Quantikine® HS ELISA

Human TNF-α Immunoassay

Catalog Number HSTA00E
SSTA00E
PHSTA00E

For the quantitative determination of human Tumor Necrosis Factor alpha (TNF-α) concentrations in 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

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

Tumor Necrosis Factor alpha (TNF-α), also known as cachectin and TNFSF1A, is the prototypic ligand of the TNF superfamily (1). It is a pleiotropic molecule that plays a central role in inflammation, immune system development, apoptosis, and lipid metabolism (2-5). TNF-α is also involved in a number of pathological conditions including asthma, Crohn's disease, rheumatoid arthritis, neuropathic pain, obesity, type 2 diabetes, septic shock, autoimmunity, and cancer (5-11).

Human TNF-α is synthesized as a 26 kDa type II transmembrane protein that consists of a 35 amino acid (aa) cytoplasmic domain, a 21 aa transmembrane segment, and a 177 aa extracellular domain (ECD) (12, 13). Within the ECD, human TNF-α shares 97% aa sequence identity with rhesus monkey, and 71%-92% aa identity with bovine, canine, cotton rat, equine, feline, mouse, porcine, and rat TNF-α. It is produced by a wide variety of immune, epithelial, endothelial, and tumor cells. TNF-α is assembled intracellularly to form a noncovalently linked homotrimer which is expressed on the cell surface (14). Cell surface TNF-α can both induce the lysis of tumor cells and virus infected cells, and generate its own downstream cell signaling following ligation by soluble TNF RI (15, 16). Shedding of membrane bound TNF-α by TACE/ADAM17 releases the bioactive cytokine, a 55 kDa soluble trimer of the TNF-α extracellular domain (17-19).

TNF-α binds the ubiquitous 55-60 kDa TNF RI (20, 21) and the hematopoietic cell-restricted 78-80 kDa TNF RII (22, 23), both of which are also expressed as homotrimers (1, 24). Both type I and type II receptors bind TNF-α with comparable affinity and can promote NFκB activation (25-28). Only TNF RI, however, contains a cytoplasmic death domain which triggers the activation of apoptosis (3, 29). Soluble forms of both types of receptors are released into human serum and urine and can neutralize the biological activity of TNF-α (30-32).

The Quantikine® HS Human TNF-α Immunoassay is a 4.0 hour solid phase ELISA designed to measure human TNF-α in serum and plasma. It contains *E. coli*-derived recombinant human TNF-α and antibodies raised against the recombinant factor. It has been shown to accurately quantitate recombinant human TNF-α. Results obtained with natural human TNF-α samples 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 natural human TNF-α.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human TNF-α has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TNF-α present is bound by the immobilized antibody. After washing away any unbound substances, a biotinylated polyclonal antibody specific for human TNF-α is added to the wells. Following a wash to remove any unbound antibody-biotin reagent, an enzyme-linked streptavidin is added to the wells. After washing away any unbound streptavidin-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of TNF-α 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.
- Any variation in 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

- To ensure accurate results, bring liquids to room temperature and mix to homogeneity prior to pipetting or aliquoting.
- When mixing protein solutions, always avoid foaming.
- 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.

PRECAUTIONS

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.

MATERIALS PROVIDED & STORAGE CONDITIONS

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

PART	PART #	CATALOG # HSTA00E	CATALOG # SSTA00E	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human TNF-α HS Microplate	898530	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human TNF-α.	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.*
Human TNF-α HS Standard	898532	2 vials	12 vials	Recombinant human TNF-α in a buffered protein base with preservatives; lyophilized. *Refer to vial label for reconstitution volume.*	Use a new standard for each assay. Discard after use.
Human TNF-α HS Conjugate	898531	1 vial	6 vials	21 mL/vial of a polyclonal antibody specific for human TNF-α conjugated to biotin with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-40	895513	1 vial	6 vials	12 mL/vial of a buffered protein base with preservatives.	
Calibrator Diluent RD6S	895142	1 vial	6 vials	21 mL/vial of animal serum with preservatives.	
Wash Buffer Concentrate	895003	2 vials	12 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Stop Solution	895032	1 vial	6 vials	6 mL/vial of 2 N sulfuric acid.	
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).	
Streptavidin Polymer-HRP Diluent	898387	1 vial	6 vials	21 mL/vial of a solution with preservatives.	
Streptavidin Polymer-HRP (100X)	898350	1 vial	6 vials	0.3 mL/vial of Streptavidin Polymer-HRP in a buffer with preservative.	
Plate Sealers	N/A	8 strips	48 strips	Adhesive strips.	

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

HSTA00E contains sufficient materials to run an ELISA on one 96 well plate.
SSTA00E (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems®, Catalog # PHSTA00E). 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 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 1000 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.
- Human TNF-α Controls (optional; R&D Systems®, Catalog # QC232).

