GE Healthcare

Leukotriene B_4 Biotrak Enzymeummunoassay (EIA) System

Product Booklet

Codes: RPN223 – 96 wells
RPN22310 – 960 wells

Page finder

1. Legal

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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 four weeks from the date of dispatch.

3. Components of the assay system

RPN223 contains 1 x each component listed.

RPN22310 contains 10 x each component listed.

Microplate
Plate containing 12 x 8 well strips coated with donkey anti-rabbit IgG, ready for use after thawing. Store at -15°C to -30°C.

Leukotriene B_4 peroxidase conjugate
Leukotriene B_4-horseradish peroxidase, lyophilised. Store at -15°C to -30°C.

Standard
Leukotriene B_4 lyophilised. On reconstitution this bottle contains 4 ng/ml. Store at -15°C to -30°C.

Antiserum
Rabbit anti-leukotriene B_4 lyophilised. Store at -15°C to -30°C.

TMB substrate
Enzyme substrate containing 3,3', 5,5'-tetramethyl-benzidine(TMB)/ hydrogen peroxide. Ready for use after thawing. Store at -15°C to -30°C.

Assay buffer concentrate
Assay buffer concentrate with preservative. On dilution this bottle contains 0.1 M phosphate buffer, pH 7.5 containing 0.9% sodium chloride, 0.1% bovine serum albumin and 0.05% preservative. Store at -15°C to -30°C.

Wash buffer concentrate
Wash buffer concentrate. 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. Description

- Specific for leukotriene B_4
- High sensitivity ~0.3 pg/well, 6 pg/ml
- 3.5 hour protocol
- Non isotopic
- Ready to use substrate
- Colour coded reagents

The Biotrak™ leukotriene B_4 Enzymeimmunoassay System from GE Healthcare has been specifically designed for research purposes. It combines the use of a peroxidase labelled leukotriene B_4 conjugate, a specific antiserum which can be immobilised on to precoated microplates, and a one pot stabilised substrate solution. This provides a rapid and sensitive non-isotopic method for the determination of leukotriene B_4 in the range 0.3 pg to 40 pg/well.

Each pack of RPN223 contains sufficient material for 96 wells. This permits the construction of one standard curve and 37 unknown in duplicate. Each pack of RPN22310 contains sufficient material for 960 wells. This permits the construction of one standard curve and 37 unknown in duplicate per plate.

The assay is based on the competition between unlabelled leukotriene B_4 and a fixed quantity of peroxidase labelled leukotriene B_4 for a limited number of binding sites on a leukotriene B_4 specific antibody. With fixed amounts of antibody and peroxidase labelled leukotriene B_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 immobilised 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 B_4 bound to the antibody is determined by addition of a 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 B^4 in a sample is determined by interpolation from a standard curve.

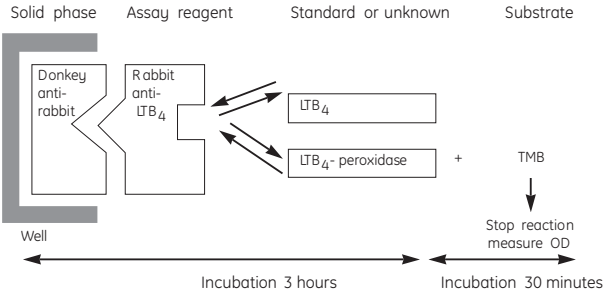

Figure 1.

5. 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.
- Keep the plates covered with lids during the incubation steps.
- The total dispensing time for each plate should not exceed 20 minutes.
- Use only coated wells from the same reagent batch for each assay.

6. Additional materials and equipment required

The following materials and equipment are required but not supplied:

- Pipettes or pipetting equipment with disposable tips (50µl, 100µl, 150µl, 200µl, 800µl and 6 ml)
- Disposable polypropylene test tubes
- Measuring cylinders 50 ml and 500 ml
- Distilled or deionised water
- Spectrophotometer plate reader capable of measuring at 450 nm
- 1.0 M sulphuric acid
- Microplate shaker

7. Specimen collection and sample preparation

7.1. Sample preparation

Leukotriene B_4 is present in many different types of biological material. It is unlikely that a single sample preparation procedure will prove suitable for all samples. This section provides information on sample purification procedures which have been reported in the literature to be useful. This information is provided for guidance only. It remains the responsibility of individual researchers to satisfy themselves that the sample preparation they are employing is appropriate to their particular situation.

