ab133040 – Leukotriene B_4 ELISA Kit

Instructions for Use

For quantitative detection of Leukotriene B_4 in tissue culture media, plasma, urine and saliva.

This product is for research use only and is not intended for diagnostic use.

Version 5d Last Updated 18 March 2021

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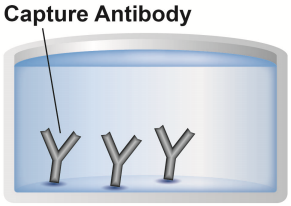
1. BACKGROUND

Abcam's Leukotriene B_4 *in vitro* competitive ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the accurate quantitative measurement of Leukotriene B_4 (LTB_4) in tissue culture media, plasma, urine and saliva.

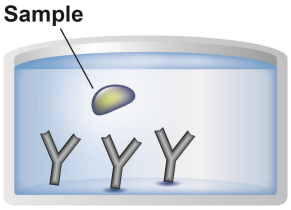
A goat anti-rabbit IgG antibody has been precoated onto 96-well plates. Standards or test samples are added to the wells, along with an alkaline phosphatase (AP) conjugated-LTB_4 antigen and a polyclonal rabbit antibody specific to LTB_4. After incubation the excess reagents are washed away. pNpp substrate is added and after a short incubation the enzyme reaction is stopped and the yellow color generated is read at 405 nm. The intensity of the yellow coloration is inversely proportional to the amount of LTB_4 captured in the plate.

Leukotriene B_4 is a major product of arachidonic acid metabolism and is formed via the 5-lipoxygenase pathway. Leukotriene B_4 stimulates leukocyte functions including lysosomal enzyme release, adhesion, and aggregation of polymorphonuclear leukocytes. Leukotriene B_4 has been implicated as a potent mediator of inflammatory diseases and immunoregulation.

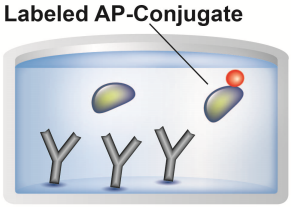
2. ASSAY SUMMARY



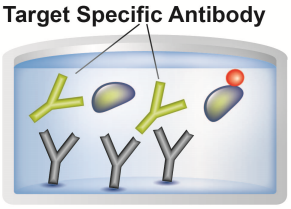
Prepare all reagents and samples as instructed.



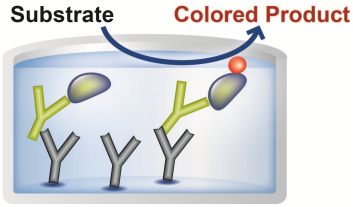
Add standards and samples to appropriate wells.



Add prepared labeled AP-conjugate to appropriate wells.



Add LTB_4 antibody to appropriate wells. Incubate at room temperature.



Add pNpp substrate to each well. Incubate at 37°C. Add Stop Solution to each well. Read immediately.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

- Some kit components contain azide, which may react with lead or copper plumbing. When disposing of reagents always flush with large volumes of water to prevent azide build-up
- Stop Solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in use
- The activity of the alkaline phosphatase conjugate is dependent on the presence of Mg^{2+} and Zn^{2+} ions. The activity of the conjugate is affected by concentrations of chelators (>10 mM) such as EDTA and EGTA
- We test this kit's performance with a variety of samples, however it is possible that high levels of interfering substances may cause variation in assay results
- The Leukotriene B_4 Standard provided, is supplied in ethanolic buffer at a pH optimized to maintain Leukotriene B4 integrity. Care should be taken handling this material because of the known and unknown effects of Leukotriene B_4.

4. STORAGE AND STABILITY

Store kit at 4°C immediately upon receipt, apart from the AP Conjugate, which should be stored at -20°C. Avoid multiple freeze-thaw cycles.

Refer to list of materials supplied for storage conditions of individual components.

