Leukotriene C₄/D₄/E₄
ELISA Kit Instructions

Please read all instructions carefully before beginning this assay

PRODUCT #406410
For Research Use Only.

Storage Conditions:
Lyophilized conjugate: -20°C or less
Do not freeze kit reconstituted conjugate
All other kit components: 4°C

DESCRIPTION

Leukotriene C₄/D₄/E₄ (LTC₄/LTD₄/LTE₄) are potent mediators of immediate hypersensitivity, bronchoconstriction, smooth muscle contraction, increased vascular permeability and epithelial mucous secretion. They are derived from arachidonic acid through the 5-lipoxygenase pathway. The monoclonal antisera in this kit recognizes LTC₄, LTD₄ and LTE₄ for a combined quantitative value.

PRINCIPLE OF ASSAY

This is an ELISA (Enzyme-Linked ImmunoSorbent Assay) for the quantitative analysis of Leukotriene C₄/D₄/E₄ levels in biological fluid. This test kit operates on the basis of competition between the enzyme conjugate and Leukotriene C₄/D₄/E₄ in the sample for a limited number of binding sites.

First, the sample or standard solution is added to the microplate. Next, the diluted enzyme conjugate is added and the mixture is shaken and incubated at room temperature for one hour. During the incubation, competition for binding sites is taking place. The plate is then washed, removing all the unbound material. The bound enzyme conjugate is detected by the addition of substrate which generates an optimal color after 30 minutes. Quantitative test results may be obtained by measuring and comparing the absorbance reading of the wells of the samples against the standards with a microplate reader at 650 nm. The extent of color development is inversely proportional to the amount of Leukotriene C₄/D₄/E₄ in the sample or standard. For example, the absence of Leukotriene C₄/D₄/E₄ in the sample will result in a bright blue color, whereas the presence of Leukotriene C₄/D₄/E₄ will result in decreased or no color development.
PRINCIPLE OF ASSAY (continued)

MATERIALS PROVIDED

1. **EIA BUFFER**: 30 mL. Provided to dilute enzyme conjugate and Leukotriene C₄ standards. **NOTE: PLEASE ONLY USE THE EIA BUFFER THAT IS PROVIDED WITH THIS KIT LOT.**

2. **WASH BUFFER (10X)**: 20 mL. Dilute 10-fold with deionized water. Diluted wash buffer is used to wash all unbound enzyme conjugate, samples and standards from the plate after the one hour incubation.

3. **K-BLUE SUBSTRATE**: 20 mL. Stabilized 3,3', 5,5' Tetramethylbenzidine (TMB) plus Hydrogen Peroxide (H₂O₂) in a single bottle. It is used to develop the color in the wells after they have been washed. Keep refrigerated. **LIGHT SENSITIVE**.

4. **EXTRACTION BUFFER (5X)**: 30mL. Dilute 5-fold with deionized water. This is used for diluting extracted and non-extracted samples.


6. **LEUKOTRIENE C₄ STANDARD**: 100 µL. Leukotriene C₄ standard at the concentration of 1 µg/mL. Green capped vial.

7. **LEUKOTRIENE C₄/D₄/E₄ ANTIBODY-COATED MICROPLATE**: A 96 well Maxisorp™ Nunc microplate with anti-LTC₄/D₄/E₄ monoclonal rat antibody precoated on each well. The plate is ready to use as is. **DO NOT WASH!**

MATERIALS NEEDED BUT NOT PROVIDED

1. 300 mL deionized water for diluting wash buffer, extraction buffer and reconstituting lyophilized conjugate.

2. Precision pipettes that range from 10 µL - 1000 µL and disposable tips.

**NOTE**: If all or several strips are to be used at one time, it is suggested that a multichannel pipette be used.

3. Clean test tubes used to dilute the standards and conjugate.

4. Graduated cylinder to dilute and mix wash buffer and extraction buffer.

5. Microplate reader with 650 nm filter.

6. Plate cover of plastic film to cover plate during incubation.
### OPTIONAL MATERIALS:

7. 1 N HCl or Neogen’s Red Stop Solution.
8. Microplate shaker.

If performing an extraction on samples, the following will be required:

9. 1N HCl
10. Ethanol
11. C18 Sep-Pak® light column (Waters® Corporation)
12. Petroleum ether
13. Methanol
14. Nitrogen gas
15. Centrifuge

### WARNINGS AND PRECAUTIONS

1. **DO NOT** use components beyond expiration date.
2. **DO NOT** mix any reagents or components of this kit with any reagents or components of any other kit. This kit is designed to work properly as provided.
3. **DO NOT** pipette reagents by mouth.
4. Always pour substrate out of the bottle into a clean test tube. **DO NOT** pipette out of the bottle. If the pipette tip is unclean, this could result in contamination of the substrate.
5. All specimens should be considered potentially infectious. Exercise proper handling precautions.
6. **DO NOT** smoke, eat or drink in areas where specimens or reagents are being handled.
7. Use aseptic technique when opening and removing reagents from vials and bottles.
8. Keep plate covered except when adding reagents, washing or reading.
9. Kit components should be refrigerated at all times when not in use; lyophilized conjugate, frozen.
10. Ensure that the conjugate is completely reconstituted before use. Each vial, when reconstituted, provides sufficient reagent to perform 64 assays (8 strips). If more than 64 assays are to be run, reconstitute both vials and pool the reconstituted conjugate.

