Quantikine® ELISA

Human ENPP-2/Autotaxin Immunoassay

Catalog Number DENP20

For the quantitative determination of human ectonucleotide pyrophosphatase/ phosphodiesterase 2 (ENPP-2) concentrations in cell culture supernates, serum, plasma, urine, and human milk.

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

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INTRODUCTION

ENPP-2 (ectonucleotide pyrophosphatase/phosphodiesterase 2), also known as autotaxin (ATX), is a secreted glycoprotein that belongs to the ectonucleotide pyrophosphatase/phosphodiesterase (NPP) family (1-5). While NPPs generally hydrolyze phosphates from nucleotides and their derivatives, ENPP-2 uniquely exhibits a lysophospholipase D activity (6). ENPP-2 is not a type II transmembrane protein like other NPPs, but is a preproprotein with an N-terminal signal sequence and a proprotein convertase cleavage site (3, 7, 8). The mature active protein includes two somatomedin-B-like (SMB) cysteine knot domains, a catalytic domain, and an inactive C-terminal nuclease-like domain with an EF-hand-like motif that is important in cell motility, and a region involved in ENPP-2 secretion (1, 7, 9-11). The SMB domain also contains an RGD motif for integrin interaction. Both the SMB and C-terminal domains interact with the catalytic domain and are essential for enzymatic activity. Three isoforms have been described in mouse and human (8, 12). Most circulating ENPP-2 is the 863 amino acid (aa) β form (isoform 1, or "ter", found in teratocarcinoma). The 915 aa α form (isoform 2, or "mel" found in melanoma cells), which contains an inserted exon after aa 324, is rapidly cleaved and degraded. The 888 aa γ form (isoform 3) contains an inserted exon after aa 593 and is expressed mainly in brain oligodendrocytes.

ENPP-2 activity is the extracellular source of the bioactive phospholipid LPA (lysophosphatidic acid) from LPC (lysophosphatidylcholine) (13-17). ENPP-2 also produces minor amounts of S1P (sphingosine 1-phosphate) and cPA (cyclic phosphatidic acid); cPA can antagonize many of the tumorigenic properties of LPA (4, 14). ENPP-2 activity can be inhibited by its products LPA and S1P (8, 18). The hydrolysis of nucleotides and lysophospholipids by ENPP-2 is mediated by a catalytic site at Thr210 (1, 6, 13, 15). Mutation of this site in mice is lethal, as is deletion of ENPP-2 (14-17). LPA production, which is dependent upon ENPP-2 activity, is essential for formation and stability of embryonic and extraembryonic blood vessels and for closure of the neural tube (16, 17, 19, 20). LPA acts through a family of G-protein-coupled receptors (LPA1-6), some of which are termed EDG, of which LPA1/EDG2 is prominent. LPA receptors activate PLC, Ras-MAPK, PI3K and RhoA signaling cascades, and inhibit adenylate cyclase (8, 19).

ENPP-2 stimulates tumor cell motility and enhances invasion and metastasis (5, 6, 8, 22, 23). It is upregulated in melanoma, glioblastoma, breast and lung carcinoma, follicular lymphoma and other cancers, promoting inflammation, proliferation, migration and angiogenesis, and is a potential target for treatment (2, 8, 13, 23-26). ENPP-2 is normally widely expressed (8, 12). ENPP-2 expression is elevated in reactive astrocytes adjacent to sites of neurotrauma, and in rheumatoid arthritis synoviocytes, Alzheimer's disease frontal cortex, and multiple sclerosis cerebrospinal fluid (8, 20). It is thought to play a role in neuropathic pain (2, 3, 8). In oligodendrocyte precursors prior to myelination, it promotes process branching and focal adhesion organization (8, 10, 21). ENPP-2 production by adipocytes enhances pre-adipocyte proliferation and may be elevated in obesity (8, 27). ENPP-2 is also expressed in high endothelial venules of lymphoid organs, promoting actin reorganization, chemotaxis, and entry of lymphocytes into lymph nodes, spleen and Peyer's patches (28, 29). ENPP-2 binds platelets via integrin interaction, inducing platelet activation, but also attenuating aggregation and thrombosis (30). During wound healing, it promotes re-epithelialization (8, 10). ENPP-2 is present in blood, urine, saliva, seminal and cerebrospinal fluids (2, 3). Since plasma ENPP-2 is cleared by the liver, it is elevated in liver disease (3, 31). Normal serum or plasma ENPP-2 concentration is reported to be slightly higher in females than in males, and highest in pregnant females (2, 31).

