Leukotriene E$_4$ ELISA

Enzyme immunoassay for the quantitative determination of Leukotriene E$_4$ (LTE$_4$) in urine, serum, plasma, whole blood, and other sample matrices.

REF CM520411

Σ 96/480

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Distributed by:

IBL INTERNATIONAL GMBH
Flughafenstrasse 52a
D-22335 Hamburg, Germany
Phone: +49 (0)40-53 28 91-0
Fax: +49 (0)40-53 28 91-11
IBL@IBL-International.com
www.IBL-International.com
Leukotriene E₄ EIA Kit
Catalog No. 520411 (Strip Plate)
Catalog No. 520411.1 (Solid Plate)
### Materials Supplied

<table>
<thead>
<tr>
<th>Catalog Number</th>
<th>Item</th>
<th>96 wells Quantity/Size</th>
<th>480 wells Quantity/Size</th>
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<tr>
<td>420412</td>
<td>Leukotriene E_{4} EIA Antiserum</td>
<td>1 vial/100 dtn</td>
<td>1 vial/500 dtn</td>
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<tr>
<td>420410</td>
<td>Leukotriene E_{4} AChE Tracer</td>
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<td>420414</td>
<td>Leukotriene E_{4} EIA Standard</td>
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<td>400060</td>
<td>EIA Buffer Concentrate (10X)</td>
<td>2 vials/10 ml</td>
<td>4 vials/10 ml</td>
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<tr>
<td>400062</td>
<td>Wash Buffer Concentrate (400X)</td>
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<td>1 vial/12.5 ml</td>
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<tr>
<td>400035</td>
<td>Tween 20</td>
<td>1 vial/3 ml</td>
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<td>400005</td>
<td>Mouse Anti-Rabbit IgG Coated Plate</td>
<td>1 plate</td>
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<td>400012</td>
<td>Plate Cover</td>
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<td>5 covers</td>
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<tr>
<td>400050</td>
<td>Ellman's Reagent</td>
<td>3 vials/100 dtn</td>
<td>6 vials/250 dtn</td>
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<td>400040</td>
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<td>EIA Antiserum Dye</td>
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If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 975-3999. We cannot accept any returns without prior authorization.
**WARNING:** Not for human or animal disease diagnosis or therapeutic drug use.

**Precautions**

**Please read these instructions carefully before beginning this assay.**

The reagents in this kit have been tested and formulated to work exclusively with Cayman Chemical’s ACE™ EIA Kits. This kit may not perform as described if any reagent or procedure is replaced or modified.

For research use only. Not for human or diagnostic use.

**If You Have Problems**

**Technical Service Contact Information**

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888
Fax: 734-971-3641
E-Mail: techserv@caymanchem.com
Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

**Storage and Stability**

This kit will perform as specified if stored as directed at -80°C and used before the expiration date indicated on the outside of the box.

**Materials Needed But Not Supplied**

1. A plate reader capable of measuring absorbance between 405-420 nm.
2. Adjustable pipettes and a repeat pipettor.
3. A source of ‘UltraPure’ water. Water used to prepare all EIA reagents and buffers must be deionized and free of trace organic contaminants (‘UltraPure’). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for EIA. **NOTE: UltraPure water is available for purchase from Cayman (Catalog No. 400000).**
4. Materials used for Sample Preparation (see page 13).
INTRODUCTION

Biochemistry of Leukotriene E<sub>4</sub>

Leukotriene E<sub>4</sub> (LTE<sub>4</sub>) is a product of the 5-lipoxygenase (5-LO) pathway in activated mast cells, eosinophils, and monocytes. Leukotriene A<sub>4</sub> (LTA<sub>4</sub>), the primary 5-LO metabolite, is converted to LTC<sub>4</sub> and sequentially to LTD<sub>4</sub> and LTE<sub>4</sub> in the host cell, or by transcellular metabolism in erythrocytes, platelets, or neutrophils (see Figure 1, page 7). This metabolism is rapid and complete, in that plasma levels of LTC<sub>4</sub> are virtually undetectable. Exogenously administered LTC<sub>4</sub> is recovered in the urine as LTE<sub>4</sub> (5-13%) and two prominent oxidized metabolites resulting from several cycles of β-oxidation. The plasma half-life of LTE<sub>4</sub> is about 7 minutes. Plasma LTE<sub>4</sub> levels are likewise <2 pg/ml as a consequence of the low rate of production and rapid elimination.

Normal human urine contains low but detectable amounts of LTE<sub>4</sub>, ranging from 10-60 pg/ml. Asthmatic patients in an acute episode of bronchoconstriction may have elevations of urinary LTE<sub>4</sub> to several hundred pg/ml, but their baseline LTE<sub>4</sub> levels are not consistently abnormal. Methods for the rapid isolation and detection of LTE<sub>4</sub> from human urine have been developed.

About This Assay

Cayman’s LTE<sub>4</sub> EIA Kit is a competitive assay that can be used for quantification of LTE<sub>4</sub> in urine, plasma, serum, whole blood, as well as other heterogeneous mixtures such as lavage fluids and aspirates and other sample matrices. The EIA typically displays an IC<sub>50</sub> (50% B/B<sub>0</sub>) of approximately 100 pg/ml and a detection limit (80% B/B<sub>0</sub>) of approximately 25 pg/ml.
Introduction

Biochemistry of Acetylcholinesterase

The electric organ of the electric eel, *Electrophorus electricus*, contains an avid acetylcholinesterase (AChE) capable of massive catalytic turnover during the generation of its electrochemical discharges. The electric eel AChE has a clover leaf-shaped tertiary structure consisting of a triad of tetramers attached to a collagen-like structural fibril. This stable enzyme is capable of high turnover (64,000 s⁻¹) for the hydrolysis of acetylthiocholine.

