New Wash Buffer

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Correlate-EIA™

Cysteinyl Leukotriene

Enzyme Immunoassay Kit

Catalog No. 900-070

96 Well Kit

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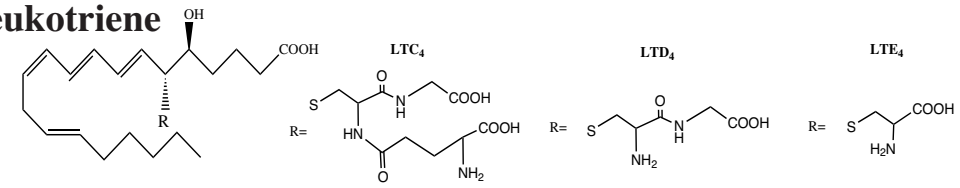
Description

The Assay Designs' Correlate-EIA™ Cysteinyl Leukotriene kit is a competitive immunoassay for the quantitative determination of Cysteinyl Leukotrienes in tissue culture media and urine. Please read the complete kit insert before performing this assay. The kit measures three of the major metabolites of LTA_4; LTC_4, LTD_4 and LTE_4. The kit uses a monoclonal antibody to the Cysteinyl Leukotrienes (LTC_4, LTD_4 and LTE_4) to bind, in a competitive manner, the Cysteinyl Leukotrienes in the sample or an alkaline phosphatase molecule which has LTC_4 covalently attached to it. After incubation at room temperature the excess reagents are washed away and substrate is added. After a second incubation, the enzyme reaction is stopped and the yellow color generated is read on a microplate reader at 405nm. The intensity of the bound yellow color is inversely proportional to the concentration of LTC_4 in the standards or Cysteinyl Leukotrienes in the samples. The measured optical density of LTC_4 is used to calculate the concentration of Cysteinyl Leukotrienes in the sample. For further explanation of the principles and practice of immunoassays please see the excellent books by Chard[1] or Tijssen[2].

Introduction

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 LTA_4[3]. The conversion of LTA_4 to LTC_4 by the enzyme LTC_4 synthase limits the rate of Cysteinyl Leukotriene formation[4]. The Cysteinyl Leukotrienes are potent lipid mediators in inflammation[5]. They cause smooth muscle contractions and increased capillary permeability[6]. Monitoring the levels of Cysteinyl Leukotrienes has been useful in several studies, such as Hepatitis B, Helicobacter pylori infections, cerebral vasospasms and blood-brain cell contact, as well as asthma and other inflammatory responses[7-13].

Cysteinyl Leukotriene



Precautions

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1. 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.
2. Stop Solution is a solution of trisodium phosphate. This solution is caustic; care should be taken in use.
3. 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.
4. 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.
5. The Cysteinyl Leukotriene Standard provided, Catalog No. 80-0868, is supplied in ethanolic buffer at a pH optimized to maintain integrity. Care should be taken in handling this material because of the known and unknown effects of Leukotrienes.

Materials Supplied

1. **Goat anti-Mouse IgG Microtiter Plate, One Plate of 96 Wells, Catalog No. 80-0050**
   A plate using break-apart strips coated with goat antibody specific to mouse IgG.
2. **Cysteinyl Leukotriene EIA Conjugate, 6 mL, Catalog No. 80-0865**
   A blue solution of alkaline phosphatase conjugated with LTC_4.
3. **Cysteinyl Leukotriene EIA Antibody, 6 mL, Catalog No. 80-0864**
   A yellow solution of a mouse monoclonal antibody to LTC_4, D_4, E_4.
4. **Assay Buffer, 30 mL, Catalog No. 80-1548**
   Tris buffered saline, containing proteins and sodium azide as preservative.
5. **Wash Buffer Concentrate, 30 mL, Catalog No. 80-1286**
   Tris buffered saline containing detergents.
6. **Cysteinyl Leukotriene Standard, 0.5 mL, Catalog No. 80-0868**
   A solution of 25,000 pg/mL LTC_4.
7. **pNpp Substrate, 20 mL, Catalog No. 80-0075**
   A solution of p-nitrophenylphosphate in buffer. Ready to use.
8. **Stop Solution, 6 mL, Catalog No. 80-0247**
   A solution of trisodium phosphate in water. Keep tightly capped. Caution: **Caustic.**
9. **Cysteinyl Leukotriene Assay Layout Sheet, 1 each, Catalog No. 30-0153**
10. **Plate Sealer, 1 each, Catalog No. 30-0012**

Storage

All components of this kit, **except the conjugate and standard**, are stable at 4 °C until the kit's expiration date. The conjugate and standard **must** be stored frozen at -20 °C.