The Quantikine Human ENPP-2/Autotaxin Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human ENPP-2 in cell culture supernates, serum, plasma, urine, and human milk. It contains NS0-expressed recombinant human ENPP-2 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human ENPP-2 showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that this kit can be used to determine relative mass values for naturally occurring human ENPP-2.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for ENPP-2 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any ENPP-2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for ENPP-2 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of ENPP-2 bound in the initial step. The color development is stopped and the intensity of the color is measured..

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, further dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- 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 the Quantikine Immunoassay, 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.
- 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.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
ENPP-2 Microplate	893995	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody against ENPP-2.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
ENPP-2 Conjugate	893996	21 mL of a polyclonal antibody against ENPP-2 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
ENPP-2 Standard	893997	100 ng of recombinant human ENPP-2 in a buffer with preservatives; lyophilized.	
Assay Diluent RD1-34	895265	11 mL of a buffer with preservatives.	
Calibrator Diluent RD5-10	895266	21 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

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

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm 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 and samples.
- Human ENPP-2 Controls (optional; available from R&D Systems).

PRECAUTIONS

ENPP-2 is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

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

Some components in this kit contain ProClin® 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. Please 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.

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

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.

Plasma - Collect plasma using 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.

Note: *EDTA and citrate plasma are not recommended for use in this assay due to their chelating properties.*

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Human Milk - Centrifuge for 15 minutes at 1000 x g at 2-8 °C. Collect the aqueous fraction and repeat this process a total of 3 times. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Serum and heparin plasma samples require a 20-fold dilution. A suggested 20-fold dilution is 20 µL of sample + 380 µL of Calibrator Diluent RD5-10.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

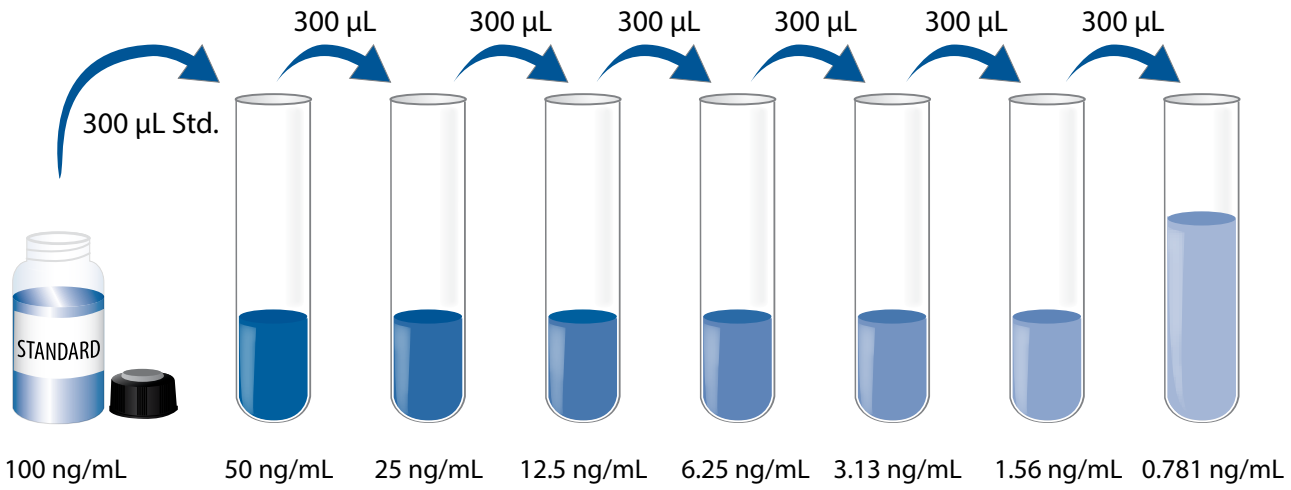
Note: *High concentrations of ENPP-2 are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

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 µL of the resultant mixture is required per well.

