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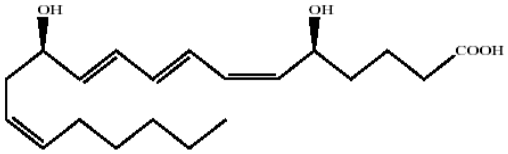
Leukotriene B_4 EIA

ProductInformation

Product Number **CS0220**
Storage Temperature –20 °C

Technical Bulletin

Product Description



LTB_4

Leukotriene B_4 (LTB_4) EIA is a four-hour competitive enzyme immunoassay (EIA) for the quantitative determination of leukotriene B_4 (LTB_4) concentrations in cell culture supernatants, plasma, saliva, urine and other biological fluids. LTB_4 present in the samples or standards competes with the fixed amount of LTB_4 conjugated to alkaline phosphatase for the limited number of binding sites on the anti-rabbit LTB_4 antibody. During the first incubation, the antigen-antibody complex binds to the anti-rabbit IgG antibody-coated multiwell plate. The excess conjugate and unbound sample are washed away and a substrate is added. During the second incubation the bound enzyme reacts with the substrate. The enzyme reaction is stopped and absorbance read at 405 nm. The intensity of the yellow color is inversely proportional to the concentration ofLTB4 in the standards or the samples. The concentration is calculated on the basis of optical reading of standard dilutions.

Leukotriene B_4 (LTB_4) is a major product of arachidonic acid metabolism and is formed via the 5-lipoxygenase pathway. LTB_4 is a potent chemoattractant that is primarily involved in inflammation, immune responses, and host defense against infection.[1] LTB_4 stimulates leukocyte functions including lysosomal enzyme release, adhesion and aggregation of polymorpho-nuclear leukocytes. LTB_4 has been implicated as a potent mediator of inflammatory diseases and immunoregulation. LTB_4 can also bind and activate the intranuclear transcription factor PPAR-alpha resulting in the activation of genes that terminate inflammatory processes. Increased LTB_4 levels were found in asthma, diabetes, inflammatory conditions, sepsis, psoriasis, and in high cholesterol diets.[2-5]

Reagents

- **Leukotriene B_4 (LTB_4) Standard, 1 vial, Product No. L 3917** – 0.5 mL (120,000 pg/mL) in a buffer with preservative.
- **Anti-Rabbit IgG-coated 96 well plate, 1 plate, Product No. I 6283.**
- **Assay Buffer, 30 mL, Product No. A 4228 –** contains sodium azide. Ready to use.
- **Anti-LTB_4, 5 mL, Product No. L 3667 –** from rabbit, contains sodium azide, yellow dye. Ready to use.
- **Leukotriene B4 (LTB_4)-Alkaline Phosphatase conjugate, 5 mL, Product No. L 3792 -** contains sodium azide, blue dye. Ready to use.
- **p-Nitrophenylphosphate (pNpp) Substrate, 20 mL, Product No. N 6911 -** Ready to use.
- **Wash Buffer Concentrate, 10X, 30 mL, Product No. W 3139 -** contains sodium azide.
- **Stop Solution, 5 mL, Product No. S 9443 –** a solution of trisodium phosphate. Ready to use.
- **Plate sealer, 1 each, Product No. P 1496**

Reagents and Equipment required but not provided

- Multiwell plate reader capable of readings at 405 nm, preferably with corrections between 570 and 590 nm.
- Horizontal orbital multiwell plates shaker capable of maintaining a speed of 500 +/- 50 rpm.
- Calibrated adjustable precision pipettes for volumes between 5 µL and 1,000 µL.
- Cell extraction materials (see recommended extraction procedure).
- Deionized or distilled water.
- Plate washer (optional), use squirt bottle, manifold dispenser, etc.
- Glass or plastic 1.0 – 1.5 mL tubes for diluting and aliquoting standard.
- Absorbent paper towels to blot the plate.
- Calibrated beakers and graduated cylinders in various sizes.
- Vortex mixer.

