

Quantikine[®]

Rhesus Macaque TNF- α Immunoassay

Catalog Number RHMTA0

For the quantitative determination of rhesus macaque Tumor Necrosis Factor alpha (TNF- α) 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

Contents	Page
INTRODUCTION	2
PRINCIPLE OF THE ASSAY.	3
LIMITATIONS OF THE PROCEDURE	3
PRECAUTIONS	3
TECHNICAL HINTS	4
MATERIALS PROVIDED.	4
STORAGE	5
OTHER SUPPLIES REQUIRED	5
SAMPLE COLLECTION AND STORAGE	5
REAGENT PREPARATION	6
ASSAY PROCEDURE	7
PROCEDURE SUMMARY AND CHECKLIST	8
CALCULATION OF RESULTS	9
TYPICAL DATA	9
PRECISION	10
RECOVERY.	10
LINEARITY	11
SENSITIVITY	11
CALIBRATION	11
SAMPLE VALUES	12
SPECIFICITY	12
REFERENCES	13
PLATE LAYOUT	14

MANUFACTURED AND DISTRIBUTED BY:

R&D Systems, Inc.
614 McKinley Place NE
Minneapolis, MN 55413
United States of America

TELEPHONE:
FAX:
E-MAIL:

(800) 343-7475
(612) 379-2956
(612) 656-4400
info@RnDSystems.com

DISTRIBUTED BY:

R&D Systems Europe, Ltd.
19 Barton Lane
Abingdon Science Park
Abingdon, OX14 3NB
United Kingdom

TELEPHONE:
FAX:
E-MAIL:

+44 (0)1235 529449
+44 (0)1235 533420
info@RnDSystems.co.uk

R&D Systems China Co. Ltd.
24A1 Hua Min Empire Plaza
726 West Yan An Road
Shanghai PRC 200050

TELEPHONE:
FAX:
E-MAIL:

+86 (21) 52380373
+86 (21) 52371001
info@RnDSystemsChina.com.cn

INTRODUCTION

Rhesus macaque tumor necrosis factor alpha (TNF- α), also known as TNFSF1A, is a 51 kDa homotrimeric protein that plays a central role in inflammation, metabolism and apoptosis (1 - 4). It is synthesized as a 26 kDa, type II transmembrane protein that is 233 amino acids in length (5), with a 35 amino acid (aa) cytoplasmic domain, a 21 aa transmembrane (TM) segment, and a 177 aa extracellular region that is divided into a short membrane proximal "linker-region", and a 17 kDa extended mature segment (6, 7). TNF- α is assembled internally as a non-covalent homotrimer, and exists in this form on the cell surface (3, 8). Metalloproteinase cleavage releases soluble trimeric TNF- α (3). Based on human studies, rhesus macaque TNF- α will not be glycosylated. It will, however, show myristoylation (9) and phosphorylation (10) on its cytoplasmic domain. Rhesus macaque soluble TNF- α shows 98% aa sequence identity with the human (11). Soluble mature TNF- α from mouse (12), baboon (13), squirrel monkey (14), crab-eating macaque (15), chimpanzee (16) and canine (17) also shows 79%, 99%, 89%, 99%, 96% and 94% aa sequence identity, respectively to soluble rhesus macaque TNF- α . A partial sequence from owl monkey reveals 92% aa sequence identity (18). Mammalian cells known to express TNF- α include B cells (19), colonic columnar epithelial cells (20), NK and CD3⁺CD56⁺ natural T cells (21), macrophages (22), monocytes and monocyte-derived dendritic cells (23), CD4⁺ and CD8⁺ T cells (24), mast cells (25), neutrophils (26), keratinocytes (27), plasma cells (28), adipocytes (29) and astrocytes (30).

There are two receptors for TNF- α . Neither have been reported in rhesus macaque. The first receptor (TNF RI or TNFRSF1A) in human is a 55 - 60 kDa type I TM glycoprotein that binds TNF- α with a high affinity (31, 32). It is characterized by the presence of a cytoplasmic death domain that may mediate apoptosis. The second receptor (TNF RII or TNFRSF1B) in human is a 75 - 80 kDa type I TM glycoprotein that also binds TNF- α with high affinity (2, 32). This receptor has little homology to TNF RI. Although each receptor can individually mediate TNF- α activity, physiological activities often require the presence and interaction of both receptors (33 - 35).

