Parameter™

LTB_4 Assay

Catalog Number KGE006B
SKGE006B
PKGE006B

For the quantitative determination of Leukotriene B_4 (LTB_4) concentrations in cell culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

TABLE OF CONTENTS

Contents Page

INTRODUCTION 2
PRINCIPLE OF THE ASSAY . . . 3
LIMITATIONS OF THE PROCEDURE 3
TECHNICAL HINTS . . . 3
MATERIALS PROVIDED 4
STORAGE . . . 4
OTHER SUPPLIES REQUIRED 5
PRECAUTIONS . . . 5
SAMPLE COLLECTION AND STORAGE 5
REAGENT PREPARATION . . . 6
ASSAY PROCEDURE 7
ASSAY PROCEDURE SUMMARY . . . 8
CALCULATION OF RESULTS 9
TYPICAL DATA . . . 9
PRECISION 10
RECOVERY . . . 10
LINEARITY 11
SENSITIVITY . . . 11
SAMPLE VALUES 12
SPECIFICITY . . . 12
REFERENCES 13
PLATE LAYOUT . . . 14

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INTRODUCTION

Leukotriene B4 (LTB_4) is a potent pro-inflammatory molecule that belongs to a family of eicosanoid lipid mediators (1, 2). It is synthesized from arachidonic acid that is generated from nuclear membrane phospholipid (1, 3). In general, and upon cell activation, phospholipase A2 is directed to the nuclear membrane. Here, it generates arachidonic acid that is bound by one 18 kDa membrane-bound protein termed Five-Lipooxygenase Activating Protein (FLAP). FLAP-bound arachidonic acid is subjected to two distinct, sequential actions by 80 kDa 5-lipooxygenase (5-LO). The first 5-LO action generates 5(S)-HPETE/5-HPETE, an oxygenated form of arachidonic acid. The newly generated 5-HPETE is then acted on again by 5-LO to generate LTA_4 (1 - 4). The LTB_4 synthetic pathway has two key checkpoints. The first is at the level of arachidonic acid formation, where COX enzymes offer an alternative to 5-LO in the generation of prostaglandins. The second is at the level of LTA_4, where 69 kDa LTA_4 hydrolase activity will generate LTB_4 (a pro-inflammatory molecule), and 18 kDa LTC_4 synthetase activity will generate LTC/D/E4 (smooth muscle contractants) (4, 5). The key enzyme in LTB_4 synthesis is 5-LO, which has limited cell expression. Cells known to express 5-LO include B cells, macrophages, monocytes, mast cells, neutrophils, neurons, eosinophils, and dendritic cells (1, 2). Notably, all cells are potential sources for LTB_4 production. This is due to the fact that LTA_4 hydrolase is ubiquitously expressed, and excess LTA_4 generated by 5-LO expressing cells can diffuse into neighboring LTA_4 hydrolase-containing cells for conversion into LTB_4 (5).

LTB_4 has two known membrane receptors and one intracellular receptor. The membrane receptors are named BLT1 and BLT2 (2, 6). Both are 7-transmembrane receptors that induce Ca^{2+} flux when activated. BLT1 is a high-affinity receptor for LTB_4 expressed by hematopoietic cells and keratinocytes, while BLT2 is a low-affinity receptor that is widely expressed (6, 7). The intracellular receptor is PPAR-γ, which becomes active upon binding to LTB_4. This activation promotes the expression of LTB_4 degradative enzymes, creating a negative feedback loop for LTB_4 production (1, 8).

LTB_4 functions are considered pro-inflammatory but vary depending upon the target cell. On hematopoietic stem cells, LTB_4 may induce differentiation into CFU-GEMM and CFU-GM progenitor cells (9). B cells are induced to differentiate by LTB_4, and in the presence of IL-4, to release IgE (2). LTB_4 is also considered essential for Th2-type responses. It drives IL-4 and IL-5 production, and promotes $CD4^+$ cell proliferation while inhibiting $CD8^+$ cell expansion (2, 6, 10). Under the influence of LTB_4, endothelial cells bind and promote neutrophil transmigration (1, 6). LTB_4 is perhaps best known for its activity on neutrophils. It promotes polymorphonuclear (PMN) cell migration, blocks PMN apoptosis, and induces neutrophil granule release in conjunction with reactive oxygen species generation (2, 6). Finally, it activates macrophage phagocytosis and drives mononuclear pro-inflammatory cytokine release (2, 6).

