Quantikine® ELISA

Porcine TNF-α Immunoassay

Catalog Number PTA00

For the quantitative determination of porcine 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.

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INTRODUCTION

Tumor necrosis factor alpha (TNF-α), also known as cachectin, is a member of the TNF ligand superfamily and has been designated TNFSF1A. It binds to the same cell surface receptors, and shares some biological functions with TNF-β/TNFSF1B. TNF-α inhibits the growth of certain tumors. It also plays a critical role in normal host resistance to infection, serving as an immunomodulator and as a mediator of inflammatory responses. Over-production of TNF has been implicated in a number of pathological conditions, including cachexia, septic shock, and autoimmune disorders (1-4). TNF-α is produced primarily by activated macrophages (1-6). Various other porcine cell types, including NK cells (6), keratinocytes (7), vascular smooth muscle cells (8), and granulosa lutein cells (9) are also known to produce TNF-α.

The porcine TNF-α gene product is a 232 amino acid (aa) residue type II membrane glycoprotein containing a 35 aa cytoplasmic domain, a 21 aa transmembrane domain and a 178 aa extracellular domain (10-13). The 156 aa residue soluble TNF-α is released from the C-terminus of the membrane protein by TNF-α converting enzyme (TACE, ADAM17), a member of the ADAM (a disintegrin and metalloprotease domain) family of metalloproteases (10, 11, 14). The biologically active TNF-α has been shown to exist as a trimer (1-4). Porcine TNF-α is active on mouse cells and shares 89% and 79% aa sequence identity with human and mouse TNF-α, respectively (10, 14).

Two distinct TNF receptors, referred to as type I (type B, p55, or TNFRSF1A) and type II (type A, p75, or TNFRSF1B), that specifically bind TNF-α and TNF-β with equal affinities are known (15-17). The two TNF receptors share aa sequence homology in their extracellular but not their cytoplasmic domains, suggesting that the two receptors employ different signal transduction pathways. Soluble forms of both types of receptors have been found in human and mouse serum (18-20). These soluble receptors are capable of neutralizing the biological activities of the TNFs and may serve to modulate the activities of TNF. Porcine TNF RI shares 79% and 72% aa homology with the human and mouse TNF RI, respectively (21-23).

The Quantikine® Porcine TNF-α Immunoassay is a 4.5 hour solid phase ELISA designed to measure porcine TNF-α in cell culture supernates, serum, and plasma samples. It contains *E. coli*-expressed recombinant porcine TNF-α and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately measure recombinant porcine TNF-α. Results obtained using natural porcine TNF-α show dose response curves that are 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 porcine TNF-α.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for porcine TNF-α has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any TNF-α present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for porcine 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 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 the appropriate calibrator diluent and repeat the assay.
- 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

- 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.
- It is recommended that samples be pipetted within 15 minutes.
- 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.
- 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.

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
Porcine TNF-α Microplate	890868	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for porcine TNF-α.	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.*
Porcine TNF-α Standard	890869	2 vials recombinant porcine TNF-α in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard within 8 hours of reconstitution. Use a new standard and control for each assay.
Porcine TNF-α Control	890188	2 vials of recombinant porcine TNF-α in a buffered protein base with preservatives; lyophilized. The assay value of the control should be within the range specified on the label.	
Porcine TNF-α Conjugate	890870	12 mL of a monoclonal antibody specific for porcine TNF-α conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-63	895352	12 mL of a buffered protein solution with preservatives.	
Calibrator Diluent RD5T	895175	21 mL of a buffered protein solution with preservatives. *For cell culture supernate samples.*	
Calibrator Diluent RD6-33	895349	21 mL of diluted animal serum with preservatives. *For serum/plasma samples.*	
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	895174	23 mL of diluted hydrochloric 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.
- **Polypropylene** test tubes for dilution of standards.

PRECAUTIONS

Calibrator Diluent RD6-33 contains sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

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.

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

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 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 20 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 has not been validated for use in this assay.*

REAGENT PREPARATION

Bring all reagents to room temperature before use.