- Graph paper: linear, log-log, or semi-log, as desired.
- Prostaglandin synthetase inhibitor: Indomethacin (Product No. I 8280) or meclofenamic acid (Prod. No. M 4531).

Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Sample Preparation

- The assay is validated for cell culture supernatants, human saliva, urine and EDTA plasma, which may be assayed directly or after extraction.
- Samples containing rabbit IgG may interfere with the assay, due to the fact that the wells are coated with anti-rabbit IgG.
- Cell culture supernatants should be separated from the cells and frozen with the addition of synthetase inhibitor, if not analyzed shortly after collection.
- Extract LTB_4 as described in Protocol for Sample Extraction
- Cell culture samples may be assayed undiluted or diluted in cell culture media.
- Use the same cell culture media for blanks, controls and standard dilutions.
- EDTA Plasma, urine and sera may be assayed directly or after 1:2 – 1:4 dilution in Assay Buffer.
- To ensure accurate results, always dilute the standards and blanks in the same diluent as the samples.
- If samples are not assayed immediately, a prostaglandin synthetase inhibitor, such as indomethacin (Product No. I 8280), or meclofenamic acid should be added to all samples at approximately 10 µg/mL before storage.

Materials Required for Extraction

- 2 N HCl
- Ethanol
- Hexane
- Ethyl acetate
- 200 mg C_{18} Reverse Phase Column
- High specific activity tritiated leukotriene (for determination of extraction efficiency). Activity should be > 3.5 TBq/mMol

Protocol for Sample Extraction

1. Acidify sample to pH 3.5 with 2N HCl. Needs ~ 50 µL of HCl per 1 mL of plasma.
2. Equilibrate at 4°C for 15 minutes.
3. Centrifuge samples in a microcentrifuge for 2 minutes to remove any precipitate.
4. Prepare the C_{18} reverse phase column by washing with 10 mL of ethanol followed by 10 mL of deionized water.
5. Apply the sample under a slight positive pressure to obtain a flow rate of about 0.5 mL/minute.
6. Wash the column with 10 mL of water, followed by 10 mL of 15% ethanol, and finally 10 mL hexane.
7. Elute the sample from the column by addition of 10 ml ethyl acetate.
8. If analysis is to be carried out immediately, evaporate samples under a stream of nitrogen.
9. Add 50 µL ethanol to the dried samples and reconstitute sample with at least 200 µL of Assay Buffer or cell culture media.
10. If analysis is to be delayed, store samples as the eluted ethyl acetate solutions at -70°C until the immunoassay is to be run.
11. Repeat steps 8 and 9.

References 6 and 7 contain detailed extraction protocols.

Reagent Preparation

LTB_4 Standard

1. The LTB_4 Standard is supplied in ethanol buffer at a pH optimized to maintain LTB_4 integrity. Handle with care.
2. Equilibrate the 120,000 pg/mL stock standard solution to room temperature.
3. Prepare serial standard dilutions as follows:

Tube #	Assay Buffer or Cell Culture Media mL	Standard from tube #: mL	Final Standard Concentration pg/mL
0	Standard vial 120,000 pg/mL		
1	0.9 mL	0.1 mL (0)	12,000
2	0.75 mL	0.25 mL (1)	3,000
3	0.75 mL	0.25 mL (2)	750
4	0.75 mL	0.25 mL (3)	188
5	0.75 mL	0.25 mL (4)	46.9

4. Diluted standards should be used within 60 minutes of preparation.

LTB_4 -Alkaline Phosphatase conjugate

1. The activity of LTB_4 Conjugate is depended on the presence of MG^{2+} and Zn^{2+} ions and is affected by high concentration of chelators, such as EDTA and EGTA. Samples containing >10 mg/mL chelators should be diluted prior to assay.
2. Equilibrate conjugate to room temperature.
3. Add 50 µL conjugate to the appropriate wells as indicated in the Assay Layout Sheet.
4. Aliquot the remaining conjugate and freeze at –70 °C
5. For Total Activity (TA) wells: dilute 50 µL conjugate in 450 µL Assay Buffer – add 5 µL to TA wells.