Materials Needed but Not Supplied

1. Deionized or distilled water.
2. Precision pipets for volumes between 5 µL and 1,000 µL.
3. Repeater pipets for dispensing 50 µL and 200 µL.
4. Disposable beakers for diluting buffer concentrates.
5. Graduated cylinders.
6. A microplate shaker.
7. Adsorbent paper for blotting.
8. A 37 °C incubator.
9. Microplate reader capable of reading at 405 nm, preferably with correction between 570 and 590 nm.

Sample Handling

The Assay Designs' Correlate-EIA™ Cysteinyl Leukotriene enzyme immunoassay is compatible with samples in a wide range of matrices. Samples diluted sufficiently into Assay Buffer can be read directly from the standard curve. Please refer to the Sample Recovery recommendations on page 11 for details of suggested dilutions. However, the end user **must verify** that the recommended dilutions are appropriate for their samples. **Samples containing mouse 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. Users should only use standard curves generated in media or buffer to calculate concentrations of Cysteinyl Leukotriene in the appropriate matrix. For tissue and urine samples, prostaglandin synthetase inhibitors, such as, indomethacin or meclofenamic acid at concentrations up to 10 µg/mL should be added to either the tissue homogenate or urine samples.

Some samples normally have very low levels of Cysteinyl Leukotrienes present and extraction may be necessary for accurate measurement. Extraction is required for plasma and serum samples. A suitable extraction procedure is outlined below:

Materials Needed

1. LTC_4 Standard to allow extraction efficiency to be accurately determined.
2. 2M hydrochloric acid, deionized water, ethanol, hexane and ethyl acetate.
3. 200 mg C_{18} Reverse Phase Extraction Columns.

Procedure

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.
2. Prepare the C_{18} reverse phase column by washing with 10 mL of ethanol followed by 10 mL of deionized water.
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.
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 in 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.

Please refer to references 14-17 for details of extraction protocols.

Procedural Notes

1. Do not mix components from different kit lots or use reagents beyond the kit expiration date.
2. Allow all reagents to warm to room temperature for at least 30 minutes before opening.
3. Standards can be made up in either glass or plastic tubes.
4. Pre-rinse the pipet tip with reagent, use fresh pipet tips for each sample, standard and reagent.
5. Pipet standards and samples to the bottom of the wells.
6. Add the reagents to the side of the well to avoid contamination.
7. 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.
8. **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.
9. **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.**

Reagent Preparation

1. Cysteinyl Leukotriene Standard

Allow the 25,000 pg/mL LTC_4 standard solution to warm to room temperature. Label six 12x75 mm glass tubes #1 through #6. Pipet 900 µL of standard diluent (Assay Buffer or Tissue Culture Media) into tube #1. Pipet 500 µL of standard diluent into tubes #2 - #6. Add 100 µL of the 25,000 pg/mL standard to tube #1. Vortex thoroughly. Add 500 µL of tube #1 to tube #2 and vortex thoroughly. Add 500 µL of tube #2 to tube #3 and vortex thoroughly. Continue this for tubes #4 through #6.

The concentration of LTC_4 in tubes #1 through #6 will be 2,500, 1,250, 625, 312.5, 156.3 and 78.1 pg/mL, respectively. See Cysteinyl Leukotriene Assay Layout Sheet for dilution details.

Diluted standards should be used within 60 minutes of preparation.

2. Cysteinyl Leukotriene Conjugate

Allow the Conjugate to warm to room temperature. Any unused Conjugate should be aliquoted and re-frozen at or below -20 °C. Avoid repeated freeze-thaws of the aliquots.

3. Conjugate 1:10 Dilution for Total Activity Measurement

Prepare the Conjugate 1:10 Dilution by diluting 50 µL of the supplied conjugate with 450 µL of Assay Buffer. The dilution should be used within 3 hours of preparation. **This 1:10 dilution is intended for use in the Total Activity wells ONLY**.

4. Wash Buffer

Prepare the Wash Buffer by diluting 5 mL of the supplied concentrate with 95 mL of deionized water. This can be stored at room temperature until the kit expiration date, or for 3 months, whichever is earlier.

Assay Procedure

Bring all reagents to room temperature for at least 30 minutes prior to opening.

All standards and samples should be run in duplicate.

1. Refer to the Assay Layout Sheet to determine the number of wells to be used and put any remaining wells with the desiccant back into the pouch and seal the ziploc. Store unused wells at 4 °C.

2. Pipet 100 µL of standard diluent (Assay Buffer or Tissue Culture Media) into the NSB and the Bo (0 pg/mL Standard) wells.

