GE Healthcare

Amersham Leukotriene $C_4/D_4/E_4$ Biotrak Enzymeimmunoassay (EIA) System

96 wells

Product Booklet

Code: RPN224

Page finder

1. Legal

www.gehealthcare.com/lifesciences

GE Healthcare UK Limited
Amersham Place, Little Chalfont,
Buckinghamshire, HP7 9NA UK

2. Handling

2.1. Safety warnings and precautions

Warning: *For research use only.*

Not recommended or intended for diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.

2.2. Storage

Store at -15°C to -30°C.

2.3. Expiry

The expiry date is stated on the package and will be at least 4 weeks from the date of despatch.

3. Components

Microplate: Plate containing 12 × 8 well strips coated with goat anti-rat IgG, ready to use after thawing. Store at -15°C to -30°C.

Peroxidase conjugate: Leukotriene C_4-Horseradish Peroxidase, lyophilized. Store at -15°C to -30°C.

Standard: Leukotriene C_4 standard 50 ng in 250 µl of 0.05 M Phosphate buffer, pH 6.9:Ethanol (2:1). Store at -15°C to -30°C.

Antiserum: Rat anti-leukotriene C_4/D_4/E_4, lyophilized. Store at -15°C to -30°C.

TMB substrate: Enzyme substrate containing 3,3', 5,5'-Tetramethylbenzidine (TMB) Hydrogen Peroxide Ready for use after thawing. Store at -15°C to -30°C.

Assay buffer concentrate: Assay buffer concentrate, 5 ml. On dilution this bottle contains 0.1 M Phosphate buffer, pH 7.5 containing 0.9% Sodium Chloride, 0.1% Bovine Serum Albumin and 0.5% preservative. Store at -15°C to -30°C.

Wash buffer concentrate: Wash buffer concentrate, 12.5 ml. On dilution the reagent contains 0.01 M phosphate buffer, pH 7.5 containing 0.05% Tween™20. Store at -15°C to -30°C.

4. Other materials required

The following materials and equipment are required but not supplied:

- Pipettes or pipetting equipment with disposable tips (50 µl, 100 µl, 150 µl, 500 µl, and 6 ml)
- Disposable polypropylene test tubes
- Measuring cylinders 50 ml and 500 ml
- Distilled or deionized water
- Spectrophotometer plate reader capable of measuring at 450 nm
- 1.0 M Sulfuric acid
- Microplate shaker

5. Description

- High sensitivity ~0.5 pg/well for Leukotriene C_4/D_4, 10 pg/ml
- 5.5 hour protocol
- Non isotopic
- Ready to use substrate
- Colour coded reagents
- Choice of protocol

The Leukotriene $C_4/D_4/E_4$ Biotrak™ Enzymeimmunoassay system from GE Healthcare has been specifically designed for research purposes. It combines the use of a Peroxidase labelled Leukotriene C_4 conjugate, a peptido Leukotriene specific antiserum which can be immobilized on to pre-coated microplates, and a one pot stabilized substrate solution. This provides a rapid and sensitive non-isotopic method for the determination of Leukotriene $C_4/D_4/E_4$ in the range 0.77 pg to 49.5 pg/well.

Each pack contains sufficient material for 96 wells. This permits the construction of one standard curve and 39 unknowns in duplicate. The assay is based on the competition between unlabelled Leukotriene C_4 and a fixed quantity of Peroxidase labelled Leukotriene C_4 for a limited number of binding sites on a peptido leukotriene specific antibody. With fixed amounts of antibody and Peroxidase labelled Leukotriene C_4 the amount of Peroxidase labelled ligand bound by the antibody will be inversely proportional to the concentration of added unlabelled ligand.

The Peroxidase ligand that is bound to the antibody is immobilized on to polystyrene microplate wells precoated with second antibody, as demonstrated in figure 1. Thus any unbound ligand can be removed from the well by simple washing procedures.

The amount of Peroxidase labelled Leukotriene C_4 bound to the antibody is determined by addition of Tetramethylbenzidine (TMB)/ Hydrogen Peroxide single pot substrate (1). The reaction is stopped by addition of an acid solution, and the resultant colour read at 450 nm in a microplate spectrophotometer.