### PROCEDURAL NOTES

1. It is not necessary to allow reagents to warm to room temperature before use.
2. Desiccant bag must remain in foil pouch with unused strips. Keep zip-lock pouch sealed when not in use to maintain a dry environment.
3. Always use clean pipette tips for the buffer, enzyme conjugate, standards and samples.
4. Before pipetting a reagent, rinse the pipette tip three times with that reagent (i.e. fill the tip with the desired amount of reagent and dispense back into the same vial - repeat 2 times). Now the tip is properly rinsed and ready to dispense the reagent into your well or test tube.
5. When pipetting into the wells, **DO NOT** allow the pipette tip to touch the inside of the well, or any of the reagents already in the well. This can result in cross contamination.
6. Standards and samples should be assayed in duplicate.
7. To quantitate, always run samples alongside a standard curve. If testing a sample that is not extracted, dilute standards in the same type of medium being tested. This medium should be known to be negative.
8. Gently mix specimens and reagents before use. Avoid vigorous agitation.
9. Before opening the lyophilized conjugate vial, examine the vial to ensure that lyophilized material has not been trapped in the cap. If material is in the cap, gently tap the upright vial to dislodge the trapped material.
10. To reconstitute the lyophilized conjugate, add 75 µL of deionized water to a vial. Rehydrate the conjugate by gently rotating the vial. Do not vortex or shake the contents. Avoid excess foaming. After the solid material has gone into solution, allow the conjugate to incubate at least 15 minutes before dilution. Write the date of reconstitution on the label. Concentrated, reconstituted conjugate has a shelf life of at least one week.
11. The enzyme conjugate is most stable in its concentrated form. Dilute only the volume necessary for the amount of strips currently being used.
12. Before taking an absorbance reading, wipe the outside bottom of the wells with a lint-free wiper to remove dust and fingerprints.
SAMPLE PREPARATION

This assay is non-species specific. Usually, urine and tissue culture supernatant can be assayed directly by diluting them with the diluted extraction buffer. Dilute specimens may require extraction in order to concentrate LTC_4/D_4/E_4. Plasma and most other mediums will require extraction.

EXTRACTION OF LEUKOTRIENE C_4/D_4/E_4

1. For 1 mL plasma or urine: Acidify to pH 3.5 with 1N HCl (1 mL of plasma requires about 150 µL of 1N HCl).
   For tissue: Homogenize the tissue in ethanol (5 mL/gm) and centrifuge to obtain supernatant. Dilute 1 mL of the supernatant with 5 mL of water and acidify to pH 3.5 with 1N HCl.
2. Precondition the C₁₈ Sep-Pak® light column (Waters® Corporation) by washing the column with 2 mL of ethanol followed by 2 mL of water.
3. Apply the above sample to the column and adjust the flow rate to 1 mL per minute. Reducing the flow rate to 0.5 mL per minute may increase extraction efficiencies. Some samples may clog the column. These samples may be diluted 1:5 in water to improve the flow rate.
4. Wash the column with 1 mL of water followed by 1 mL of petroleum ether.
5. Elute eicosanoid with 2 mL of methanol.
7. Dissolve the residue in an appropriate volume of diluted extraction buffer and assay for eicosanoid content.

NOTE: Extraction buffer must be diluted 5-fold with deionized water before use. Any precipitant present must be brought into solution before dilution.

TEST PROCEDURES

1. Prepare standards as follows:

<table>
<thead>
<tr>
<th>Standard</th>
<th>Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>stock solution 1 µg/mL (this is provided)</td>
</tr>
<tr>
<td>B</td>
<td>take 20 µL of A, add to 980 µL of EIA buffer and mix=20 ng/mL</td>
</tr>
<tr>
<td>C</td>
<td>take 200 µL of B, add to 1.8 mL of EIA buffer and mix=2 ng/mL</td>
</tr>
<tr>
<td>D</td>
<td>take 200 µL of C, add to 1.8 mL of EIA buffer and mix=0.2 ng/mL</td>
</tr>
</tbody>
</table>

Continue standard preparation following Scheme I.