R&D Systems' LTB_4 Immunoassay is a 4.5 hour forward sequential competitive enzyme immunoassay designed to measure LTB_4 in cell culture supernates, serum, and plasma.

PRINCIPLE OF THE ASSAY

This assay is based on the forward sequential competitive binding technique in which LTB_4 present in a sample competes with a fixed amount of horseradish peroxidase (HRP)-labeled LTB_4 for sites on a chicken polyclonal antibody. During the incubations, the chicken polyclonal antibody becomes bound to the rabbit anti-chicken antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. Following color development, the assay is stopped, and the absorbance is read at 450 nm. The intensity of the color is inversely proportional to the concentration of LTB_4 in the sample.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in this assay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- Pre-rinse the pipette tip when pipetting.
- Pipette standards and samples to the bottom of the wells. Add all other reagents to the side of the wells to avoid contamination.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution. Gently tap the side of the plate to ensure thorough mixing.

MATERIALS PROVIDED

Description	Part #	Cat. # KGE006B	Cat. # SKGE006B
LTB_4 Microplate - 96 well polystyrene microplate (12 strips of 8 wells) coated with a rabbit anti-chicken polyclonal antibody.	893074	1 plate	6 plates
LTB_4 Conjugate - 6 mL/vial of LTB_4 conjugated to horseradish peroxidase with red dye and preservatives.	893641	1 vial	6 vials
LTB_4 Standard - 25 ng/vial of LTB_4 in buffer with preservatives; lyophilized.	893077	1 vial	6 vials
Primary Antibody Solution - 6 mL/vial of a chicken polyclonal antibody to LTB_4 in buffer with blue dye and preservatives.	893642	1 vial	6 vials
Calibrator Diluent RD5-52 - 21 mL/vial of a buffered protein base with preservatives.	895925	1 vial	6 vials
Wash Buffer Concentrate - 21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservatives.	895003	1 vial	6 vials
Color Reagent A - 12.5 mL/vial of stabilized hydrogen peroxide.	895000	1 vial	6 vials
Color Reagent B - 12.5 mL/vial of stabilized chromogen (tetramethylbenzidine).	895001	1 vial	6 vials
Stop Solution - 11 mL/vial of 2 N sulfuric acid.	895926	1 vial	6 vials
Plate Covers - Adhesive strips.	——	4 strips	24 strips

KGE006B contains sufficient materials to run an ELISA on one 96 well plate.
SKGE006B (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PKGE006B). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at ≤ -20° C in a manual defrost freezer. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="9">Opened/ Reconstituted Reagents</td><td>Diluted Wash Buffer</td><td rowspan="6">May be stored for up to 1 month at 2 - 8° C.*</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Calibrator Diluent RD5-52</td></tr>
<tr><td>Unmixed Color Reagent A</td></tr>
<tr><td>Unmixed Color Reagent B</td></tr>
<tr><td>Primary Antibody Solution</td></tr>
<tr><td>Conjugate</td><td rowspan="2">Aliquot and store for up to 1 month at ≤ -20° C in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.</td></tr>
<tr><td>Standard</td></tr>
<tr><td>Microplate Wells</td><td>Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*</td></tr>
</table>

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution.
- LTB_4 Controls (optional; available from R&D Systems).

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

Care should be taken when handling the LTB_4 Standard because of the known and unknown effects of eicosinoids.

