NEOGEN®
CORPORATION
ISO 9001

Leukotriene B_4 ELISA Kit Instructions

Please read all instructions carefully before beginning this assay

PRODUCT #406110
For research use only.

Storage Conditions:
Lyophilized conjugate: -20°C or less.
Do not freeze kit components.
Do not freeze reconstituted conjugate.

DESCRIPTION

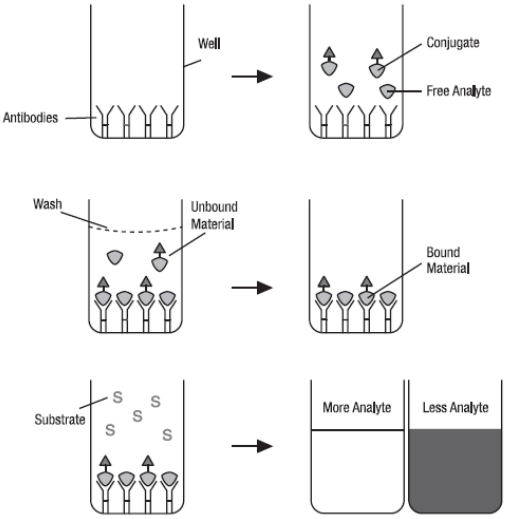
Leukotriene B_4 (LTB_4) is derived from LTA_4 which in turn is biosynthesized from arachidonic acid. LTB_4 is produced in phagocytic cells such as leukocytes and macrophages. It is the most potent chemotactic substance ever described for leukocytes. It also stimulates vascular permeability and may play a significant role in mediating inflammatory responses. The availability of an ELISA for LTB_4 will facilitate the evaluation of the physiological and pathological roles of this lipid mediator.

PRINCIPLE OF ASSAY

This is an ELISA (Enzyme-Linked Immunosorbent Assay) for the quantitative analysis of Leukotriene B_4 levels in biological fluid. This test kit operates on the basis of competition between the enzyme conjugate and the Leukotriene B_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 Leukotriene B_4 in the sample or standard. For example, the absence of Leukotriene B_4 in the sample will result in a bright blue color, whereas the presence of Leukotriene B_4 will result in decreased or no color development.

PRINCIPLE OF ASSAY (continued)



MATERIALS PROVIDED

1. ***EIA BUFFER:*** 30 mL. Provided to dilute enzyme conjugate and Leukotriene B_4 standards
2. ***WASH BUFFER (10X):*** 20 mL. To be diluted 10-fold with deionized water. This is used to wash all unbound enzyme conjugate, samples and standards from the plate after the one hour incubation.
3. ***K-BLUE 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 they have been washed. **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. ***LEUKOTRIENE B_4 ENZYME CONJUGATE:*** Two vials of lyophilized LTB_4 horseradish peroxidase conjugate. Reconstitution with 75 µL of deionized water results in a 50:1 concentrate. Blue capped vial.
6. ***LEUKOTRIENE B_4 STANDARD:*** 100 µL. Leukotriene B_4 standard at the concentration of 1 µg/mL. Green capped vial.
7. ***LEUKOTRIENE B_4 ANTIBODY-COATED MICROPLATE:*** A 96 well MaxiSorp™ Nunc microplate with anti-LTB_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 lyophilized 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. Plate cover or plastic film to cover plate during incubation.

OPTIONAL MATERIALS:

7. 1 N HCl or Neogen's Red Stop Solution.
8. Microplate shaker.

   If performing an extraction on samples, the following will be required:
9. 1N HCl
10. Ethanol
11. C_{18} Sep-Pak® light column (Waters® Corporation)
12. Petroleum ether
13. Methyl Formate
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. If the pipette tip is unclean, this could result in contamination of 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 wells (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 zip-lock pouch sealed when not in use to maintain a dry environment.
3. Always use new 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 samples alongside a standard curve. If testing a sample that is not extracted, standards should be diluted 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 4°C.
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

This assay is non-species specific. 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 LTB_4. Plasma and most other mediums require extraction.

EXTRACTION OF LEUKOTRIENE B_4

1. For 1 mL plasma or urine: Acidify to pH 3.5 with 1N HCl (1 mL of plasma requires about 150 µL of 1N HCl).
   For tissue: Homogenize the tissue in ethanol (5mL/gm) and centrifuge to obtain supernatant. Dilute 1 mL of the supernatant with 5 mL of water and acidify to pH 3.5 with 1N 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 into 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 eicosanoid 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 extraction buffer and assay for eicosanoid 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 (provided in green capped vial.)
B	take 20 µL of A, add to 980 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

Standards	*ng/mL*	*EIA buffer (µL added)*	*B standard µL*	*C standard µL*	*D standard µL*
S_0	0	as is	-	-	-
S_1	0.04	800	-	-	200
S_2	0.1	500	-	-	500
S_3	0.2	-	-	-	as is
S_4	0.4	800	-	200	-
S_5	1	500	-	500	-
S_6	2	-	-	as is	-
S_7	4	800	200	-	-

2. Determine the number of wells to be used.
3. Dilute the Leukotriene B_4 enzyme conjugate. Add 1µL of enzyme conjugate into 50 µL total volume of EIA buffer for each well assayed. Mix the solution thoroughly (avoid foaming). For the entire plate, add 110 µL of the Leukotriene B_4 enzyme conjugate into 5.5 mL total volume of EIA Buffer. Mix the solution thoroughly (avoid foaming).

