GE Healthcare

Amersham 6-Keto-Prostaglandin $F_{1\alpha}$ Enzymeimmunoassay Biotrak (EIA) System

96 wells

Product Booklet

Code: RPN221

Page finder

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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 2–8°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: 12 x 8 well strips coated with donkey anti-rabbit IgG, ready for use.

6-keto-prostaglandin $F_{1\alpha}$ Peroxidase conjugate:
6-keto-prostaglandin $F_{1\alpha}$-Horseradish Peroxidase, lyophilized

Standard: 6-keto-prostaglandin $F_{1\alpha}$ 2.56 ng, lyophilized. On reconstitution this bottle contains 1.28 ng/ml

Antiserum: Rabbit anti-
6-keto-prostaglandin $F_{1\alpha}$, lyophilized

TMB Substrate:
Enzyme substrate containing 3,3',5,5'-Tetramethylbenzidine (TMB)/ Hydrogen Peroxide, Ready for use.

Assay buffer concentrate:
20 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 preservative.

Wash buffer concentrate:
12.5 ml. On dilution the reagent contains 0.01 M Phosphate Buffer, pH 7.5 containing 0.05% Tween™20.

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, 2 ml and 6 ml)
- Disposable polypropylene test tubes
- Measuring cylinders
  50 ml and 500 ml
- Distilled or deionized water
- Spectrophotometric plate reader capable of measuring at 450 nm
- 1.0 M Sulfuric acid
- Microplate shaker

Optional Equipment: Assays may be performed with commercially available microplate washers to aid convenience and assay throughput

5. Description

6-keto-prostaglandin $F_{1\alpha}$ Enzymeimmunoassay Biotrak System from GE Healthcare has been specifically designed for research purposes. It combines the use of a Peroxidase labelled 6-keto-prostaglandin $F_{1\alpha}$ conjugate, a 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 6-keto-prostaglandin $F_{1\alpha}$ in the range 0.5 to 64 pg/well.

Each pack contains sufficient material for 96 wells. This permits the construction of one standard curve and 37 unknowns in duplicate.

- Specific for 6-keto-prostaglandin $F_{1\alpha}$
- High sensitivity ~ 0.2 pg
- 2.5 hour protocol
- Non isotopic
- Ready to use substrate
- Color coded reagents

6. Introduction

A diverse array of mammalian cells and tissues enzymatically oxidize Arachidonic acid to physiologically active compounds. These compounds include prostacyclin, thromboxanes, prostaglandins and leukotrienes.

Prostacyclin, also known as prostaglandin I_2 (PGI_2), is an unstable vinyl ether formed from the prostaglandin endoperoxide, prostaglandin H_2 (PGH_2). This conversion of PGH_2 to prostacyclin is catalyzed by prostacyclin synthetase, the two primary sites of synthesis being the veins and arteries, Prostacyclin has biological properties opposing the effect of thromboxane A_2. Prostacyclin is a vasodilator and a potent inhibitor of platelet aggregation (1) while thromboxane A_2 is a vasoconstrictor and a promoter of platelet aggregation (2,3). A physiological balance between the activities of these two effectors is probably important to maintaining a healthy vascular bed.

Prostacyclin is unstable and it undergoes a spontaneous hydrolysis to 6-keto-prostaglandin $F_{1\alpha}$. Study of this reaction *in vitro* established that prostacyclin has a half-life of approximately 3 minutes (4). This half-life increases to 9-23 minutes in platelet-poor plasma (5). Due to this spontaneous hydrolysis of prostacyclin, the quantitation of 6-keto-prostaglandin $F_{1\alpha}$ is accepted by many researchers as a measure of prostacyclin formation (6).

The development of specific immunoassays has facilitated the measurement of 6-keto-prostaglandin $F_{1\alpha}$. Radioimmunoassay techniques are the most widely used methods of measuring 6-keto-prostaglandin $F_{1\alpha}$ (7,8,9). Early enzymeimmunoassays suffered from poor sensitivity, however recent improvements in non-isotopic assay design and chromogens have shown assay performance to be equal or superior to radioisotopic methods (10,11,12).

