CALCULATIONS."

Note: Absorbance readings will approximately double when stopped with acid. If absorbance readings are too high for measuring with your microplate reader, decrease the substrate incubation approximately 10 minutes but no more than 15 minutes.

Scheme II

	1	2	3	4	5	6	7	8	9	10	11	12
A	S_0	S_0	U_1	U_1	U_9	U_9	U_{17}	U_{17}	U_{25}	U_{25}	U_{33}	U_{33}
B	S_1	S_1	U_2	U_2	U_{10}	U_{10}	U_{18}	U_{18}	U_{26}	U_{26}	U_{34}	U_{34}
C	S_2	S_2	U_3	U_3	U_{11}	U_{11}	U_{19}	U_{19}	U_{27}	U_{27}	U_{35}	U_{35}
D	S_3	S_3	U_4	U_4	U_{12}	U_{12}	U_{20}	U_{20}	U_{28}	U_{28}	U_{36}	U_{36}
E	S_4	S_4	U_5	U_5	U_{13}	U_{13}	U_{21}	U_{21}	U_{29}	U_{29}	U_{37}	U_{37}
F	S_5	S_5	U_6	U_6	U_{14}	U_{14}	U_{22}	U_{22}	U_{30}	U_{30}	U_{38}	U_{38}
G	S_6	S_6	U_7	U_7	U_{15}	U_{15}	U_{23}	U_{23}	U_{31}	U_{31}	U_{39}	U_{39}
H	S_7	S_7	U_8	U_8	U_{16}	U_{16}	U_{24}	U_{24}	U_{32}	U_{32}	U_{40}	U_{40}

CALCULATIONS

1. After the substrate background has been subtracted from all absorbance values, average all of your duplicate well absorbance values.
2. The average of your two S_0 values is now your B_0 value. (S_1 now becomes B_1, etc.)
3. Next, find the percent of maximal binding (%B/B_0 value). To do this, divide the averages of each standard absorbance value (now known as B_1 through B_7) by the B_0 absorbance value and multiply by 100 to achieve percentages.

4. Graph your standard curve by plotting the $\%B/B_0$ for each standard concentration on the ordinate (y) axis against concentration on the abscissa (x) axis. Draw a curve by using a curve-fitting routine (i.e. 4-parameter or linear regression).
5. Divide the averages of each sample absorbance value by the B_0 value and multiply by 100 to achieve percentages.
6. Using the standard curve, the concentration of each sample can be determined by comparing the $\%B/B_0$ of each sample to the corresponding concentration of 15-epi-Lipoxin A_4 standard.
7. If the samples were diluted, the concentration determined from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

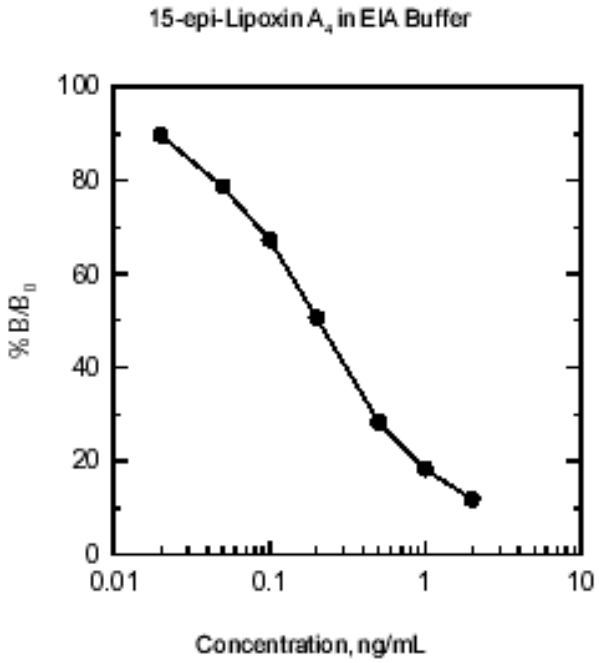
Note: "Typical data" is a representation. Variances in data will occur. Optical density readings may fluctuate during the shelf-life of the kit, but the $\%B/B_0$ should remain comparable. Measuring wavelength: 650 nm

Standard	Standard Concentration (ng/mL)	Optical Density (Absorbance Value)	$\%B/B_0$
S_0 (B_0)	0	1.057	100
S_1 (B_1)	0.02	0.977	92
S_2 (B_2)	0.05	0.833	79
S_3 (B_3)	0.1	0.715	68
S_4 (B_4)	0.2	0.539	51
S_5 (B_5)	0.5	0.301	29
S_6 (B_6)	1	0.193	18
S_7 (B_7)	2	0.125	12

CROSS REACTIVITY

15-epi-Lipoxin A_4	100.0%
Lipoxin A_4	3%
15(R)-HETE	0.8%
5(S)-HETE	<0.01%
12(S)-HETE	<0.01%
15(S)-HETE	<0.01%

TYPICAL STANDARD CURVE



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2. Takano T, Clish CB, Gronert K, Petasis N, Serhan CN. Neutrophil-mediated changes in vascular permeability are inhibited by topical applications of aspirin-triggered 15-epi-Lipoxin A_4 and novel Lipoxin B_4 stable analogues. *J. Clin. Invest.* **101:** 819-826 (1998).

TECHNICAL SUPPORT

If you need technical information or assistance with assay procedures, call our Technical Support Department at 1-800-692-4633 or 1-248-852-8815. Our staff will be happy to answer your questions about this or any other product in the Oxford Biomedical line.

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