

Parameter™

LTB₄ Assay

Catalog Number KGE006B

SKGE006B

PKGE006B

For the quantitative determination of Leukotriene B₄ (LTB₄) concentrations in cell culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

TABLE OF CONTENTS

SECTION	PAGE
INTRODUCTION	1
PRINCIPLE OF THE ASSAY	2
LIMITATIONS OF THE PROCEDURE	2
TECHNICAL HINTS	2
MATERIALS PROVIDED & STORAGE CONDITIONS	3
OTHER SUPPLIES REQUIRED	4
PRECAUTIONS	4
SAMPLE COLLECTION & STORAGE	4
REAGENT PREPARATION	5
ASSAY PROCEDURE	6
CALCULATION OF RESULTS	7
TYPICAL DATA	7
PRECISION	8
RECOVERY	8
LINEARITY	8
SENSITIVITY	9
SAMPLE VALUES	9
SPECIFICITY	10
REFERENCES	10

MANUFACTURED AND DISTRIBUTED BY:

USA & Canada | R&D Systems, Inc.

614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.

19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.

24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

INTRODUCTION

Leukotriene B₄ (LTB₄) is a potent pro-inflammatory molecule that belongs to a family of eicosanoid lipid mediators (1, 2). It is synthesized from arachidonic acid that is generated from nuclear membrane phospholipid (1, 3). In general, and upon cell activation, phospholipase A2 is directed to the nuclear membrane. Here, it generates arachidonic acid that is bound by one 18 kDa membrane-bound protein termed Five-Lipoxygenase Activating Protein (FLAP). FLAP-bound arachidonic acid is subjected to two distinct, sequential actions by 80 kDa 5-lipoxygenase (5-LO). The first 5-LO action generates 5(S)-HPETE/5-HPETE, an oxygenated form of arachidonic acid. The newly generated 5-HPETE is then acted on again by 5-LO to generate LTA₄ (1-4). The LTB₄ synthetic pathway has two key checkpoints. The first is at the level of arachidonic acid formation, where COX enzymes offer an alternative to 5-LO in the generation of prostaglandins. The second is at the level of LTA₄, where 69 kDa LTA₄ hydrolase activity will generate LTB₄ (a pro-inflammatory molecule), and 18 kDa LTC₄ synthetase activity will generate LTC/D/E4 (smooth muscle contractants) (4, 5). The key enzyme in LTB₄ synthesis is 5-LO, which has limited cell expression. Cells known to express 5-LO include B cells, macrophages, monocytes, mast cells, neutrophils, neurons, eosinophils, and dendritic cells (1, 2). Notably, all cells are potential sources for LTB₄ production. This is due to the fact that LTA₄ hydrolase is ubiquitously expressed, and excess LTA₄ generated by 5-LO expressing cells can diffuse into neighboring LTA₄ hydrolase-containing cells for conversion into LTB₄ (5).

LTB₄ has two known membrane receptors and one intracellular receptor. The membrane receptors are named BLT1 and BLT2 (2, 6). Both are 7-transmembrane receptors that induce Ca²⁺ flux when activated. BLT1 is a high-affinity receptor for LTB₄ expressed by hematopoietic cells and keratinocytes, while BLT2 is a low-affinity receptor that is widely expressed (6, 7). The intracellular receptor is PPAR-γ, which becomes active upon binding to LTB₄. This activation promotes the expression of LTB₄ degradative enzymes, creating a negative feedback loop for LTB₄ production (1, 8).

LTB₄ functions are considered pro-inflammatory but vary depending upon the target cell. On hematopoietic stem cells, LTB₄ may induce differentiation into CFU-GEMM and CFU-GM progenitor cells (9). B cells are induced to differentiate by LTB₄, and in the presence of IL-4, to release IgE (2). LTB₄ is also considered essential for Th2-type responses. It drives IL-4 and IL-5 production, and promotes CD4⁺ cell proliferation while inhibiting CD8⁺ cell expansion (2, 6, 10). Under the influence of LTB₄, endothelial cells bind and promote neutrophil transmigration (1, 6). LTB₄ is perhaps best known for its activity on neutrophils. It promotes polymorphonuclear (PMN) cell migration, blocks PMN apoptosis, and induces neutrophil granule release in conjunction with reactive oxygen species generation (2, 6). Finally, it activates macrophage phagocytosis and drives mononuclear pro-inflammatory cytokine release (2, 6).