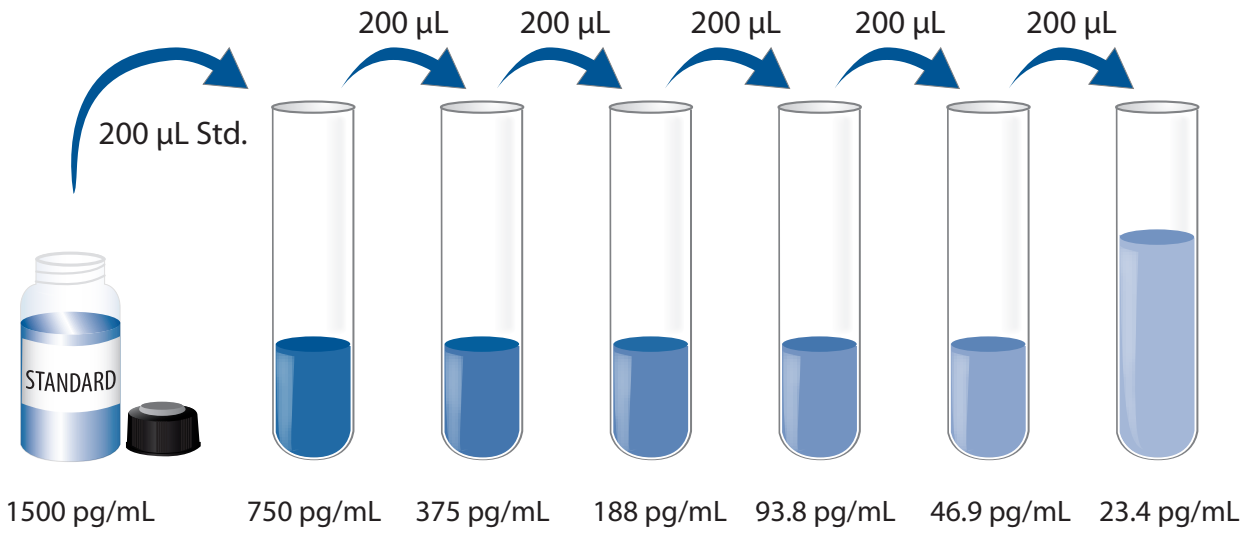
Porcine TNF-α Control - Reconstitute the control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the control undiluted.

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 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. 100 µL of the resultant mixture is required per well.

Porcine TNF-α Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Porcine TNF-α Standard with Calibrator Diluent RD5T (*for cell culture supernate samples*) or Calibrator Diluent RD6-33 (*for serum/plasma samples*). Do not substitute other diluents. This reconstitution produces a stock solution of 1500 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Use polypropylene tubes. Pipette 200 µL of the Calibrator Diluent RD5T (*for cell culture supernate samples*) or Calibrator Diluent RD6-33 (*for serum/plasma samples*) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Porcine TNF-α Standard (1500 pg/mL) serves as the high standard. The appropriate calibrator diluent 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 standards, control, and samples be assayed in duplicate.

1. Prepare reagents, samples, and standard dilutions as directed by 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 to record standards and samples 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 Porcine 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.

CALCULATION OF RESULTS

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

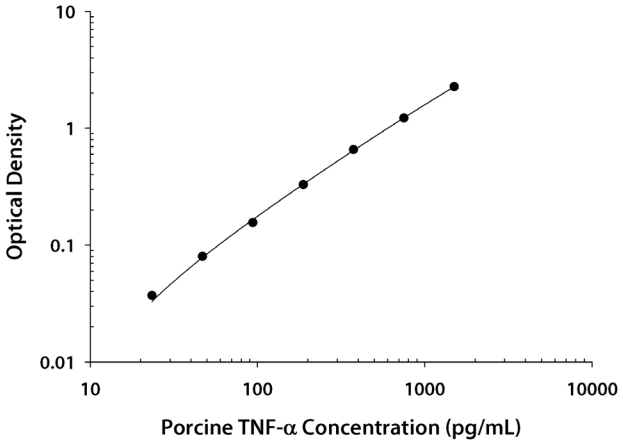
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the porcine 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

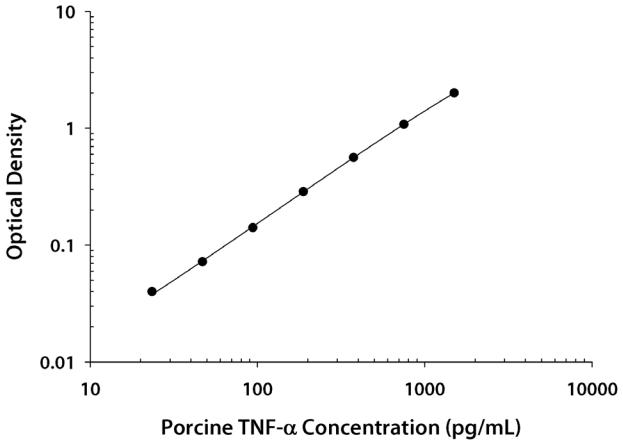
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.059 0.060	0.059	—
23.4	0.096 0.097	0.096	0.037
46.9	0.139 0.139	0.139	0.080
93.8	0.211 0.220	0.215	0.156
188	0.376 0.401	0.388	0.329
375	0.687 0.744	0.715	0.656
750	1.230 1.330	1.280	1.221
1500	2.252 2.392	2.322	2.263

SERUM/PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.046 0.047	0.046	—
23.4	0.083 0.089	0.086	0.040
46.9	0.118 0.119	0.118	0.072
93.8	0.183 0.192	0.187	0.141
188	0.333 0.334	0.333	0.287
375	0.592 0.622	0.607	0.561
750	1.116 1.134	1.125	1.079
1500	1.993 2.108	2.050	2.004

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.

