Quantikine® ELISA

Canine TNF-α Immunoassay

Catalog Number CATA00

For the quantitative determination of canine 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

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

Canine TNF-α, also known as TNFSF1A, is a pleiotropic cytokine that plays a central role in inflammation, metabolism and apoptosis (1-4). Canine TNF-α is a type II transmembrane protein that contains 233 amino acid (aa) residues (5). It has an N-terminal 35 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 exists as non-covalent homotrimers on the cell surface (3, 8). The soluble trimeric TNF-α is derived from the C-terminal extracellular domain through the activity of a metalloproteinase (3). Canine TNF-α shares 90% and 94% aa sequence identity with the feline and human protein, respectively (9, 10). Mammalian cells that are known to express TNF-α include B cells (11), colonic columnar epithelial cells (12), NK and $CD3^+CD56^+$ natural T cells (13), macrophages (14), monocytes and monocyte-derived dendritic cells (15), $CD4^+$ and $CD8^+$ T cells (16), mast cells (17), neutrophils (18), keratinocytes (19), plasma cells (20), adipocytes (21) and astrocytes (22).

There are two known receptors for TNF-α. The partial DNA sequence has been reported for only one of the canine receptors. Both TNF RI/TNFRSF1A and TNF RII/TNFRSF1B are type I TM glycoproteins that bind TNF-α with high affinity (23, 24). The two receptors share limited aa sequence homology in their extracellular domains but show no homology in their intracellular domains. Although the two receptors can individually mediate TNF-α activities utilizing different signaling mechanisms, the two receptors can cooperate to enhance cellular responses (25-27). TNF receptor(s) can elicit intracellular signals in cells expressing TNF-α, which acts as a receptor to transmit reverse signals (28).

TNF-α is involved in a number of physiological and pathophysiological processes. It is a prototypical pro-inflammatory molecule 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 activate phagocyte killing mechanisms (via increased $NO_2^-/O_2^-/H_2O_2$) (29). Cachexia, or 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, 30).

The Quantikine Canine TNF-α Immunoassay is a 4.5 hour solid-phase ELISA designed to measure canine TNF-α in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant canine TNF-α and antibodies raised against the recombinant protein. Results obtained for naturally occurring canine TNF-α 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 canine TNF-α.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for canine TNF-α has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any canine TNF-α present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for canine 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 canine TNF-α bound in the initial step. The sample values are then read from 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 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.
- It is recommended that the samples be pipetted within 15 minutes.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Allow the plate to soak for 10-15 seconds between washes to 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.

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
Canine TNF-α Microplate	892966	One 96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for canine 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.*
Canine TNF-α Standard	892968	2 vials of recombinant canine TNF-α in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Use a new Standard and Control for each assay.
Canine TNF-α Control	892969	2 vials of recombinant canine TNF-α in a buffered protein base with preservatives; lyophilized. The concentration range of recombinant canine TNF-α after reconstitution is shown on the vial label. The assay value of the Control should be within the range specified on the Control label.	
Canine TNF-α Conjugate	892967	12 mL of a polyclonal antibody against canine TNF-α conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-38	895301	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-17	895512	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	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

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. 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 30 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 30 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.

