Quantikine® ELISA

Canine IFN-γ Immunoassay

Catalog Number CAIF00

For the quantitative determination of canine Interferon gamma (IFN-γ) 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

Interferon-gamma (IFN-γ), also known as type II interferon, is an important immunoregulatory cytokine that was originally identified through its anti-viral activity (1). It plays a key role in host defense by exerting anti-viral, anti-proliferative and immunoregulatory activities (2-5). This includes both pro-inflammatory and anti-inflammatory activity (6, 7). On many cell types, IFN-γ induces the production of cytokines and upregulates the expression of various membrane proteins including class I and II MHC antigens, Fc receptors, leukocyte adhesion molecules and B7 family antigens. IFN-γ is a potent initial primer of macrophage effector function (8). It potentiates the secretion of immunoglobulins by B cells and directs the synthesis of IgG. IFN-γ also influences T-helper cell phenotype development by inhibiting Th2 differentiation and stimulating Th1 development (2-6).

IFN-γ is produced by a number of cell types, including dendritic cells (9), keratinocytes (10), peripheral blood γδ T cells (11), mast cells (12), neurons (13), $CD8^+$ T cells (14), macrophages (15), B cells (16), neutrophils (17), NK cells (18), $CD4^+$ T cells (19), and testicular spermatids (20). The production of IFN-γ is upregulated synergistically by IL-12, IL-18, and IL-23 (21-26). The canine IFN-γ cDNA encodes a 166 amino acid (aa) residue precursor protein that contains a 20 aa signal sequence that is cleaved to generate a 146 aa residue mature canine IFN-γ (27). Canine IFN-γ is presumably a non-covalently linked homodimer (3). In the mature segment, canine IFN-γ shares 87%, 65%, 43%, 72%, and 80% aa identity to feline (28), human (29), mouse (30), porcine (31), and equine (32) IFN-γ, respectively.

The functional IFN-γ receptor complex consists of two distinct subunits (33). The α-subunit (IFN-γ R1) binds IFN-γ with high affinity and species specificity. The β-subunit (IFN-γ R2, also known as AF-1 or accessory factor-1) interacts with the IFN-γ occupied α-subunit in a species-specific manner and participates in JAK-STAT mediated signal transduction. Although the functional receptor is suggested to consist of homodimeric IFN-γ in combination with two α-chains, and two β-chains (33, 34), it has been suggested that additional subunits may be involved (35, 36). Whereas the α-chain is expressed constitutively on many cell types, the cellular regulation of the β-chain correlates with an IFN-γ responsive state and is tightly regulated (33).

The Quantikine® Canine IFN-γ Immunoassay is a 4.0 hour solid-phase ELISA designed to measure canine IFN-γ in cell culture supernates, serum, and plasma. It contains *E. coli*-expressed recombinant canine IFN-γ and antibodies raised against the recombinant factor. Results obtained for naturally occurring canine IFN-γ 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 IFN-γ.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for canine IFN-γ has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any IFN-γ present is bound by the immobilized antibody. After washing away any unbound substances, a biotinylated mouse monoclonal antibody specific for canine IFN-γ is added to the wells. Following a wash to remove any unbound antibody, streptavidin conjugated to HRP is added to the wells. Followed by a wash to remove any unbound streptavidin-HRP, 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 IFN-γ 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 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.
- 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 IFN-γ Microplate	892488	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for canine IFN-γ.	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 IFN-γ Biotin Conjugate	892489	12 mL of a biotinylated monoclonal antibody specific for canine IFN-γ with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Canine IFN-γ Streptavidin-HRP	892679	12 mL of streptavidin conjugated to horseradish peroxidase with preservatives.	
Canine IFN-γ Standard	892490	Recombinant canine IFN-γ in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Canine IFN-γ Control	892491	Recombinant canine IFN-γ in a buffered protein base with preservatives; lyophilized. The assay value of the control should be within the range specified on the control label.	
Assay Diluent RD1-63	895352	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD6-12	895214	21 mL of diluted animal serum with preservatives.	
Wash Buffer Concentrate	895003	2 vials (21 mL/vial) 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	8 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.
- 1000 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 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 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.

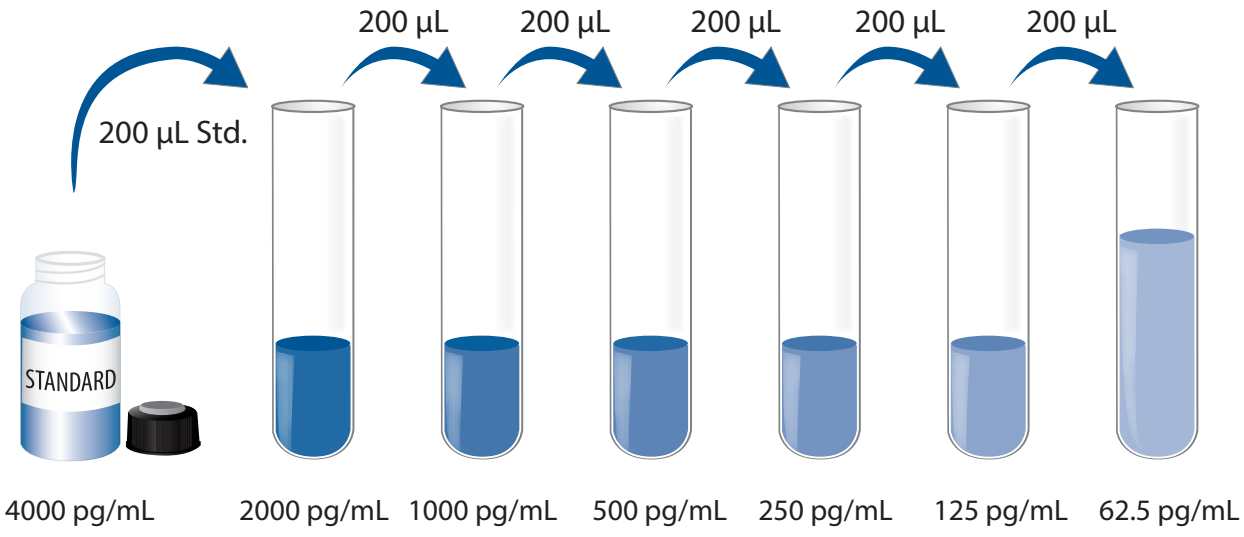
Canine IFN-γ Control - Reconstitute the control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 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. 100 µL of the resultant mixture is required per well.