It may be possible to assay leukotriene B_4 in some samples directly, that is without prior extraction and chromatography. For example, the concentration of leukotriene B_4 in supernatants derived from incubations of human polymorphonuclear leukocytes (5×10^6/ml) with either the calcium ionophore A23187 or serum-treated zymosan can be determined directly (2). Since the level of leukotriene B_4 in these incubations may be high, the supernatants may have to be diluted with EIA buffer prior to assay. It is possible that *in vitro* incubations of other cells and tissues which generate leukotriene B_4 could also be assayed directly. In addition, the concentration of leukotriene B_4 in exudate derived from an animal model of acute inflammation has been determined reliably by direct RIA (3,4).

7.2. Extraction

Extraction and/or chromatography of other samples may be required however, prior to EIA, either to improve specificity or increase sensitivity. If extraction of leukotriene B_4 prior to EIA is considered necessary then the following methods may be suitable:

Solid phase extraction

Solid phase extraction techniques using commercially available (for example Amprep™ mini-columns from GE Healthcare) reverse-phase silica cartridges are generally considered to give the best results. The scheme outlined by Powell(5) has proved to be very efficient and reproducible.

Before applying the sample, precondition a 100 mg C2 reverse phase column (Amprep, code RPN 1903) with 2 ml of methanol followed by 2 ml of distilled or deionised water. The columns can be used to purify a range of samples. Plasma samples are acidified to pH 3. Tissue samples are homogenised in 0.1 M phosphate buffer pH 7.4, centrifuged and the supernatant retained. With all suspensions, mix 0.25 ml of 1 M HCl with each 1 ml of suspension.

The samples are applied to the column and eluted in turn with 5 ml of water, 5 ml of 10% ethanol and 5 ml of hexane (or 30-40° petroleum ether) to remove interfering compounds. The samples are then eluted with 5 ml of methyl formate. The methyl formate fractions are taken to dryness and reconstituted in assay buffer. Typical recoveries are in the order of >90%. In our experience lower recoveries are obtained with the C18 columns.

Liquid extraction

Alternatively, liquid extraction such as the following method can be used.

Adjust pH of sample to 3–4 and extract leukotriene B_4 into ethyl acetate (2 x 2 volumes). The organic solvent should be removed under nitrogen and the residue dissolved in EIA buffer and assayed or subjected to further purification.

If the samples contain a high concentration of proteins and lipids it is probably advisable to initially precipitate the protein with ice cold acetone (2 volumes). The pH of the aqueous-acetone should then be adjusted to pH 10–11 with 1 M sodium hydroxide and neutral lipids

extracted into petroleum ether (40-60) or hexane, which can then be discarded. The remaining aqueous-acetone should then be acidified (pH 3–4) with 1 M hydrochloric acid and leukotriene B_4 extracted into either ethyl acetate (upper layer) or chloroform (lower layer) (3).

For samples which contain high levels of impurities further purification by high pressure liquid chromatography prior to EIA may be necessary. Chromatography of leukotriene B_4 has proved successful on a variety of reverse-phase columns using methanol: water:acetic acid (for example 70:30:0.01 adjusted to an apparent pH of 5.7) and other solvent mixtures (6).

8. Enzymeimmunoassay procedure

8.1. 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 reconstituted peroxidase conjugate, antiserum and standard should be stored at -15°C to -30°C and re-used within two weeks. Repeated freezing and thawing should be avoided.

The coated microplate 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 pH7.5 containing 0.9% sodium chloride, 0.1% bovine serum albumin and 0.05% preservative.

Standard

Carefully add 2.5 ml diluted assay buffer and replace the stopper. Mix the contents of the bottle until completely dissolved. The final solution should contain leukotriene B_4 at a concentration of 4 ng/ml.

Leukotriene B_4 peroxidase conjugate

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

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.

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 pH7.5 containing 0.05% Tween 20.