7. Summary of the assay

The assay is based on the competition between unlabelled 6-keto-prostaglandin $F_{1\alpha}$ and a fixed quantity of Peroxidase labelled 6-keto-prostaglandin $F_{1\alpha}$ for a limited number of binding sites on a 6-keto-prostaglandin $F_{1\alpha}$ specific antibody. With fixed amounts of antibody and Peroxidase labelled 6-keto-prostaglandin $F_{1\alpha}$ 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 microtitre 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 6-keto-prostaglandin $F_{1\alpha}$ bound to the antibody is determined by addition of a Tetramethylbenzidine (TMB)/Hydrogen Peroxide single pot substrate (13). The reaction is stopped by additon of an acid solution, and the resultant color read at 450 nm in a microplate photometer.

The concentration of unlabelled 6-keto-prostaglandin $F_{1\alpha}$ in a sample is determined by interpolation from a standard curve.

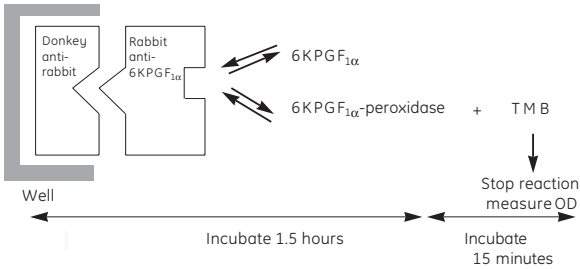


Figure 1.

8. Protocol

8.1. Specimen collection and sample preparation

Collect the blood in a tube with an anticoagulant, centrifuge the blood immediately and rapidly freeze the plasma. If blood-samples cannot be rapidly processed it is recommended that indomethacin or aspirin are added to the anticoagulant. Either of these compounds will inhibit the subsequent metabolism of Arachidonic acid to prostaglandins. One effective method is to collect the blood in a tube containing EDTA and Indomethacin. Mix 0.95 ml of an EDTA solution (2 g Disodium EDTA and 0.8 g NaCl adjusted to pH 7.4 with NaOH and made to a final volume of 100 ml in distilled water) with 0.05 ml of a 0.04 M Indomethacin solution (50 mg indomethacin dissolved in 3.5 ml absolute Ethanol) for the treatment of 10 ml of blood. The EDTA solution must be mixed while the indomethacin is being added or the indomethacin will precipitate. Store the plasma samples at -15°C to -30°C until the assay is conducted.

For excellent information about conducting prostaglandin immunoassays, three relevant articles are recommended (14,15,16). It is well estabished that non-esterified fatty acids can interfere with the assay (2,17). If samples contain these interfering compounds, several methods are available for separating the prostaglandins from the fatty acids (18,19,20).

In addition, Dray has evaluated blood drawing procedures (21).

Solid phase extraction procedures have become the method of choice for many researchers, giving high recovery and clean samples. However liquid phase extraction techniques are also still used. Representative procedures for the extraction of 6-keto-prostaglandin $F_{1\alpha}$ from plasma using Amprep™ minicolumns and by solvent extraction are described below. This information is provided

for guidance only. It remains the investigators' responsibility to validate their chosen procedure with their own samples.

Amprep extraction

1. Column type
Amprep C2 100 mg code RPN1903

2. Sorbent conditioning
Rinse the column with 2 ml Methanol
Rinse the column with 2 ml water

3. Sample treatment
Plasma - Acidify 1 ml plasma to pH 3. Apply to the column

4. Interference elution
Wash column with 5 ml water
Wash column with 5 ml 10% Ethanol
Wash column with 5 ml Hexane (or Petroleum Ether 30–40°C)

5. Elution
Elute 6-keto-prostaglandin $F_{1\alpha}$ with 5 ml Methyl Formate

Notes

1. The Methyl Formate should be dried under Nitrogen or vacuum and the extract redissolved in assay buffer before estimation.

2. The typical recovery of [^{3}H] 6-keto-prostaglandin $F_{1\alpha}$ is 90%.

3. For further details of the Amprep range of products see your GE Healthcare representative.

Liquid phase (21)

1. Add tritiated 6-keto-prostaglandin $F_{1\alpha}$ to 1 ml biological sample for estimation of recovery.

2. Add 2 ml Acetone and shake for 2 minutes.

3. Centrifuge at 4°C.

4. Transfer the supernant to a separate tube and add 2 ml hexane or petroleum ether.

5. Shake for 2 minutes and centrifuge at 4°C.
6. Discard the upper hexane layer.
7. Adjust the pH of the lower layer to 3.0–4.0 with 1 M Citric acid.
8. Add 2 ml Chloroform and shake for 2 minutes.
9. Centrifuge at 4°C.
10. The lower Chloroform layer contains the extracted 6-keto-prostaglandin $F_{1\alpha}$. Separate and re-extract the top layer with 2 ml Chloroform.
11. Combine the Chloroform extracts, dry under Nitrogen or vacuum and estimate the 6-keto-prostaglandin $F_{1\alpha}$ recovered.