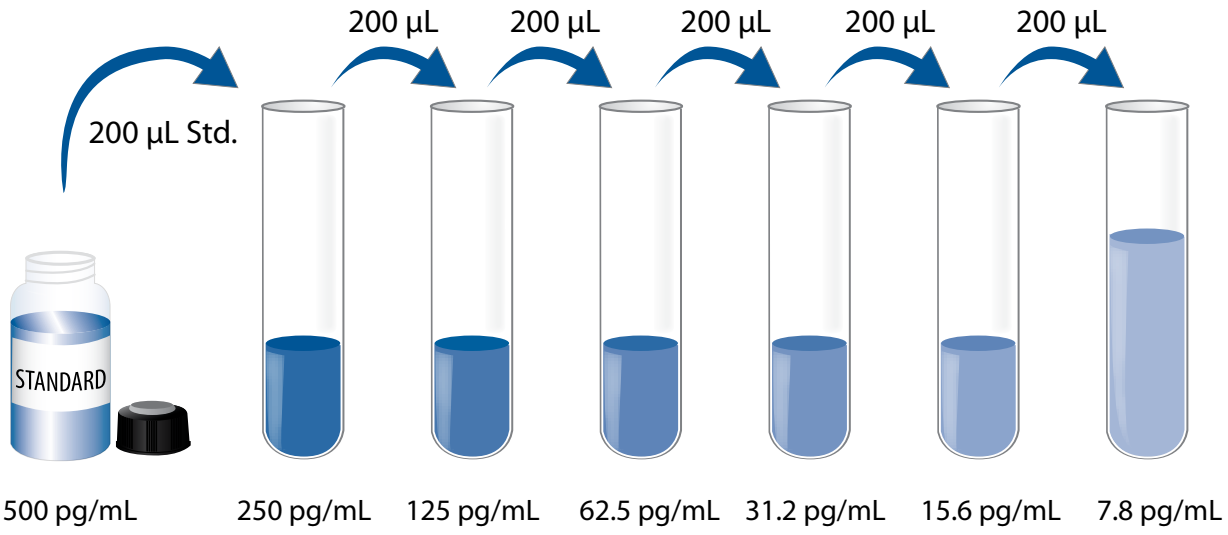
Canine TNF-α Control - Reconstitute the Control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the Control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. 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.

Canine TNF-α Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the canine TNF-α Standard with Calibrator Diluent RD5-17. Do not substitute other diluents. This reconstitution produces a stock solution of 500 pg/mL. Mix the standard to ensure complete reconstitution and 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 Calibrator Diluent RD5-17 into each tube. Use the standard stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted canine TNF-α Standard serves as the high standard (500 pg/mL). Calibrator Diluent RD5-17 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 all reagents, standard dilutions, control, 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 50 µL of Assay Diluent RD1-38 to each well.
4. Add 50 µL of Standard, Control, or sample per well within 15 minutes. Mix by gently tapping the plate frame for 1 minute. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
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 canine 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

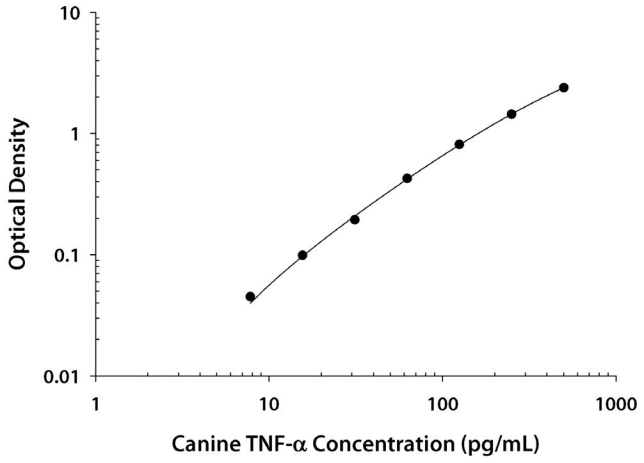
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 canine 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	0.058 0.066	0.062	—
7.8	0.103 0.110	0.107	0.045
15.6	0.157 0.165	0.161	0.099
31.2	0.253 0.258	0.256	0.194
62.5	0.481 0.495	0.488	0.426
125	0.859 0.886	0.873	0.811
250	1.496 1.510	1.503	1.441
500	2.447 2.455	2.451	2.389

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 separate assays to assess inter-assay precision.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	100	66	106
Mean (pg/mL)	18.6	123	201	20.9	54.0	149
Standard deviation	1.5	6.5	15.4	2.1	5.2	13.8
CV (%)	8.1	5.3	7.7	10.0	9.6	9.3