SAMPLE COLLECTION AND STORAGE

Samples containing chicken IgY may interfere with this assay.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Note: *Animal serum used in the preparation of cell culture media may contain high levels of LTB_4. For best results, do not use animal serum for growth of cell cultures when assaying for LTB_4 production. If animal serum is used as a supplement in the media, the appropriate media control should be prepared and tested in the immunoassay to determine the baseline concentration of LTB_4.*

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles to minimize the conversion of LTA_4 to LTB_4 (refer to the note on page 12).

Plasma - Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles to minimize the conversion of LTA_4 to LTB_4 (refer to the note on page 12).

Note: *Citrate plasma has not been validated for use in this assay. Do not use lipemic samples in this assay.*

REAGENT PREPARATION

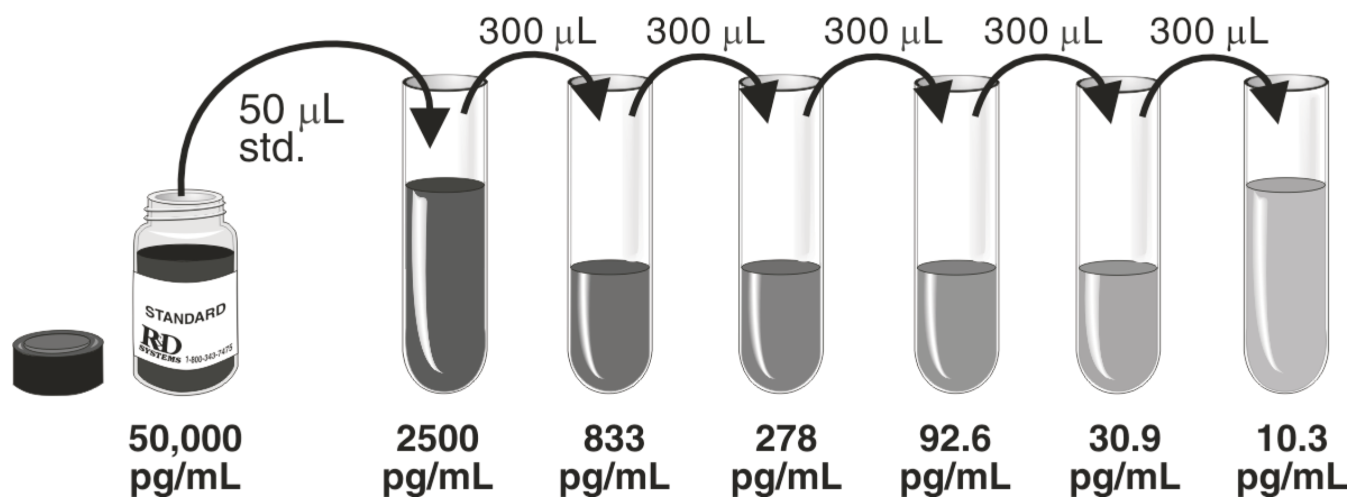
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μL of the resultant mixture is required per well.

LTB_4 Standard - Reconstitute the LTB_4 Standard with 0.5 mL of deionized or distilled water. This reconstitution produces a stock solution of 50,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 950 μL of Calibrator Diluent RD5-52 into the 2500 pg/mL tube. Pipette 600 μL of Calibrator Diluent RD5-52 into the remaining tubes. Use the 50,000 pg/mL standard stock to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 2500 pg/mL standard serves as the high standard and Calibrator Diluent RD5-52 serves as the zero standard (B_0) (0 pg/mL). **Use diluted standards within 60 minutes of preparation.**



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.

2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.

3. Add 100 μL of Calibrator Diluent RD5-52 to the non-specific binding (NSB) wells.

4. Add 50 μL of Calibrator Diluent RD5-52 to the zero standard (B_0) wells.

5. Add 50 μL of Standard, Control, or sample to the remaining wells.

6. Add 50 μL of the Primary Antibody Solution to each well **(*excluding the NSB wells*).** All wells, except the NSBs, will now be blue in color. Cover with the adhesive strip provided. Incubate for 1 hour at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.

