



Cysteinyl Leukotriene Enzyme Immunoassay Kit

Catalog No. 133-16462

96 Well Kit

Table of Contents

Description	2
Introduction	2
Precautions	2
Materials Supplied	2
Storage	2
Materials Needed but Not Supplied	2
Sample Handling	3
Reagent Preparation	3
Assay Procedure	4
Data Analysis	5
Assay Performance Characteristics	5
References	7
Limited Warranty	7

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

Description

Sapphire's Cysteinyl Leukotriene kit is a competitive immunoassay for the quantitative determination of cysteinyl leukotrienes in biological samples. It measures all three cysteinyl leukotriene metabolites, LTC₄, LTD₄ and LTE₄. This assay is based on the competition between cysteinyl leukotrienes and a cysteinyl leukotriene-alkaline phosphatase tracer for a limited amount of cysteinyl leukotriene-specific monoclonal antibody. Because of the competition between cysteinyl leukotrienes in the sample and cysteinyl leukotriene tracer for the cysteinyl leukotriene antibody, the signal obtained with the assay will be inversely proportional to the amount of cys LT in each sample. This equilibration is performed in the wells of a 96-well plate pre-coated with goat polyclonal anti-mouse IgG, which binds all of the cysLT monoclonal antibody added to the well. After the equilibration step, the plate is washed, and a solution of *para*-nitrophenyl phosphate (*p*NPP), a substrate for alkaline phosphatase, is added. The product of this enzymatic reaction has a distinct yellow color which absorbs at 412 nm, allowing quantification of cysteinyl leukotriene in each sample.

Introduction

Leukotrienes C₄, D₄, and E₄ (LTC₄, LTD₄, and LTE₄) are collectively referred to as cysteinyl leukotrienes (CysLTs). LTC₄ is produced in large quantities by activated leukocytes such as mast cells, eosinophils and macrophages. Biosynthesis of LTC₄ is initiated by the conversion of arachidonic acid to LTA₄ by the enzyme 5-lipoxygenase (5-LO) with 5(S)-HpETE formed as an intermediate. Subsequent conjugation of glutathione to LTA₄ by LTC₄ synthase produces LTC₄.¹⁻⁴ Metabolism of LTC₄ to LTD₄ and LTE₄ occurs rapidly in the circulation by stepwise cleavage of glutamate and glycine from the glutathione adduct by γ -glutamyltransferase and dipeptidase.¹⁻⁴ LTC₄ and LTD₄ are potent mediators of asthma and hypersensitivity acting *via* a pair of G protein-coupled receptors, CysLT₁ and CysLT₂.⁴⁻⁸ They induce bronchoconstriction, increase microvascular permeability, and are vasoconstrictors of coronary arteries.^{5,9} Montelukast, zafirlukast, and pranlukast are selective CysLT₁ receptor antagonists marketed for the treatment of asthma as well as for the symptoms associated with allergic rhinitis.¹⁰ LTE₄ has weak affinity for the CysLT receptors and therefore low biological activity. However, its presence indicates the prior existence of LTC₄ and LTD₄.

Precautions

1. Please read all instructions carefully before beginning this assay.
2. Some reagents contain azide, which may react with lead or copper plumbing. If disposing of reagents in the sanitary sewer, please flush with a large volume of water to prevent azide build-up.
3. This kit is for research use only. It is not intended for human or diagnostic use.

Materials Supplied

1. Cysteinyl Leukotriene Antibody	1 vial	133-64623
2. Cysteinyl Leukotriene AP Tracer	1 vial	133-64622
3. Cysteinyl Leukotriene Standard	1 vial	133-64621
4. Goat Anti-mouse IgG Coated Plate	1 plate	133-00007
5. Tris Buffer Concentrate	2 vials	133-00003
6. AP Wash Buffer Concentrate	1 vial	133-00004
7. DEA Buffer Concentrate	1 vial	133-00001
8. <i>p</i> NPP Tablets	5 tablets	133-00002
9. Plate Cover	1 cover	133-00005

Storage

This kit should be stored at -80°C and used before the expiration date printed on the box.