8.2. Preparation of working standards

1. Label 8 polypropylene tubes 40 pg, 20 pg, 10 pg, 5 pg, 2.5 pg, 1.25 pg, 0.62 pg and 0.31 pg.
2. Pipette 800 µl assay buffer into the 40 pg tube.
3. Into the remaining marked standard tubes pipette 500 µl assay buffer.
4. Pipette 200 µl of the stock standard (4 ng/ml) into the 40 pg tube and mix thoroughly.
5. Transfer 500 µl from the 40 pg tube to the 20 pg tube and mix thoroughly.
6. Repeat this doubling dilution successively with the remaining tubes.
7. 50 µl aliquots from each serial dilution will give rise to 8 standard levels of leukotriene B_4 ranging from 40 pg to 0.31 pg per well.

Note: Working standards should be prepared within one hour of performing the enzymeimmunoassay so as to minimise any effect of leukotriene B_4 adsorption to the walls of the test tubes.

8.3. 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 (0–40 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 50µl of antiserum to all wells except the blank and NSB wells.
7. Cover the plate with the lid provided and incubate at room temperature (15–25°C) by shaking for 2 hours on a microplate shaker.
8. Pipette 50 µl leukotriene B_4 peroxidase conjugate into all wells except the blank.
9. Cover the plate with the lid provided and incubate at room temperature (15–25°C) by shaking for 1 hour on a microplate shaker.
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 colour 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 sulphuric 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.

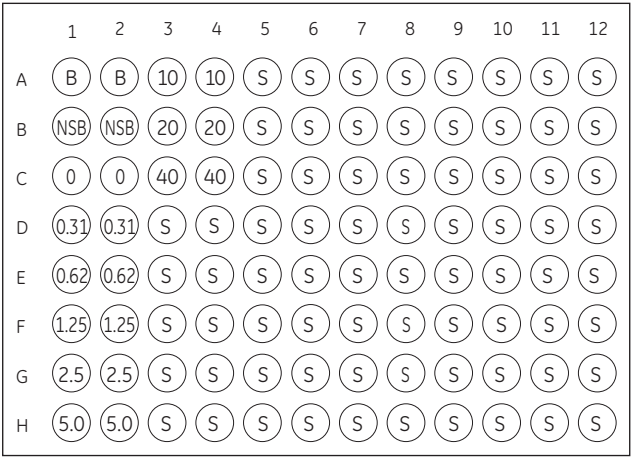

Figure 2. Recommended positioning of standard (0–40 pg/ml) and sample (S) wells.

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 15–25°C for 2 hours while shaking.					
Peroxidase conjugate	–	50	50	50	50
Cover plate, incubate at room temperature for exactly 1 hour while shaking.					
Aspirate, wash all wells four times with 300 µl wash buffer					
Substrate	150	150	150	150	150
Cover plate, incubate at room temperature 15–25°C for exactly 30 minutes while shaking.					
1.0 M Sulphuric 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.

9. Data processing

9.1. 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:

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

A standard curve may be generated by plotting the percent B/B_0 as a function of the log leukotriene B_4 concentration.

Plot % B/B_0 (y axis) against pg leukotriene B_4 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.

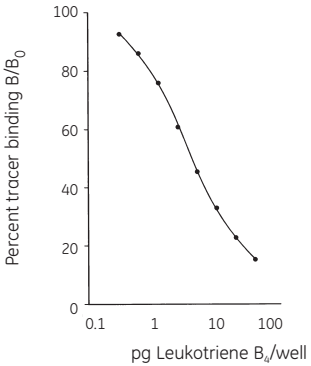

Figure 3. Leukotriene B_4 standard curve

Table 2. Typical assay data

Standard (pg/well)	Optical density (OD) at 450 nm	Mean OD at 450 nm	Mean OD - NSB	%B/B$_0$
Substrate blank	0.042 0.043	0.043		
NSB	0.070 0.068	0.069		
0	1.050 1.056	1.053	0.984	
0.31	0.989 0.971	0.980	0.911	92.6
0.62	0.906 0.925	0.916	0.847	86
1.25	0.817 0.814	0.816	0.747	75.9
2.5	0.671 0.664	0.668	0.599	60.8
5	0.521 0.510	0.516	0.447	45.4
10	0.397 0.389	0.393	0.324	32.9
20	0.286 0.299	0.293	0.224	22.7
40	0.217 0.220	0.218	0.149	15.2

10. Additional information

10.1. 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.