SCHEME I

<table>
<thead>
<tr>
<th>Standards</th>
<th>ng/mL</th>
<th>EIA buffer</th>
<th>C standard µL</th>
<th>D standard µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>S₀</td>
<td>0</td>
<td>as is</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S₁</td>
<td>0.04</td>
<td>800</td>
<td>-</td>
<td>200</td>
</tr>
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<td>S₂</td>
<td>0.1</td>
<td>500</td>
<td>-</td>
<td>500</td>
</tr>
<tr>
<td>S₃</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
<td>as is</td>
</tr>
<tr>
<td>S₄</td>
<td>0.4</td>
<td>800</td>
<td>200</td>
<td>-</td>
</tr>
<tr>
<td>S₅</td>
<td>0.8</td>
<td>600</td>
<td>400</td>
<td>-</td>
</tr>
<tr>
<td>S₆</td>
<td>1</td>
<td>500</td>
<td>500</td>
<td>-</td>
</tr>
<tr>
<td>S₇</td>
<td>2</td>
<td>-</td>
<td>as is</td>
<td>-</td>
</tr>
</tbody>
</table>

2. Determine the number of wells to be used.
3. Dilute the Leukotriene C\(_4\)/D\(_4\)/E\(_4\) enzyme conjugate. Add 1 µL of conjugate to 50 µL total volume of EIA buffer for each well assayed. Mix the solution thoroughly (avoid foaming). For the entire plate, add 110 µL of the enzyme conjugate into 5.5 mL total volume of EIA Buffer. Mix the solution thoroughly (avoid foaming).

**NOTE:** If more concentrated conjugate is needed than is contained in the first conjugate vial, reconstitute and use the second vial. Do not use the separate contents of both vials in the same assay as some assay variability may result. If the contents of both vials are required for an assay, pool the concentrated conjugates. Use the expiration date of the oldest reconstituted vial for the pool. Alternatively, prepare the necessary volumes of diluted conjugate and pool before using in the assay.

4. Add 50 µL of standard (S) or unknown (U) (some samples may require diluting) to the appropriate wells in duplicate. See Scheme II for suggested template design.

5. Add 50 µL of the diluted enzyme conjugate to each well. Use 8-channel pipette or 12-channel pipette for rapid addition.

6. Mix by shaking plate gently. A microplate shaker may be used.

7. Cover plate with plastic film or plate cover and incubate at room temperature for one hour. **NOTE:** Keep plate away from drafts and temperature fluctuations.

8. Dilute concentrated wash buffer with deionized water (i.e. 20 mL of wash buffer plus 180 mL of deionized water). Mix thoroughly.

9. After incubation, dump out the contents of the plate. Tap out contents thoroughly on a clean lint-free towel.

10. Wash each well with 300 µL of the diluted wash buffer. Repeat for a total of three washings. An automated plate washer can be used, however, increase wash cycles from three to five.

11. Add 150 µL of substrate to each well. Use multichannel pipette for best results. Mix by shaking plate gently.

12. Incubate at room temperature for 30 minutes.

13. Gently shake plate before taking a reading to ensure uniform color throughout each well.

14. Plate is read in a microplate reader at 650 nm. If a dual wavelength is used, set W\(_1\) at 650 nm and W\(_2\) at 490 nm.

15. If accounting for substrate background, use 2 to 8 wells as blanks with only substrate in the wells (150 µL/well). Subtract the average of these absorbance values from the absorbance values of the wells being assayed. **NOTE:** Some microplate readers can be programmed to do the background subtractions automatically when reading the plate. Consult your instrument manual.

**OPTIONAL TEST PROCEDURES**

16. Add 50-100 µL of 1 N HCl or Neogen’s Red Stop Solution to each well to stop enzyme reaction.

17. Read plate at 450 nm, if 1N HCl solution was used. Read plate at 650 nm, if Red Stop Solution was used.

18. Plot the standard curve and estimate the concentrations of the samples from the curve. See "CALCULATIONS."

**NOTE:** Absorbance readings will approximately double when stopped with acid. If absorbance readings are too high for measuring with your microplate reader, decrease the substrate incubation by approximately 10 minutes but no more than 15 minutes.