Procedural notes

1. Allow samples and all reagents to reach room temperature prior to performing the assay.
2. Mix samples and all reagents thoroughly before use.
3. Avoid excessive foaming of reagents.
4. Avoid handling the tops of the wells both before and after filling.
5. Keep the wells covered with lids except when adding reagents and reading.
6. Standards and samples should be assayed in duplicate.
7. Run a separate standard curve for each microplate.
8. The total dispensing time for each plate should not exceed 20 minutes.

8.2. Enzymeimmunassay procedure

8.2.1. Reagent preparation

Note: All reagents must be allowed to equilibrate to room temperature prior to use.

All reagents should be stored at 2–8°C. Once reconstituted components should be stored at 2–8°C and re-used within seven days.

The coated microplate and enzyme substrate are provided ready for use.

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 preservative.

Standard

Carefully add 2.0 ml diluted assay buffer and replace the stopper. Mix the contents of the bottle until completely dissolved. The final solution should contain 6-keto-prostaglandin $F_{1\alpha}$ at a concentration of 1.28 ng/ml.

6-keto-prostaglandin $F_{1\alpha}$ 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 6-keto-prostaglandin $F_{1\alpha}$- Horseradish Peroxidase in Phosphate Buffer containing 0.9% Sodium Chloride, 0.1% Bovine Serum Albumin and 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-6-keto-prostaglandin $F_{1\alpha}$ serum in Phosphate Buffer containing 0.9% Sodium Chloride, 0.1% Bovine Serum Albumin and preservative.

Wash buffer
Transfer the contents of the bottle to a 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.

8.2.2. Preparation of working standards

1. Label 7 polypropylene tubes 0.5 pg, 1 pg, 2 pg, 4 pg, 8 pg, 16 pg, and 32 pg.

2. Pipette 500 µl assay buffer into all tubes.

3. Pipette 500 µl of the stock standard (1.28 ng/ml) into the 32 pg tube and mix thoroughly.

4. Transfer 500 µl from the 32 pg tube to the 16 pg tube and mix thoroughly.

5. Repeat this doubling dilution successively with the remaining tubes.

6. 50 µl aliquots from each serial dilution together with the stock solution will give rise to 8 standard levels of 6-keto-prostaglandin $F_{1\alpha}$ ranging from 0.5 to 64 pg per well.

Note: Working standards should be prepared within one hour of performing the enzymeimmunoassay so as to minimize any effect of
6-keto-prostaglandin $F_{1\alpha}$ adsorption to the walls of the test tubes.

8.2.3. Assay 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 (0–64) 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 non specific binding wells.

7. Cover the plate with the lid provided and incubate at room temperature (15–30°C) by shaking for 30 minutes on a microplate shaker.

8. Pipette 50 µl 6-keto-prostaglandin $F_{1\alpha}$ Peroxidase conjugate into all wells except the blank.

9. Cover the plate with the lid provided and incubate at room temperature (15–30°C) by shaking for 1 hour on a microplate shaker.

10. Aspirate and wash all wells four times with 400 µl wash buffer.

11. Immediately dispense 150 µl enzyme substrate into all wells, cover the plate and mix on a microplate shaker for exactly 15 minutes at room temperature (15–30°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.

Table 1. Enzymeimmunoassay protocol (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 room temperature 15–30°C for 30 minutes while shaking					
Peroxidase conjugate	—	50	50	50	50
Cover plate, incubate at room temperature 15–30°C for 1 hour while shaking. Aspirate, wash all wells four times with 400 µl wash buffer					
Substrate	150	150	150	150	150
Cover plate, incubate at room temperature 15–30°C for exactly 15 minutes while shaking					
1.0 M Sulfuric acid**	100	100	100	100	100
Shake to mix contents and determine optical density at 450 nm.					