CELL CULTURE SUPERNATE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	79	260	646	88	279	679
Standard deviation	4.9	9.2	27	8.8	24	44
CV (%)	6.2	3.5	4.2	10.0	8.6	6.5

SERUM/PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	104	358	880	106	348	866
Standard deviation	7.2	13	36	9.7	32	72
CV (%)	6.9	3.6	4.1	9.2	9.2	8.3

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture supernate (n=6)	99	91-105%
Serum (n=6)	105	91-120%
EDTA plasma (n=6)	98	83-111%
Heparin plasma (n=4)	91	82-99%

SENSITIVITY

Four assays were evaluated and the minimum detectable dose (MDD) of porcine TNF-α ranged from 2.8-5.0 pg/mL. The mean MDD was 3.7 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.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with various concentrations of porcine TNF-α were diluted with the appropriate calibrator diluent and assayed.

		Cell culture samples (n=6)	Serum (n=6)	EDTA plasma (n=6)	Heparin plasma (n=6)
1:2	Average % of Expected	105	102	100	106
	Range (%)	102-108	98-109	97-104	103-111
1:4	Average % of Expected	106	107	100	104
	Range (%)	99-110	100-116	94-108	96-110
1:8	Average % of Expected	110	108	103	99
	Range (%)	102-117	100-115	91-112	96-103
1:16	Average % of Expected	114	113	104	104
	Range (%)	107-117	106-118	92-119	99-109

CALIBRATION

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

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for detectable levels of porcine TNF-α in the assay.

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Serum (n=20)	63	100	25-120
EDTA plasma (n=19)	75	95	ND-237
Heparin plasma (n=4)	145	100	64-351

ND=Non-detectable

Cell Culture Supernates:

Porcine peripheral blood lymphocytes (PBLs) (5 x 10^6 cells/mL) were cultured for 6 days in DMEM supplemented with 10% fetal bovine serum and stimulated twice with 100 ng/mL LPS at day 0 and day 4. An aliquot of the cell culture supernate was removed, assayed for porcine TNF-α, and measured 1100 pg/mL.

Porcine PBLs (5 x 10^6 cells/mL) were cultured for 4 days in DMEM supplemented with 10% fetal bovine serum and stimulated with 50 ng/mL PMA and 500 ng/mL calcium ionomycin for 4 days. An aliquot of the cell culture supernate was assayed, removed for porcine TNF-α, and measured 8200 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant porcine 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 prepared at 50 ng/mL in a mid-range porcine TNF-α control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant porcine:

GM-CSF
IFN-γ
IL-1α
IL-1β
IL-1ra
IL-2
IL-4
IL-6
IL-8
IL-10
IL-12
TGF-β1

Recombinant mouse:

TNF-α
TNF RI
TNF RII

Recombinant human:

TNF-β
TNF RI

Recombinant rat:

TNF-α

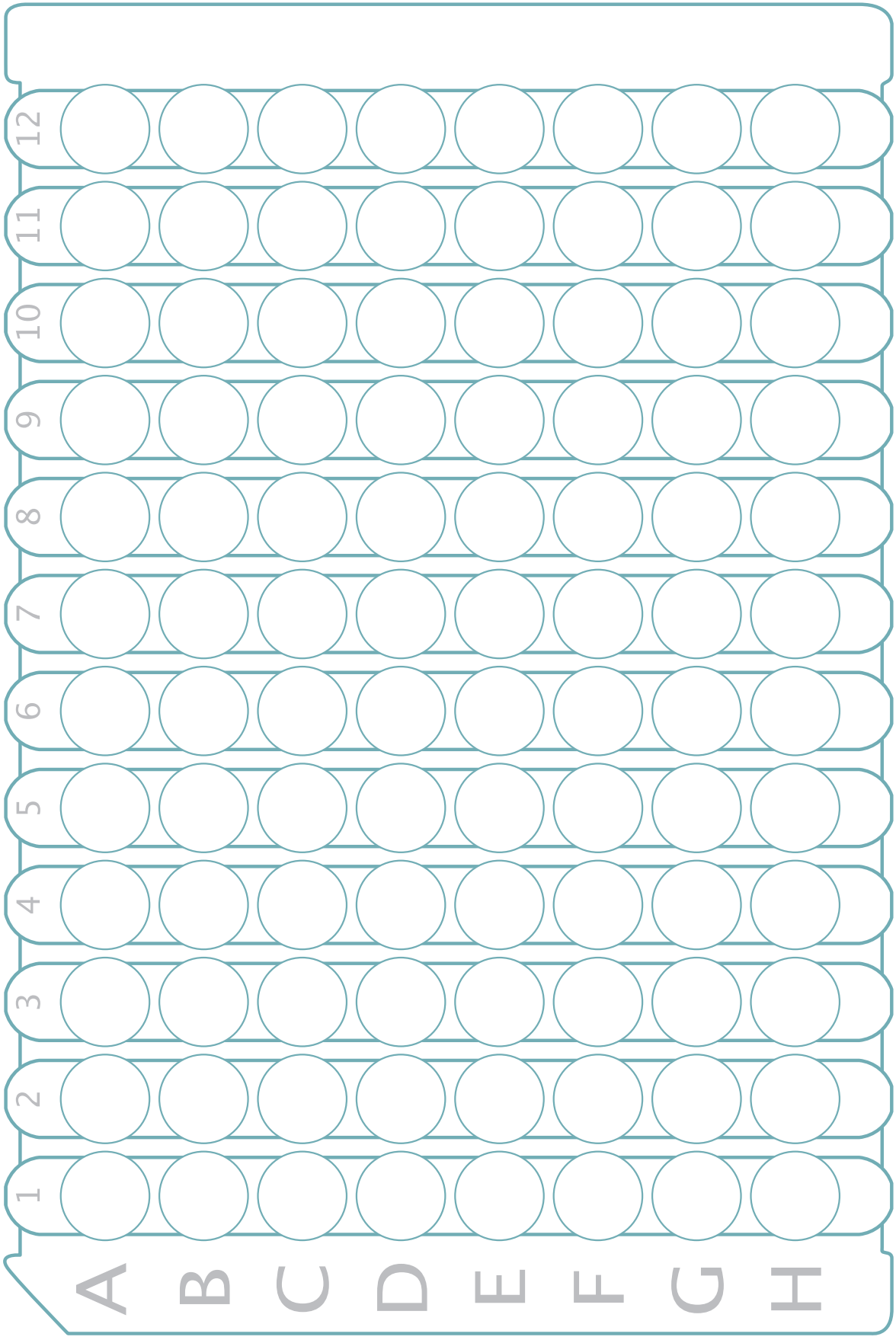
Recombinant human TNF-α cross-reacts approximately 0.24% in this assay.

REFERENCES

1. Vilcek, J. and T.H. Lee (1991) J. Biol. Chem. **266**:7313.
2. Ware, C.F. *et al.* (1996) J. Cellular Biochemistry **60**:47.
3. Tumor Necrosis Factor: *Structure, Function and Mechanism of Action*, Aggarwal, B.B. and J. Vilcek, eds. (1991) Marcel Dekker, Inc., New York.
4. Beutler, B. and A. Cerami (1989) Annu. Rev. Immunol. **7**:625.
5. Zhao, Y. *et al.* (1998) Biol. Reprod. **59**:1385.
6. Trebichavsky, I. *et al.* (1995) Folia Microbiol. **40**:417.
7. Allen, D.G. *et al.* (2001) Toxicol. Lett. **119**:209.
8. Newman, W.H. *et al.* (1998) J. Surg. Res. **80**:129.
9. Vezina, S-A. *et al.* (1995) Clin. Diag. Lab. Immunol. **2**:665.
10. Pauli, U. (1995) Vet. Immunol. Immunopathol. **47**:187.
11. Pauli, U. *et al.* (1989) Gene **81**:185.
12. Von Niederhausen, B. *et al.* (1993) Vet. Immunol. Immunopathol. **38**:57.
13. Kuhnert, P. *et al.* (1991) Gene **102**:171.
14. GenBank: Accession # P23563 (1999).
15. Tartaglia, L.A. and D.V. Goeddel (1992) Immunol. Today **13**:151.
16. Aggarwal, B. and S. Reddy (1994) in *Guidebook to Cytokines and their Receptors*, N.A. Nicola ed., Oxford University Press, New York, p. 110.
17. Baker, S.J. and E.P. Reddy (1998) Oncogene **17**:3261.
18. Seckinger, P. *et al.* (1989) J. Biol. Chem. **264**:11966.
19. Olsson, K. *et al.* (1989) Eur. J. Haematol. **42**:270.
20. Engelmann, H. *et al.* (1990) J. Biol. Chem. **265**:1531.
21. Suter, B. and U. Pauli (1995) Gene **163**:263.
22. Lewis, M. *et al.* (1991) Proc. Natl. Acad. Sci. USA **88**:2830.
23. Goodwin, R.G. *et al.* (1991) Mol. Cell. Biol. **11**:3020.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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