The concentration of unlabelled Leukotriene C_4 in a sample is determined by interpolation from a standard curve.

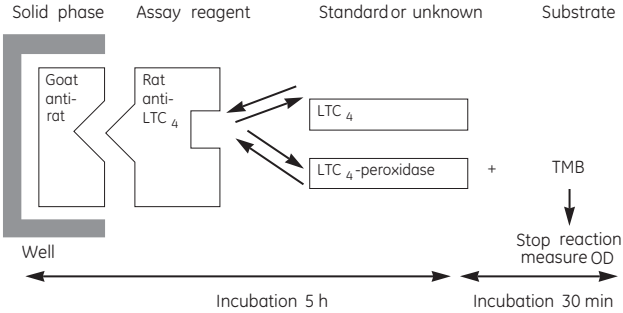


Figure 1.

6. Critical parameters

The following points are critical.

- Working standards should be freshly prepared before each assay, and not re-used.
- Mix samples and all reagents thoroughly before use.
- Avoid excessive foaming of reagents.
- Avoid handling the tops of the wells both before and after filling.
- Keep the wells covered with lids except when adding reagents and reading.
- Standards and samples should be assayed in duplicate.
- Run a separate standard curve for each microplate.
- The total dispensing time for each plate should not exceed 20 minutes.
- Use only coated wells from the same reagent batch for each assay.

7. Protocol

7.1. Specimen collection and sample preparation

7.1.1. Sample preparation

Peptido-leukotrienes are present in a wide variety of biological material. It is unlikely that a single sample preparation procedure will prove suitable for all samples. This section provides information about sample purification procedures which have been reported in the literature. This information is provided for guidance only. It remains the investigators responsibility to validate their chosen sample preparation procedure. A number of investigators have reported techniques for purifying Peptido-leukotrienes (2–5).

7.1.2. Extraction

Solid phase extraction procedures such as the one described by Powell (6) have been reported to be the method of choice.

The antiserum in this product reacts with Leukotrienes C_4, D_4 and E_4. All three Leukotrienes can be quantitated provided they are resolved before the assay. A suitable HPLC system is described below.

Samples are extracted prior to HPLC separation following the method of Anderson *et al* (7). Plasma samples are mixed with four times their volume of Absolute Ethanol and left at 2–8°C for 30 minutes. The resulting precipitate is removed by centrifugation at 3000 × g for 30 minutes. The Leukotriene containing Ethanolic supernatant is collected. The Ethanol is then removed by rotary evaporation under vacuum and the samples stored under Argon at -50°C prior to separation by reverse phase HPLC.

7.1.3. HPLC separation

Appropriate conditions for HPLC separation of Peptido-leukotrienes are:

Solvent: Acetonitrile:water:Acetic acid (40:60:0.1)

Flow rate: 1 ml/minute

Column: C18 µbondapak 30 cm × 3.9 mm internal diameter 10 mm particle size

Typical elution times:

Standard	Collection band	
LTC_4	7.6 minutes	7–10 minutes
LTD_4	10.2 minutes	10–12.25 minutes
LTE_4	14.0 minutes	12.25–16 minutes

Typical values obtained using this procedure are described in a paper by Hammond *et al*(8).

7.2. EIA procedure

7.2.1. Reagent preparation

All reagents should be stored at -15°C to -30°C. Once thawed, assay buffer, wash buffer, unused microtitre wells and substrate may be stored at 2–8°C and re-used within seven days. The standard, reconstituted antiserum and peroxidase conjugate should be stored at -15°C to -30°C and re-used within two weeks.

The coated microtitre plate and enzyme substrate are provided ready for use after thawing.

Assay buffer

Transfer the contents of the bottle to a 50 ml graduated cylinder by repeated washing with distilled water. Adjust the final volume to 50 ml with distilled water and mix thoroughly. The diluted buffer contains 0.1 M Phosphate Buffer pH 7.5 containing 0.9% Sodium Chloride, 0.1% Bovine Serum Albumin and 0.5% preservative.

