Leukotriene C4 ELISA Kit

Cat. No.:DEIA5003
Pkg.Size:96T

Intended use

The Leukotriene C4 ELISA Kit is a competitive assay that can be used for quantification of LTC4 in plasma, culture media, and other sample matrices. The ELISA typically displays an IC50 (50% B/B0) of approximately 45 pg/ml and a detection limit (80% B/B0) of approximately 10 pg/ml.

General Description

The leukotrienes (LTs) were discovered in 1979 as a group of acute inflammatory mediators derived from arachidonic acid in leukocytes. Their biosynthesis was shown to proceed via the 5-lipoxygenase (5-LO) pathway. LT biosynthesis has subsequently been demonstrated in other bone marrow-derived cells expressing 5-LO including eosinophils, mast cells, and macrophages. 5-LO converts arachidonic acid into LTA4 with 5(S)-HpETE as an intermediate. The conjugation of glutathione to LTA4 results in the formation of LTC4. LTC4 is rapidly metabolized to LTD4 and LTE4. This metabolism is essentially complete within 10 minutes in the human lung. LTC4, LTD4, and LTE4 are collectively referred to as cysteinyl leukotrienes (CysLTs). LTC4 and LTD4 are potent mediators of asthma and hypersensitivity. They induce bronchoconstriction, increase microvascular permeability, and are vasoconstrictors of coronary arteries. The biological activity of LTE4 is much lower in most systems studied, but its presence reflects the prior existence of LTC4 and LTD4.

Principle Of The Test

Description of Competitive ELISA

This assay is based on the competition between LTC4 and an LTC4-acetylcholinesterase (AChE) conjugate (LTC4 tracer) for a limited amount of LTC4 antiserum. Because the concentration of the LTC4 tracer is held constant while the concentration of LTC4 varies, the amount of LTC4 tracer that is able to bind to the LTC4 antiserum will be inversely proportional to the concentration of LTC4 in the well. This antibody-LTC4 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 LTC4 tracer bound to the well, which is inversely proportional to the amount of free LTC4 present in the well during the incubation; or Absorbance ∝ [Bound LTC4 Tracer] ∝ 1/[LTC4]

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 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. 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 re-development 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.

Reagents And Materials Provided

1. Leukotriene C4 ELISA Antiserum: 1 vial/100 dtn
2. Leukotriene C4 AChE Tracer: 1 vial/100 dtn
3. Leukotriene C4 ELISA Standard: 1 vial
4. ELISA Buffer Concentrate (10X): 2 vials/10 ml
5. Wash Buffer Concentrate (400X): 1 vial/5 ml
6. Polysorbate 20: 1 vial/3 ml
7. Mouse Anti-Rabbit IgG Coated Plate: 1 plate
8. 96-Well Cover Sheet: 1 cover
9. Ellman's Reagent: 3 vials/100 dtn
10. ELISA Tracer Dye: 1 vial
11. ELISA Antiserum Dye: 1 vial

Materials Required 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 ELISA 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 ELISA. NOTE: UltraPure water is available for purchase from CD.

Storage

This kit will perform as specified if stored as directed at -20°C and used before the expiration date indicated on the outside of the box.

Specimen Collection And Handling

Sample Preparation
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 10 and 500 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 LTC4 concentration, purification is not required. If you do not see good correlation of serial dilutions, purification is advised. The Purification Protocol below is one such method. NOTE: We recommend using CD’s Cysteinyl Leukotriene Affinity Sorbent or for purification of LTC4 from biological samples prior to ELISA 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; they will be stable for approximately six months.
• Samples of rabbit origin may contain antibodies which interfere with the assay by binding to the mouse anti-rabbit plate. We recommend that all rabbit samples be purified prior to use in this assay.

**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 Express ELISA Kit. NOTE: The complex nature of these samples makes purification mandatory in order to achieve accurate results.

**Culture Medium**
Cultured cells synthesizing LTC4 will generally release it into the medium where it will accumulate without further metabolism. Consequently, analysis of these fluids is the optimal application of this assay. Culture medium, in general, can be assayed without purification. For best results, the standard curve should also be diluted in culture medium.

**Plasma**
Plasma samples should be collected in vacutainers containing sodium heparin, EDTA, or sodium citrate. Plasma is a complex matrix that contains many substances that can interfere with this assay. Metabolism of LTC4 in plasma is rapid and complete, resulting in plasma levels of LTC4 that are very low (<10 pg/ml). By purifying a large volume of sample (5-10 ml), the LTC4 content of plasma can be concentrated into as little as 0.5 ml of ELISA Buffer. This may be sufficient to bring the LTC4 concentration into the readable range of the standard curve.