Functionally, TNF- α is involved in a number of pathophysiological processes. It is considered one of the prototypical pro-inflammatory molecules and is induced in macrophages by gram-negative bacteria (LPS). TNF- α is reported to promote inflammatory cell infiltration by upregulating leukocyte adhesion molecules on endothelial cells, serve as a chemotactic agent for monocytes, and also activate phagocyte killing mechanisms (via increased NO₂⁻/O₂⁻/H₂O₂) (36). Cachexia (whole body wasting) has also been associated with long-term circulating TNF- α . This may well be a consequence of the role TNF- α is known to play in modulating adipocyte function, impacting both carbohydrate and lipid metabolism (4, 37).

The Quantikine Rhesus Macaque TNF- α Immunoassay is a 4.5 hour solid phase ELISA designed to measure rhesus macaque TNF- α levels in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant rhesus macaque TNF- α and antibodies raised against recombinant rhesus macaque TNF- α . This immunoassay has been shown to quantitate the recombinant rhesus macaque TNF- α accurately. Results obtained using natural rhesus macaque TNF- α showed dose curves that were parallel to the standard curves obtained using the recombinant Quantikine kit standards. These results indicate that the Quantikine rhesus macaque TNF- α Immunoassay kit can be used to determine relative mass values for natural rhesus macaque TNF- α .

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for rhesus macaque TNF- α has been pre-coated onto a microplate. Standards, Control, and samples are pipetted into the wells and any rhesus macaque TNF- α present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for rhesus macaque TNF- α is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of rhesus macaque TNF- α bound in the initial step. The sample values are then read off the standard curve.

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 operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors or other binding proteins present in biological samples. Until all receptors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

Wear gloves and take appropriate biohazard precautions when working with samples.

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.
- For best results, pipette reagents and samples into the center of each well.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- 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. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.
- Stop Solution should be added to the plate in the same order as the Substrate Solution.

MATERIALS PROVIDED

Rhesus Macaque TNF- α Microplates (Part 892676) - One 96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for rhesus macaque TNF- α .

Rhesus Macaque TNF- α Conjugate (Part 892677) - 12.5 mL of a polyclonal antibody against rhesus macaque TNF- α conjugated to horseradish peroxidase with preservatives.

Rhesus Macaque TNF- α Standard (Part 892678) - 2 vials (2 ng/vial) of recombinant rhesus macaque TNF- α in a buffered protein solution with preservatives; lyophilized.

Rhesus Macaque TNF- α Control (Part 892779) - 2 vials of recombinant rhesus macaque TNF- α in a buffered protein solution with preservatives; lyophilized. The concentration range of rhesus macaque TNF- α after reconstitution is shown on the vial label. The assay value of the Control should be within the range specified on the label.

Assay Diluent RD1-63 (Part 895352) - 12.5 mL of a buffered protein solution with preservatives.

Calibrator Diluent RD5-16 (Part 895302) - 21 mL of a buffered protein solution with preservatives.

Wash Buffer Concentrate (Part 895024) - 50 mL of a 25-fold concentrated solution of a buffered surfactant with preservative.

Color Reagent A (Part 895000) - 12.5 mL of stabilized hydrogen peroxide.

Color Reagent B (Part 895001) - 12.5 mL of stabilized chromogen (tetramethylbenzidine).

Stop Solution (Part 895174) - 23 mL of a diluted hydrochloric acid solution.

Plate Covers (Part 640197) - 4 adhesive plate sealers.

STORAGE

Unopened Kit	Store at 2 - 8° C. Do not use beyond kit expiration date.	
Opened/ Reconstituted Reagents	Diluted Wash Buffer	May be stored for up to 1 month at 2 - 8° C.*
	Stop Solution	
	Calibrator Diluent RD5-16	
	Assay Diluent RD1-63	
	Rhesus Macaque TNF- α Conjugate	
	Unmixed Color Reagent A	
	Unmixed Color Reagent B	Use a new Standard and Control for each assay.
	Rhesus Macaque TNF- α Standard (1000 pg/mL)	
	Rhesus Macaque TNF- α Control	
Microplate Wells	Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*	

*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.
- 100 mL and 1000 mL graduated cylinders.
- Test tubes for serial dilution.