The Parameter™ LTB₄ Assay is a 4.5 hour forward sequential competitive enzyme immunoassay designed to measure LTB₄ in cell culture supernates, serum, and plasma.

PRINCIPLE OF THE ASSAY

This assay is based on the forward sequential competitive binding technique in which LTB₄ present in a sample competes with a fixed amount of horseradish peroxidase (HRP)-labeled LTB₄ for sites on a chicken polyclonal antibody. During the incubations, the chicken polyclonal antibody becomes bound to the rabbit anti-chicken antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound enzyme activity. Following color development, the assay is stopped, and the absorbance is read at 450 nm. The intensity of the color is inversely proportional to the concentration of LTB₄ in the sample.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with calibrator diluent and repeat the assay.
- Any variation in diluent, operator, pipetting technique, incubation time or temperature, and kit age can cause assay variability.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in this assay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- Pre-rinse the pipette tips when pipetting.
- Pipette standards and samples to the bottom of the wells. Add all other reagents to the side of the wells to avoid contamination.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of wash buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution. Gently tap the side of the plate to ensure thorough mixing.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at $\leq -20\text{ }^{\circ}\text{C}$ in a manual defrost freezer.

Do not use past kit expiration date.

PART	PART #	CATALOG # KGE006B	CATALOG # SKGE006B	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
LTB ₄ Microplate	893074	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a rabbit anti-chicken polyclonal antibody.	Return unused wells to the storage bag. May be stored at 2-8 °C for up to 1 month.*
LTB ₄ Conjugate	893641	1 vial	6 vials	6 mL/vial of LTB ₄ conjugated to horseradish peroxidase with red dye and preservatives.	Aliquot and store at $\leq -20\text{ }^{\circ}\text{C}$ for up to 1 month in a manual defrost freezer.* Avoid repeated freeze-thaw cycles.
LTB ₄ Standard	893077	1 vial	6 vials	LTB ₄ in a buffer with preservatives; lyophilized. Refer to the vial label for reconstitution volume.	
Primary Antibody Solution	893642	1 vial	6 vials	6 mL/vial of a chicken polyclonal antibody to LTB ₄ in a buffer with blue dye and preservatives.	May be stored for up to 1 month at 2-8 °C.*
Calibrator Diluent RD5-52	895925	1 vial	6 vials	21 mL/vial of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative. May turn yellow over time.	
Color Reagent A	895000	1 vial	6 vials	12 mL/vial of stabilized hydrogen peroxide.	
Color Reagent B	895001	1 vial	6 vials	12 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895926	1 vial	6 vials	11 mL/vial of 2 N sulfuric acid.	
Plate Sealers	N/A	4 strips	24 strips	Adhesive strips.	

* Provided this is within the expiration date of the kit.

KGE006B contains sufficient materials to run an ELISA on one 96 well plate.

SKGE006B (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems®, Catalog # PKGE006B). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Refer to the literature accompanying your order for specific vial counts.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards.
- LTB₄ Controls (optional; R&D Systems®, Catalog # QC116).

PRECAUTIONS

Care should be taken when handling the LTB₄ Standard because of the known and unknown effects of eicosanoids.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Samples containing chicken IgY may interfere with this assay.

Cell Culture Supernates - Remove particulates by centrifugation. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Animal serum used in the preparation of cell culture media may contain high levels of LTB₄. For best results, do not use animal serum for growth of cell cultures when assaying for LTB₄ production. If animal serum is used as a supplement in the media, the appropriate media control should be prepared and tested in the immunoassay to determine the baseline concentration of LTB₄.*

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles to minimize the conversion of LTA₄ to LTB₄ (refer to the note on page 9).

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles to minimize the conversion of LTA₄ to LTB₄ (refer to the note on page 9).