7. **Do not wash the plate.** Add 50 μL of LTB_4 Conjugate to each well. All wells will now be violet in color except the NSB wells, which will be pink. Cover with a new adhesive strip. Incubate for 3 hours at room temperature on the shaker.

8. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

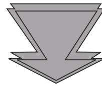
9. Add 200 μL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**

10. Add 100 μL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

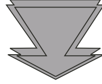
11. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

ASSAY PROCEDURE SUMMARY

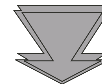
1. Prepare reagents, samples, and standards as instructed.



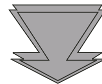
2. Add 100 µL Calibrator Diluent RD5-52 to the NSB wells.



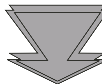
3. Add 50 µL Calibrator Diluent RD5-52 to the zero standard (B_0) wells.



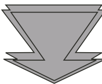
4. Add 50 µL Standard, control, or sample to the remaining wells.



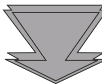
5. Add 50 µL Primary Antibody Solution to each well (excluding the NSB wells). Incubate for 1 hour on the shaker at RT.



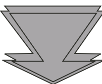
6. Do not wash the plate. Add 50 µL LTB_4 Conjugate to each well. Incubate for 3 hours on the shaker at RT.



7. Aspirate and wash 4 times.



8. Add 200 µL Substrate Solution to each well. Incubate for 30 minutes at RT **on the benchtop. Protect from light.**



9. Add 100 µL Stop Solution to each well. Read at 450 nm within 30 minutes. λ correction 540 or 570 nm

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample then subtract the NSB optical density.

Create a standard curve by reducing the data using computer software capable of generating a four paramater logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on a linear y-axis against the concentration on a logarithmic x-axis and draw the best fit curve through the points on the graph. Do not include the B_0 in the standard curve.

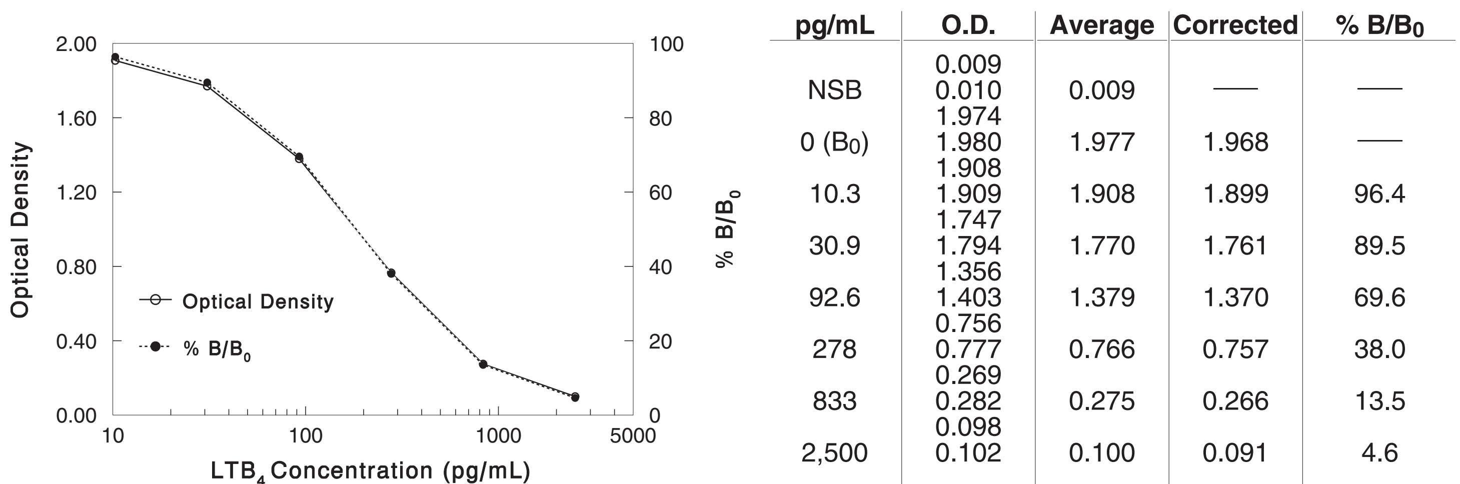
If desired, % B/B_0 can be calculated by dividing the corrected OD for each standard or sample by the corrected B_0 OD and multiplying by 100.