SAMPLE COLLECTION AND STORAGE

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Serum - Allow blood samples to clot for 2 hours at room temperature or overnight at 2 - 8° C before centrifuging for 20 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA as an anticoagulant. Centrifuge for 20 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Note: *Heparin and citrate plasma have not been validated for use in this assay.*

REAGENT PREPARATION

Bring all reagents to room temperature before use.

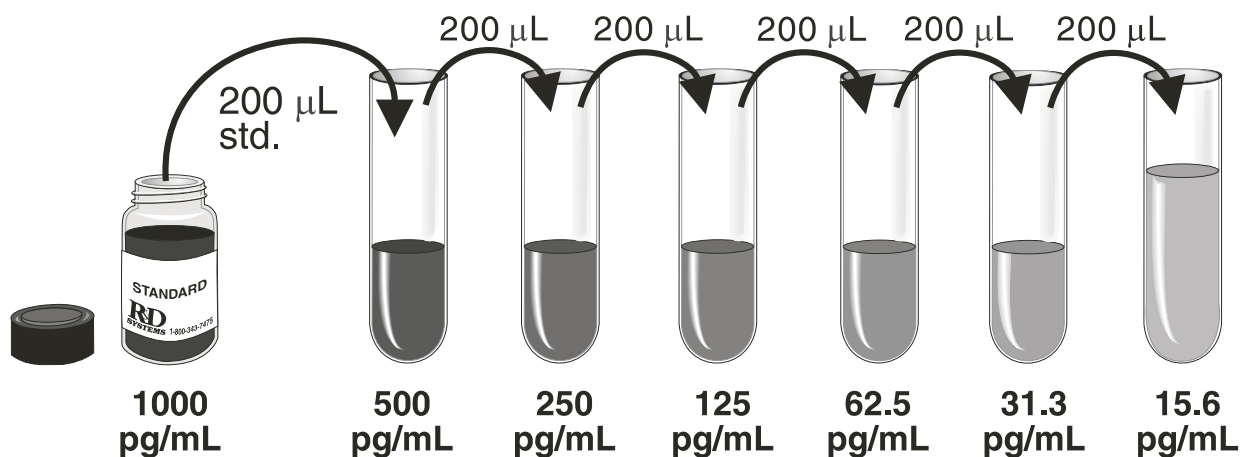
Rhesus Macaque TNF- α Kit Control - Reconstitute the Kit Control with 1.0 mL deionized or distilled water. Assay the Control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough Wash Buffer for one plate, add 25 mL Wash Buffer Concentrate into deionized or distilled water to prepare 625 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. 100 μ L of the resultant mixture is required per well.

Rhesus Macaque TNF- α Standard - Reconstitute the rhesus macaque TNF- α Standard with 2.0 mL of Calibrator Diluent RD5-16. Do not substitute other diluents. This reconstitution produces a stock solution of 1000 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Pipette 200 μ L of Calibrator Diluent RD5-16 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted rhesus macaque TNF- α Standard serves as the high standard (1000 pg/mL). Calibrator Diluent RD5-16 serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

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

1. Prepare reagents and standard dilutions 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 50 μL of Assay Diluent RD1-63 to each well.
4. Add 50 μL of Standard, Control, or sample per well. Mix by gently tapping the plate frame for 1 minute. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided as a record of samples and standards assayed.
5. Aspirate each well and wash, repeating the process four times for a total of five 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 100 μL of Rhesus Macaque TNF- α Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 μL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 100 μL of Stop Solution to each well. 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.

PROCEDURE SUMMARY AND CHECKLIST

1. Bring all reagents to room temperature.
 Prepare reagents and samples as instructed.
 Return unused components to storage temperature as indicated in the instructions.

2. Add 50 μL Assay Diluent to each well.

3. Add 50 μL Standard, Control, or sample to each well.
 Tap plate gently for one minute.
 Cover the plate and incubate for 2 hours at room temperature.