Table 3.

Analyte	% Cross-reactivity
Leukotriene B_4	100.0
20-OH-leukotriene B_4	2.0
6-trans-leukotriene B_4	25.5
Leukotriene C_4	0.011
Leukotriene D_4	<0.010
5-Hydroxyeicosatetraenoic acid (5-HETE)	0.008
12-HETE	<0.034
15-HETE	<0.002
Prostaglandin $F_{2\alpha}$	<0.002
Thomboxane B_2	<0.002
6-Keto-prostaglandin $F_{1\alpha}$	<0.002
Arachidonic acid	<0.002
12-OH-5,8,10 heptadecatetrienoic acid	0.009

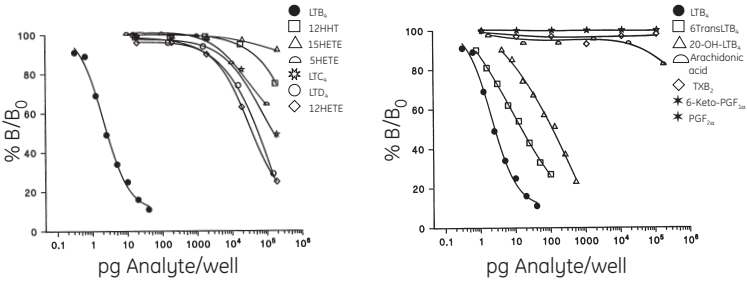

Figure 4. (a) Cross-reactivity profile **(b)** Cross-reactivity profile

10.2. Sensitivity

The sensitivity, defined as the amount of leukotriene B_4 needed to reduce zero dose binding by two standard deviations, was 0.3 pg/well, which is equivalent to 6 pg/ml.

10.3. 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	1.14 ± 0.1	9.1	12
B	5.85 ± 0.44	7.56	12
C	28.92 ± 2.09	7.22	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	0.549 ± 0.06	11.5	10
B	3.24 ± 0.31	9.5	10
C	9.84 ± 0.61	6.2	10

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 the peroxidase conjugate addition may lead to a reduction in sensitivity. Assay temperature will also influence OD readings. However, important assay parameters such as sensitivity and curve shape are not changed between 15 and 25°C.

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.

Table 6.

Standard (pg/well)	Standard deviation	%CV
0.31	0.046	15.5
0.62	0.049	8.81
1.25	0.033	2.80
2.5	0.07	2.94
5	0.393	8.27
10	0.397	4.18
20	2.14	11.2
40	6.51	16.9

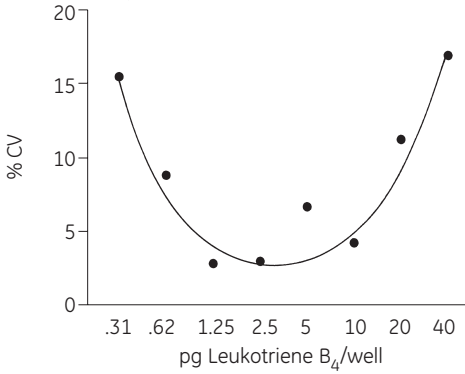

Figure 5.

11. 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.

Leukotriene B_4 is a dihydroxy metabolite of arachidonic acid. It is a potent stimulator of both directional (chemotactic) and random (chemokinetic) movement of polymorphonuclear leukocytes (7,8,9). It induces degranulation and release of lysosomal enzymes from human and rabbit polymorphonuclear leukocytes (10,11). Other reports indicate that leukotriene B_4 induces adhesion of leukocytes to endothelial cells of post-capillary venules (11) and aggregation of polymorphonuclear leukocytes (12).

These results have lead to speculation that leukotriene B_4 is a potent mediator of the inflammatory response.

The availability of the Biotrak high sensitivity leukotriene B_4 enzymeimmunoassay will facilitate the evaluation of the role of this important arachidonic acid metabolite.