A molecule of the analyte covalently attached to a molecule of AChE serves as the tracer in ACE™ enzyme immunoassays. Quantification of the tracer is achieved by measuring its AChE activity with Ellman’s Reagent. This reagent consists of acetylthiocholine and 5,5’-dithio-bis-(2-nitrobenzoic acid). Hydrolysis of acetylthiocholine by AChE produces thiocholine (see Figure 3, on page 10). The non-enzymatic reaction of thiocholine with 5,5’-dithio-bis-(2-nitrobenzoic acid) produces 5-thio-2-nitrobenzoic acid, which has a strong absorbance at 412 nm (ε = 13,600).

AChE has several advantages over other enzymes commonly used for enzyme immunoassays. Unlike horseradish peroxidase, AChE does not self-inactivate during turnover. This property of AChE also allows redevelopment of the assay if it is accidentally splashed or spilled. In addition, the enzyme is highly stable under the assay conditions, has a wide pH range (pH 5-10), and is not inhibited by common buffer salts or preservatives. Since AChE is stable during the development step, it is unnecessary to use a ‘stop’ reagent, and the plate may be read whenever it is convenient.

Description of ACE™ Competitive EIAs

This assay is based on the competition between LTE₄ and an LTE₄-acetylcholinesterase (AChE) conjugate (LTE₄ Tracer) for a limited amount of LTE₄ Antiserum. Because the concentration of the LTE₄ Tracer is held constant while the concentration of LTE₄ varies, the amount of LTE₄ Tracer that is able to bind to the LTE₄ Antiserum will be inversely proportional to the concentration of LTE₄ in the well. This antibody-LTE₄ complex binds to a mouse monoclonal anti-rabbit IgG that has been previously attached to the well. The plate is washed to remove any unbound reagents and then Ellman’s Reagent (which contains the substrate to AChE) is added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is proportional to the amount of LTE₄ Tracer bound to the well, which is inversely proportional to the amount of free LTE₄ present in the well during the incubation; or

\[ \text{Absorbance} \propto \frac{[\text{Bound LTE}_4 \text{ Tracer}]}{[\text{LTE}_4]} \propto 1/[\text{LTE}_4] \]

A schematic of this process is shown in Figure 2, below.

![Figure 2. Schematic of the ACE™ EIA](image-url)
Definition of Key Terms

Blank: background absorbance caused by Ellman’s Reagent. The blank absorbance should be subtracted from the absorbance readings of all the other wells.

Total Activity: total enzymatic activity of the AChE-linked tracer. This is analogous to the specific activity of a radioactive tracer.

NSB (Non-Specific Binding): non-immunological binding of the tracer to the well. Even in the absence of specific antibody a very small amount of tracer still binds to the well; the NSB is a measure of this low binding.

$B_0$ (Maximum Binding): maximum amount of the tracer that the antibody can bind in the absence of free analyte.

$\%B/B_0$ ($\%$Bound/Maximum Bound): ratio of the absorbance of a particular sample or standard well to that of the maximum binding ($B_0$) well.

Standard Curve: a plot of the $\%B/B_0$ values versus concentration of a series of wells containing various known amounts of analyte.

Dtn: determination, where one dtn is the amount of reagent used per well.

Figure 3. Reaction catalyzed by acetylcholinesterase
NOTE: Water used to prepare all EIA reagents and buffers must be deionized and free of trace organic contaminants (‘UltraPure’). Use activated carbon filter cartridges or other organic scavengers. Glass distilled water (even if double distilled), HPLC-grade water, and sterile water (for injections) are not adequate for EIA. UltraPure water may be purchased from Cayman (Catalog No. 400000).

Buffer Preparation

Store all buffers at 4°C; they will be stable for about two months.

1. EIA Buffer Preparation
   Dilute the contents of one vial of EIA Buffer Concentrate (Catalog No. 400060) with 90 ml of UltraPure water. Be certain to rinse the vial to remove any salts that may have precipitated. NOTE: It is normal for the concentrated buffer to contain crystalline salts after thawing. These will completely dissolve upon dilution with water.

2. Wash Buffer Preparation
   5 ml vial Wash Buffer (96-well kit; Catalog No. 400062): Dilute to a total volume of 2 liters with UltraPure water and add 1 ml of Tween 20 (Catalog No. 400035).
   OR
   12.5 ml vial Wash Buffer (480-well kit; Catalog No. 400062): Dilute to a total volume of 5 liters with UltraPure water and add 2.5 ml of Tween 20 (Catalog No. 400035).

Smaller volumes of Wash Buffer can be prepared by diluting the Wash Buffer Concentrate 1:400 and adding Tween 20 (0.5 ml/liter of Wash Buffer).

NOTE: Tween 20 is a viscous liquid and cannot be measured by a regular pipette. A positive displacement pipette or a syringe should be used to deliver small quantities accurately.