Leukotriene C_4 Peroxidase conjugate

Carefully add 6.0 ml diluted assay buffer and replace the stopper. Mix the contents of the bottle until completely dissolved. The solution

will contain Leukotriene C_4-Horseradish Peroxidase in Phosphate buffer containing 0.9% Sodium Chloride, 0.1% Bovine Serum Albumin and 0.5% preservative.

Antiserum

Carefully add 6.0 ml diluted assay buffer and replace the stopper. Gently mix the contents of the bottle by inversion and swirling until a complete solution is obtained. Vigorous agitation and foaming should be avoided. The contents will contain anti-leukotriene serum in Phosphate Buffer containing 0.9% Sodium Chloride, 0.1% Bovine Serum Albumin and 0.5% preservative.

Wash buffer

Transfer the contents of the bottle to 500 ml graduated cylinder by repeated washings with distilled water. Adjust the final volume to 500 ml with distilled water and mix thoroughly. The diluted wash buffer contains 0.01 M Phosphate Buffer pH 7.5 containing 0.05% Tween 20.

Standard

Dilute the standard concentrate by removing exactly 50 µl of the vial contents and add to exactly 5 ml of assay buffer in a polypropylene vessel. The tube contents should be thoroughly mixed. The resulting solution contains 1.98 ng/ml of Leukotriene C_4 and serves as the stock solution to prepare a series of standards as follows.

7.2.2. Preparation of working standards

1. Label 7 polypropylene tubes 49.5 pg, 24.8 pg, 12.4 pg, 6.2 pg, 3.1 pg, 1.55 pg and 0.77 pg.

2. Pipette 500 µl assay buffer into all the tubes.

3. Pipette 500 µl of the stock standard (1.98 ng/ml) into the 49.5 pg tube and mix thoroughly.

4. Transfer 500 µl from the 49.5 pg tube into the 24.8 pg tube and mix thoroughly.

5. Repeat this doubling dilution successively with the remaining tubes.
6. 50 µl aliquots from each serial dilution will give rise to 7 standard levels of Leukotriene C_4 ranging from 0.77 pg to 49.5 pg per well.

Note: To minimize any effect of leukotriene adsorption to the walls of the test tube the user is recommended to mix the standards using a pipette and to avoid vigorous vortex mixing. A new pipette tip should be used to dispense each standard. The standards should also be prepared within one hour of performing the enzyme immunoassay.

7.2.3. Assay protocol

Two enzyme immunoassay protocols are provided with assays performed at 4–10°C or 15–25°C. It is recommended that the user examines the cross-reactivity data (see additional information) before choosing the most appropriate protocol.

1. Prepare assay reagents and working standards as described in the previous sections.
2. Set up the microplate with sufficient wells to enable the running of all blanks, standards and samples as required (see table 1). If the last row is incomplete make up to 12 with clear blank wells, ensuring the base of the wells is flush with the strip holder. Recommended positioning of blank (B), non-specific binding (NSB), standard (0–49.5 pg) and sample (S) wells is shown in figure 2
3. Pipette 100 µl assay buffer into the non-specific binding (NSB) wells.
4. Pipette 50 µl assay buffer into the zero standard wells (B_0).
5. Starting with the most dilute, pipette 50 µl of each standard or unknown sample into the appropriate wells.
6. Pipette 50 µl of antiserum to all wells except the blank and NSB wells.
7. Cover the plate with the lid provided and incubate at 4–10°C for 2 hours.

8. Pipette 50 µl Leukotriene C_4 Peroxidase conjugate into all wells except the blank.

9. Cover the plate with the lid provided and incubate at 4–10°C for 3 hours.

10. Aspirate and wash all wells four times with 300 µl wash buffer.

11. Immediately dispense 150 µl enzyme substrate into all wells, cover the plate and mix on a microplate shaker for exactly 30 minutes at room temperature (15–25°C). A blue color will develop which can be read at 630 nm. However we do recommend halting the reaction prior to end point determination as follows:

12. Pipette 100 µl 1 M Sulfuric acid into each well, mix the contents of the plate and determine the optical density in a plate reader at 450 nm within 30 minutes.

