Quantikine® ELISA

Mouse Fas Ligand/TNFSF6 Immunoassay

Catalog Number MFL00

For the quantitative determination of mouse Fas Ligand concentrations in cell culture supernates, serum, and plasma.

Note: The standard reconstitution method has changed. Please read this package insert in its entirety before using this product.

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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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

Mouse Fas Ligand is a member of the TNF superfamily of molecules that induces apoptosis in susceptible cells expressing the fibroblast associated (Fas) receptor (1). Mouse Fas Ligand is expressed on a number of cell types, including endothelial cells (2), macrophages (3), $CD4^+$ and $CD8^+$ T cells (4, 5), neutrophils (6, 7), Sertoli cells and Leydig cells (8), thyroid epithelium (9, 10), fibroblasts (11), microglia (12), corneal epithelium (13), renal proximal epithelium (14), monocytes (15), mammary epithelium (16), and myeloid dendritic cells (17, 18). The cDNA for mouse Fas Ligand codes for a 279 amino acid (aa) residue, 40 kDa type II transmembrane protein that contains a 179 aa extracellular region, a 22 aa transmembrane segment, and a 78 aa cytoplasmic domain (19-23). A point mutation at residue 273 of the extracellular domain results in a molecule with a generalized lymphoproliferative disease (gld) phenotype (20, 23). Membrane-bound Fas Ligand can be cleaved by metalloproteinases to produce a 150 aa, 26 kDa soluble form that circulates as a nondisulfide-linked trimer (19-25). An alternate splice form also exists that is 16 kDa, 69 aa long, and constitutively expressed. This form is non-apoptotic, as it blocks anti-Fas activation (26). Both membrane-bound and soluble Fas Ligand are bioactive, with the highest activity associated with membrane-bound Fas Ligand (24, 27). Both mouse and human Fas Ligand are active on mouse and human cells (28). Mouse soluble Fas Ligand shares 94% and 85% aa identity to rat and human Fas Ligand, respectively (28, 29).

The signaling receptor for Fas Ligand is Fas, a 45 kDa type I transmembrane glycoprotein that is 245 aa in length (30). There is also a second reported receptor for Fas Ligand termed DcR3 (decoy receptor 3) which is known only in soluble form (31). Both Fas and DcR3 bind Fas Ligand with equal affinity (31).

Fas-mediated apoptosis plays a critical role during development, in liver hemostasis, and in immunological reactions. In the immune system, Fas Ligand is suggested to play an important role in T cell activation. During an immune response, naive T cells are exposed to antigens. This leads to T cell proliferation and differentiation. To insure that proliferation is self-limiting, Fas Ligand is upregulated on activated T cells and interacts with Fas on either the same cell or bystander cells such as unstimulated T cells and macrophages. Fas-induced apoptosis of these cells interrupts the cycle of cytokine stimulation and proliferation (19, 32, 33). In addition to activating Fas, Fas Ligand can also be activated by Fas, transmitting a reverse signal that results in proliferation of $CD4^+$ naive T cells (5, 33).

The Quantikine Mouse Fas Ligand/TNFSF6 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure mouse Fas Ligand in cell culture supernates, serum, and plasma. It contains NS0-expressed recombinant mouse Fas Ligand and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate the recombinant factor. Results obtained using natural mouse Fas Ligand 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 naturally occurring mouse Fas Ligand.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse Fas Ligand has been pre-coated onto a microplate. Standards, Control, and samples are pipetted into the wells and any mouse Fas Ligand present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse Fas Ligand 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 mouse Fas Ligand 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 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.
- For best results, pipette reagents and samples into the center of each well.
- 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.
- 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
Mouse Fas Ligand Microplate	891009	96 well microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse Fas Ligand.	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.*
Mouse Fas Ligand Standard	891011	2 vials of recombinant mouse Fas Ligand 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. Discard within 8 hours of reconstitution.
Mouse Fas Ligand Control	891012	2 vials of recombinant mouse Fas Ligand in a buffered protein base with preservatives; lyophilized. The assay value of the Control should be within the range specified on the label.	
Mouse Fas Ligand Conjugate	891010	12 mL of a polyclonal antibody specific for mouse Fas Ligand conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-47	895524	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5T	895175	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.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- **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 20 minutes at 2000 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 as a anticoagulant. Centrifuge for 20 minutes at 2000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

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

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REAGENT PREPARATION

Bring all reagents to room temperature before use.

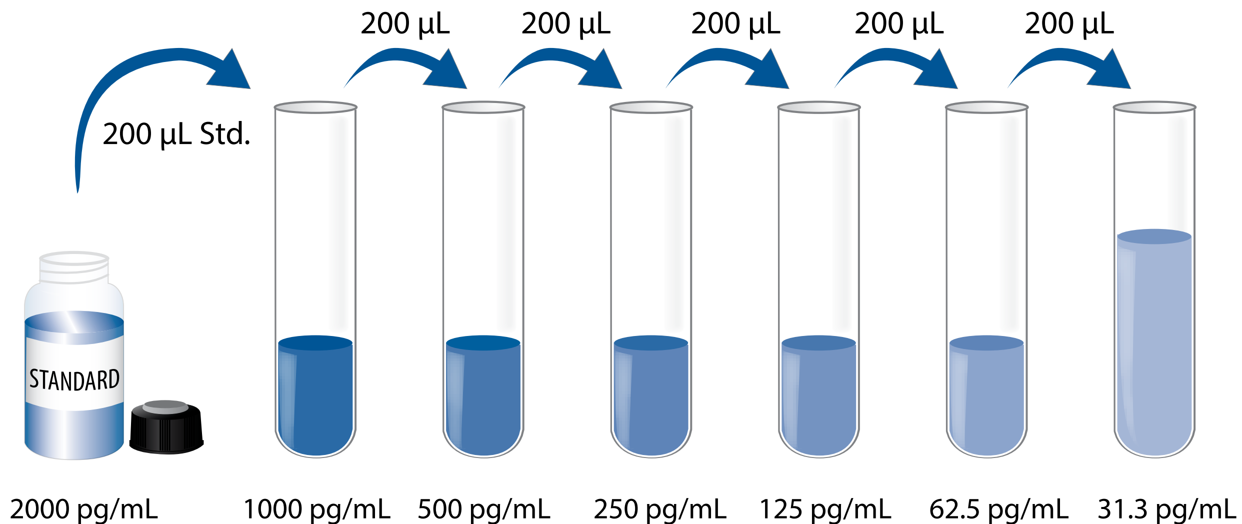
Mouse Fas Ligand Control - Reconstitute the Control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the Control undiluted.