Sample Preparation

Urine, plasma, serum, whole blood, as well as other heterogeneous mixtures such as lavage fluids and aspirates often contain contaminants which can interfere in the assay. The presence of rabbit IgG in the sample will interfere in the assay. It is best to check for interference before embarking on a large number of sample measurements. To test for interference, dilute one or two test samples to at least two different dilutions between approximately 25 and 1,000 pg/ml (i.e., between 20-80% B/B₀). If the two different dilutions of the sample show good correlation (differ by 20% or less) in the final calculated LTE₄ concentration, purification is not required. If you do not see good correlation of serial dilutions, purification is advised. The Purification Protocol, on page 15, is one such method. NOTE: We recommend using Cayman’s Cysteinyl Leukotriene Affinity Sorbent (Catalog No. 420509) or Cayman’s Cysteinyl Leukotriene Column (Catalog No. 10005362) for purification of LTE₄ from biological samples prior to EIA analysis.

General Precautions

• All samples must be free of organic solvents prior to assay.
• Samples should be assayed immediately after collection; samples that cannot be assayed immediately should be stored at -80°C.
Lavage Fluids and Aspirates

CysLTs can accumulate to relatively high concentrations in the effusion fluids associated with inflammation (e.g., ascites fluid, synovial fluid, pleural effusion, pericardial or cerebral intraventricular aspirates). Since LT metabolism is incomplete in these circumstances, substantial amounts of LTC₄, LTD₄, and LTE₄ may be present (e.g., bronchoalveolar lavage fluid from asthmatic subjects may contain 700-1,000 pg/ml CysLTs comprised mainly of LTC₄ and LTD₄). Consequently, analysis of these fluids is the optimal application of the Cysteinyl Leukotriene EIA Kit (Catalog No. 520501). \textit{Note: The complex nature of these samples makes purification mandatory in order to achieve accurate results.}^{9,10}

Urine

CysLTs are excreted in urine as intact LTE₄ (~9-12%) and LTE₄ metabolites. Levels of intact LTE₄ in human urine are 50-80 pg/mg creatinine (about 10-50 pg/ml). Since LTC₄ and LTD₄ are virtually absent from urine, CysLT measurement in urine samples is often best accomplished by measuring LTE₄ using this kit. Unpurified urine samples show a linear but elevated concentration of CysLTs. Therefore, to increase the accuracy of the data generated, urine samples should be purified prior to assay.\textsuperscript{9}

Culture Media Samples

Cultured cells synthesizing LTC₄ will generally release it into the medium where it will accumulate without further metabolism. Thus, samples of this type are best analyzed by the measurement of LTC₄ specifically (LTC₄ EIA Kit, Catalog No. 520211).

Plasma

Metabolism of LTC₄ in plasma is rapid and complete, after which the metabolites are eliminated and recovered in the urine as LTE₄. Plasma levels of LTE₄ are therefore very low (<2 pg/ml) (see \textit{Biochemistry of Leukotriene E₄} for additional details). Plasma samples should be collected in vacutainers containing sodium heparin, EDTA, or sodium citrate. Due to the low levels of LTE₄ in plasma, as well as it being a complex matrix that contains many substances that can interfere with this assay, purification is necessary prior to performing the assay.

Sample Purification

Determination of Recovery

Determination of percent recovery is recommended when any sample purification is performed. Detailed below are two methods that can be employed to monitor the recovery. If the \textbf{Hot Spike} method is used, 10,000 cpm of tritium-labeled LTE₄ is added directly to the sample and 10% is removed for scintillation counting after purification. If the \textbf{Cold Spike} method is used, the sample must be split prior to purification and an appropriate amount of LTE₄ added to one aliquot. The spiked sample is then assayed \textit{via} EIA alongside the unspiked sample. Calculations for each method are found in the \textit{Analysis} section on page 24.

Purification Protocol

Cayman Chemical highly recommends the use of our affinity purification reagents for the rapid and easy purification of cysteinyl leukotrienes from biological samples (Cysteinyl Leukotriene Affinity Sorbent - Catalog No. 420509; Cysteinyl Leukotriene Affinity Column - Catalog No. 10005362). These reagents are easier to use and provide higher purity with better recovery than solid phase extraction (SPE) chromatography. In particular, urinary LTE₄ cannot be sufficiently purified using SPE (C-18). Impurities in urine co-elute with LTE₄ from SPE (C-18) cartridges and interfere in the EIA measurement. Therefore, the options for purification of urinary LTE₄ are to either use the affinity reagents or use SPE (C-18) followed by HPLC/TLC. The protocol for affinity purification accompanies those reagents when purchased.
1. Aliquot a known amount of each sample into a clean test tube (500 µl is recommended). If your samples need to be concentrated, a larger volume should be used (e.g., a 5 ml sample will be concentrated by a factor of 10, a 10 ml sample will be concentrated by a factor of 20, etc.).

2. Add 10,000 cpm of tritium-labeled LTE_4 ([^3]H-LTE_4). Use a high specific activity tracer to minimize the amount of radioactive LTE_4 as the EIA will be able to detect the added LTE_4.

   **Cold Spike**
   1. Aliquot a known amount of each sample into each of two tubes (500 µl is recommended). Label the first tube ‘sample #’ and the second ‘sample # + spike’. If your samples need to be concentrated, a larger volume should be used (e.g., a 5 ml sample will be concentrated by a factor of 10, a 10 ml sample will be concentrated by a factor of 20, etc.).

   2. Add a cold spike of LTE_4 to the ‘sample + spike’ tubes. Follow the procedure below for both spiked and unspiked samples.