Materials Needed but Not Supplied

1. A source of Millipore water
2. Adjustable pipettors
3. Materials used for sample preparation
4. An orbital shaker
5. A plate reader capable of measuring absorbance between 405-420 nm

Sample Handling

[*Note: Samples should be stored at -80°C if unable to be assayed immediately following collection.*]

1. Culture medium

Cultured cells producing LTC₄ will release it into the medium where it will accumulate without further metabolism. These samples can generally be assayed without purification. For the best results, dilute the standard curve in the same tissue culture medium as the samples are collected in.

2. Lavage fluids and effusion fluids associated with inflammation

Cysteinyl leukotrienes may be found at relatively high concentrations in lavage fluids and the effusion fluids associated with inflammation, such as synovial fluid and pleural effusion. These matrices often contain a mixture of cysteinyl leukotrienes, which can be measured effectively with this kit. These complex samples generally require purification in order to obtain accurate results.

3. Plasma samples

Plasma is a very complex sample matrix that generally contains very low levels of cysteinyl leukotrienes. In order to detect these compounds in plasma, it is generally necessary to purify and concentrate them.

4. Urine samples

Urine contains LTE₄ and metabolites of LTE₄. The levels of intact LTE₄ found in urine are approximately 50-80 pg/mg creatinine.¹¹

Materials needed for sample purification

1. Cysteinyl leukotriene standard to use in determination of recovery
2. 1.0 M HCl, methanol, Millipore water and hexane
3. 6 ml SPE C-18 cartridges

Purification Procedure

While the following purification protocol works well for many samples, it may not be sufficient for the purification of cysteinyl leukotrienes from all samples. The level of purification required for measurement of urinary LTE₄ varies according to published work.^{12,13}

1. Split samples into two equal parts and place each sample into clean test tubes.
2. Spike one set of samples with cysteinyl leukotriene.
3. Perform the following steps on all samples:
 - a. Acidify the sample to pH < 4.0 by the addition of 1.0 M HCl.
 - b. Prepare a C-18 SPE cartridge by conditioning it first with 5 ml of methanol followed by 5 ml Millipore water.
 - c. Apply the sample and allow it to flow through the column.
 - d. Rinse the column with 5 ml Millipore water, followed by 5 ml hexane. Allow the column to become dry following the hexane wash.
 - e. Elute the cysteinyl leukotrienes with 5 ml methanol. If unable to run the assay at this time, store the samples in methanol at -80°C.
 - f. Dry the sample under a stream of nitrogen. Reconstitute the sample in Tris Buffer and assay both spiked and unspiked samples in the assay.

Reagent Preparation

All diluted buffers should be stored at 4°C. When stored in this manner, they will be stable for approximately two months.

1. Tris Buffer

Dilute the contents of one vial of Tris Buffer Concentrate with 90 ml of Millipore water. It is common for the concentrated buffer to contain crystalline salts after thawing. It is important to rinse the vial to obtain any salts that may have precipitated.

2. Wash Buffer

Dilute the 5 ml vial of Wash Buffer to a final volume of 750 ml with Millipore water.

3. DEA Buffer

Dilute the 2.5 ml vial of DEA Buffer Concentrate to a final volume of 25 ml with Millipore water.

4. Cysteinyl Leukotriene Standard

[*Note: If assaying culture medium samples that have not been diluted with Tris Buffer, the culture medium rather than Tris Buffer should*

be used for dilution of the standard curve.]

Equilibrate a pipet tip in ethanol, by repeatedly filling and expelling. Use the equilibrated pipet tip to transfer 100 µl of the CysLT standard into a clean test tube. Dilute with 900 µl Millipore water. The concentration of this standard is 100 ng/ml. Label eight glass test tubes #1 - #8. Pipet 900 µl of Tris Buffer into tube #1, and 500 µl into tubes #2 - #8. Transfer 100 µl of the 100 ng/ml standard into tube #1 and vortex to mix. Transfer 250 µl from tube #1 to tube #2. Vortex to mix. Transfer 250 µl from tube #2 to tube #3. Vortex to mix. Continue this process for standard tubes #4 - #8. These diluted standards should be used within twenty-four hours.