**Sample Purification**
Cayman highly recommends the use of our affinity purification reagents for the rapid and easy purification of Cysteinyl Leukotrienes from biological samples. These reagents are easier to use and provide higher purity with better recovery than solid phase extraction (SPE) chromatography. The protocol for affinity purification accompanies those reagents when purchased.
Additional items required for affinity purification include Eicosanoid Affinity Column Buffer (5X) and Eicosanoid Affinity Column Elution Solution.

**SPE (C-18) Purification Protocol**
The following protocol is a suggestion only. You may choose a different protocol based on your own requirements, sample type, and expertise. If desired, recovery may be tracked by spiking samples with tritium-labeled LTC4 ([3H]-LTC4) and follow the spiked-sample recovery calculations. Otherwise, omit steps 2 and 9.

**Materials Needed**
1. Tritium-labeled LTC4 (optional)
2. 1 M acetate buffer (pH 4), deionized water, and methanol
3. C-18 solid phase extraction (SPE) columns

**Protocol**
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 LTC4 ([3H]-LTC4). Use a high specific activity tracer to minimize the amount of radioactive LT as the ELISA will be able to detect the added LTC4.
3. Precipitation of proteins using ethanol is optional and may not be needed if samples are clean enough to flow through the SPE Cartridge (C-18). Body fluids such as plasma and urine can typically be applied directly to the SPE Cartridge (C-18) after the acidification step (step 4) below. To precipitate proteins, add methanol (2-4X the sample volume) to the sample and vortex. Incubate at 4°C for five minutes, then centrifuge at 3,000 x g for 10 minutes to remove precipitated proteins. Pour the
supernatant into a clean container. Evaporate the methanol either by vacuum centrifugation or under a gentle stream of nitrogen.

4. Adjust the pH to ~4 by the addition of 1 M acetate buffer (citrate buffer or dilute HCl). To avoid having to measure the pH of each individual sample, adjust the pH of an equivalent volume of sample matrix to pH 4 using 1 M acetate buffer. Add this volume of buffer to each sample. NOTE: For samples of different volumes, the amount of buffer should be adjusted to maintain this ratio of buffer to sample. If the samples are cloudy or contain precipitate, either filter or centrifuge to remove the precipitate. Particulate matter in the sample may clog the SPE Cartridge.

5. Activate a 6 ml SPE Cartridge (C-18) by rinsing with 5 ml methanol followed by 5 ml UltraPure water. Do not allow the SPE Cartridge (C-18) to dry.

6. Pass the sample through the SPE Cartridge (C-18). Rinse the cartridge with 5 ml UltraPure water. Discard the wash. Elute the LTC4 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.*

7. 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 ELISA.

8. Add 500 µl of ELISA Buffer and vortex. The sample is now ready for use in the ELISA. It is common for an insoluble precipitate to remain after the addition of ELISA Buffer; this will not affect the assay.

9. Use 50 µl of the resuspended sample for scintillation counting.

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

---

**Plate Preparation**

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): Reconstitute with 20 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.

---

**Reagent Preparation**

NOTE: Water used to prepare all ELISA 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 ELISA. UltraPure water may be purchased from CD.

**Buffer Preparation**

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

1. ELISA Buffer Preparation

   Dilute the contents of one vial of ELISA Buffer Concentrate (10X) 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 Concentrate (400X) (96-well kit): Dilute to a total volume of 2 liters with UltraPure water and add 1 ml of Polysorbate 20.
Smaller volumes of Wash Buffer can be prepared by diluting the Wash Buffer Concentrate 1:400 and adding Polysorbate 20 (0.5 ml/liter of Wash Buffer).

NOTE: Polysorbate 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.

**Leukotriene C4 ELISA 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 LTC4 ELISA Standard 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 media samples that have not been diluted with ELISA Buffer, culture medium should be used in place of ELISA Buffer for dilution of the standard curve.

To prepare the standard for use in ELISA: Obtain eight clean test tubes and number them #1 through #8. Aliquot 900 µl ELISA Buffer to tube #1 and 500 µl ELISA 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.

**Leukotriene C4 AChE Tracer**

Reconstitute the LTC4 AChE Tracer as follows:

100 dtm LTC4 AChE Tracer (96-well kit): Reconstitute with 6 ml ELISA Buffer.

Transfer the reconstituted tracer to a polypropylene tube or vial, store at 4°C (do not freeze), and use within one week. A 20% surplus per vial of tracer has been included to account for any incidental losses.

NOTE: The 480-well size kit contains two 500 dtm vials of tracer so that the kit may be used for more than one week. Each vial of tracer will be stable for one week from reconstitution.

**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).

**Leukotriene C4 ELISA Antiserum**

Reconstitute the LTC4 ELISA Antiserum as follows:

100 dtm LTC4 ELISA Antiserum (96-well kit): Reconstitute with 6 ml ELISA Buffer.