   **Hot Spike**
   1. Pass the sample through the SPE (C-18) cartridge. Rinse the cartridge with 5 ml UltraPure water followed by 5 ml of HPLC grade hexane (allow the cartridge to become dry after this step). Discard both washes. Elute the LTE_4 with 5 ml methanol. Higher recovery and better reproducibility may be obtained if the sample is applied and eluted by gravity. The wash steps may be performed under vacuum or pressure.

   2. Evaporate the methanol to dryness either by vacuum centrifugation or by evaporation under a stream of dry nitrogen. It is imperative that all of the organic solvent be removed as even trace quantities will adversely affect the EIA.

   3. Add 500 µl of EIA Buffer and vortex. Use this for EIA analysis. It is common for an insoluble precipitate to remain after the addition of EIA buffer; this will not affect the assay. Use 50 µl of this sample for scintillation counting to determine recovery.

   * If it is necessary to stop during this purification, samples may be stored in the methanol solution at -80°C.

3. Precipitation of proteins using ethanol is optional and may not be needed if samples are clean enough to flow through the SPE (C-18) cartridge. Body fluids such as plasma and urine can typically be applied directly to the SPE (C-18) cartridge after the acidification step (step 4) below. To precipitate proteins, add methanol (approximately four times the sample volume) to each tube. Vortex to mix thoroughly. Incubate samples at 4°C for five minutes, then centrifuge a 3,000 x g for 10 minutes to remove precipitated proteins. Transfer the supernatant to a clean test tube. Evaporate the methanol either by vacuum centrifugation or under a gentle stream of nitrogen. Adjust the pH of the sample to 4.0 using 1.0 M acetate, citrate buffer (pH 4.0), or dilute HCl.

4. Activate a 6 ml SPE (C-18) cartridge (Catalog No. 400020) by rinsing with 5 ml methanol and followed by 5 ml UltraPure water. Do not allow the SPE (C-18) cartridge to dry.

5. Pass the sample through the SPE (C-18) cartridge. Rinse the cartridge with 5 ml UltraPure water followed by 5 ml of HPLC grade hexane (allow the cartridge to become dry after this step). Discard both washes. Elute the LTE_4 with 5 ml methanol. Higher recovery and better reproducibility may be obtained if the sample is applied and eluted by gravity. The wash steps may be performed under vacuum or pressure.

6. Evaporate the methanol to dryness either by vacuum centrifugation or by evaporation under a stream of dry nitrogen. It is imperative that all of the organic solvent be removed as even trace quantities will adversely affect the EIA.

7. Add 500 µl of EIA Buffer and vortex. Use this for EIA analysis. It is common for an insoluble precipitate to remain after the addition of EIA buffer; this will not affect the assay. Use 50 µl of this sample for scintillation counting to determine recovery.

Proceed to step 3, below

3. Precipitation of proteins using ethanol is optional and may not be needed if samples are clean enough to flow through the SPE (C-18) cartridge. Body fluids such as plasma and urine can typically be applied directly to the SPE (C-18) cartridge after the acidification step (step 4) below. To precipitate proteins, add methanol (approximately four times the sample volume) to each tube. Vortex to mix thoroughly. Incubate samples at 4°C for five minutes, then centrifuge a 3,000 x g for 10 minutes to remove precipitated proteins. Transfer the supernatant to a clean test tube. Evaporate the methanol either by vacuum centrifugation or under a gentle stream of nitrogen. Adjust the pH of the sample to 4.0 using 1.0 M acetate, citrate buffer (pH 4.0), or dilute HCl.

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5. Pass the sample through the SPE (C-18) cartridge. Rinse the cartridge with 5 ml UltraPure water followed by 5 ml of HPLC grade hexane (allow the cartridge to become dry after this step). Discard both washes. Elute the LTE_4 with 5 ml methanol. Higher recovery and better reproducibility may be obtained if the sample is applied and eluted by gravity. The wash steps may be performed under vacuum or pressure.

6. Evaporate the methanol to dryness either by vacuum centrifugation or by evaporation under a stream of dry nitrogen. It is imperative that all of the organic solvent be removed as even trace quantities will adversely affect the EIA.

7. Add 500 µl of EIA Buffer and vortex. Use this for EIA analysis. It is common for an insoluble precipitate to remain after the addition of EIA buffer; this will not affect the assay. Use 50 µl of this sample for scintillation counting to determine recovery.

   *If it is necessary to stop during this purification, samples may be stored in the methanol solution at -80°C.

8. Use 50 µl of the resuspended sample for scintillation counting.
**Preparation of Assay-Specific Reagents**

**Leukotriene E₄ EIA Standard**

Equilibrate a pipette tip in ethanol by repeatedly filling and expelling the tip with ethanol several times. Using the equilibrated pipette tip, transfer 100 µl of the LTE₄ Standard (Catalog No. 420414) into a clean test tube, then dilute with 900 µl UltraPure water. The concentration of this solution (the bulk standard) will be 10 ng/ml.

**NOTE:** If assaying culture medium samples that have not been diluted with EIA Buffer, culture medium should be used in place of EIA Buffer for dilution of the standard curve.

To prepare the standard for use in EIA: Obtain 8 clean test tubes and number them #1 through #8. Aliquot 900 µl EIA Buffer to tube #1 and 500 µl EIA Buffer to tubes #2-8. Transfer 100 µl of the bulk standard (10 ng/ml) to tube #1 and mix thoroughly. Serially dilute the standard by removing 500 µl from tube #1 and placing in tube #2; mix thoroughly. Next, remove 500 µl from tube #2 and place it into tube #3; mix thoroughly. Repeat this process for tubes #4-8. These diluted standards should not be stored for more than 24 hours.