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.

Mouse Fas Ligand Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse Fas Ligand Standard with Calibrator Diluent RD5T. Do not substitute other diluents. This reconstitution produces a stock solution of 2000 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 Calibrator Diluent RD5T into each tube. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Mouse Fas Ligand Standard (2000 pg/mL) serves as the high standard. Calibrator Diluent RD5T 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 RD1-47 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 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 Mouse Fas Ligand Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
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 **on the benchtop. 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

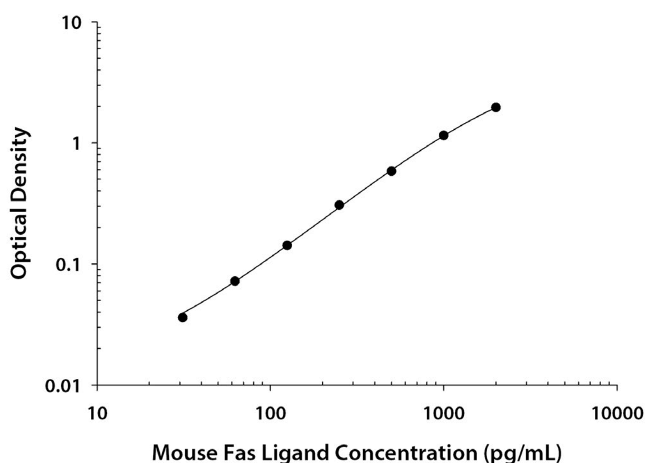
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 mouse Fas Ligand 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	0.063 0.066	0.064	—
31.3	0.100 0.101	0.100	0.036
62.5	0.133 0.138	0.136	0.072
125	0.203 0.208	0.206	0.142
250	0.368 0.371	0.370	0.306
500	0.637 0.656	0.646	0.582
1000	1.210 1.217	1.214	1.150
2000	1.938 2.107	2.022	1.958

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)	84	261	675	76	243	663
Standard deviation	8.1	9.0	20	6.8	12	33
CV (%)	9.6	3.4	3.0	8.9	4.9	5.0

RECOVERY

The recovery of mouse Fas Ligand spiked to three levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture supernates (n=6)	107	84-120%
Serum (n=6)	98	86-111%
EDTA plasma (n=6)	99	87-110%

LINEARITY

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

		Cell culture supernates (n=6)	Serum (n=6)	EDTA plasma (n=6)
1:2	Average % of Expected	100	104	102
	Range (%)	97-103	101-109	101-104
1:4	Average % of Expected	102	107	107
	Range (%)	96-107	101-115	104-110
1:8	Average % of Expected	104	106	108
	Range (%)	96-110	99-115	104-118
1:16	Average % of Expected	103	99	104
	Range (%)	93-108	90-114	93-115

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SENSITIVITY

Two assays were evaluated and the minimum detectable dose (MDD) of mouse Fas Ligand ranged from 3.1-3.9 pg/mL. The mean MDD was 3.6 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 highly purified NS0-expressed recombinant mouse Fas Ligand produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of mouse Fas Ligand in this assay.

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Serum (n=20)	83	15%	ND-138
EDTA plasma (n=20)	73	50%	ND-231

ND=Non-detectable

Cell Culture Supernates:

Mouse thymocytes (from one mouse thymus) were cultured for 3 days in RPMI supplemented with 10% fetal calf serum and 50 µM β-mercaptoethanol and stimulated with 10 ng/mL recombinant human IL-2. The plate in which the cells grew was pre-treated with 10 µg/mL of hamster anti-mouse CD3. An aliquot of the cell culture supernate was removed, assayed for mouse Fas Ligand, and measured 243 pg/mL.

Mouse splenocytes (1 x 10^6 cells/mL) were cultured for 3 days in RPMI supplemented with 10% fetal calf serum and 50 µM β-mercaptoethanol, and stimulated with 10 ng/mL recombinant human IL-2. An aliquot of the cell culture supernate was removed, assayed for mouse Fas Ligand, and measured 133 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant mouse Fas Ligand.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the same factors at 50 ng/mL in a mid-range mouse Fas Ligand control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:

C10
Eotaxin
E-Selectin
Fas
Flt-3 Ligand
G-CSF
GM-CSF
IFN-γ
IL-1α
IL-1β
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-9
IL-10
IL-10 R
IL-12 p70
IL-13
IL-17
IL-18
JE/MCP-1
KC
L-Selectin
Leptin
LIF
MARC
MCP-5
MIP-1α
MIP-2
OPG
OSM
RANK Ligand
RANTES
TARC
TNF-α
TNF RI
TNF RII
Tpo
VEGF R2

Recombinant human:

Fas
Fas Ligand

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

NOTES

NOTES

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