Note: *Citrate plasma has not been validated for use in this assay. Do not use lipemic samples.*

REAGENT PREPARATION

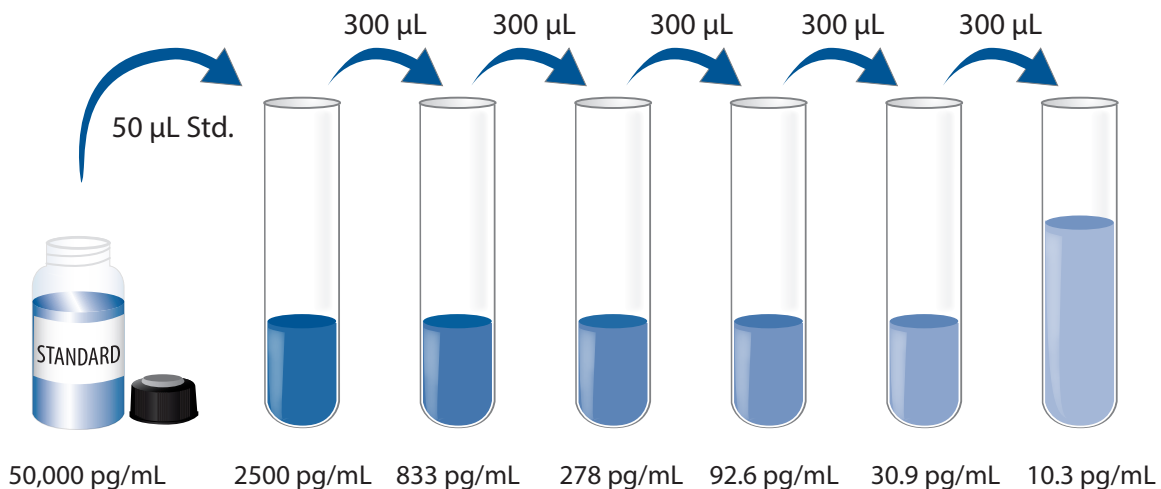
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μ L of the resultant mixture is required per well.

LTB₄ Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the LTB₄ Standard with deionized or distilled water. This reconstitution produces a stock solution of 50,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 950 μ L of Calibrator Diluent RD5-52 into the 2500 pg/mL tube. Pipette 600 μ L into the remaining tubes. Use the 50,000 pg/mL standard stock to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 2500 pg/mL standard serves as the high standard. Calibrator Diluent RD5-52 serves as the zero standard (B₀) (0 pg/mL). **Use diluted standards within 60 minutes of preparation.**



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 μL of Calibrator Diluent RD5-52 to the non-specific binding (NSB) wells.
4. Add 50 μL of Calibrator Diluent RD5-52 to the zero standard (B_0) wells.
5. Add 50 μL of standard, control, or sample to the remaining wells.
6. Add 50 μL of the Primary Antibody Solution to each well (**excluding the NSB wells**). All wells, except the NSBs, will now be blue in color. Cover with the adhesive strip provided. Incubate for 1 hour at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
7. **Do not wash the plate.** Add 50 μL of LTB_4 Conjugate to each well. All wells will now be violet in color except the NSB wells, which will be pink. Cover with a new adhesive strip. Incubate for 3 hours at room temperature on the shaker.
8. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
9. Add 200 μL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
10. Add 100 μL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample then subtract the average NSB optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on a linear y-axis against the concentration on a logarithmic x-axis and draw the best fit curve through the points on the graph. Do not include the B_0 in the standard curve.

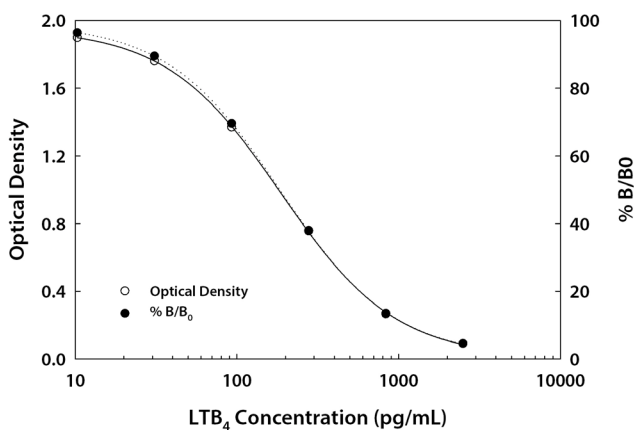
If desired, % B/ B_0 can be calculated by dividing the corrected O.D. for each standard or sample by the corrected B_0 O.D. and multiplying by 100.