Figure 4. Preparation of the LTE₄ standards

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**Leukotriene E₄ AChE Tracer**

Reconstitute the LTE₄ Tracer as follows:

- **100 dtn LTE₄ AChE Tracer (96-well kit; Catalog No. 420410):** Reconstitute with 6 ml EIA Buffer.

  - OR

- **500 dtn LTE₄ AChE Tracer (480-well kit; Catalog No. 420410):** Reconstitute with 30 ml EIA Buffer.

Store the reconstituted LTE₄ Tracer at 4°C (*do not freeze!*) and use within four weeks. A 20% surplus of LTE₄ Tracer has been included to account for any incidental losses.

**Tracer Dye Instructions (optional)**

This dye may be added to the tracer, if desired, to aid in visualization of tracer-containing wells. Add the dye to the reconstituted tracer at a final dilution of 1:100 (add 60 µl of dye to 6 ml tracer or add 300 µl of dye to 30 ml of tracer).

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**Leukotriene E₄ EIA Antiserum**

Reconstitute the LTE₄ Antiserum as follows:

- **100 dtn LTE₄ Antiserum (96-well kit; Catalog No. 420412):** Reconstitute with 6 ml EIA Buffer.

  - OR

- **500 dtn LTE₄ Antiserum (480-well kit; Catalog No. 420412):** Reconstitute with 30 ml EIA Buffer.

Store the reconstituted LTE₄ Antiserum at 4°C. It will be stable for at least four weeks. A 20% surplus of LTE₄ Antiserum has been included to account for any incidental losses.

**Antiserum Dye Instructions (optional)**

This dye may be added to the antiserum, if desired, to aid in visualization of antiserum-containing wells. Add the dye to the reconstituted antiserum at a final dilution of 1:100 (add 60 µl of dye to 6 ml antiserum or add 300 µl of dye to 30 ml of antiserum).
Plate Set Up

The 96-well plate(s) included with this kit is supplied ready to use. It is not necessary to rinse the plate(s) prior to adding the reagents. **NOTE:** If you do not need to use all the strips at once, place the unused strips back in the plate packet and store at 2-4°C. Be sure the packet is sealed with the desiccant inside.

Each plate or set of strips must contain a minimum of two blanks (Blk), two non-specific binding wells (NSB), two maximum binding wells (B₀), and an eight point standard curve run in duplicate. **NOTE:** Each assay must contain this minimum configuration in order to ensure accurate and reproducible results. Each sample should be assayed at two dilutions and each dilution should be assayed in duplicate. For statistical purposes, we recommend assaying samples in triplicate.

A suggested plate format is shown in Figure 5, below. The user may vary the location and type of wells present as necessary for each particular experiment. The plate format provided below has been designed to allow for easy data analysis using a convenient spreadsheet offered by Cayman (see page 24, for more details). We suggest you record the contents of each well on the template sheet provided (see page 35).

![Plate Format](image)

Figure 5. Sample plate format

Performing the Assay

Pipetting Hints

- Use different tips to pipette the buffer, standard, sample, tracer, and antiserum.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (i.e., slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

Addition of the Reagents

1. **EIA Buffer**

Add 100 µl EIA Buffer to Non-Specific Binding (NSB) wells. Add 50 µl EIA Buffer to Maximum Binding (B₀) wells. If culture medium was used to dilute the standard curve, substitute 50 µl of culture medium for EIA Buffer in the NSB and B₀ wells (i.e., add 50 µl culture medium to NSB and B₀ wells and 50 µl EIA Buffer to NSB wells).

2. **Leukotriene E₄ EIA Standard**

Add 50 µl from tube #8 to both of the lowest standard wells (S8). Add 50 µl from tube #7 to each of the next two standard wells (S7). Continue with this procedure until all the standards are aliquoted. The same pipette tip should be used to aliquot all the standards. Before pipetting each standard, be sure to equilibrate the pipette tip in that standard.

3. **Samples**

Add 50 µl of sample per well. Each sample should be assayed at a minimum of two dilutions. Each dilution should be assayed in duplicate (triplicate recommended).

4. **Leukotriene E₄ AChE Tracer**

Add 50 µl to each well except the Total Activity (TA) and the Blank (Blk) wells.

5. **Leukotriene E₄ EIA Antiserum**

Add 50 µl to each well except the Total Activity (TA), the Non-Specific Binding (NSB), and the Blank (Blk) wells.
### Well EIA Buffer Standard/ Sample Tracer Antibody

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**Table 1. Pipetting summary**

### Incubate the Plate

Cover each plate with plastic film (Catalog No. 400012) and incubate 18 hours at room temperature.

### Develop the Plate

1. Reconstitute Ellman’s Reagent immediately before use (20 ml of reagent is sufficient to develop 100 wells):

   **100 dtn vial Ellman’s Reagent (96-well kit; Catalog No. 400050):** Reconstitute with 20 ml of UltraPure water.

   OR

   **250 dtn vial Ellman’s Reagent (480-well kit; Catalog No. 400050):** Reconstitute with 50 ml of UltraPure water.

   **NOTE:** Reconstituted Ellman’s Reagent is unstable and should be used the same day it is prepared; protect the Ellman’s Reagent from light when not in use. Extra vials of the reagent have been provided should a plate need to be re-developed or multiple assays run on different days.