**SCHEME II**

<table>
<thead>
<tr>
<th></th>
<th>1</th>
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<th>3</th>
<th>4</th>
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<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
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<tbody>
<tr>
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<td>$^0$</td>
<td>$^1$</td>
<td>$^1$</td>
<td>$^9$</td>
<td>$^9$</td>
<td>$^{17}$</td>
<td>$^{17}$</td>
<td>$^{25}$</td>
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<td>$^{33}$</td>
</tr>
<tr>
<td>B</td>
<td>$^1$</td>
<td>$^1$</td>
<td>$^2$</td>
<td>$^2$</td>
<td>$^{10}$</td>
<td>$^{10}$</td>
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</tr>
<tr>
<td>F</td>
<td>$^5$</td>
<td>$^5$</td>
<td>$^6$</td>
<td>$^6$</td>
<td>$^{14}$</td>
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<tr>
<td>G</td>
<td>$^6$</td>
<td>$^6$</td>
<td>$^7$</td>
<td>$^7$</td>
<td>$^{15}$</td>
<td>$^{15}$</td>
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<td>$^{31}$</td>
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</tr>
<tr>
<td>H</td>
<td>$^7$</td>
<td>$^7$</td>
<td>$^8$</td>
<td>$^8$</td>
<td>$^{16}$</td>
<td>$^{16}$</td>
<td>$^{24}$</td>
<td>$^{24}$</td>
<td>$^{32}$</td>
<td>$^{32}$</td>
<td>$^{40}$</td>
<td>$^{40}$</td>
</tr>
</tbody>
</table>
CALCULATIONS

1. After the substrate background has been subtracted from all absorbance values, average all of your duplicate well absorbance values.

2. The average of your two \( S_0 \) values is now your \( B_0 \) value. \( (S_i \) now becomes \( B_i \), etc.)

3. Next, find the percent of maximal binding (%B/\( B_0 \) value). To do this, divide the averages of each standard absorbance value (now known as \( B_1 \) through \( B_7 \)) by the \( B_0 \) absorbance value and multiply by 100 to achieve percentages.

4. Graph your standard curve by plotting the %B/\( B_0 \) for each standard concentration on the ordinate (y) axis against concentration on the abscissa (x) axis. Draw a curve by using a curve-fitting routine (i.e. 4-parameter or linear regression).

5. Divide the averages of each sample absorbance value by the \( B_0 \) value and multiply by 100 to achieve percentages.

6. Using the standard curve, the concentration of each sample can be determined by comparing the %B/\( B_0 \) of each sample to the corresponding concentration of Leukotriene C\(_4\) standard.

7. If the samples were diluted, the concentration determined from the standard curve must be multiplied by the dilution factor.

TYPICAL STANDARD CURVE

\[ \text{LTC}_4/D/E_4 \text{ in EIA Buffer} \]
TYPICAL DATA

NOTE: "Typical data" is a representation. Variances in data will occur. Optical density readings may fluctuate during the shelf-life of the kit, but the %B/B₀ should remain comparable. Measuring wavelength: 650 nm

<table>
<thead>
<tr>
<th>Standard (Bᵢ)</th>
<th>Standard Concentration (ng/mL)</th>
<th>Optical Density (Absorbance Value)</th>
<th>%B/B₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>S₀ (B₀)</td>
<td>0</td>
<td>1.155</td>
<td>100</td>
</tr>
<tr>
<td>S₁ (B₁)</td>
<td>0.04</td>
<td>0.991</td>
<td>86</td>
</tr>
<tr>
<td>S₂ (B₂)</td>
<td>0.1</td>
<td>0.807</td>
<td>70</td>
</tr>
<tr>
<td>S₃ (B₃)</td>
<td>0.2</td>
<td>0.548</td>
<td>47</td>
</tr>
<tr>
<td>S₄ (B₄)</td>
<td>0.4</td>
<td>0.327</td>
<td>28</td>
</tr>
<tr>
<td>S₅ (B₅)</td>
<td>0.8</td>
<td>0.200</td>
<td>17</td>
</tr>
<tr>
<td>S₆ (B₆)</td>
<td>1</td>
<td>0.150</td>
<td>13</td>
</tr>
<tr>
<td>S₇ (B₇)</td>
<td>2</td>
<td>0.103</td>
<td>9</td>
</tr>
</tbody>
</table>

CROSS REACTIVITY

LEUKOTRIENE C₄ ............................................................... 100.0%
LEUKOTRIENE D₄ ............................................................... 80.0%
LEUKOTRIENE E₄ ............................................................... 80.0%
LEUKOTRIENE A₄ ............................................................... 2.0%
LEUKOTRIENE B₄ ............................................................... <1.0%
PROSTAGLANDIN B₂ .......................................................... <0.01%
PROSTAGLANDIN D₂ .......................................................... <0.01%
PROSTAGLANDIN E₂ .......................................................... <0.01%
PROSTAGLANDIN F₂ₐ .......................................................... <0.01%
6-KETO-PROSTAGLANDIN F₁₉ ............................................. <0.01%
5-HETE ................................................................. <0.01%
12-HETE ................................................................. <0.01%
15-HETE ................................................................. <0.01%
6-TRANS-LEUKOTRIENE B₄ ............................................. <0.01%
20-OH-LEUKOTRIENE B₄ .............................................. <0.01%
THROMBOXANE B₂ ...................................................... <0.01%
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