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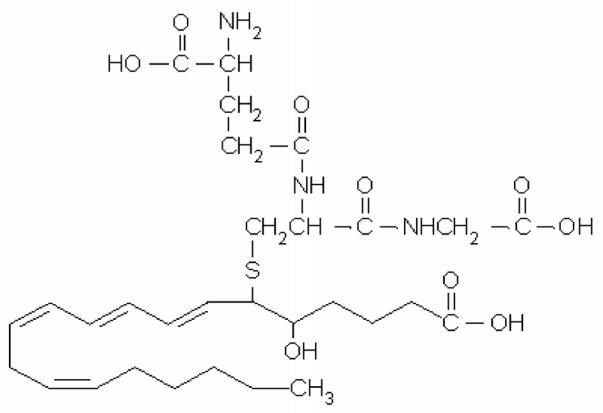
Cysteinyl leukotriene EIA

Product Number **CS0230**
Storage Temperature –20 °C

ProductInformation

Technical Bulletin

Product Description



LTC_4

Cysteinyl Leukotriene is a four-hour competitive enzyme immunoassay (EIA) for the quantitative determination of cysteinyl leukotrienes (LTC4, LTD4, and LTE4) concentrations in cell culture supernatants, urine and other biological fluids. Cysteinyl leukotriene present in a sample competes with the fixed amount of cysteinyl leukotriene conjugated to alkaline phosphatase for the limited number of binding sites on the monoclonal anti-cysteinyl leukotriene antibody. During the first incubation, the antigen-antibody complex binds to the monoclonal anti-mouse 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 of cysteinyl leukotriene in the standards or the samples. The concentration is calculated on the basis of optical reading of standard dilutions.

The cysteinyl leukotrienes (LTC_4, LTD_4, and LTE_4) are metabolites of arachidonic acid. They are formed via the 5-lipoxygenase pathway by addition of cysteine derivatives to leukotriene A_4 hydrolase (LTA_4).[1] The conversion of LTA_4 to LTC_4 by the enzyme LTC_4 synthase limits the rate of cysteinyl leukotriene formation.[2] The cysteinyl leukotrienes are potent lipid mediators in inflammation.[3] They cause smooth muscle contractions and increased capillary permeability.[4] Monitoring the levels of cysteinyl leukotrienes has been useful in several studies, such as those involving Hepatitis B and *Helicobacter pylori* infections, cerebral vasospasms and blood-brain cell contact, as well as asthma and other inflammatory responses.[5-8] Urinary LTE_4 a are useful markers of airway inflammation and can be helpful in guiding asthma management.[9]

Reagents

- **Cysteinyl Leukotriene (LTC_4) Standard, 1 vial, Product No. C 0990** – 0.5 mL (25,000 pg/mL) in phosphate buffer with preservative.
- **Anti-Mouse IgG-coated 96 well plate, 1 plate, Product No. I 6033.**
- **Assay Buffer, 30 mL, Product No. A 4353 –** contains sodium azide. Ready to use.
- **Monoclonal Anti Cysteinyl Leukotriene, 5 mL, Product No. C 0740 –** contains sodium azide, yellow dye. Ready to use.
- **Cysteinyl Leukotriene (LTC_4)-Alkaline phosphatase conjugate, 5 mL, Product No. C 0865-** 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 sealers, 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 shaker 500 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.
- Indomethacin (Product No. I 8280) or other prostaglandin synthetase inhibitor.

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 was validated for cell culture supernatants and urine samples, which may be assayed directly or after extraction.
- Cell culture supernatants should be separated from the cells and frozen, if not analyzed shortly after collection.
- Cell culture samples may be assayed undiluted or diluted in cell culture media.
- Use the same media for blanks, controls and standard dilutions.
- Cell culture samples and urines containing low levels of cysteinyl leukotriene require extraction.
- Urine samples may be analysed without extraction.
- Urine samples require at least 1:5 dilution in Assay Buffer before assay.
- To ensure accurate quantitation, always dilute the standards and blanks in the same diluent as the samples.
- Plasma and sera samples require extraction before assaying by EIA.
- A prostaglandin synthetase inhibitor Indomethacin, should be added to all samples at approximately 10 µg/mL.

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 mL of HCl per 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 C18 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 8,9.

References 10-12 contain detailed extraction protocols.

Reagent Preparation

Cysteinyl Leukotriene (LTC_4) Standard

1. Equilibrate standard solution to room temperature.
2. 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 25,000 pg/mL		
1	0.9 mL	0.1 mL (0)	2,500
2	0.5 mL	0.5 mL (1)	1,250
3	0.5 mL	0.5 mL (2)	625.0
4	0.5 mL	0.5 mL (3)	312.5
5	0.5 mL	0.5 mL (4)	156.3
6	0.5 mL	0.5 mL (5)	78.1

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

Cysteinyl Leukotriene (LTC_4)-Alkaline phosphatase conjugate

1. Equilibrate to room temperature.
2. Add conjugate to the appropriate wells.
3. Aliquot the remaining conjugate and freeze at –70 °C
4. For Total Activity: Dilute 50 µL conjugate with 450 µL Assay Buffer – use in Total Activity wells.

Wash Buffer

1. Warm to room temperature.
2. Dilute 30 mL concentrate and 270 mL deionized water.
3. Label Working Wash buffer
4. May be stored at room temperature for 3 months.