Calculate the concentration of LTB_4 corresponding to the mean absorbance from the standard curve.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

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



pg/mL	O.D.	Average	Corrected	% B/B_0
NSB	0.009 0.010	0.009	—	—
0 (B_0)	1.974 1.980	1.977	1.968	—
10.3	1.908 1.909	1.908	1.899	96.4
30.9	1.747 1.794	1.770	1.761	89.5
92.6	1.356 1.403	1.379	1.370	69.6
278	0.756 0.777	0.766	0.757	38.0
833	0.269 0.282	0.275	0.266	13.5
2,500	0.098 0.102	0.100	0.091	4.6

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in forty separate assays to assess inter-assay precision.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	170	481	847	164	516	879
Standard deviation	10	19	34	14.1	36.6	73.6
CV (%)	5.8	4.0	4.0	8.6	7.1	8.4

RECOVERY

The recovery of LTB_4 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample	Average % Recovery	Range
Cell culture media (n=4)	95	86 - 114%
Serum (n=4)	98	82 - 118%
EDTA plasma (n=4)	91	82 - 112%
Heparin plasma (n=4)	98	82 - 115%

LINEARITY

To assess the linearity of the assay, samples spiked with high concentrations of LTB_4 were serially diluted with Calibrator Diluent RD5-52 to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum (n=4)	Heparin plasma (n=4)	EDTA plasma (n=4)
1:2	Average % of Expected	101	89	95	97
	Range (%)	93 - 107	79 - 100	89 - 103	88 - 102
1:4	Average % of Expected	105	102	108	113
	Range (%)	98 - 110	97 - 108	104 - 116	108 - 120
1:8	Average % of Expected	108	101	102	110
	Range (%)	101 - 115	91 - 118	95 - 111	99 - 120
1:16	Average % of Expected	113	102	102	108
	Range (%)	101 - 124	99 - 106	96 - 106	100 - 117

SENSITIVITY

Fifty assays were evaluated and the minimum detectable dose (MDD) of LTB_4 ranged from 3.7 - 10.9 pg/mL. The mean MDD was 8.2 pg/mL.

The MDD was determined by subtracting two standard deviations from the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of LTB_4 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Serum (n=35)	138	100	33.5 - 564
EDTA plasma (n=35)	38.2	97	ND - 72.8
Heparin plasma (n=35)	54.3	100	11.5 - 129

ND = Non-detectable.

Note: *LTA_4 hydrolase, a metal dependent enzyme present in serum and plasma, converts LTA_4 to LTB_4 (11). In samples collected in the presence of EDTA, this conversion is inhibited at the time the blood is drawn resulting in a more accurate measurement of circulating LTB_4. Other samples, especially serum samples, typically have higher levels of LTB_4 that result from the conversion of LTA_4 after the sample is drawn.*

Cell Culture Supernates - Human peripheral blood cells (1×10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 μg/mL PHA. Aliquots of the cell culture supernates were removed and assayed for levels of LTB_4.

Condition	Day 1 (pg/mL)	Day 6 (pg/mL)
Unstimulated	61.8	56.6
Stimulated	222	96.8

Note: *Samples values have not been corrected for LTB_4 present in media.*

SPECIFICITY

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range LTB_4 control were assayed for interference. No significant cross-reactivity or interference was observed.

5(S)-HETE
12(S)-HETE
15(S)-HETE
Arachidonic acid
Arachidonyl ethanolamide
LTC_4
LTD_4

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PLATE LAYOUT

Use this plate layout as a record of standards and samples assayed.

	A	B	C	D	E	F	G	H
12								
11								
10								
9								
8								
7								
6								
5								
4								
3								
2								
1								

NOTES

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