4. Aspirate and wash each well five times.

5. Add 100 μL Conjugate to each well.
 Cover the plate and incubate for 2 hours at room temperature.

6. Aspirate and wash each well five times.

7. Add 100 μL Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**

8. Add 100 μL Stop Solution to each well.

9. Read Optical Density at 450 nm (correction wavelength set at 540 nm or 570 nm).

CALCULATION OF RESULTS

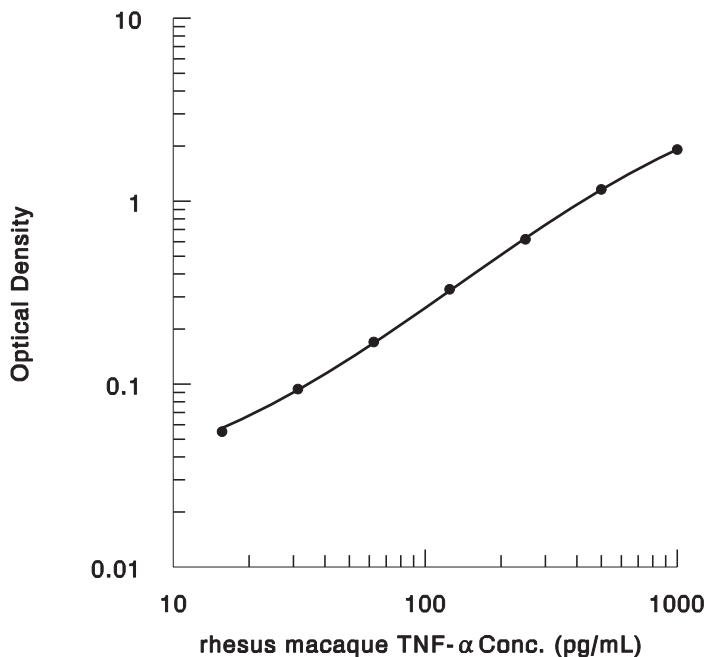
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

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 the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the rhesus macaque TNF- α concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

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
0	0.076 0.076	0.076	—
15.6	0.129 0.133	0.131	0.055
31.3	0.166 0.173	0.170	0.094
62.5	0.244 0.247	0.246	0.170
125	0.405 0.406	0.406	0.330
250	0.692 0.698	0.695	0.619
500	1.183 1.288	1.236	1.160
1000	1.930 2.052	1.991	1.915

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.

Sample	Intra-assay Precision			Inter-assay Precision		
	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	61	123	492	56	111	465
Standard deviation	3.6	7.4	35.7	5.6	7.2	41.8
CV (%)	5.9	6.0	7.3	10.0	6.5	9.0

RECOVERY

The recovery of rhesus macaque TNF- α spiked to three levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range (%)
Cell culture supernates (n = 4)	110	104 - 120%
Serum (n = 4)	98	90 - 109%
EDTA plasma (n = 3)	94	89 - 99%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with various concentrations of rhesus macaque TNF- α in each matrix were diluted with Calibrator Diluent RD5-16 and then assayed.

		Cell culture supernates (n = 5)	Serum (n = 4)	EDTA plasma (n = 3)
1:2	Average % of Expected	96	101	98
	Range (%)	87 - 105	96 - 104	85 - 106
1:4	Average % of Expected	91	95	102
	Range (%)	81 - 98	88 - 100	90 - 112
1:8	Average % of Expected	92	99	98
	Range (%)	81 - 111	91 - 109	87 - 110
1:16	Average % of Expected	89	100	101
	Range (%)	80 - 95	85 - 113	96 - 107

SENSITIVITY

Thirteen assays were evaluated and the minimum detectable dose (MDD) of rhesus macaque ranged from 2.6 - 6.2 pg/mL. The mean MDD was 4.1 pg/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 *E. coli*-expressed recombinant rhesus macaque TNF- α produced at R&D Systems. The 157 amino acid residue protein has a calculated molecular mass of approximately 17.4 kDa.

The protein concentration of the recombinant rhesus macaque TNF- α was determined by the method of Bradford (38) using purified bovine serum albumin as a standard.

SAMPLE VALUES

Serum/Plasma - Four serum and three EDTA plasma samples were evaluated for detectable levels of rhesus macaque TNF- α in this assay. All serum and plasma samples tested measured below the lowest standard, 15.6 pg/mL.

Cell Culture Supernates - Rhesus macaque peripheral blood mononuclear cells (1×10^6 cells/mL) were cultured for 24 and 96 hours in RPMI supplemented with 10% fetal calf serum, and stimulated with 0.5 μ g/mL calcium ionomycin and 0.05 μ g/mL PMA. Aliquots of the cell culture supernate were removed, tested for levels of natural rhesus macaque TNF- α and measured 2856 pg/mL and 946 pg/mL, respectively.