5. Cysteinyl Leukotriene Alkaline Phosphatase Tracer

Reconstitute the Cysteinyl Leukotriene Alkaline Phosphatase Tracer with 6 ml of Tris buffer. Vortex to mix.

Store this reconstituted tracer at 4°C and use within four weeks.

6. Cysteinyl Leukotriene Monoclonal Antibody

Reconstitute the Cysteinyl Leukotriene Monoclonal Antibody with 6 ml of Tris Buffer. Vortex to mix.

Store this reconstituted antibody at 4°C and use within four weeks.

Assay Procedure

[Note: All reagents should be allowed to warm to room temperature before use.]

1. Tris Buffer

Pipet 150 µl Tris Buffer into non-specific binding (NSB) wells, and 100 µl Tris Buffer into zero standard (B₀) wells. If tissue culture medium was used to dilute the standard curve, substitute 100 µl of this same medium for the Tris Buffer in the NSB and B₀ wells.

2. Cysteinyl Leukotriene Standard

Pipet 100 µl of standards into the appropriate wells.

3. Samples

Pipet 100 µl of samples into the appropriate wells. Each sample should be assayed in duplicate or triplicate.

4. Cysteinyl Leukotriene Alkaline Phosphatase Tracer

Pipet 50 µl of tracer into each well except the blank wells and total activity (TA) wells.

5. Cysteinyl Leukotriene Antibody

Pipet 50 µl of antibody into each well except the blank wells, TA wells and NSB wells.

Well	Tris Buffer	Std/Sample	Tracer	Antibody
Blank				
TA			5 µl (at development)	
NSB	150 µl		50 µl	
B ₀	100 µl		50 µl	50 µl
Std/Sample		100 µl	50 µl	50 µl

Pipetting Summary

6. Incubate the plate

Cover each plate with a plate cover and incubate for two hours at room temperature on an orbital shaker.

7. Prepare the pNPP Solution

Dissolve 5 pNPP tablets in 25 ml DEA Buffer (25 ml is sufficient to develop 100 wells). *[Note: Reconstituted pNPP is not stable, so we recommend that you make only the amount that you need at any one time.]*

8. Wash the plate

Empty the wells and rinse five times with Wash Buffer. After the final wash, firmly tap the inverted plate on a paper towel to remove any recalcitrant drops of buffer.

9. Develop the plate

Add 200 µl pNPP solution to each well including blank and TA wells. Add 5 µl of tracer to the TA wells. Cover the plate and allow to develop in the dark on an orbital shaker. This assay typically develops in approximately 60-90 minutes.

10. Read the plate

Wipe the bottom of the plate with a paper towel to remove any finger prints, smudges or dirt which may interfere with obtaining an accurate reading of absorbance. Remove the plate cover, and read the plate at a wavelength between 405 and 420 nm.

Data Analysis

Most plate readers come with data reduction software that plots data automatically. Alternatively a spreadsheet program can be used. The data should be plotted as %B/B₀ versus log concentration of standard using either a 4-parameter logistic or a log-logit curve fit.

1. Prepare the Data

The following procedure is recommended to prepare the data prior to graphing (subtract the absorbance of the blank wells from all wells on the plate, if not already done).

- a. Average the absorbance readings from the NSB wells.
- b. Average the absorbance readings from the B₀ wells.
- c. Subtract the average NSB from the average B₀. This is the corrected B₀.

$$\text{Corrected } B_0 = \text{Average } B_0 - \text{Average NSB}$$

- d. Calculate the %B/B₀ for each standard and sample. To do this, subtract the average NSB absorbance from the absorbance and divide by the corrected B₀ (from step c). Multiply by 100 to obtain %B/B₀. Repeat for all wells.

$$\%B/B_0 = \left[\frac{\text{Absorbance} - \text{Average NSB}}{\text{Corrected } B_0} \right] \times 100$$

2. Plot the Standard Curve

Plot %B/B₀ for all standards *versus* CysLT concentration using log (x) and linear (y) axes, and fit the data to a four parameter logistic equation. Alternatively, the data can be linearized using a logit transformation. [**Note:** Do not use %B/B₀ in this calculation.]

$$\text{Logit } (B/B_0) = \ln [B/B_0 / (1 - B/B_0)]$$

Plot the data as logit (B/B₀) *versus* log concentration of standard and perform a linear regression fit.