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition
Goat anti-rabbit IgG Microplate (12 x 8 wells)	96 Wells	+4ºC
Leukotriene B_4 Alkaline Phosphatase Conjugate	5 mL	-20ºC
Leukotriene B_4 Antibody	5 mL	+4ºC
Leukotriene B_4 Standard	500 µL	+4ºC
Assay Buffer	27 mL	+4ºC
20X Wash Buffer Concentrate	27 mL	+4ºC
pNpp Substrate	20 mL	+4ºC
Stop Solution	5 mL	+4ºC

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Standard microplate reader - capable of reading at 405 nm, preferably with correction between 570 and 590 nm
- Automated plate washer (optional)
- Adjustable pipettes and pipette tips. Multichannel pipettes are recommended when large sample sets are being analyzed
- Eppendorf tubes
- Microplate Shaker
- Absorbent paper for blotting
- 200 mg C_{18} Reverse Phase Extraction Columns (only required for extraction of samples containing low levels of Leukotriene B_4)
- 2M hydrochloric acid (only required for extraction of samples containing low levels of Leukotriene B_4)
- Deionized Water
- Ethanol
- Hexane
- Ethyl Acetate
- A 37°C Incubator

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted

8. TECHNICAL HINTS

- Standards can be made up in either glass or plastic tubes
- Pre-rinse the pipette tip with the reagent, use fresh pipette tips for each sample, standard and reagent
- Pipette standards and samples to the bottom of the wells
- Add the reagents to the side of the well to avoid contamination
- This kit uses break-apart microtiter strips, which allow the user to measure as many samples as desired. Unused wells must be kept desiccated at 4°C in the sealed bag provided. The wells should be used in the frame provided
- Care must be taken to minimize contamination by endogenous alkaline phosphatase. Contaminating alkaline phosphatase activity, especially in the substrate solution, may lead to high blanks. Care should be taken not to touch pipet tips and other items that are used in the assay with bare hands
- Prior to addition of substrate, ensure that there is no residual wash buffer in the wells. Any remaining wash buffer may cause variation in assay results
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions**

9. REAGENT PREPARATION

Equilibrate all reagents and samples to room temperature (18 - 25°C) prior to use.

9.1 Leukotriene B_4 AP Conjugate

Allow the Leukotriene B_4 AP Conjugate to equilibrate to room temperature. Any unused conjugate should be aliquoted and re-frozen at or below -20°C.

9.2 1X Wash Buffer

Prepare the 1X Wash Buffer by diluting 5 mL of the 20X Wash Buffer Concentrate with 95 mL of deionized water.

10. STANDARD PREPARATIONS

Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use. Diluted standards should be used within 60 minutes of preparation.

10.1 Allow the 120,000 pg/mL Leukotriene B_4 **Stock Standard** solution to warm to room temperature.

10.2 Label five tubes with #1 – #5.

10.3 Add 975 µL appropriate diluent (Assay Buffer or Tissue Culture Media) to tube #1.

10.4 Add 750 µL of the appropriate diluent (Assay Buffer or Tissue Culture Media) to tube #2 - #5.

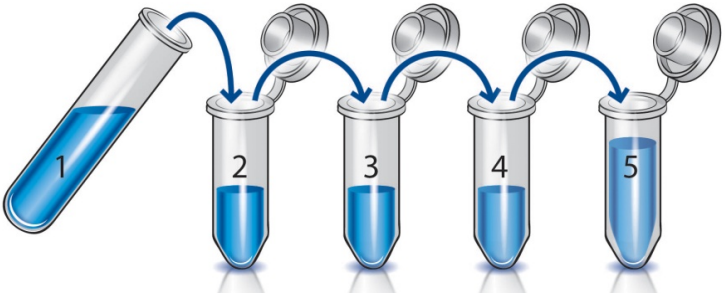
10.5 Prepare a 3,000 pg/mL **Standard 1** by adding 25 µL of the 120,000 pg/mL Leukotriene B_4 standard solution to tube 1. Vortex thoroughly.