Store the reconstituted LTC4 ELISA Antiserum at 4°C. It will be stable for at least two weeks. A 20% surplus of 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).

**Assay Steps**

**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. Performing the Assay Pipetting Hints at once, place the unused strips back in the plate packet and store at 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 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 CD. We suggest you record the contents of each well on the template sheet provided. Figure 2.

**Pipetting Hints**

- Use different tips to pipette each reagent.
- 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. **ELISA Buffer**
   Add 100 µl ELISA Buffer to Non-Specific Binding (NSB) wells. Add 50 µl ELISA Buffer to Maximum Binding (B0) wells. If culture medium was used to dilute the standard curve, substitute 50 µl of culture medium for ELISA Buffer in the NSB and B0 wells (i.e., add 50 µl culture medium to NSB and B0 wells and 50 µl ELISA Buffer to NSB wells).

2. **Leukotriene C4 ELISA 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 C4 AChE Tracer
Add 50 µl to each well except the Total Activity (TA) and the Blank (Blk) wells.

5. Leukotriene C4 ELISA Antiserum
Add 50 µl to each well except the Total Activity (TA), the Non-Specific Binding (NSB), and the Blank (Blk) wells.

Table 1.

<table>
<thead>
<tr>
<th>Well</th>
<th>EIA Buffer</th>
<th>Standard/ Sample</th>
<th>Tracer</th>
<th>Antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blk</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TA</td>
<td>-</td>
<td>-</td>
<td>5 µl (at devl. step)</td>
<td>-</td>
</tr>
<tr>
<td>NSB</td>
<td>100 µl</td>
<td>-</td>
<td>50 µl</td>
<td>-</td>
</tr>
<tr>
<td>B₀</td>
<td>50 µl</td>
<td>-</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>Std/Sample</td>
<td>-</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

Incubation of the Plate
Cover each plate with plastic film and incubate 18 hours at room temperature.

Reading 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.

Calculation
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 either %B/B₀ versus log concentration using a four-parameter logistic fit or as logit B/B₀ versus log concentration using a linear fit.

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). Repeat for S2-S8 and all sample wells. (To obtain %B/B0 for a logistic four-parameter fit, multiply these values by 100.)

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. Erratic absorbance values and a low (or no) % Bound could indicate the presence of organic solvents in the buffer or other technical problems.

**Determine the Sample Concentration**

Calculate the B/B0 (or %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 indicates interference which could be eliminated by purification.

**Spiked-Sample Recovery Calculation**

Figure 3.

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

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

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

*If [3H] LTD4 or LTE4 is used for the hot spike, it is important to take into account their cross-reactivity in this assay. (e.g., 100 pg of LTD4 with a cross-reactivity of 46% will be measured as 46 pg.)*

**Typical Standard Curve**

Plot %B/B0 for standards S1-S8 versus LTC4 concentration using linear (y) and log (x) axes and perform a 4-parameter logistic fit.

Alternative Plot - The data can also be linearized using a logit transformation. The equation for this conversion is shown below.

NOTE: Do not use %B/B0 in this calculation.

\[
\text{logit} \frac{\text{B}}{\text{B0}} = \ln \left[ \frac{\text{B}}{\text{B0}(1 - \text{B}/\text{B0})} \right]
\]

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

Figure 4.
Sensitivity

10 pg/ml

Specificity

eukotriene C4: 100%
Arachidonic Acid: <0.01%
Leukotriene C5: 100%
Glutathione: <0.01%
Leukotriene D5: 48%
Leukotriene B4: <0.01%
Leukotriene D4: 46%
Leukotriene B5: <0.01%
N-acetyl Leukotriene E4: 28%
14,15-Leukotriene C4: <0.01%
Leukotriene E: 57%
tetranor-PGEM: <0.01%
Leukotriene E: 42%
tetranor-PGFM: <0.01%

Reproducibility

Intra-assay variation(%CV): 5.1% - 18.0%
Inter-assay variation(%CV): 7.8% - 18.9%

**Precautions**

1. Please read these instructions carefully before beginning this assay.
2. The reagents in this kit have been tested and formulated to work exclusively. This kit may not perform as described if any reagent or procedure is replaced or modified.
3. When compared to quantification by LC/MS or GC/MS, it is not uncommon for immunoassays to report higher analyte concentrations. While LC/MS or GC/MS analyses typically measure only a single compound, antibodies used in immunoassays sometimes recognize not only the target molecule, but also structurally related molecules, including biologically relevant metabolites. In many cases, measurement of both the parent molecule and metabolites is more representative of the overall biological response than is the measurement of a short-lived parent molecule. It is the responsibility of the researcher to understand the limits of both assay systems and to interpret their data accordingly.

**REFERENCES**