3. Determine the Concentration of your Samples

Calculate the %B/B₀ for each sample. Determine the concentration of each sample using the equations obtained from the analysis of the standard curve. Remember to account for any dilutions made to the sample prior to addition to the well. %B/B₀ values of greater than 80% or less than 20% should be re-assayed as they generally fall outside of the linear range of the standard curve.

4. Correct for Recovery (if purification was performed)

Divide the concentration determined in step #3 by the recovery factor. Correct for any volume changes of the sample which may have occurred during purification.

Recovery Factor =

$$\frac{\text{EIA value of spiked sample (pg/ml)} - \text{EIA value of unspiked sample (pg/ml)}}{\text{Concentration of spike (pg/ml)}}$$

Assay Performance Characteristics

1. Precision

Intra-assay precision was determined by measuring samples containing low, medium, and high concentrations of cysteinyl leukotriene multiple times in the same assay (eight samples per plate on a total of five plates). Inter-assay precision was determined by measuring low, medium, and high concentrations of the samples eight times using different reagents.

	CysLT (pg/ml)	Intra-assay %CV	Inter-assay %CV
Low	41.2	8.3	7.4
Medium	123.4	5.3	7.0
High	370.4	4.4	4.1

2. Specificity

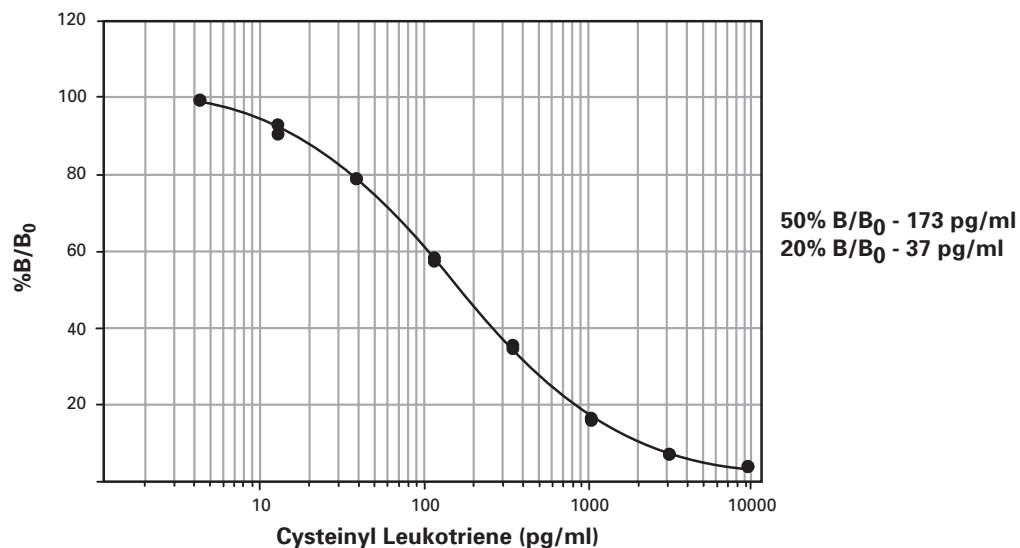
Analyte	Cross-Reactivity	Analyte	Cross-Reactivity
Leukotriene C ₄	100%	Leukotriene B ₄	<0.01%
Leukotriene D ₄	134%	Arachidonic Acid	<0.01%
Leukotriene E ₄	121%	Cysteine	<0.01%
Leukotriene C ₄ methyl ester	32%	Glutathione	<0.01%

3. Typical Results

The standard curve shown here is an example of data typically produced by this kit. Your results will vary from these, and it is therefore important that you run a standard curve each time you use the kit.