* Since the non-specific binding in this assay is so low, typically less than 0.2%, it is possible to omit the substrate blank determination, enabling the assay of an extra unknown sample. If this option is taken, the non-specific binding wells should be used to blank the plate reader.

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

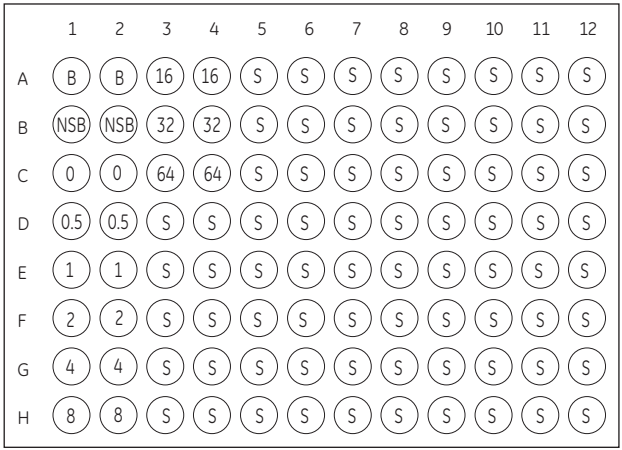

1 2 3 4 5 6 7 8 9 10 11 12
A B B 16 16 S S S S S S S S
B NSB NSB 32 32 S S S S S S S S
C 0 0 64 64 S S S S S S S S
D 0.5 0.5 S S S S S S S S S S
E 1 1 S S S S S S S S S S
F 2 2 S S S S S S S S S S
G 4 4 S S S S S S S S S S
H 8 8 S S S S S S S S S S

Figure 2.

8.3. Data processing

8.3.1. 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:-

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

A standard curve may be generated by plotting the percent B/B_0 as a function of the log 6-keto-prostaglandin $F_{1\alpha}$ concentration. Plot % B/B_0 (y axis) against pg 6-keto-prostaglandin $F_{1\alpha}$ standard 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.

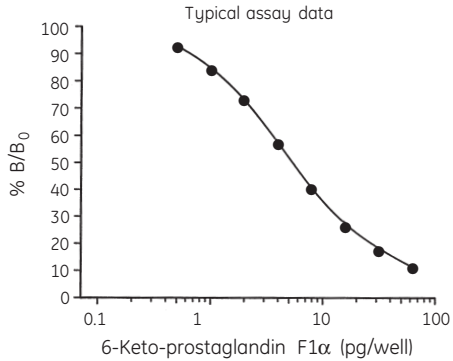


Figure 3. Typical assay data

8.3.2. Typical assay data

Table 2

Standard (pg/well)	Optical density (OD at 450 nm)	Mean OD	%B/B_0
NSB	<0.01		
0	0.935 0.920	0.920	100.0
0.5	0.840 0.884	0.862	92.9
1	0.802 0.772	0.787	84.1
2	0.681 0.675	0.678	73.1
4	0.545 0.533	0.574	57.5
8	0.381 0.370	0.376	40.5
16	0.255 0.249	0.252	27.2
32	0.175 0.165	0.169	18.2
64	0.112 0.092	0.104	11.2

* The OD value is dependent on the surrounding temperature. For example typical OD values of the zero standard curve can vary from 0.6 at 15°C to 1.3 at 30°C. Standard curve parameters remain unchanged throughout this temperature range.

The data presented in this table was obtained from an assay performed at 22°C.

8.3.3. Alternative high sensitivity protocol

Sensitivity may be further enhanced if the plate is allowed to incubate for 15–20 hours, without shaking, at 4°C after addition of the Peroxidase conjugate (after step 8). The delayed addition of the conjugate is not necessary, therefore step 7 may be disregarded. All other protocol steps (10–12) remain the same.

In order to achieve a usable standard curve the contents of the bottle containing Peroxidase conjugate should be diluted to 24 ml instead of 6 ml. ALL other components should be used at the concentrations described in the reagent preparation section. (p.13).

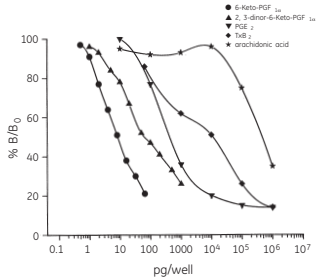
Note: This protocol is not routinely tested by our quality assurance department.