2. Empty the wells and rinse five times with Wash Buffer.
3. Add 200 µl of Ellman’s Reagent to each well.
4. Add 5 µl of tracer to the Total Activity wells.
5. Cover the plate with plastic film. Optimum development is obtained by using an orbital shaker equipped with a large, flat cover to allow the plate(s) to develop in the dark. This assay typically develops (i.e., B₀ wells ≥0.3 A.U. (blank subtracted)) in 60-90 minutes.

### Read the Plate

1. Wipe the bottom of the plate with a clean tissue to remove fingerprints, dirt, etc.
2. Remove the plate cover being careful to keep Ellman’s Reagent from splashing on the cover. **NOTE:** Any loss of Ellman’s Reagent will affect the absorbance readings. If Ellman’s Reagent is present on the cover, use a pipette to transfer the Ellman’s Reagent into the well. If too much Ellman’s Reagent has splashed on the cover to easily redistribute back into the wells, wash the plate three times with wash buffer and repeat the development with fresh Ellman’s Reagent.
3. Read the plate at a wavelength between 405 and 420 nm. The absorbance may be checked periodically until the B₀ wells have reached a minimum of 0.3 A.U. (blank subtracted). The plate should be read when the absorbance of the B₀ wells are in the range of 0.3-1.0 A.U. (blank subtracted). If the absorbance of the wells exceeds 1.5, wash the plate, add fresh Ellman’s Reagent and let it develop again.
Many plate readers come with data reduction software that plot data automatically. Alternatively a spreadsheet program can be used. The data should be plotted as %B/B₀ versus log concentration using either a 4-parameter logistic or log-logit curve fit. NOTE: Cayman has a computer spreadsheet available for data analysis. Please contact Technical Service or visit our website (www.caymanchem.com/analysis/eia) to obtain a free copy of this convenient data analysis tool.

Calculations

Preparation of the Data

The following procedure is recommended for preparation of the data prior to graphical analysis.

NOTE: If the plate reader has not subtracted the absorbance readings of the blank wells from the absorbance readings of the rest of the plate, be sure to do that now.

1. Average the absorbance readings from the NSB wells.
2. Average the absorbance readings from the B₀ wells.
3. Subtract the NSB average from the B₀ average. This is the corrected B₀ or corrected maximum binding.
4. Calculate the %B/B₀ (% Sample or Standard Bound/Maximum Bound) for the remaining wells. To do this, subtract the average NSB absorbance from the S1 absorbance and divide by the corrected B₀ (from Step 3). Multiply by 100 to obtain %B/B₀. Repeat for S2-S8 and all sample wells.

NOTE: The total activity (TA) values are not used in the standard curve calculations. Rather, they are used as a diagnostic tool; the corrected B₀ divided by the actual TA (10X measured absorbance) will give the % Bound. This value should closely approximate the % Bound that can be calculated from the Sample Data (see page 27). Erratic absorbance values and a low (or no) % Bound could indicate the presence of organic solvents in the buffer or other technical problems (see page 31 for Troubleshooting).

Plot the Standard Curve

Plot %B/B₀ for standards S1-S8 versus LTE₄ concentration using linear (y) and log (x) axis and fit the data to a 4-parameter logistic equation.

Alternative Plot - The data can also be linearized using a logit transformation. The equation for this conversion is as follows, NOTE: Do not use %B/B₀ in this calculation:

\[
\text{logit} \left(\frac{B}{B_0}\right) = \ln \left(\frac{B}{B_0}/(1 - B/B_0)\right)
\]

Plot the data as logit (B/B₀) versus log concentrations and perform a linear regression fit.

Determine the Sample Concentration

Calculate the %B/B₀ value for each sample. Determine the concentration of each sample using the equation obtained from the standard curve plot. NOTE: Remember to account for any concentration or dilution of the sample prior to the addition to the well. Samples with %B/B₀ values greater than 80% or less than 20% should be re-assayed as they generally fall out of the linear range of the standard curve. A 20% or greater disparity between the apparent concentration of two different dilutions of the same sample would indicate interference which could be eliminated by purification.

Hot Spike Method

\[
\text{Recovery Factor} = \frac{10 \text{ cpm of sample}}{[\text{³H}]-\text{LTE}_4 \text{ added to sample (cpm)}}
\]

\[
\text{LTE}_4 \text{ (pg) in purified sample} = \left[ \frac{\text{Value from EIA (pg/ml)}}{\text{Recovery Factor}} \right] \times 0.5 \text{ ml} - \text{ added [³H]-LTE}_4 \text{ (pg)}
\]

\[
\text{Total LTE}_4 \text{ in sample (pg/ml)} = \frac{\text{LTE}_4 \text{ (pg) in purified sample}}{\text{Volume of sample used for purification (ml)}}
\]
Cold Spike Method

The original concentration of the sample and recovery factor can be determined by the following method:

\[ V = \text{EIA determined concentration of the unspiked sample (pg/ml)} \]

\[ S = \text{concentration of the spike (pg/ml)} \]

\[ Y = \text{EIA determined concentration of the spiked sample (pg/ml)} \]

\[ \text{Purification Recovery Factor} = \left( \frac{Y - V}{S} \right) \]

\[ \text{LTE}_4 (\text{pg)} \text{ in purified sample} = \left( \frac{V}{\text{Recovery Factor}} \right) \times \text{reconstituted volume of sample} \]

\[ \text{LTE}_4 \text{ in original sample (pg/ml)} = \frac{\text{LTE}_4 \text{ (pg)} \text{ in purified sample}}{\text{Volume of sample used for purification (ml)}} \]

Performance Characteristics

Sample Data

The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. You must run a new standard curve. Do not use the data below to determine the values of your samples. Your results could differ substantially.