3. Pipet 100 µL of Standards #1 through #6 into the appropriate wells.

4. Pipet 100 µL of the Samples into the appropriate wells.

5. Pipet 50 µL of Assay Buffer into the NSB wells.

6. Pipet 50 µL of blue Conjugate into each well, except the Total Activity (TA) and Blank wells.

7. Pipet 50 µL of yellow Antibody into each well, except the Blank, TA and NSB wells.

NOTE: Every well should be **Green** in color except the NSB wells which should be **Blue**. The Blank and TA wells are empty at this point and have no color.

8. Incubate the plate at room temperature on a plate shaker for 2 hours at ~500 rpm.

9. Empty the contents of the wells and wash by adding 400 µL of wash solution to every well. Repeat the wash 2 more times for a total of **3 Washes.**

10. 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.

11. Add 5 µL of the light blue Conjugate 1:10 Dilution **(see step 3, Reagent Preparation, on page 5)** to the TA wells.

12. Add 200 µL of the pNpp Substrate solution to every well. Seal the plate and incubate at 37 °C for 2 hours.

13. Add 50 µL of Stop Solution to every well. This stops the reaction and the plate should be read immediately.

14. Blank the plate reader against the Blank wells, read the optical density 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.

Calculation of Results

Several options are available for the calculation of the concentration of Cysteinyl Leukotriene in the samples. We recommend that the data be handled by an immunoassay software package utilizing a 4 parameter logistic curve fitting program. If data reduction software is not readily available, the concentration of Cysteinyl Leukotriene can be calculated as follows:

1. Calculate the average Net Optical Density (OD) bound for each standard and sample by subtracting the average NSB OD from the average OD bound:

   Average Net OD = Average Bound OD - Average NSB OD

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

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

3. Using Logit-Log paper plot Percent Bound versus Concentration of Cysteinyl Leukotriene for the standards. Approximate a straight line through the points. The concentration of Cysteinyl Leukotriene in the unknowns can be determined by interpolation.

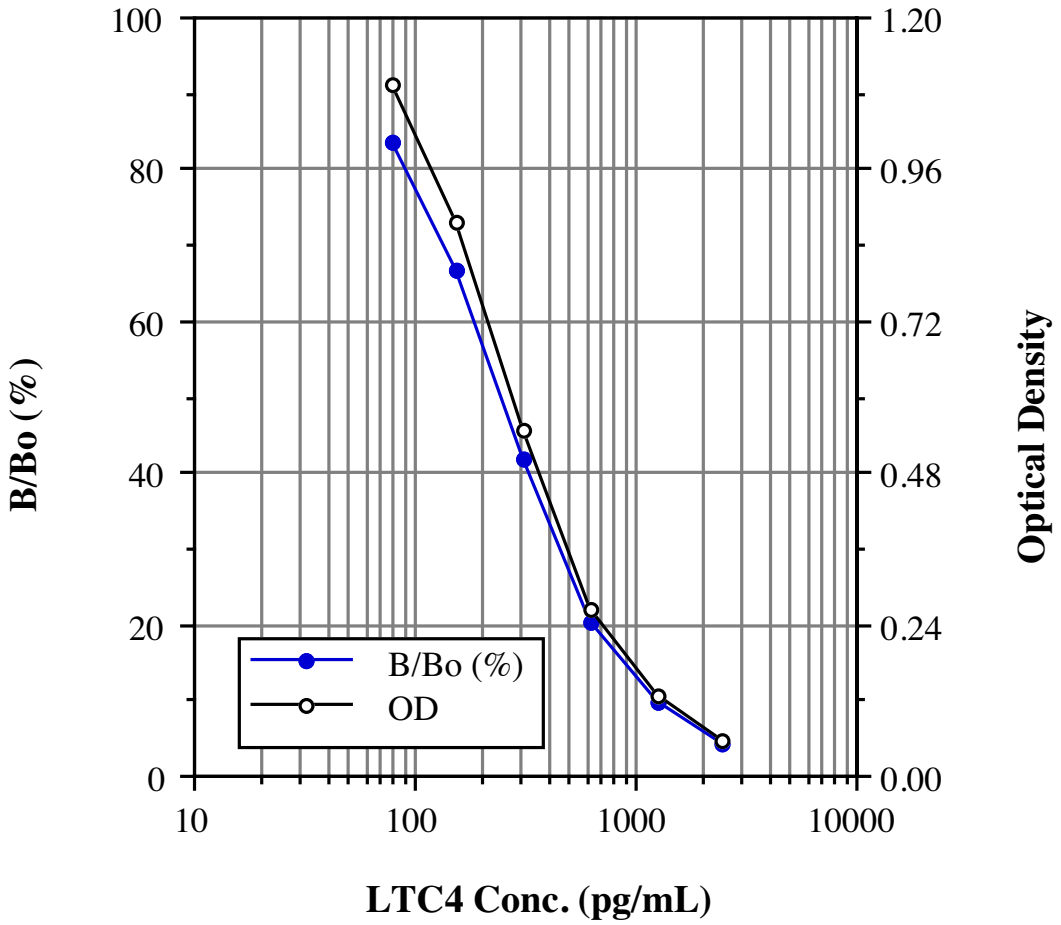
Typical Results

The results shown below are for illustration only and **should not** be used to calculate results from another assay.