Canine IFN-γ Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Canine IFN-γ Standard with Calibrator Diluent RD6-12. Do not substitute other diluents. This reconstitution produces a stock solution of 4000 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 RD6-12 into each tube. Use the standard stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Canine IFN-γ Standard (4000 pg/mL) serves as the high standard. Calibrator Diluent RD6-12 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 RD1-63 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.
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 IFN-γ Biotin Conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 µL of Canine IFN-γ Streptavidin-HRP to each well. Cover with a new adhesive strip. Incubate for 30 minutes at room temperature.
9. Repeat the aspiration/wash as in step 5.
10. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
11. Add 100 µL of Stop Solution to each well. 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

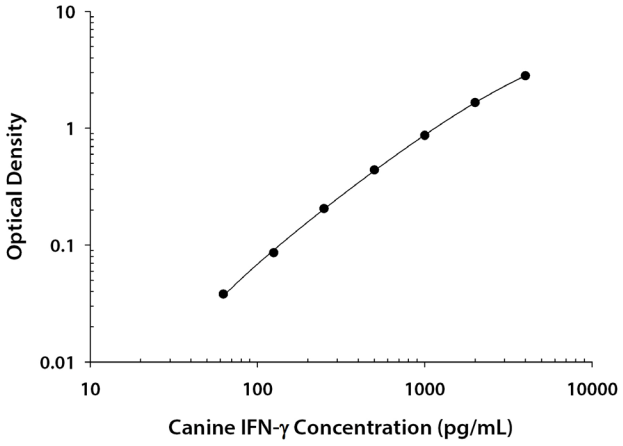
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 canine IFN-γ 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	0.069 0.076	0.072	—
62.5	0.108 0.113	0.110	0.038
125	0.156 0.160	0.158	0.086
250	0.274 0.280	0.277	0.205
500	0.506 0.516	0.511	0.439
1000	0.924 0.955	0.940	0.868
2000	1.684 1.773	1.728	1.656
4000	2.877 2.892	2.884	2.812

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)	111	459	1234	119	457	1315
Standard deviation	10.1	35.1	92.4	10.1	21.5	64.8
CV (%)	9.1	7.6	7.5	8.5	4.7	4.9

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture supernates (n=4)	103	92-111%
Serum (n=4)	96	83-107%
EDTA plasma (n=4)	96	86-102%
Heparin plasma (n=4)	98	93-106%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of canine IFN-γ were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates (n=5)	Serum (n=6)	EDTA plasma (n=4)	Heparin plasma (n=6)
1:2	Average % of Expected	97	95	96	100
	Range (%)	95-97	91-99	93-98	98-101
1:4	Average % of Expected	95	98	95	101
	Range (%)	92-100	93-100	92-98	100-103
1:8	Average % of Expected	94	100	92	103
	Range (%)	91-100	91-107	86-97	100-107
1:16	Average % of Expected	91	106	87	97
	Range (%)	86-94	97-111	82-91	90-101

SENSITIVITY

Seventeen assays were evaluated and the minimum detectable dose (MDD) of canine IFN-γ ranged from 8.0-60 pg/mL. The mean MDD was 25 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 canine IFN-γ produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma - Eighteen samples were evaluated for detectable levels of canine IFN-γ in this assay. One serum sample measured 97 pg/mL. All other samples measured below the lowest standard, 62.5 pg/mL.

Cell Culture Supernates - Canine peripheral blood cells (1 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal bovine serum and unstimulated or stimulated with 10 µg/mL Concanavalin A. Aliquots of the cell culture supernates were removed and assayed for canine IFN-γ.

Condition	Day 1 (pg/mL)	Day 3 (pg/mL)	Day 4 (pg/mL)
Stimulated	3082	4654	30,523
Unstimulated	0	———	———

SPECIFICITY

This assay recognizes natural and recombinant canine IFN-γ.

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 canine IFN-γ control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant canine:

GM-CSF
IL-4
IL-6
IL-8
IL-10
MCP-1
TNF-α
VEGF

Recombinant mouse:

IFN-γ
IFN-γ R1
IFN-γ R2
IFN-γ α/β R2
IL-5

Recombinant human:

IFN-γ
IFN-γ R1
IFN-γ R2

Other recombinants:

cotton rat IFN-γ
feline IFN-γ
porcine IFN-γ
rat IFN-γ
rhesus macaque IFN-γ

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