10.6 Prepare **Standard 2** by transferring 250 µL from tube #1 to tube #2. Vortex thoroughly and gently.

10.7 Prepare **Standard 3** by transferring 250 µL from tube #2 to tube #3. Vortex thoroughly and gently.

10.8 Using the table below as a guide, repeat for tubes #4 and #5.

Standard	Sample to Dilute	Volume to Dilute (µL)	Volume of Diluent (µL)	Starting Conc. (pg/mL)	Final Conc. (pg/mL)
1	Stock	25	975	120,000	3,000
2	Standard 1	250	750	3,000	750
3	Standard 2	250	750	750	188
4	Standard 3	250	750	188	46.9
5	Standard 4	250	750	46.9	11.7



11. SAMPLE COLLECTION AND STORAGE

- The Leukotriene B_4 kit is compatible with Vasopressin samples in a wide range of matrices after dilution in Assay Buffer. However, the end user must verify that the recommended dilutions are appropriate for their samples. Samples containing rabbit IgG may interfere with the assay.
- Samples in the majority of tissue culture media, including those containing fetal bovine serum, can also be read in the assay, provided the standards have been diluted into the tissue culture media instead of Assay Buffer. There will be a small change in binding associated with running the standards and samples in media.

Some samples normally have very low levels of Leukotriene B_4 present and extraction may be necessary for accurate measurement. A suitable extraction procedure is outlined below:

11.1 Materials needed

11.1.1 LTB_4 Standard to allow extraction efficiency to be accurately determined.

11.1.2 2M hydrochloric acid, deionized water, ethanol, hexane and ethyl acetate.

11.1.3 200 mg C18 Reverse Phase Extraction Columns.

11.2 Procedure

11.2.1 Acidify the plasma, urine or tissue homogenate by addition of 2M HCl to pH of 3.5. Approximately 50 µL of HCl will be needed per mL of plasma. Allow to sit at 4 °C for 15 minutes. Centrifuge samples in a microcentrifuge for 2 minutes to remove any precipitate.

11.2.2 Prepare the C18 reverse phase column by washing with 10 mL of ethanol followed by 10 mL of deionized water.

11.2.3 Apply the sample under a slight positive pressure to obtain a flow rate of about 0.5 mL/minute. Wash the column with 10 mL of water, followed by 10 mL of 15% ethanol, and finally 10 mL hexane. Elute the sample from the column by addition of 10 mL ethyl acetate.

11.2.4 If analysis is to be carried out immediately, evaporate samples under a stream of nitrogen. Add at least 250 µL of Assay Buffer to the dried samples. Vortex well, then allow to sit for five minutes at room temperature. Repeat twice more. If analysis is to be delayed, store samples as the eluted ethyl acetate solutions at -80 °C until the immunoassay is to be run. Evaporate the organic solvent under a stream of nitrogen prior to running assay and reconstitute as above.

12. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents
- Unused well strips should be returned to the plate packet and stored at 4°C
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates)
- Well effects have not been observed with this assay.

	1	2	3	4
A	B_s	Std 1	Std 5	Sample 1
B	B_s	Std 1	Std 5	Sample 1
C	TA	Std 2	Std 6	Sample 2
D	TA	Std 2	Std 6	Sample 2
E	NSB	Std 3	Std 7	etc
F	NSB	Std 3	Std 7	etc
G	B_0	Std 4	Std 8	
H	B_0	Std 4	Std 8	

Plate layout shows controls, blanks and standards required for each assay. Use additional strips of wells to assay all your samples.

Key:
B_s = Blank; contains substrate only.
TA = Total Activity; contains conjugate (5 µL) and substrate.
NSB = Non-specific binding; contains standard diluent, assay buffer, conjugate and substrate.