Sample	Mean OD(-Blank)	Average Net OD	Percent Bound	**LTC_4 (pg/mL)**
Blank OD	(0.134)			
TA	0.323			
NSB	0.001	0.000		
Bo	1.312	1.311	100%	**0**
S1	0.059	0.058	4.4%	**2,500**
S2	0.129	0.128	9.8%	**1,250**
S3	0.265	0.264	20.1%	**625**
S4	0.548	0.547	41.7%	**312.5**
S5	0.876	0.875	66.7%	**156**
S6	1.094	1.093	83.4%	**78.1**
Unknown 1	0.162	0.161	12.3%	**966**
Unknown 2	0.851	0.850	64.8%	**165**

Typical Standard Curve

A typical standard curve is shown below. This curve **must not** be used to calculate Cysteinyl Leukotriene concentrations; each user must run a standard curve for each assay.



Typical Quality Control Parameters

Total Activity Added	=	0.323 x 10 x 10 = 32.3
%NSB	=	0.0%
%Bo/TA	=	4.06%
Quality of Fit	=	1.000 (Calculated from 4 parameter logistic curve fit)
20% Intercept	=	636 pg/mL
50% Intercept	=	250 pg/mL
80% Intercept	=	94.1 pg/mL

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[18].

Sensitivity

Sensitivity was calculated by determining the average optical density bound for sixteen (16) wells run as Bo, and comparing to the average optical density for sixteen (16) wells run with Standard #6. The detection limit was determined as the concentration of Cysteinyl Leukotriene measured at two (2) standard deviations from the zero along the standard curve.

Average Optical Density for the Bo =	1.064 $\pm$ 0.023 (2.2%)
Average Optical Density for the Standard #6 =	0.929 $\pm$ 0.024 (2.5%)
Delta Optical Density (0-78.1 pg/mL) =	0.135
2 SD's of the Zero Standard = 2 x 0.023 =	0.046

Sensitivity = $\frac{0.046}{0.135}$ x 78.1 pg/mL = **26.6 pg/mL**

Linearity

A sample containing 2,448 pg/mL Cysteinyl Leukotriene was serially diluted 4 times 1:2 in the kit Assay Buffer and measured in the assay. The data was plotted graphically as actual Cysteinyl Leukotriene concentration versus measured Cysteinyl Leukotriene concentration.

The line obtained had a slope of 0.9866 and a correlation coefficient of 0.999.

Precision

Intra-assay precision was determined by taking samples containing low, medium and high concentrations of Cysteinyl Leukotriene 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 Cysteinyl Leukotriene in multiple assays (n=8).

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

	LTC_4 (pg/mL)	Intra-assay %CV	Inter- assay %CV
Low	295	4.6	
Medium	544	4.7	
High	1,036	4.6	
Low	253		4.6
Medium	617		6.2
High	941		8.3

Cross Reactivities

The cross reactivities for a number of related compounds was determined by dissolving the cross reactant (purity checked by N.M.R. and other analytical methods) in Assay Buffer at concentrations from 25,000 to 2.5 pg/mL. These samples were then measured in the Cysteinyl Leukotriene assay and the measured Cysteinyl Leukotriene 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
LTC_4	100%
LTD_4	115.12%
LTE_4	62.74%
LTB_4	1.16%
Arachidonic Acid	0.61%
PGE_2	0.48%
6-trans-LTB_4	0.45%
TXB_2	0.28%
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%

Sample Recoveries

Please refer to pages 4 and 5 for Sample Handling recommendations and Standard preparation.

Cysteinyl Leukotriene concentrations were measured in Tissue Culture Media and human urine. For samples in tissue culture media, ensure that the standards have been diluted into the same media. For urine, Cysteinyl Leukotriene was spiked into the undiluted samples which were diluted with the kit Assay Buffer and then assayed in the kit. The following results were obtained:

Sample	% Recovery*	Recommended Dilution*
Tissue Culture Media	101.0	1:2
Human Urine	107.6	no dilution

* See Sample Handling instructions on page 4 for details.

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Material Safety Data Sheet (MSDS) available on our website or by fax.

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