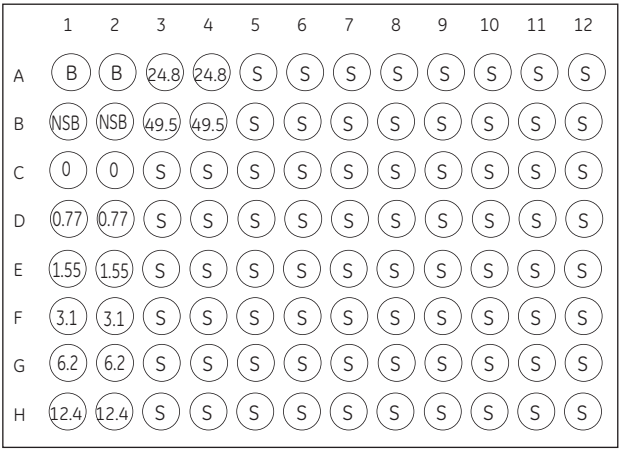


Figure 2. Recommended positioning of standard (0.77–49.5 ng/ml) and sample (S) wells

Table 1: Enzymeimmunoassay protocol 4–10°C (All volumes are in microlitres)

	Substrate blank	Non-specific binding (NSB)	Zero standard (B_0)	Standards	Samples
Buffer	–	100	50	–	–
Standard	–	–	–	50	–
Sample	–	–	–	–	50
Antiserum	–	–	50	50	50
Cover plate, incubate at 4–10°C for 2 hours.					
Peroxidase conjugate	–	50	50	50	50
Cover plate, incubate at 4–10°C for exactly 3 hours.					
Aspirate, wash all wells four times with 300 ml wash buffer					
Substrate	150	150	150	150	150
Cover plate, incubate at room temperature 15–25°C for 30 minutes while shaking.					
1.0 M Sulfuric acid **	100	100	100	100	100
Shake to mix contents and determine optical density at 450 nm					

** Reaction can be read at 630 nm before acidification but halting reaction prior to end point determination is recommended.

7.2.4. Alternative room temperature protocol

The assay may be performed at room temperature. This reduces the assay time to 3.5 hours. However, the cross-reactivity of Leukotriene E_4 is reduced to 30% at 15–25°C.

After addition of antiserum, the microplate should be shaken at 15–25°C for two hours. Peroxidase conjugate is added and the plate shaken for a further one hour at 15–25°C. The plate is then washed and substrate added as described in the 4–10°C assay protocol.

7.3. Data processing

7.3.1. Calculation of results

The assay data collected should be similar to the data shown in table 2.

1. Calculate the average optical density (OD) for each set of replicate wells.
2. Calculate the percent bound for each standard and sample using the following relationship:

$$\%B/B_0 = \frac{(\text{standard or sample OD-NSB OD}) \times 100}{(B_0 \text{ OD - NSB OD})}$$

A standard curve may be generated by plotting the percent B/B_0 as a function of the log Leukotriene C_4 concentration. Plot % B/B_0 (y axis) against Leukotriene C4 per well (x axis). The curve shape should be similar to figure 3, if plotted on semi-log paper.

The pg/well value of samples can be read directly from the graph.

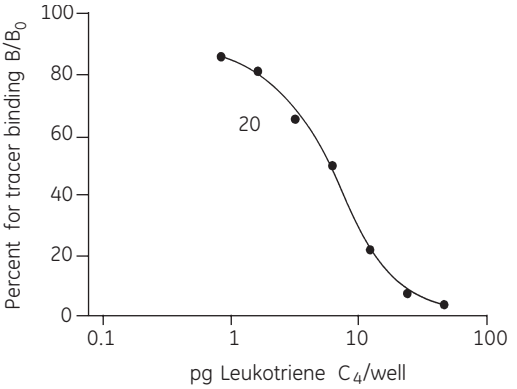


Figure 3. Leukotriene C_4 standard curve

Provided the individual Peptido-leukotrienes are separated using a suitable HPLC system, the concentration of Leukotriene D_4 can be determined directly from the Leukotriene C_4 curve. For the accurate quantitation of Leukotriene E_4 the user is recommended to prepare a Leukotriene E_4 standard curve in the range 1 pg to 50 pg/well. However, an estimate of the Leukotriene E_4 concentration may be obtained from the Leukotriene C_4 curve as follows:

Leukotriene E_4 fraction derived by HPLC

value obtained from Leukotriene C_4 standard curve = 10 pg/well

cross reactivity of Leukotriene E_4 (4–10°C) = 70%

Leukotriene E_4 concentration $= 10 \times \frac{100}{70}$ = 14.3 pg/well

Table 2. Typical assay data

Standard (pg/well)	Optical (OD) at 450 nm	Mean OD at 450 nm	Mean OD - NSB	%B/B_0
Substrate blank	0.042 0.044	0.043		
NSB	0.078 0.080	0.079		
0	1.096 1.099	1.097	1.018	
0.77	1.038 1.033	1.036	0.957	94.0
1.55	1.001 1.035	1.018	0.939	92.2
3.1	0.935 0.954	0.944	0.865	85.0
6.2	0.749 0.748	0.753	0.674	66.2
12.4	0.487 0.508	0.498	0.419	41.2
24.8	0.257 0.236	0.247	0.168	16.5
49.5	0.130 0.132	0.131	0.052	5.1

8. Additional information

8.1. Specificity

The cross-reactivity, as determined by the concentration giving 50% B/B_0, with a number of related compounds is shown in the table below and graphically in figure 4.

The cross-reactivity of the 11-transisomers of Leukotriene C_4 and Leukotriene D_4 in the enzymeimmunoassay performed at 4–10°C are high (figure 4a). However, if these cross-reactivities cause concern the assay may be performed at room temperature (15–25°C) which results in reduced cross-reactivities (figure 4b).

Table 3.

Analyte	**% Cross-reactivity 4–10°C**	**% Cross-reactivity 15–25°C**
Leukotriene C_4	100	100
Leukotriene D_4	100	100
Leukotriene E_4	91	75
Leukotriene B_4	<1.35	
Prostaglandin $F_2\alpha$	<0.006	
Prostaglandin D_2	<0.006	
Prostaglandin E_2	<0.006	
6-Keto-prostaglandin $F_1\alpha$	<0.006	
Thromboxane B_2	<0.006	
Glutathione	<0.006	

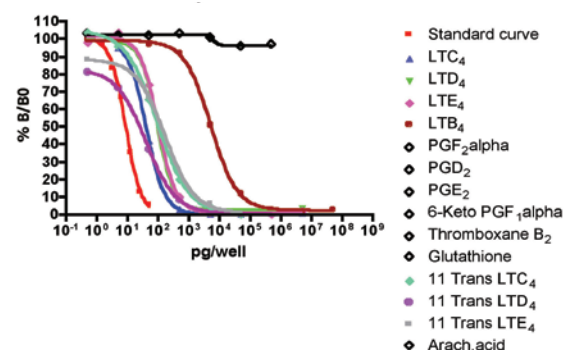
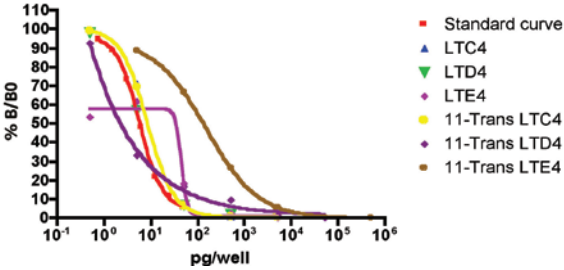


Figure 4. (a) Cross-reactivity profile at 4–10°C



(b) Cross-reactivity profile at 15–25°C

8.2. Sensitivity

The sensitivity, defined as the amount of Leukotriene C_4 needed to reduce zero dose binding by two standard deviations, was 0.5 pg/well, which is equivalent to 10 pg/ml.