SAMPLE COLLECTION & STORAGE

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

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 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.

Note: *Citrate plasma is not validated for use in this assay.*
Grossly hemolyzed samples are not suitable for use in this assay.
High albumin samples are not suitable for use in this assay.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

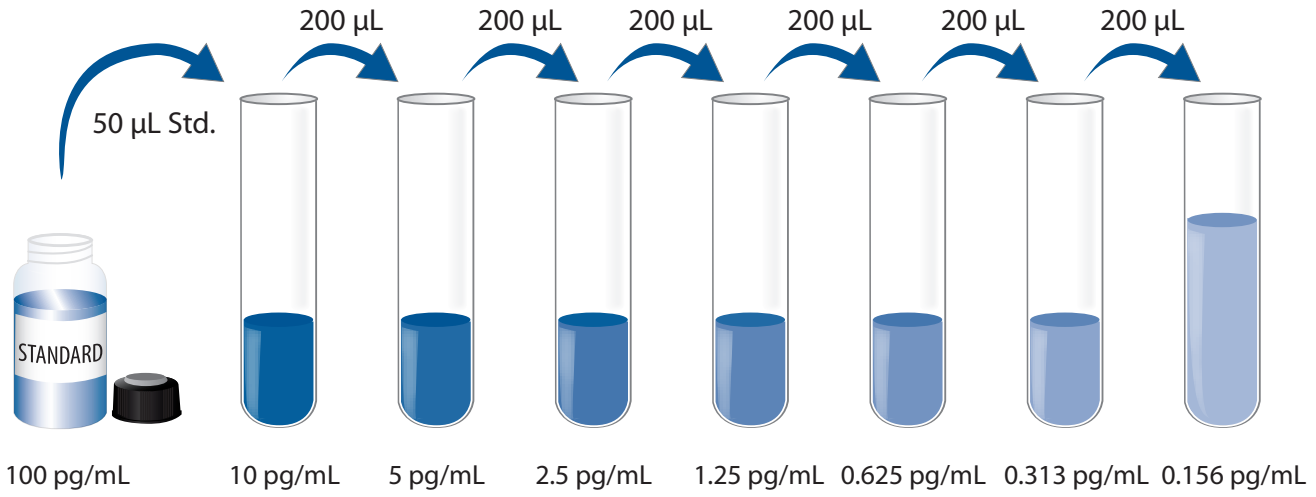
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 40 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 1000 mL of Wash Buffer.

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.

Streptavidin Polymer-HRP (1X) - Add 0.215 mL of Streptavidin Polymer-HRP (100X) directly to the Streptavidin Polymer-HRP Diluent. Mix well.

Human TNF-α HS Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human TNF-α HS Standard with deionized or distilled water. This reconstitution produces a stock solution of 100 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle agitation prior to making dilutions.

Pipette 450 µL of Calibrator Diluent RD6S into the 10 pg/mL tube. Pipette 200 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 10 pg/mL standard serves as the high standard. Calibrator Diluent RD6S 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 and standards be assayed in duplicate.

1. Prepare all reagents and working standards 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 RD1-40 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. A plate layout is provided to record standards and samples assayed.
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 Human TNF-α HS Conjugate to each well. Cover with a new adhesive strip. Incubate for **1 hour** at room temperature on the shaker.
7. Repeat the wash as in step 5.
8. Add 200 µL of Streptavidin Polymer-HRP (1X) to each well. Cover with a new adhesive strip. Incubate for **30 minutes** at room temperature on the shaker.
9. Repeat the wash as in step 5.
10. Add 200 µL of Substrate Solution to each well. Incubate for **30 minutes** at room temperature **on the benchtop. Protect from light.**
11. 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.
12. 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

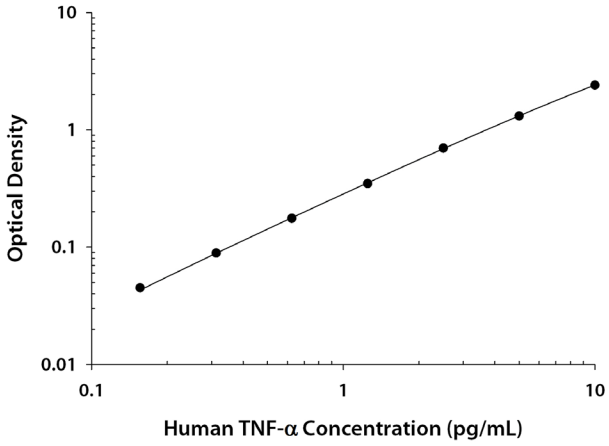
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a 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 human 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 measured concentrations 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	0.072 0.073	0.073	—
0.156	0.117 0.119	0.118	0.045
0.313	0.160 0.164	0.162	0.089
0.625	0.247 0.251	0.249	0.176
1.25	0.417 0.424	0.421	0.348
2.5	0.769 0.773	0.771	0.698
5	1.383 1.388	1.386	1.313
10	2.480 2.484	2.482	2.409