9. Additional Information

9.1. 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.

Compound	% Cross-reactivity
6-keto-prostaglandin $F_{1\alpha}$	100.0
2,3-dinor-6-keto-prostaglandin $F_{1\alpha}$	10.5
6,15-diketo-13,14-dihydro-prostaglandin $F_{1\alpha}$	0.2
6-keto-prostaglandin E_1	9.2
Prostaglandin D_2	0.5
Prostaglandin E_2	2.8
Prostaglandin $F_{1\alpha}$	2.1
Prostaglandin $F_{2\alpha}$	1.4
Thromboxane B_2	0.03
Arachidonic acid	0.01



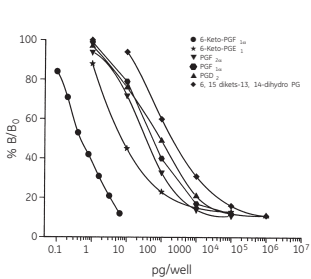


Figure 4 (a) Cross-reactivity profile **(b)** Cross-reactivity profile

9.2. Typical assay parameters

Sensitivity

The sensitivity, defined as the amount of 6-keto-prostaglandin $F_{1\alpha}$ needed to reduce zero dose binding by two standard deviations was 0.15 pg/well, which is equivalent to 3.0 pg/ml.

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	38.0 ± 2.5	6.6	25
B	13.0 ± 0.6	4.6	25
C	4.4 ± 0.2	4.5	35

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
D	20.8 ± 3.1	14.9	23
E	7.3 ± 1.2	16.4	24
F	2.7 ± 0.4	14.8	32

Precision profile

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

Table 6

Standard (pg/well)	Standard deviation	% CV
0.5	0.14	26.9
1.0	0.14	13.3
2.0	0.26	12.6
4.0	0.39	9.4
8.0	0.53	6.5
16.0	1.64	9.9
32.0	4.07	12.5
64.0	8.56	12.9

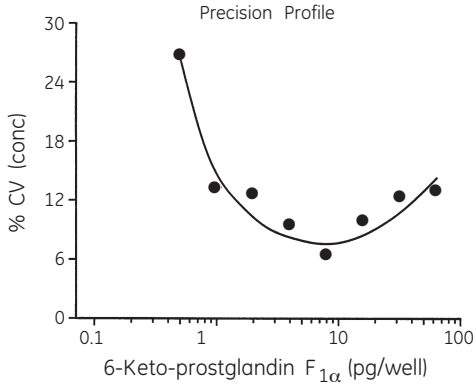


Figure 5. Precision profile

Effect of time and temperature on assay performance

It is important that all incubation processes are performed for the times stated in the assay protocol. Prolonged incubation following TMB substrate addition may lead to elevated OD values outside the spectrophotometer's accurate measurement range. Assay temperature will also influence OD readings. However important assay parameters such as sensitivity and curve shape are not changed between 15 and 30°C.

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11. Related products

Assay systems	EIA	SPA	^{3}H charcoal	^{125}I
6-Keto-Prostaglandin $F_{1\alpha}$				RPA515
Thromboxane B_2	RPN220			RPA516
Prostaglandin D_2			TRK890	
Leukotriene B_4)	RPN223			
Leukotriene $C_4/D_4/E_4$	RPN224			
Prostaglandin E_2	RPN222			RPA530
Platelet Activating Factor (PAF)		TRK990		

GE Healthcare's range also includes leukotrienes, prostaglandins and thromboxanes, labelled arachidonic acids and other fatty acids, specifically 2-labelled arachidonyl phospholipids and labelled steroidal and non-steroidal anti-inflammatory drugs.

Amprep C2 100 mg	pack of 100	RPN1903
Amprep C2 500 mg	pack of 50	RPN1913
Amprep SAX Trimethyl Amino-Propyl 100 mg	pack of 100	RPN1908
Amprep SAX Trimethyl Amino-Propyl 500 mg	pack of 50	RPN1918
Amprep C18 100 mg	pack of 100	RPN1900
Amprep C18 500 mg	pack of 50	RPN1910
Amprep C8 100 mg	pack of 100	RPN1902
Amprep PH Phenyl 100 mg	pack of 100	RPN1904
Amprep SI Silica 100 mg	pack of 100	RPN1906

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