Wash Buffer

1. Warm to room temperature.
2. Dilute 10 mL wash concentrate with 90 mL deionized water.
3. Label as Working Wash buffer
   May be stored at room temperature for 3 months or for the shelf life of the kit.

Storage/Stability

The kit is shipped on dry ice and should be stored at <–20 °C until use.
After opening:

- Conjugate must remain frozen at –20 °C.
- The rest of the components may be stored at 2-8 °C.

Refer to the Certificate of Analysis for kit shelf life. To obtain C of A go to www sigma-aldrich.com

Procedure

Precautions

- 20-30 minutes before use equilibrate kit and all reagents to room temperature (15-30 ºC).
- Use only the coated 96 multiwell capture plate provided with the kit.
- Multiwell plate should be equilibrated to room temperature in unopened foil bag. Remove desired number of strips, reseal the bag and refrigerate at 2 - 8°C to maintain plate integrity.
- When not in use all kit components should be stored as described in **Storage/Stability.**
- Assay all standards, controls and samples in duplicate.
- If particulate matter is present, centrifuge or filter prior to analysis.
- A standard curve must be run with each assay
- Maintain a consistent order of components and reagents addition from well to well. This ensures equal incubation times for all wells.
- Run in-house controls with every assay. If control values fall outside pre-established ranges, the accuracy of the assay may be suspect.
- All reagents are lot-specific. Do not mix reagents from different kit lots.
- Do not use reagents after the kit expiration date.
- Standards and samples can be made up in either glass or plastic tubes.
- Minimize contamination by endogenous alkaline phosphatase, present especially in the substrate solution. Avoid touching pipette tips and other items with bare hands.
- Pre-rinse the pipette tip with the reagent and use fresh pipette tips for each sample, standard or reagent.
- Read absorbances within 2 hours of assay completion.

Washing directions

1. The purpose of washing is to remove unbound proteins and other non-specific parts of lysate.
2. Incomplete washing will adversely affect the assay and render false results.
3. Use only Wash Buffer provided in kit.
4. Washing may be performed using automated washer, manifold pipette or squirt bottle.
5. Wash cycle three times, blotting as dry as possible after the 3rd wash.
6. When washing manually, fill wells with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.
7. It is recommended to use laboratory tape to hold plate strips to the plate frame while performing the plate washing and drying procedure to avoid strips coming free of the frame.

Assay Procedure

Refer to the diagram at the end of this bulletin to view the assay layout.

The following wells are needed for each assay run:

2 substrate blank wells (SBo)
2 Total Activity wells (TA),
2 Non-specific Binding wells (NSB)
2 0 Standard (B_0) pg/mL wells
12 standard dilutions wells
2 wells for each sample to be assayed

1st Incubation

a. Remove the appropriate number of plate strips and return the unused strips to the pouch. Reseal pouch and refrigerate.
b. Add 100 µL of Assay Buffer to the NSB and standard zero blank (B_o) wells
c. Add 100 µL of standards #1-5 to the appropriate wells.
d. Add 100 µL of samples to the appropriate wells
e. Add 50 µL of Assay Buffer to NSB wells
f. Add 50 µL of LTB_4-alkaline phosphatase conjugate to all wells (except the TA and SB).
g. Add 50 µL of Anti-LTB_4 to each well (except NSB, TA and SBo wells).
h. Cover with plate cover and incubate 2 hours at room temperature on orbital shaker set at 500 rpm.
i. All wells should be green, except NSB wells, which are blue.
j. Wash wells for a total of 3 times following washing instructions.
k. After the final wash, blot dry on a lint free paper towel to remove any remaining wash buffer.