<table>
<thead>
<tr>
<th>Dose (pg/ml)</th>
<th>Raw Data</th>
<th>Average</th>
<th>Corrected</th>
<th>%B/B_0</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,000</td>
<td>0.072</td>
<td>0.069</td>
<td>0.069</td>
<td>0.066</td>
</tr>
<tr>
<td>500</td>
<td>0.094</td>
<td>0.098</td>
<td>0.091</td>
<td>0.095</td>
</tr>
<tr>
<td>250</td>
<td>0.131</td>
<td>0.134</td>
<td>0.128</td>
<td>0.131</td>
</tr>
<tr>
<td>125</td>
<td>0.165</td>
<td>0.166</td>
<td>0.162</td>
<td>0.163</td>
</tr>
<tr>
<td>62.5</td>
<td>0.209</td>
<td>0.206</td>
<td>0.206</td>
<td>0.203</td>
</tr>
<tr>
<td>31.3</td>
<td>0.249</td>
<td>0.261</td>
<td>0.246</td>
<td>0.258</td>
</tr>
<tr>
<td>15.6</td>
<td>0.299</td>
<td>0.304</td>
<td>0.296</td>
<td>0.301</td>
</tr>
<tr>
<td>7.8</td>
<td>0.322</td>
<td>0.347</td>
<td>0.319</td>
<td>0.344</td>
</tr>
</tbody>
</table>

Table 2. Typical results
ANALYSIS

Evaluate data cautiously

Use data with confidence

Leukotriene E4 (pg/ml)

50% B/B₀ - 100 pg/ml
Detection Limit (80% B/B₀) - 25 pg/ml

Figure 6. Typical standard curve

ANALYSIS

Precision:
The intra- and inter-assay CV’s have been determined at multiple points on the standard curve. These data are summarized in the graph on page 28.

<table>
<thead>
<tr>
<th>Dose (pg/ml)</th>
<th>%CV* Intra-assay variation</th>
<th>%CV* Inter-assay variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,000</td>
<td>4.5</td>
<td>28.3</td>
</tr>
<tr>
<td>500</td>
<td>6.4</td>
<td>9.2</td>
</tr>
<tr>
<td>250</td>
<td>6.5</td>
<td>10.0</td>
</tr>
<tr>
<td>125</td>
<td>7.1</td>
<td>7.9</td>
</tr>
<tr>
<td>62.5</td>
<td>8.0</td>
<td>9.0</td>
</tr>
<tr>
<td>31.3</td>
<td>11.0</td>
<td>10.0</td>
</tr>
<tr>
<td>15.6</td>
<td>53.3</td>
<td>25.9</td>
</tr>
<tr>
<td>7.8</td>
<td>146.6</td>
<td>68.8</td>
</tr>
</tbody>
</table>

Table 3. Intra- and inter-assay variation
*%CV represents the variation in concentration (not absorbance) as determined using a reference standard curve.
Specificity:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukotriene ( E_4 )</td>
<td>100%</td>
</tr>
<tr>
<td>Leukotriene ( E_5 )</td>
<td>100%</td>
</tr>
<tr>
<td>11-trans Leukotriene ( E_4 )</td>
<td>66.3%</td>
</tr>
<tr>
<td>N-acetyl Leukotriene ( E_4 )</td>
<td>20%</td>
</tr>
<tr>
<td>Leukotriene ( C_4 )</td>
<td>10%</td>
</tr>
<tr>
<td>Leukotriene ( D_4 )</td>
<td>7%</td>
</tr>
<tr>
<td>Leukotriene ( C_5 )</td>
<td>2%</td>
</tr>
<tr>
<td>Arachidonic Acid</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>Leukotriene ( B_6 )</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>Leukotriene ( B_7 )</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>Leukotriene ( D_5 )</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>tetranor-PGEM</td>
<td>&lt;0.01%</td>
</tr>
<tr>
<td>tetranor-PGFM</td>
<td>&lt;0.01%</td>
</tr>
</tbody>
</table>

Table 4. Specificity of the LTE\(_4\) Antiserum

**Troubleshooting**

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Causes</th>
<th>Recommended Solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erratic values; dispersion of duplicates</td>
<td>A. Trace organic contaminants in the water source</td>
<td>A. Replace activated carbon filter or change source of UltraPure water</td>
</tr>
<tr>
<td></td>
<td>B. Poor pipetting/technique</td>
<td></td>
</tr>
<tr>
<td>High NSB (&gt;0.035)</td>
<td>A. Poor washing</td>
<td>A. Rewash plate and redevelop</td>
</tr>
<tr>
<td></td>
<td>B. Exposure of NSB wells to specific antibody</td>
<td></td>
</tr>
<tr>
<td>Very low ( B_0 )</td>
<td>A. Contamination of water with organic solvents</td>
<td>A. Replace activated carbon filter or change source of UltraPure water</td>
</tr>
<tr>
<td></td>
<td>B. Plate requires additional development time</td>
<td>B. Return plate to shaker and reread later</td>
</tr>
<tr>
<td></td>
<td>C. Dilution error in preparing reagents</td>
<td></td>
</tr>
<tr>
<td>Low sensitivity (shift in dose response curve)</td>
<td>Standard is degraded</td>
<td>Replace standard</td>
</tr>
<tr>
<td>Analyses of two dilutions of a biological sample do not agree (i.e., more than 20% difference)</td>
<td>Interfering substances are present</td>
<td>Purify sample prior to analysis by EIA(^1)</td>
</tr>
<tr>
<td>Only Total Activity (TA) wells develop</td>
<td>Trace organic contaminants in the water source</td>
<td>Replace activated carbon filter or change source of UltraPure water</td>
</tr>
</tbody>
</table>
**Additional Reading**

Go to [www.caymanchem.com/520411/references](http://www.caymanchem.com/520411/references) for a list of publications citing the use of Cayman Chemical's LTE<sub>4</sub> EIA Kit.