ENPP-2 Standard - Reconstitute the ENPP-2 Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 100 ng/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 300 µL of Calibrator Diluent RD5-10 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 50 ng/mL standard serves as the high standard. Calibrator Diluent RD5-10 serves as the zero standard (0 ng/mL).



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.

Note: *ENPP-2 is found in saliva. It is recommended that a face mask and gloves are used to protect kit reagents from contamination.*

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 Assay Diluent RD1-34 to each well.
4. Add 50 µL of Standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
5. 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.
6. Add 200 µL of ENPP-2 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 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.
10. 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.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

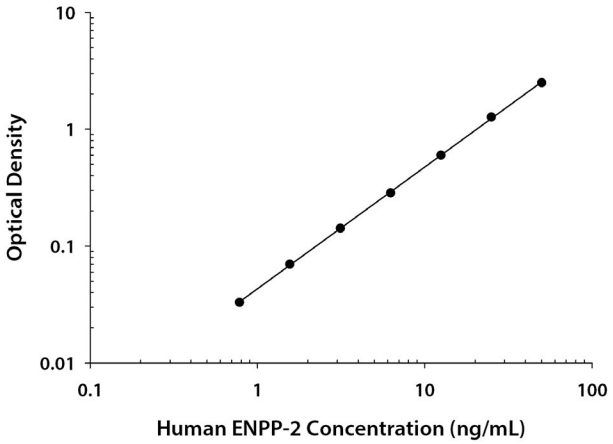
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the human ENPP-2 concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

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.



(ng/mL)	O.D.	Average	Corrected
0	0.052 0.054	0.053	—
0.781	0.085 0.087	0.086	0.033
1.56	0.122 0.124	0.123	0.070
3.13	0.192 0.198	0.195	0.142
6.25	0.337 0.338	0.338	0.285
12.5	0.651 0.653	0.652	0.599
25	1.315 1.328	1.322	1.269
50	2.503 2.603	2.553	2.500

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 twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	6.5	13.8	28.9	6.8	14.0	28.9
Standard deviation	0.18	0.51	0.74	0.32	0.55	0.83
CV (%)	2.8	3.7	2.6	4.7	3.9	2.9

RECOVERY

The recovery of ENPP-2 spiked to levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	102	93-109%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of ENPP-2 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)	Heparin plasma* (n=4)	Human milk (n=4)
1:2	Average % of Expected	102	103	100	95
	Range (%)	99-108	98-108	97-102	89-99
1:4	Average % of Expected	97	102	98	96
	Range (%)	93-101	96-111	94-102	88-104
1:8	Average % of Expected	94	97	95	———
	Range (%)	90-99	91-102	88-106	———
1:16	Average % of Expected	91	92	90	———
	Range (%)	85-97	86-100	82-99	———

*Samples were diluted prior to assay as directed in the Sample Preparation section.

SENSITIVITY

Twenty-two assays were evaluated and the minimum detectable dose (MDD) of ENPP-2 ranged from 0.055-0.157 ng/mL. The mean MDD was 0.093 ng/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant human ENPP-2 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma/Urine/Human Milk - Samples from apparently healthy volunteers were evaluated for the presence of ENPP-2 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum, male (n=11)	218	175-274	30.2
Serum, female (n=25)	345	217-1330	219
Heparin plasma, male (n=11)	218	167-286	34.3
Heparin plasma, female (n=25)	337	212-1309	217
Urine (n=10; 60% detectable)	1.40	ND-1.78	——
Human milk (n=4)	3.63	3.33-4.08	0.328

ND=Non-detectable

Cell Culture Supernates:

Hs 294T human melanoma cells were cultured in DMEM supplemented with 10% fetal bovine serum and grown to confluency. An aliquot was removed, assayed for natural human ENPP-2, and measured 5.31 ng/mL.