B_0 = 0 pg/mL standard; contains standard diluent, conjugate, antibody and substrate

13. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use**
- **It is recommended to assay all standards and samples in duplicate**
- **Refer to the recommended plate layout in Section 12 before proceeding with the assay**

13.1 Add 100 µL of appropriate diluent* into the NSB (non-specific binding) wells. (*Use the same diluent used to prepare standards in section 10, either Assay Buffer or Tissue Culture Media).

13.2 Add 100 µL appropriate diluent (Assay Buffer or tissue culture media) into the B_0 (0 pg/mL standard) wells.

13.3 Add 100 µL of prepared standards and 100 µL diluted samples to appropriate wells.

13.4 Add 50 µL of Assay Buffer into the NSB wells.

13.5 Add 50 µL of Leukotriene B_4 AP Conjugate (blue) into NSB, B_0, standard and sample wells, i.e. not the Total Activity (TA) and B_s wells.

13.6 Add 50 µL of Leukotriene B_4 Antibody (yellow) into B_0, standard and sample wells, i.e. not B_s, TA and NSB well

Note: Every well used should be green except the NSB wells which should be blue. The B_s and TA wells are empty at this point and have no color.

13.7 Incubate the plate at room temperature on a plate shaker for 2 hours at ~500 rpm. The plate may be covered with the plate sealer provided.

13.8 Empty the contents of the wells and wash by adding 400 µL of 1X Wash Buffer to every well. Repeat the wash 2 more times for a total of 3 washes. After the final wash, empty or aspirate the wells, and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer.

13.9 Add 5 µL of the Leukotriene B_4 Alkaline Phosphatase Conjugate to the TA wells only.

13.10 Add 200 µL of the pNpp Substrate solution to every well. Incubate at 37°C for 2 hours without shaking.

13.11 Add 50 µL Stop Solution into each well. The plate should be read immediately.

13.12 Blank the plate reader against the B_s wells, read the O.D. absorbance at 405 nm, preferably with correction between 570 and 590 nm. If the plate reader is not able to be blanked against the blank wells, manually subtract the mean optical density of the blank wells from all readings.

14. CALCULATIONS

14.1 Calculate the average net absorbance measurement (Average Net OD) for each standard and sample by subtracting the average NSB absorbance measurement from the average absorbance measurement (Average OD) for each standard and sample.

Average Net OD = Average Bound OD - Average NSB OD

14.2 Calculate the binding of each pair of standard wells as a percentage of the maximum binding wells (Bo), using the following formula

$$\text{Percent Bound} = \frac{\text{Average Net OD}}{\text{Average Net } B_0 \text{ OD}} \times 100$$

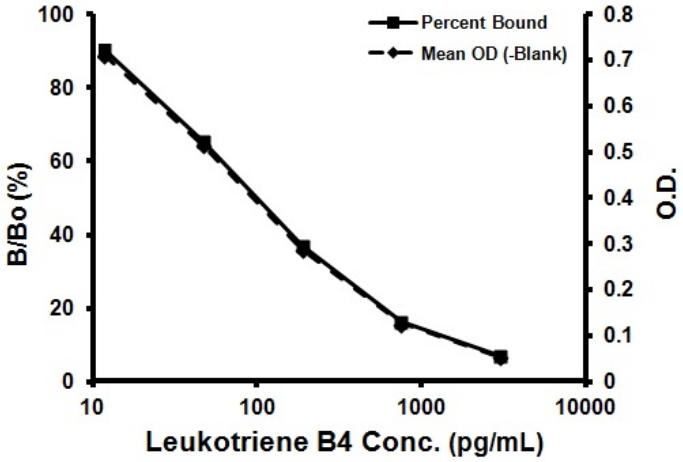
14.3 Plot the Percent Bound (B/B_0) and the net OD versus concentration of Leukotriene B4 for the standards. The concentration of Leukotriene B4 in the unknowns can be determined by interpolation of net OD values.