**References**


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Cayman Chemical Company makes no warranty or guarantee of any kind, whether written or oral, expressed or implied, including without limitation, any warranty of fitness for a particular purpose, suitability and merchantability, which extends beyond the description of the chemicals hereof. Cayman warrants only to the original customer that the material will meet our specifications at the time of delivery. Cayman will carry out its delivery obligations with due care and skill. Thus, in no event will Cayman have any obligation or liability, whether in tort (including negligence) or in contract, for any direct, indirect, incidental or consequential damages, even if Cayman is informed about their possible existence. This limitation of liability does not apply in the case of intentional acts or negligence of Cayman, its directors or its employees.

Buyer’s exclusive remedy and Cayman’s sole liability hereunder shall be limited to a refund of the purchase price, or at Cayman’s option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to Cayman within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

For further details, please refer to our Warranty and Limitation of Remedy located on our website and in our catalog.
Symbols / Symbole / Symbôles / Símbolos / Σύμβολα

REF Cat.-No.: / Kat.-Nr.: / No.- Cat.: / Cat.-No.: / N.º Cat.: / N.-Cat.: / Αριθμός-Κατ.:
LOT Lot-No.: / Chargen-Bez.: / No. Lot: / Lot-No.: / Lote N.º: / Lotto n.: / Αριθμός-Παραγωγή:
Use by: / Verwendbar bis: / Utiliser à: / Usado por: / Usar até: / Da utilizzare entro:
CONC Concentrate / Konzentrat / Concentré / Concentrar / Concentrado / Concentrato / Συμπύκνωμα
LYO Lyophilized / Lyophilisat / Lyophilisé / Liofilizado / Liofilizado / Liofilizzato / Λύοφιλισµένο
IVD In Vitro Diagnostic Medical Device. / In-vitro-Diagnostikum. / Appareil Médical pour Diagnostics In Vitro. / Equipamiento Médico de Diagnóstico In Vitro. / Dispositivo Medico Diagnostic In vitro. / Ιατρική συσκευή για In-Vitro Διάγνωση.
Evaluation kit. / Nur für Leistungsbewertungszwecke. / Kit pour évaluation. / Juego de Reactivos para Evaluación. / Kit de evaluación. / Kit di evaluazione. / Κιτ Αξιολόγησης.
Read instructions before use. / Arbeitsanleitung lesen. / Lire la fiche technique avant emploi. / Lea las instrucciones antes de usar. / Leggere le istruzioni prima dell’uso. / Διαβάστε τις οδηγίες πριν την χρήση.
Keep away from heat or direct sunlight. / Vor Hitze und direkter Sonneneinstrahlung schützen. / Garder à l’abri de la chaleur et de toute exposition lumineuse. / Manténgase alejado del calor o la luz solar directa. / Monter longe do calor ou luz solar directa. / Να φυλάσσεται μακριά από θερμότητα και άμεση επαφή με το φως του ηλίου.
Store at: / Lagern bei: / Stocker à: / Almacene a: / Armazenar a: / Conservare a: / Αποθήκευση στους:
Manufacturer: / Hersteller: / Fabricant: / Productor: / Fabricante: / Fabricriante: / Παραγωγός:
Caution! / Vorsicht! / Attention! / ¡Precaución! / Cuidado! / Attenzione! / Προσοχή!
Symbols of the kit components see MATERIALS SUPPLIED.
Die Symbole der Komponenten sind im Kapitel KOMPONENTEN DES KITS beschrieben.
Voir MATERIEL FOURNI pour les symbôles des composants du kit.
Símbolos de los componentes del juego de reactivos, vea MATERIALES SUMINISTRADOS.
Para símbolos dos componentes do kit ver MATERIAIS FORNECIDOS.
Per i simboli dei componenti del kit si veda COMPONENTI DEL KIT.
Για τα σύµβολα των συστατικών του κιτ συµβουλευτείτε το ΠΑΡΕΧΟΜΕΝΑ ΥΛΙΚΑ.

IBL AFFILIATES WORLDWIDE

IBL International GmbH
Flughafenstr. 52A, 22335 Hamburg, Germany
Tel.: + 49 (0) 40 532891 -0 Fax: -11
E-MAIL: IBL@IBL-International.com
WEB: http://www.IBL-International.com

IBL International B.V.
Zutphenseweg 55, 7418 AH Deventer, The Netherlands
Tel.: + 49 (0) 40 532891 -0 Fax: -11
E-MAIL: IBL@IBL-International.com
WEB: http://www.IBL-International.com

IBL International Corp.
194 Wildcat Road, Toronto, Ontario M3J 2N5, Canada
Tel.: +1 (416) 645 -1703 Fax: -1704
E-MAIL: Sales@IBL-International.com
WEB: http://www.IBL-International.com

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