Storage/Stability

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

After the kit is open:

- Standard and conjugate must be 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: equilibrate 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 shelf life.
- Standards and samples can be made up in either glass or plastic tubes.
- 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

- The purpose of washing is to remove unbound proteins and other non-specific parts of lysate.
- Incomplete washing will adversely affect the assay and render false results.
- Use only Wash Buffer provided in kit.
- Washing may be performed using automated washer, manifold pipette or squirt bottle.
- Wash cycle three times, blotting as dry as possible after the 3rd wash.
- When washing manually, fill wells with Wash Buffer, aspirate thoroughly and tap dry on absorbent tissue.
- 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 procedure set up.

The following wells are needed for each assay run:
2 substrate blank wells (SB)
2 Total Activity wells (TA),
2 Non-specific Binding wells (NSB)
2 0 pg/mL standard wells (B_0)
12 standard dilutions wells
2 wells for each sample to be assayed

1st Incubation

a. Remove the appropriate number of multiwell strips and return the unused strips to the pouch. Reseal pouch and refrigerate.
b. Add 100 µL of Assay Buffer to the zero standard (B_o) wells
c. Add 150 µL of Assay Buffer to the NSB wells.
d. Add 100 µL of standards or samples to all remaining wells.
e. Add 50 µL of Cysteinyl leukotriene-alkaline phosphatase conjugate to all wells (except the TA and SB).
f. Add 50 µL of Monoclonal Anti-Cysteinyl Leukotriene to each well (except NSB, TA and SB wells).
g. Cover with plate cover and incubate 2 hours at room temperature on orbital shaker set at 500 rpm.
h. All wells should be green, except NSB wells, which are blue.
i. Wash wells for a total of 3 times following washing instructions.
j. 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 Cysteinyl Leukotriene 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

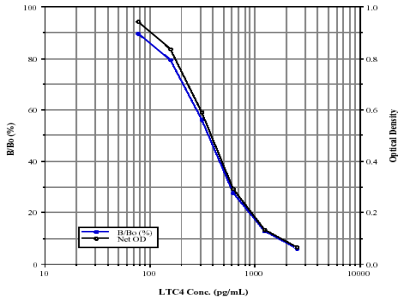
a. Add 50 µL of Stop Solution to each well.
b. Yellow color develops immediately and can be read in the multiwell plate reader at 405 nm with corrections at 570 or 590 nm. Subtract the readings at 590 nm from the readings at 405 nm, to correct for optical imperfection of the plate.

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 paramater 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 by dividing the corrected OD for each standard or sample by the corrected B_0 OD and multiplying by 100.
5. Calculate the concentration of cysteinyl leukotriene 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 LTC_4 EIA

pg/mL	Net OD 405 nm	%B/Bo
NSB	0.106	
Bo	1.160	100
78.1	1.049	89.5
156.2	0.942	79.3
312.5	0.698	56.1
625	0.399	27.7
1250	0.241	12.7
2500	0.171	6.08

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 the Cysteinyl Leukotriene 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

Substrate Blank (OD) = 0.105
Total Activity Trzech (TA x 100) = 49.2
% NSB (NSB/TA x 100) = 0.0%
% B0 (B0/TA x 100) = 2.14%
Quality of Fit = 0.999

Recovery

The recovery of cysteinyl leukotriene spiked into samples in various matrices was evaluated.

	Average % Recovery
Cell Culture Media	104%
Human Urine (diluted 1:5 in Assay Buffer)	108%

Linearity

In order to evaluate linearity, serial 2-fold dilutions of Assay Buffer containing cysteinyl leukotriene were assayed.

Dilution	Observed pg/mL	Expected pg/mL	% Observed : Expected
Neat	2448	-	-
1:2	1165	1224	95.2%
1:4	562	612	91.8%
1:8	279	306	91.1%

Sensitivity

The minimum detectable dose of the cysteinyl leukotriene is typically less than 26.6 pg/mL. The minimum detectable dose was determining by subtracting two standard deviations from the mean optical density value of 16 zero standard (B_0) replicates and calculating the corresponding concentration.

Specificity

The assay is specific for cysteinyl leukotriene. Other compounds were tested in this EIA at the concentrations of 2.5 pg/mL to 25,000 pg/mL. The cross reactivity was calculated at 50% B/B_0.

Cross Reactivity

Compound	% Cross reactivity
LTC_4	100
LTD_4	115
LTE_4	62.7
LTB_4	1.2
Arachidonic acid	0.6
PGE_2	0.5
6-trans-LTB_4	0.5
TXB_2	0.3
20-OH-LTB_4	<0.2
$PGF_{2\alpha}$	<0.2
6-keto-$PGF_{1\alpha}$	<0.2
PGD_2	<0.2
5-HETE	<0.2
12-HETE	<0.2
15-HETE	<0.2

References

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Cysteinyl Leukotriene Assay Flow Chart (CS0230)

	Blank	**TA**	**NSB**	**B_o**	**Standards**	**Samples**
Well ID	**A1, B1**	**C1, D1**	**E1, F1**	**G1, H1**	**A2-D3**	**E3-H12**
Assay Buffer			150 µL	100 µL		
Standard and/or Sample					100 µL	100 µL
LTC_4 Conjugate			50 µL	50 µL	50 µL	50 µL
Monoclonal Anti-CysLeuk Antibody				50 µL	50 µL	50 µL

Incubate 2 hours @ RT with shaking
Wash 3X

pNpp Substrate 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

Conjugate for TA must be diluted 1:10 in Assay Buffer: 450 µL Assay Buffer + 50 µL Cysteinyl Leukotriene