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture supernates (n=7)	99	83-109%
Serum (n=6)	92	83-106%
EDTA plasma (n=6)	94	82-103%
Heparin plasma (n=6)	94	88-103%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of canine TNF-α were serially diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture samples (n=8)	Serum (n=6)	EDTA plasma (n=6)	Heparin plasma (n=6)
1:2	Average % of Expected	95	103	105	99
	Range (%)	89-101	99-106	101-111	94-104
1:4	Average % of Expected	98	97	105	98
	Range (%)	93-109	94-98	102-112	94-100
1:8	Average % of Expected	93	97	101	99
	Range (%)	87-101	92-102	97-107	92-103
1:16	Average % of Expected	97	99	94	109
	Range (%)	82-114	84-107	84-106	94-118

SENSITIVITY

Twenty-four assays were evaluated and the minimum detectable dose (MDD) of canine TNF-α ranged from 0.9-4.2 pg/mL. The mean MDD was 2.4 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 canine TNF-α produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Six samples were evaluated for detectable levels of canine TNF-α in this assay. All the samples read below the lowest standard, 7.8 pg/mL.

Cell Culture Supernates - Canine peripheral blood cells (1 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal calf serum and stimulated with 3 µg/mL PHA for 24 hours. An aliquot of the cell culture supernate was assayed for canine TNF-α and measured 163 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant canine TNF-α.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent RD5-17 and assayed for cross-reactivity. Preparations of the following factors at the same concentration in a mid-range canine TNF-α control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant canine:
GM-CSF
IFN-γ
IL-4
IL-6
IL-8
IL-10
MCP-1
VEGF

Recombinant human:
TNF-β
TNF RI
TNF RII

Recombinant mouse:
TNF-α
TNF-β
TNF RI
TNF RII
TROY/TNFRSF19

Other recombinants:
cotton rat TNF-α
equine TNF-α
rat TNF-α

This assay shows cross-reactivity with the following:

Recombinant Factor	Cross-reactivity
Rhesus macaque TNF-α	23%
Human TNF-α (truncated)	3.8%
Human TNF-α	2.8%
Porcine TNF-α	0.2%

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. Hehlgans, T. and D.N. Mannel (2002) Biol. Chem. **383**:1581.
4. Warne, J.P. (2003) J. Endocrinol. **177**:351.
5. Zucker, K. *et al.* (1994) Lymphokine Cytokine Res. **13**:191.
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. McGraw, R.A. *et al.* (1990) Nucleic Acids Res. **18**:5563.
10. Pennica, D. *et al.* (1984) Nature **312**:724.
11. Corione, A. *et al.* (1997) Blood **90**:4493.
12. Jung, H.C. *et al.* (1995) J. Clin. Invest. **95**:55.
13. Doherty, D.G. *et al.* (1999) J. Immunol. **163**:2314.
14. Jovanovic, D.V. *et al.* (1998) J. Immunol. **160**:3513.
15. Avice, M-N. *et al.* (1999) J. Immunol. **162**:2748.
16. Cuturi, M.C. *et al.* (1987) J. Exp. Med. **165**:1581.
17. Lorentz, A. *et al.* (2000) J. Immunol. **164**:43.
18. Bliss, S.K. *et al.* (1999) J. Immunol. **162**:7369.
19. Ezepchuk, Y.V. *et al.* (1996) J. Invest. Dermatol. **107**:603.
20. Di Girolamo, N. *et al.* (1997) J. Leukoc. Biol. **61**:667.
21. Voros, G. *et al.* (2003) Biochim. Biophys. Acta **1625**:36.
22. Wang, Z. *et al.* (2002) Acta Pharmacol. Sin. **23**:974.
23. Aggarwal, B.B. *et al.* (1985) Nature **318**:665.
24. Schall, T.J. *et al.* (1990) Cell **61**:361.
25. Ruby, J. *et al.* (1997) J. Exp. Med. **186**:1591.
26. Lazdins, J.K. *et al.* (1997) J. Exp. Med. **185**:81.
27. Pinckard, J.K. *et al.* (1997) J. Biol. Chem. **272**:10784.
28. Eissner, G. *et al.* (2004) Cytokine Growth Factor Rev. **15**:353.
29. Vassalli, P. (1992) Annu. Rev. Immunol. **10**:411.
30. Finck, B.N. and R.W. Johnson (2000) Microsc. Res. Tech. **50**:209.