12. References

1. BOS, E.S. *et al., J.Immunol.,* **2**, pp.187-204, 1981.

2. PALMER, R.M.J. and SALMON, J.A., *Immunology,* **50**, pp.65-73, 1983.

3. SIMMONS, P.M. *et al., Biochem. Pharmacol.,* **32**, pp.1353-1359, 1983.

4. SALMON, J.A., *Prostaglandins,* **27**, pp.364-366, 1984.

5. POWELL, W.S., *Methods in Enzymology,* **86**, pp.467-477, 1982.

6. MATTHEWS, M.R. *et al., Anal. Biochem.,* **118**, pp.96-101, 1981.

7. FORD-HUTCHINSON, A.W. *et al.,* Nature, **226**, pp.264-265, 1980.

8. MALMSTEN, C. *et al., Acta Physiol. Scand.,* **110**, pp.449-451, 1980.

9. GOETZL, E.J. and PICKETT, W.C., *J.Exp. Med.,* **153**, pp.482-487, 1981.

10. BOKOCH, G.M. and REED, P.W., *J.Biol. Chem.,* **256**, pp.5317-5320, 1981.

11. RAE, S.A. and SMITH, M.J.H., *J. Pharm. Pharmacol.,* **33**, pp.616-617, 1981.

12. CUNNINGHAM, F.M. *et al., Agents Actions,* **11**, pp.583-584, 1981.

13. Related products

Eicosanoids

Thromboxane B_2	EIA	RPN220
Thromboxane B_2' [^{125}I]	RIA	RPA516
Leukotriene $C_4/D_4/E_4$	EIA	RPN224
Platelet activating factor (PAF), [^{3}H]	SPA	TRK990
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

Amprep range

Amprep C2 10 mg	pack of 100	RPN1903

GE Healthcare offices:

GE Healthcare Bio-Sciences AB
Björkgatan 30 751 84
Uppsala
Sweden

GE Healthcare Europe GmbH
Munzinger Strasse 5 D-79111
Freiburg
Germany

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

GE Healthcare Bio-Sciences Corp
800 Centennial Avenue
P.O. Box 1327
Piscataway
NJ 08855-1327
USA

GE Healthcare Bio-Sciences KK
Sanken Bldg. 3-25-1
Hyakunincho Shinjuku-ku
Tokyo 169-0073
Japan

GE Healthcare regional office contact numbers:

Asia Pacific
Tel: +85 65 62751830
Fax: +85 65 62751829

Australasia
Tel: + 61 2 8820 8299
Fax: +61 2 8820 8200

Austria
Tel: 01/57606-1613
Fax: 01/57606-1614

Belgium
Tel: 0800 73 890
Fax: 02 416 8206

Canada
Tel: 1 800 463 5800
Fax: 1 800 567 1008

Central, East, & South East Europe
Tel: +43 1 972 720
Fax: +43 1 972 722 750

Denmark
Tel: 45 70 25 24 50
Fax: 45 45 16 2424

Eire
Tel: 1 800 709992
Fax: +44 1494 542010

Finland & Baltics
Tel: +358 9 512 3940
Fax: +358 9 512 39439

France
Tel: 01 69 35 67 00
Fax: 01 69 41 98 77

Germany
Tel: 0800 9080 711
Fax: 0800 9080 712

Greater China
Tel: +852 2100 6300
Fax: +852 2100 6338

Italy
Tel: 02 26001 320
Fax: 02 26001 399

Japan
Tel: +81 3 5331 9336
Fax: +81 3 5331 9370

Korea
Tel: 82 2 6201 3700
Fax: 82 2 6201 3803

Latin America
Tel: +55 11 3933 7300
Fax: + 55 11 3933 7304

Middle East & Africa
Tel: +30 210 96 00 687
Fax: +30 210 96 00 693

Netherlands
Tel: 0800-82 82 82 1
Fax: 0800-82 82 82 4

Norway
Tel: +47 815 65 777
Fax: +47 815 65 666

Portugal
Tel: 21 417 7035
Fax: 21 417 3184

Russia, C.I.S. & N.I.S
Tel: +7 495 956 5177
Fax: +7 495 956 5176

Spain
Tel: 902 11 72 65
Fax: 935 94 49 65

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Tel: 018 612 1900
Fax: 018 612 1910

Switzerland
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Fax: 0848 8028 11

UK
Tel: 0800 515 313
Fax: 0800 616 927

USA
Tel: +1 800 526 3593
Fax: +1 877 295 8102

http://www.gehealthcare.com/lifesciences

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

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