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 50µL 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, however, increase number of wash cycles from three to five.
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 ensure 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 or Neogen's Red Stop Solution to each well to stop enzyme reaction.
17. Read plate at 450 nm, if 1 N HCl solution was used. Read plate at 650 nm, if Neogen's Red Stop Solution was used.
18. Plate 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

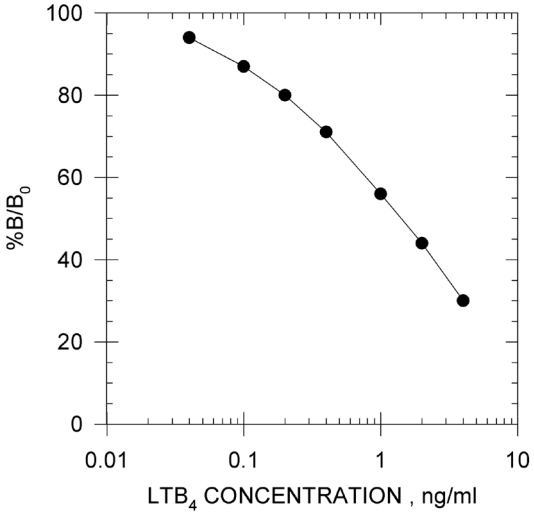
	1	2	3	4	5	6	7	8	9	10	11	12
A	S0	S0	U1	U1	U9	U9	U17	U17	U25	U25	U33	U33
B	S1	S1	U2	U2	U10	U10	U18	U18	U26	U26	U34	U34
C	S2	S2	U3	U3	U11	U11	U19	U19	U27	U27	U35	U35
D	S3	S3	U4	U4	U12	U12	U20	U20	U28	U28	U36	U36
E	S4	S4	U5	U5	U13	U13	U21	U21	U29	U29	U37	U37
F	S5	S5	U6	U6	U14	U14	U22	U22	U30	U30	U38	U38
G	S6	S6	U7	U7	U15	U15	U23	U23	U31	U31	U39	U39
H	S7	S7	U8	U8	U16	U16	U24	U24	U32	U32	U40	U40

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 Leukotriene B_4 standard.
7. If the samples were diluted, the concentration determined from the standard curve must be multiplied by the dilution factor.

TYPICAL STANDARD CURVE

LTB_4 in EIA Buffer



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	1.165	100
S_1 (B_1)	0.04	1.096	94
S_2 (B_2)	0.1	1.019	87
S_3 (B_3)	0.2	0.935	80
S_4 (B_4)	0.4	0.826	71
S_5 (B_5)	1	0.651	56
S_6 (B_6)	2	0.510	44
S_7 (B_7)	4	0.355	30

CROSS REACTIVITY

LEUKOTRIENE B_4 ..... 100.0%
6-TRANS-LTB_4 ..... 25.0%
LEUKOTRIENE B_5 ..... 14.6%
5(S),12(S) DIHETE 6.0%
LEUKOTRIENE D_4 ..... 0.96%
20-HYDROXY-LTB_4 ..... 0.50%
LEUKOTRIENE E_4 ..... 0.30%
LEUKOTRIENE C_4 ..... 0.20%
5(S)HETE 0.15%
20-CARBOXY-LTB_4 ..... <0.10%
ARACHIDONIC ACID ..... <0.10%
12(S)HETE ..... <0.10%
12(R)HETE ..... <0.10%
15-HETE ..... <0.01%
PROSTAGLANDIN A_2 ..... <0.01%
PROSTAGLANDIN B_2 ..... <0.01%
PROSTAGLANDIN D_2 ..... <0.01%
PROSTAGLANDIN E_2 ..... <0.01%
PROSTAGLANDIN $F_{2\alpha}$ ..... <0.01%
6-KETO-PROSTAGLANDIN $F_{1\alpha}$ ..... <0.01%
THROMBOXANE B_2 ..... <0.01%

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TECHNICAL ASSISTANCE

Technical assistance is available Monday-Friday, between 8:00 a.m. and 6:00 p.m. EST.

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