A four parameter algorithm (4PL) provides the best fit, though other equations can be examined to see which provides the most accurate (e.g. linear, semi-log, log/log, 4 parameter logistic). Interpolate protein concentrations for unknown samples from the standard curve plotted.

Samples producing signals greater than that of the highest standard should be further diluted and reanalyzed, then multiplying the concentration found by the appropriate dilution factor

15. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.



Sample	Mean OD ($-B_s$)	% Bound	Leukotriene B_4 (pg/mL)
B_s	(0.145)		
TA	1.466		
NSB	-0.002		
Standard 1	0.054	7.0	3,000
Standard 2	0.127	16.4	750
Standard 3	0.289	36.9	188
Standard 4	0.515	65.6	46.9
Standard 5	0.710	90.4	11.7
B_O	0.786	100	0
Unknown 1	0.465	59.3	63.5
Unknown 2	0.155	19.9	556

Typical Quality Control Parameters

Total Activity Added	= 1.466x 10 = 14.66
%Bo/TA	= 5.37%
Quality of Fit	= 1.0000 (Calculated from 4 parameter logistic curve fit)
20% Intercept	= 552 pg/mL
50% Intercept	= 98 pg/mL
80% Intercept	= 23 pg/mL

16. TYPICAL SAMPLE VALUES

SENSITIVITY –

Sensitivity was calculated by determining the average optical density bound for 16 wells run as Bo, and comparing to the average optical density for 16 wells run with Standard 5. The detection limit was determined as the concentration of Leukotriene B4 measured at two standard deviations from the zero along the standard curve and was determined to be 5.63 pg/mL.

SAMPLE RECOVERY –

Recovery was determined by Leukotriene B_4 in tissue culture media, Human saliva, serum, and urine. Mean recoveries are as follows:

Sample Type	Average % Recovery	Recommended Dilution
Tissue Culture Media	97.3	None
Human Saliva	114.1	≥1:4
Human Urine	96.9	None
Porcine Plasma (EDTA)	109.6	1:2-1:4

LINEARITY OF DILUTION –

A sample containing 1,000 pg/mL Leukotriene B_4 was diluted 6 times 1:2 in the kit Assay Buffer and measured in the assay. The data was plotted graphically as actual Leukotriene B_4 concentration versus measured Leukotriene B4 concentration.

The line obtained had a slope of 0.961 and a correlation coefficient of 0.999.

PRECISION –

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

The precision numbers listed below represent the percent coefficient of variation for the concentrations of LTB_4 determined in these assays as calculated by a 4 parameter logistic curve fitting program.

Intra- Assay

	LTB_4 (pg/mL)	%CV
Low	305	6.0
Medium	607	6.8
High	1,078	5.9

Inter-Assay

	LTB_4 (pg/mL)	%CV
Low	99	15.7
Medium	308	16.5
High	507	5.0

17. ASSAY SPECIFICITY

CROSS REACTIVITY –

The cross reactivities for a number of related compounds was determined by dissolving the cross reactant in Assay Buffer at concentrations from 40,000 to 0.4pg/mL. These samples were then measured in the LTB4 assay and the measured LTB4 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 (%)
Leukotriene B_4	100
6-trans-12-epi-Leukotriene B_4	5.50
6-trans-Leukotriene B_4	4.90
12-epi-Leukotriene B_4	0.94
PGE_2	<0.2
$PGF_{2\alpha}$	<0.2
20-OH-Leukotriene B_4	<0.2
20-COOH-Leukotriene B_4	<0.2
LTC_4	<0.2
LTD_4	<0.2
LTE_4	<0.2
5(S)-HETE	<0.2
12(S)-HETE	<0.2
15(S)-HETE	<0.2

18. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standards dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Samples give higher value than the highest standard	Starting sample concentration is too high.	Dilute the specimens and repeat the assay
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the kit	Store the all components as directed.

19. NOTES

For all technical and commercial enquires please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)