MEL-Juso human skin melanoma cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and grown to confluency. An aliquot was removed, assayed for natural human ENPP-2, and measured 0.910 ng/mL.

MRC-5 human embryonic lung fibroblast cells were cultured in MEM/NEAA supplemented with 10% fetal bovine serum and grown to confluency. An aliquot was removed, assayed for natural human ENPP-2, and measured 2.40 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human ENPP-2.

The factors listed below were prepared at 500 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 500 ng/mL in a mid-range recombinant human ENPP-2 Control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

ENPP-5
ENPP-7
LPAR1
LPAR2

Recombinant mouse:

ENPP-2

REFERENCES

1. Cimpean, A. *et al.* (2004) Biochem. J. **381**:71.
2. Nakanaga, K. *et al.* (2010) J. Biochem. **148**:13.
3. Okudaira, S. *et al.* (2010) Biochimie **92**:698.
4. Tania, M. *et al.* (2010) Biochem. Biophys. Res. Commun. **401**:493.
5. Stracke, M.L. *et al.* (1992) J. Biol. Chem. **267**:2524.
6. Umezu-Goto, M. *et al.* (2002) J. Cell Biol. **158**:227.
7. Jansen, S. *et al.* (2005) J. Cell Sci. **118**:3081.
8. van Meeteren, L.A. and W.H. Moolenaar (2007) Prog. Lipid Res. **46**:145.
9. Hausmann, J. *et al.* (2011) Nat. Struct. Mol. Biol. **18**:198.
10. Dennis, J. *et al.* (2008) Mol. Cell. Neurosci. **37**:412.
11. Jansen, S. *et al.* (2009) J. Biol. Chem. **284**:14296.
12. Giganti, A. *et al.* (2008) J. Biol. Chem. **283**:7776.
13. Gijsbers, R. *et al.* (2003) FEBS Letters. **538**:60.
14. Tsuda, S. *et al.* (2006) J. Biol. Chem. **281**:26081.
15. Ferry, G. *et al.* (2007) FEBS Lett. **581**:3572.
16. van Meeteren, L.A. *et al.* (2006) Mol. Cell. Biol. **26**:5015.
17. Tanaka, M. *et al.* (2006) J. Biol. Chem. **281**:25822.
18. van Meeteren, L.A. *et al.* (2005) J. Biol. Chem. **280**:21155.
19. Koike, S. *et al.* (2009) J. Biol. Chem. **284**:33561.
20. Fotopoulou, S. *et al.* (2010) Dev. Biol. **339**:451.
21. Savaskan, N.E. *et al.* (2007) Cell Mol. Life Sci. **64**:230.
22. Nam, S.W. *et al.* (2000) Oncogene **19**:241.
23. Nam, S.W. *et al.* (2001) Cancer Res. **61**:6938.
24. Liu, S. *et al.* (2009) Cell Cycle **8**:3695.
25. Kishi, Y. *et al.* (2006) J. Biol. Chem. **281**:17492.
26. Masuda, A. *et al.* (2008) Br. J. Haematol. **143**:60.
27. Ferry, G. *et al.* (2003) J. Biol. Chem. **278**:18162.
28. Kanda, H. *et al.* (2008) Nat. Immunol. **9**:415.
29. Nakasaki, T. *et al.* (2008) Am. J. Pathol. **173**:1566.
30. Pamuklar, Z. *et al.* (2009) J. Biol. Chem. **284**:7385.
31. Nakamura, K. *et al.* (2008) Clin. Chim. Acta **388**:51.