8.3. Precision

Within-assay precision

The within-assay precision for duplicate determinations was calculated by measuring controls in the assay. The results are shown below:

Table 4. (mean values as pg/well)

Control	Mean ± SD	% CV	n
A	1.62 ± 0.15	9.3	12
B	6.03 ± 0.44	7.3	12
C	12.00 ± 0.58	4.8	12

Between-assay precision

The between assay precision was assessed by repeated measurement of the same control in successive assays. The results are shown below:

Table 5. (mean values as pg/well)

Control	Mean ± SD	% CV	n
A	1.21 ± 0.21	17.6	10
B	4.08 ± 0.57	13.9	10
C	10.49 ± 0.6	5.7	10

Precision profile

A precision profile was generated by preparing ten replicates of each of the standards and calculating the standard deviation (SD) and percent coefficient of variation (% CV) at each concentration.

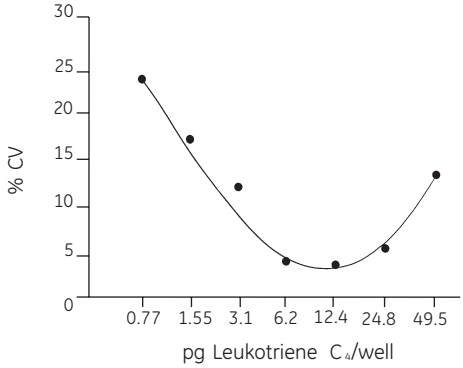


Figure 5. Precision profile

Table 6.

Standard (pg/well)	SD	% CV
0.77	0.194	24.0
1.55	0.247	17.5
3.1	0.35	12.4
6.2	0.249	4.4
12.4	0.466	4.1
24.8	1.37	6.0
49.5	6.47	13.0

8.4. Effect of time and temperature on assay performance

It is important that all incubation processes are performed for the times and at the temperatures stated in the assay protocol. Prolonged incubation following the Peroxidase conjugate addition, and assay temperatures below 4°C may lead to a reduction in sensitivity.

9. Background

A diverse array of mammalian cells and tissues enzymatically oxidise arachidonic acid to physiologically active compounds. These compounds include thromboxanes, prostacyclin, prostaglandins and leukotrienes. The leukotrienes are derived from Arachidonic Acid through the 5-Lipoxygenase enzyme pathway (9, 10). The Lipoxygenase catalyses the formation of a 5-Hydroperoxy acid, which may then form Leukotriene A_4, an unstable epoxide derivative. There are two enzymatic routes of metabolism from Leukotriene A_4. The first involves the action of an Epoxide hydrolase to produce Leukotriene B_4 (11). The second route involves the enzymatic addition of Glutathione to produce Leukotriene C_4. Subsequent metabolism of Leukotriene C_4 involves loss of Glutamic acid to produce Leukotriene D_4 and loss of Glycine to produce Leukotriene E_4.

It is now clear that the Peptido-leukotrienes (C_4, D_4, E_4) comprise the slow reacting substance of anaphylaxis, a proposed mediator of hypersensitivity reactions (12). They are potent mediators of bronchoconstriction, vascular and nonvascular smooth muscle constriction, increased vascular permeability and epithelial mucous secretion (13). It is widely considered that the peptido-leukotrienes are important mediators in asthma (14). The biological actions of the Leukotrienes have been recently reviewed (15–17)

The availability of high sensitivity Leukotriene C_4/D_4/E_4 enzymeimmunoassay from GE Healthcare will facilitate the evaluation of the physiological role and involvement in disease processes of this important group of peptido-leukotrienes.

10. References

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11. Related products

Eicosanoids

Thromboxane B_2	EIA	RPN220
Thromboxane B_2 [^{125}I]	RIA	RPA516
Leukotriene B_4	EIA	RPN223
Platelet Activating Factor (PAF), [^{3}H]	SPA	TRK990
Prostaglandin D_2 [^{3}H]	RIA	TRK890
Prostaglandin E_2	EIA	RPN222
Prostaglandin E_2 [^{125}I]	RIA/AM	RPA530
6-Keto prostaglandin $F_{1\alpha}$	EIA	RPN221
6-Keto prostaglandin $F_{1\alpha}$, [^{125}I]	RIA/AM	RPA515

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