Substrate Incubation

a. Add 5 µL of LTB_4 conjugate 1:10 dilution to the TA wells.
b. Add 200 µL of pNpp substrate to all wells. Cover.
c. Incubate 2 hours at 37 °C without shaking.

Stop Reaction

a. Add 50 µL of Stop Solution to each well.
b. Yellow color develops immediately and can be read in the multiwell plate reader at 405 nm with corrections at 570 or 590 nm. Subtract the readings at 590 nm from the readings at 405 nm, to correct for optical imperfection of the plate.

Results

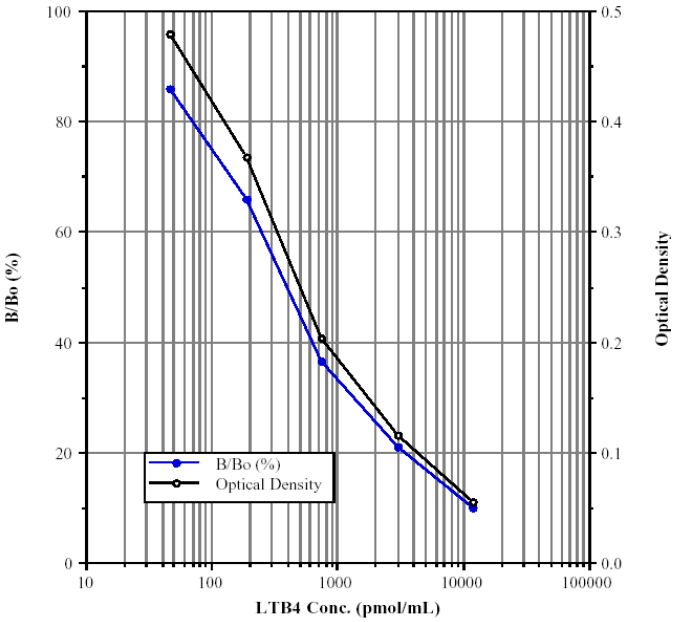
1. Average the duplicate readings for each standard and sample and subtract the average NSB optical density.
2. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit.
3. As an alternative, construct a standard curve by plotting the mean absorbance for each standard (nm) on a linear y-axis against the concentration (pg/mL) on a logarithmic x-axis and draw the best fit curve through the points on the graph.
4. % B/B_0 can be calculated as follows:

$$\% \text{ Bound} = \frac{\text{Net OD nm}}{\text{Net Bo OD}} \times 100$$

5. Calculate the concentration of LTB_4 corresponding to the mean absorbance or % B/B_0 from the standard curve. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Product Profile

Typical Results



Typical Standard Curve for LTB_4 EIA

Well	Net OD 405 nm	%Bound	LTB4 pg/mL
Blank	(0.115)		
TA	1.281		
NSB	0.002		
Bo	0.561	100%	0
S1	0.057	10.1%	12,000
S2	0.118	21.1%	3,000
S3	0.205	36.5%	750
S4	0.370	65.9%	188
S5	0.481	85.7%	46.9
Sample 1	0.316	56.2%	295
Sample 2	0.102	17.9%	3,298

Typical Results

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.

Quality Control

Each laboratory should establish a quality control program to monitor the performance of theLTB4 Immunoassay. As a part of this program, TA, NSB, B_0, and Substrate Blank wells are included in each assay. The average readings are calculated over the time. Any time the assay readings exceed the average, the assay may need to be re-run.

Typical Quality Control Parameters

Total Activity Added = 1.281X10 = 12.81
% NSB = 0.0%
B_0/TA = 4.36%
Quality of Fit = 0.9998

Performance Characteristics

The following parameters for this kit were determined using the guidelines listed in the National Committee for Clinical Laboratory Standards (NCCLS) Evaluation Protocols.

Sensitivity

- Sensitivity was calculated by determining the average optical density bound (B_0) for sixteen (16) wells, and comparing to the average optical density for sixteen (16) runs of Standard #5.
- The detection limit was determined as the concentration of LTB_4 measured at two standard deviations from the zero along the standard curve.