SPECIFICITY

This assay recognizes both recombinant and natural rhesus macaque TNF- α . The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD5-16 and assayed for cross-reactivity. Preparations of the following factors prepared at 50 ng/mL in a mid-range rhesus macaque TNF- α control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant rhesus macaque:

IFN- γ
IL-1 β
IL-4
IL-13

Recombinant mouse:

TNF- α
TNF RI
TNF RII

Recombinant human:

TNF- β
TNF RI
TNF RII/Fc Chimera

Other recombinants:

canine TNF- α
porcine TNF- α
rat TNF- α

Recombinant human TNF- α showed 100% cross-reactivity in this assay.

REFERENCES

1. Kwon, B. *et al.* (1999) *Curr. Opin. Immunol.* **11**:340.
2. Idriss, H.T. and J.H. Naismith (2000) *Microsc. Res. Tech.* **50**:184.
3. Hehlhans, T. and D.N. Mannel (2002) *Biol. Chem.* **383**:1581.
4. Warne, J.P. (2003) *J. Endocrinol.* **177**:351.
5. Villinger, F. *et al.* (1995) *J. Immunol.* **155**:3946.
6. Ishisaka, R. *et al.* (1999) *J. Biochem.* **126**:413.
7. Kriegler, M. *et al.* (1988) *Cell* **53**:45.
8. Tang, P. *et al.* (1996) *Biochemistry* **35**:8216.
9. Stevenson, F.T. *et al.* (1992) *J. Exp. Med.* **176**:1053.
10. Watts, A.D. *et al.* (1999) *EMBO J.* **18**:2119.
11. Pennica, D. *et al.* (1984) *Nature* **312**:724.
12. Pennica, D. *et al.* (1985) *Proc. Natl. Acad. Sci. USA* **82**:6060.
13. Haudek, S.B. *et al.* (1997) *Mol. Immunol.* **34**:1041.
14. Heraud, J-M. *et al.* (2002) *Immunogenetics* **54**:20.
15. Tatsumi, M. (1999) GenBank Accession #BAA19131.
16. Kulski, J.K. *et al.* (2002) *Immunol. Rev.* **190**:95.
17. Zucker, K. *et al.* (1994) *Lymphokine Res.* **13**:191.
18. Hernandez, E.C. *et al.* (2002) *Immunogenetics* **54**:645.
19. Corione, A. *et al.* (1997) *Blood* **90**:4493.
20. Jung, H.C. *et al.* (1995) *J. Clin. Invest.* **95**:55.
21. Doherty, D.G. *et al.* (1999) *J. Immunol.* **163**:2314.
22. Jovanovic, D.V. *et al.* (1998) *J. Immunol.* **160**:3513.
23. Avice, M-N. *et al.* (1999) *J. Immunol.* **162**:2748.
24. Cuturi, M.C. *et al.* (1987) *J. Exp. Med.* **165**:1581.
25. Lorentz, A. *et al.* (2000) *J. Immunol.* **164**:43.
26. Bliss, S.K. *et al.* (1999) *J. Immunol.* **162**:7369.
27. Ezepechuk, Y.V. *et al.* (1996) *J. Invest. Dermatol.* **107**:603.
28. Di Girolamo, N. *et al.* (1997) *J. Leukoc. Biol.* **61**:667.
29. Voros, G. *et al.* (2003) *Biochim. Biophys. Acta* **1625**:36.
30. Wang, Z. *et al.* (2002) *Acta Pharmacol. Sin.* **23**:974.
31. Aggarwal, B.B. *et al.* (1985) *Nature* **318**:665.
32. Schall, T.J. *et al.* (1990) *Cell* **61**:361.
33. Ruby, J. *et al.* (1997) *J. Exp. Med.* **186**:1591.
34. Lazdins, J.K. *et al.* (1997) *J. Exp. Med.* **185**:81
35. Pinckard, J.K. *et al.* (1997) *J. Biol. Chem.* **272**:10784.
36. Vassalli, P. (1992) *Annu. Rev. Immunol.* **10**:411.
37. Finck, B.N. and R.W. Johnson (2000) *Microsc. Res. Tech.* **50**:209.
38. Bradford, M.M. (1976) *Anal. Biochem.* **72**:248.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

1									
2									
3									
4									
5									
6									
7									
8									
9									
10									
11									
12									
	A	B	C	D	E	F	G	H	

NOTES