Calculate the concentration of LTB₄ corresponding to the mean absorbance from the standard curve.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(pg/mL)	O.D.	Average	Corrected	% B/ B_0
NSB	0.009 0.010	0.009	—	—
0 (B_0)	1.974 1.980	1.977	1.968	—
10.3	1.908 1.909	1.908	1.899	96.4
30.9	1.747 1.794	1.770	1.761	89.5
92.6	1.356 1.403	1.379	1.370	69.6
278	0.756 0.777	0.766	0.757	38.0
833	0.269 0.282	0.275	0.266	13.5
2,500	0.098 0.102	0.100	0.091	4.6

PRECISION

Intra-assay Precision (Precision within an assay)

Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)

Three samples of known concentration were tested in forty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	170	481	847	164	516	879
Standard deviation	10	19	34	14.1	36.6	73.6
CV (%)	5.9	4.0	4.0	8.6	7.1	8.4

RECOVERY

The recovery of LTB₄ spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	95	86-114%
Serum (n=4)	98	82-118%
EDTA plasma (n=4)	91	82-112%
Heparin plasma (n=4)	98	82-115%

LINEARITY

To assess the linearity of the assay, samples spiked with high concentrations of LTB₄ were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	101	89	97	95
	Range (%)	93-107	79-100	88-102	89-103
1:4	Average % of Expected	105	102	113	108
	Range (%)	98-110	97-108	108-120	104-116
1:8	Average % of Expected	108	101	110	102
	Range (%)	101-115	91-118	99-120	95-111
1:16	Average % of Expected	113	102	108	102
	Range (%)	101-124	99-106	100-117	96-106

SENSITIVITY

Fifty assays were evaluated and the minimum detectable dose (MDD) of LTB₄ ranged from 3.7-10.9 pg/mL. The mean MDD was 8.2 pg/mL.

The MDD was determined by subtracting two standard deviations from the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of LTB₄ in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Serum (n=35)	138	100	33.5-564
EDTA plasma (n=35)	38.2	97	ND-72.8
Heparin plasma (n=35)	54.3	100	11.5-129

ND=Non-detectable

Note: LTA₄ hydrolase, a metal dependent enzyme present in serum and plasma, converts LTA₄ to LTB₄ (11). In samples collected in the presence of EDTA, this conversion is inhibited at the time the blood is drawn resulting in a more accurate measurement of circulating LTB₄. Other samples, especially serum samples, typically have higher levels of LTB₄ that result from the conversion of LTA₄ after the sample is drawn.

Cell Culture Supernates - Human peripheral blood cells (1 x 10⁶ cells/mL) were cultured in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 µg/mL PHA. Aliquots of the cell culture supernates were removed and assayed for levels of LTB₄.

Condition	Day 1 (pg/mL)	Day 5 (pg/mL)
Stimulated	61.8	56.6
Unstimulated	222	96.8

ND=Non-detectable

Note: Samples values have not been corrected for LTB₄ present in media.

SPECIFICITY

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range LTB₄ control were assayed for interference. No significant cross-reactivity or interference was observed.

Factors:

5(S)-HETE

12(S)-HETE

15(S)-HETE

Arachidonic acid

Arachidonyl ethanolamide

LTC₄

LTD₄

REFERENCES

1. Yokomizo, T. *et al.* (2001) Arch. Biochem. Biophys. **385**:231.
2. Hedi, H. and G. Norbert (2004) J. Biomed. Biotechnol. **4**:99.
3. Radmark, O. *et al.* (2007) Trends Biochem. Sci. **32**:332.
4. Haeggstrom, J.Z. and A. Wetterholm (2002) Cell. Mol. Life Sci. **59**:742.
5. Werz, O. (2002) Curr. Drug Targets Inflamm. Allergy **1**:23.
6. Tager, A.M. and A.D. Luster (2003) Prostaglandins Leukot. Essent. Fatty Acids **69**:123.
7. Iizuka, Y. *et al.* (2005) J. Biol. Chem. **280**:24816.
8. Kikuta, Y. *et al.* (2002) Prostaglandins Other Lipid Mediat. **68-69**:345.
9. Chung, J.W. *et al.* (2005) Exp. Mol. Med. **37**:45.
10. Terawaki, K. *et al.* (2005) J. Immunol. **175**:4217.
11. Fitzpatrick, F. *et al.* (1983) Proc. Natl. Acad. Sci. USA **80**:5425.

All trademarks and registered trademarks are the property of their respective owners.