Average Optical Density for the Bo
= 0.547 ± 0.017 (3.0%)
Average Optical Density for Standard #5
= 0.464 ± 0.014 (2.9%)
Delta Optical Density (0-46.9 pg/mL)
= 0.082
2 SD's of the Zero Standard
= 2 x 0.017 = 0.034
Sensitivity = $\frac{0.034}{0.082}$ x 46.9 pg/mL = 19.4 pg/mL

Linearity

A sample containing 7,990 pg/mL LTB_4 was diluted 6 times 1:2 in the kit Assay Buffer and measured in the LTB_4 assay. The data was plotted as actual LTB_4 concentration versus measured LTB_4 concentration. The line obtained had a slope of 0.929 and a correlation coefficient of 0.998

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of LTB_4 and running these samples 16 times in the same assay.
Inter-assay precision was determined by measuring three samples with low, medium and high concentrations of LTB_4 in 8 different assays.

	LTB_4 pg/mL	Intra-Assay %CV	Inter-Assay %CV
Low	305 6.0	6.0	
Medium	607	6.8	
High	1,078	5.9	
Low	99		15.7
Medium	308		16.5
High	507		5.0

Recovery

LTB4 concentrations were measured in a variety of different samples including tissue cultures, human saliva and urine, and porcine plasma. LTB_4 was spiked into the undiluted samples, which were then diluted with the Assay Buffer and then assayed in the kit. The following results were obtained:

Sample	% Recovery	Recommended Dilution
Tissue Cultures	97.3	None
Human Saliva	114.1	>1:4
Human Urine	96.9	None
Porcine EDTA Plasma	109.6	1:2-1:4

Cross reactivity

The cross reactivities for a number of related compounds were determined by dissolving the cross reactant (purity checked by N.M.R. and other analytical methods) in Assay Buffer at concentrations from 0.4 to 40,000 pg/mL. These samples were then measured in the LTB_4 assay and the LTB_4 concentration at 50% B/Bo calculated. The % cross reactivity was calculated by comparison with the actual concentration of cross reactant in the sample and expressed as a percentage.

Compound	% Cross reactivity
LTB_4	100
6-trans-12-epi- LTB_4	5.50%
6-trans-LTB_4	4.90%
12-epi- LTB_4	0.94%
PGE_2	<0.2%
$PGF_{2\alpha}$	<0.2%
20-OH-LTB_4	<0.2%
20-COOH-LTB_4	<0.2%
LTC_4	<0.2%
LTD_4	<0.2%
LTE_4	<0.2%
$PGF_{2\alpha}$	<0.2%
6-keto-$PGF_{1\alpha}$	<0.2%
PGD_2	<0.2%
5-S-HETE	<0.2%
12-S-HETE	<0.2%
15-S-HETE	<0.2%

References

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LTB_4 Assay Flow Chart (CS0220)

Wells ID	**Blank**	**TA**	**NSB**	**B_o**	**Standards**	**Samples**
	A1, B1	**C1, D1**	**E1, F1**	**G1, H1**	**A2-B3**	**C3-H12**
Assay Buffer			150 µL	100 µL		
Standard and/or Sample*					100 µL	100 µL
LTB_4 Conjugate			50 µL	50 µL	50 µL	50 µL
Anti-LTB_4 Antibody				50 µL	50 µL	50 µL

Incubate 2 hours @ RT with shaking
Wash 3X

Conjugate 1:10 dilution		5 µL**				
pNpp Substrate	200 µL	200 µL	200 µL	200 µL	200 µL	200 µL

Incubate 2 hours @ 37 ºC without shaking

Stop Solution	50 µL	50 µL	50 µL	50 µL	50 µL	50 µL

*Sample may be diluted as needed

**Conjugate for TA must be diluted 1:10 in Standard Diluent: 450 µL Standard Diluent + 50µL LTB_4