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 (pg/mL)	1.07	3.30	6.71	1.09	3.26	6.51
Standard deviation	0.024	0.066	0.129	0.073	0.220	0.406
CV (%)	2.2	2.0	1.9	6.7	6.7	6.2

RECOVERY

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

Sample Type	Average % Recovery	Range
Serum (n=4)	94	83-103%
EDTA plasma (n=4)	97	80-111%
Heparin plasma (n=4)	96	80-111%

LINEARITY

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

		Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	96	86	89
	Range (%)	95-99	84-91	85-92
1:4	Average % of Expected	97	90	92
	Range (%)	94-99	88-93	89-98
1:8	Average % of Expected	101	89	97
	Range (%)	92-109	83-94	92-102
1:16	Average % of Expected	93	95	99
	Range (%)	81-100	87-104	89-106

SENSITIVITY

Tweny-three assays were evaluated and the minimum detectable dose (MDD) of human TNF-α ranged from 0.011-0.049 pg/mL. The mean MDD was 0.022 pg/mL.

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

CALIBRATION

This immunoassay is calibrated against a highly purified *E. coli*-expressed recombinant human TNF-α produced at R&D Systems®.

The NIBSC/WHO Human TNF-α 3rd International Standard 12/154 (rDNA derived), which was intended as a potency standard, was evaluated in this kit.

The dose response curve of this 3rd International Standard parallels the Quantikine® HS standard curve. To convert sample values obtained with the Quantikine® HS Human TNF-α kit to approximate NIBSC/WHO 12/154 values, use the equation below.

NIBSC/WHO (12/154) approximate value (IU/mL) = 0.537 x Quantikine® Human HS TNF-α value (pg/mL)

Note: *Based on data generated in December 2016.*

SAMPLE VALUES

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

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=30)	1.12	0.753-1.66	0.243
EDTA plasma (n=30)	1.12	0.679-1.61	0.244
Heparin plasma (n=30)	1.15	0.665-2.10	0.332

SPECIFICITY

This assay recognizes natural and recombinant human TNF-α.

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 recombinant human TNF-α control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:	Other recombinants:
CD40	bovine TNF-α
CD40 Ligand	canine TNF-α
Fas Ligand	cotton rat TNF-α
LIGHT	equine TNF-α
TL-1A	feline TNF-α
TNF-β	guinea pig TNF-α
TRAIL	mouse TNF-α
TRANCE	porcine TNF-α
	rat TNF-α
	rhesus macaque TNF-α

Recombinant human TNF RI interferes at concentrations > 2.0 ng/mL.