	Raw Data		Average	Corrected
Total Activity	2.974	2.959	2.966	
NSB	-0.001	-0.001	-0.001	
B ₀	0.752	0.767	0.784	0.785
	0.806	0.810		

Concentration Std (pg/ml)	Raw Data		Corrected		%B/B ₀	
10,000	0.024	0.026	0.025	0.027	3.2	3.5
3,333	0.051	0.050	0.052	0.051	6.6	6.5
1,111	0.121	0.125	0.122	0.126	15.6	16.0
370.4	0.274	0.267	0.275	0.268	35.0	34.2
123.5	0.452	0.449	0.453	0.450	57.9	57.5
41.2	0.617	0.615	0.617	0.616	78.8	78.6
13.7	0.726	0.706	0.727	0.707	92.8	90.2
4.6	0.777	0.777	0.778	0.778	99.2	99.2



References

1. Shaw, A. and Krell, R.D. Peptide leukotrienes: Current status of research. *J. Med. Chem.* **34**, 1235-1242 (1991).
2. Mayatepek, E. and Hoffmann, G.F. Leukotrienes: Biosynthesis, metabolism, and pathophysiologic significance. *Pediatr. Res.* **37**, 1-9 (1995).
3. Ford-Hutchinson, A.W. Regulation of leukotriene biosynthesis. *Cancer. Metast. Rev.* **13**, 257-267 (1994).
4. Brink, C., Dahlen, S.-E., Drazen, J., *et al.* International union of pharmacology XXXVII. Nomenclature for leukotriene and lipoxin receptors. *Pharmacol. Rev.* **55**, 195-227 (2003).
5. Samuelsson, B. Leukotrienes: Mediators of immediate hypersensitivity reactions and inflammation. *Science* **220**, 568-575 (1983).
6. Lynch, K.R., O'Neill, G.P., Liu, Q., *et al.* Characterization of the human cysteinyl leukotriene CysLT 1 receptor. *Nature* **399**, 789-793 (1999).
7. Heise, C.E., O'Dowd, B.F., Figueroa, D.J., *et al.* Characterization of the human cysteinyl leukotriene 2 receptor. *J. Biol. Chem.* **275**, 30531-30536 (2000).
8. Sarau, H.M., Ames, R.S., Chambers, J., *et al.* Identification, molecular cloning, expression, and characterization of a cysteinyl leukotriene receptor. *Mol. Pharmacol.* **56**, 657-663 (1999).
9. Dahlén, S.-E., Björk, J., Hedqvist, P., *et al.* Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: *In vivo* effects with relevance to the acute inflammatory response. *Proc. Natl. Acad. Sci. USA* **78**, 3887-3891 (1981).
10. Centanni, S. and Santus, P. Antileukotrienes in clinical development for asthma. *Expert Opin. Investig. Drugs* **11(1)**, 49-58 (2002).
11. Westcott, J.Y., Johnston, K., Batt, R.A., *et al.* Measurement of peptidoleukotrienes in biological fluids. *Am. J. Physiol.* **232**, 2640-2648 (1990).
12. Kumlin, M., Stensvad, F., Larsson, L., *et al.* Validation and application of a new simple strategy for measurements of urinary leukotriene E₄ in humans. *Clin. Exp. Allergy* **25**, 467-479 (1995).
13. Westcott, J.Y., Maxey, K.M., MacDonald, J., *et al.* Immunoaffinity resin for purification of urinary leukotriene E₄. *Prostaglandins and Other Lipid Mediators* **55**, 301-321 (1998).

Warranty and Limitation of Remedy

Sapphire Bioscience Pty Ltd ("Sapphire") makes no representation or warranty of any kind, express or implied, which extends beyond the description of the product contained herein, except that the material will meet Sapphire's specifications at the time of delivery. Sapphire's sole liability hereunder shall be limited to refund of the purchase price of, or at Sapphire's option the replacement of, all material that does not meet Sapphire's specifications. Sapphire shall not be liable otherwise or for incidental or consequential damages, including but not limited to, the costs of handling. The said refund or replacement is conditional on the Buyer giving written notice to Sapphire within thirty (30) days after arrival of the material at its destination. Failure of the buyer to give the said notice within the said thirty (30) days shall constitute a waiver by the Buyer of all claims hereunder with respect to the said material.

13/12/07