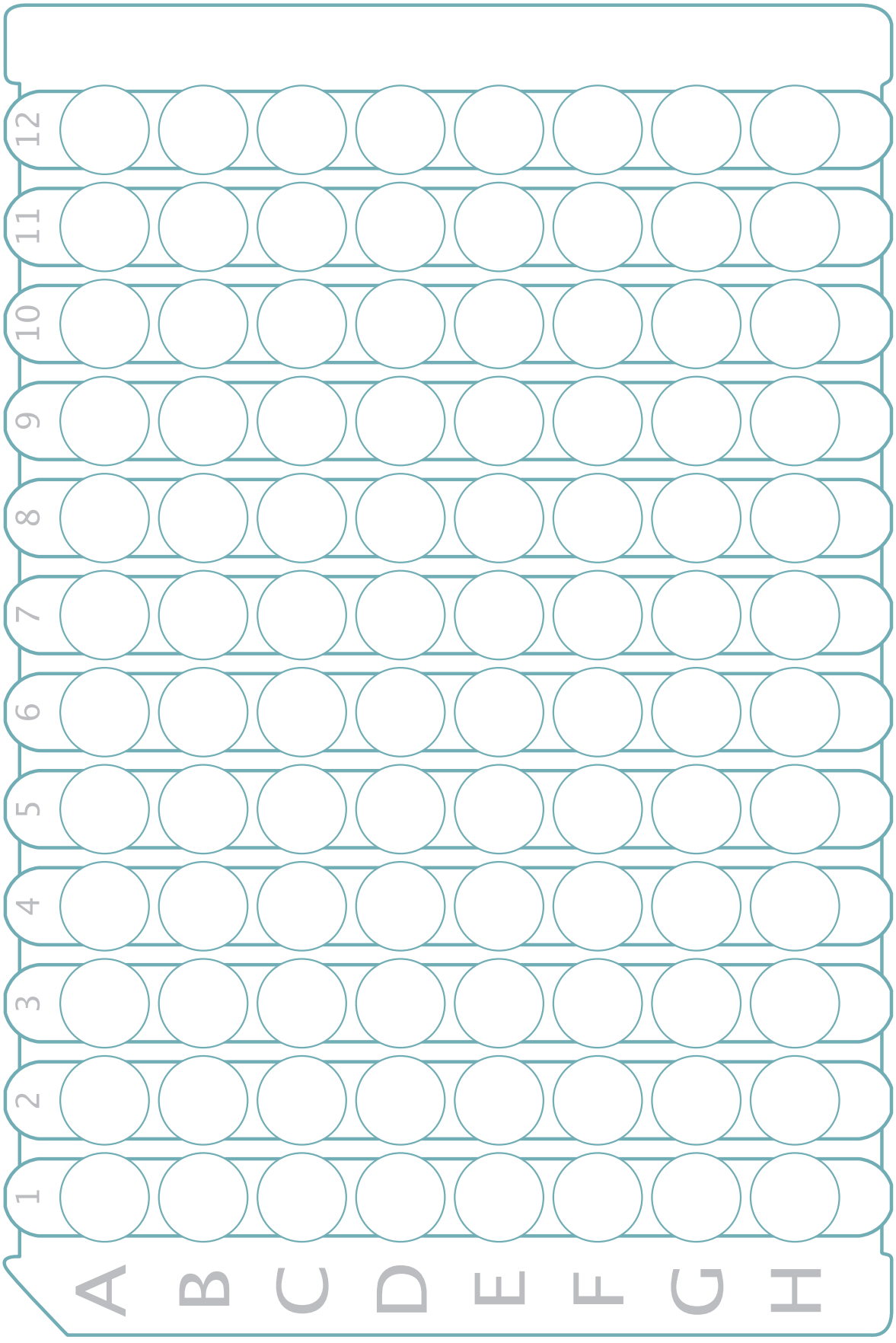
Recombinant human TNF RII interferes at concentrations > 5.0 ng/mL.

REFERENCES

1. Croft, M. *et al.* (2012) Trends Immunol. **33**:144.
2. Juhasz, K. *et al.* (2013) Expert Rev. Clin. Immunol. **9**:335.
3. Summers, de L.L and J.L. Gommerman (2012) Nat. Rev. Immunol. **12**:339.
4. Chen, X. *et al.* (2009) Cell Biochem. Funct. **27**:407.
5. Zelova, H. and J. Hosek (2013) Inflamm. Res. **62**:641.
6. Berry, M. *et al.* (2007) Curr. Opin. Pharmacol. **7**:279.
7. D'Haens, G. (2003) Curr. Pharm. Des. **9**:289.
8. Feldmann, M. and R.N. Maini (2001) Annu. Rev. Immunol. **19**:163.
9. Leung, L. and C.M. Cahill (2010) J. Neuroinflammation **7**:27.
10. Tzanavari, T. *et al.* (2010) Curr. Dir. Autoimmun. **11**:145.
11. Wu, Y. and B.P. Zhou (2010) Br. J. Cancer **102**:639.
12. Pennica, D. *et al.* (1984) Nature **312**:724.
13. Wang, A.M. *et al.* (1985) Science **228**:149.
14. Tang, P. *et al.* (1996) Biochemistry **35**:8216.
15. Perez, C. *et al.* (1990) Cell **63**:251.
16. Watts, A.D. *et al.* (1999) EMBO J. **18**:2119.
17. Black, R.A. *et al.* (1997) Nature **385**:729.
18. Moss, M.L. *et al.* (1997) Nature **385**:733.
19. Gearing, A.J.H. *et al.* (1994) Nature **370**:555.
20. Schall, T.J. *et al.* (1990) Cell **61**:361.
21. Loetscher, H. *et al.* (1990) Cell **61**:351.
22. Dembic, Z. *et al.* (1990) Cytokine **2**:231.
23. Smith, C.A. *et al.* (1990) Science **248**:1019.
24. Loetscher, H. *et al.* (1991) J. Biol. Chem. **266**:18324.
25. Rothe, M. *et al.* (1995) Science **269**:1424.
26. Ruby, J. *et al.* (1997) J. Exp. Med. **186**:1591.
27. Pinckard, J.K. *et al.* (1997) J. Biol. Chem. **272**:10784.
28. Mukhopadhyay, A. *et al.* (2001) J. Biol. Chem. **276**:31906.
29. Hsu, H. *et al.* (1995) Cell **81**:495.
30. Seckinger, P. *et al.* (1989) J. Biol. Chem. **264**:11966.
31. Olsson, I. *et al.* (1989) Eur. J. Haematol. **42**:270.
32. Engelmann, H. *et al.* (1990) J. Biol. Chem. **265**:1531.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES