Quantikine® ELISA

Human Fas Ligand/TNFSF6 Immunoassay

Catalog Number DFL00B

For the quantitative determination of human Fas Ligand concentrations in cell culture supernates, cell lysates, 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

SECTION PAGE

Manufactured and Distributed by:

USA R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413
TEL: 800 343 7475 612 379 2956
FAX: 612 656 4400
E-MAIL: info@bio-techne.com

Distributed by:

Europe | Middle East | Africa Bio-Techne Ltd.
19 Barton Lane, Abingdon Science Park
Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449
FAX: +44 (0)1235 533420
E-MAIL: info.emea@bio-techne.com

China Bio-Techne China Co., Ltd.
Unit 1901, Tower 3, Raffles City Changning Office,
1193 Changning Road, Shanghai PRC 200051
TEL: +86 (21) 52380373 (400) 821-3475
FAX: +86 (21) 52371001
E-MAIL: info.cn@bio-techne.com

INTRODUCTION

Fas ligand (FasL), also known as TNFSF6, CD178, or CD95L, is a member of the tumor necrosis factor (TNF) superfamily. Its primary role is the induction of apoptosis in cells expressing its receptor, Fas (1), also known as Apo-1, CD95, TNFRSF6. The prototypical Fas/FasL apoptosis cascade consists of Fas activation and its association with the adaptor protein, Fas-associated death domain (FADD) and caspase-8, forming the death inducing signaling complex (DISC) (2). This leads to downstream activation of several effector caspases and the initiation of apoptosis.

Human FasL was first isolated using reverse polymerase chain reaction of peripheral blood lymphocyte mRNA (1). The gene for FasL codes for a 40 kDa type II membrane spanning protein containing 281 amino acids (aa) (1). FasL is 76.9% identical to its mouse counterpart at the aa level (1) and is constitutively expressed primarily in activated T lymphocytes and immune-privileged tissues including eye, testis, and placenta (3-5). FasL may also be upregulated following exposure to UV light (6) or cytotoxic drugs (7, 8).

FasL can undergo proteolytic cleavage to liberate a 26 kDa soluble form of the molecule (sFasL), and biochemical assessment suggests that sFasL exists as a trimer (9). The liberation of sFasL is blocked by inhibitors of matrix metalloproteinases (MMPs) (10). More specifically, MMP-7 (matrilysin) and, to a lesser extent, MMP-3 (stromelysin-1) have been identified as proteases that liberate sFasL (11-13). MMP-7 cleavage sites are near the transmembrane domain and include the ELAELR sequence of human FasL, and secondarily, an SL sequence adjacent to ELAELR (13). Other cleavage points have been described suggesting the possible involvement of other proteases in the generation of human sFasL (14).

The physiological role of sFasL in the regulation of apoptosis remains unclear. Although human sFasL can stimulate apoptosis (15, 16), this activity is generally regarded to be much lower than that of membrane-associated FasL (17). Furthermore, it has been shown that non-apoptotic sFasL can compete with FasL for receptor binding and may act as a natural inhibitor of Fas signaling (18, 19). In contrast, sFasL-induced apoptosis can be markedly upregulated by the cross-linking of sFasL trimers (20).

Certain cell types do not undergo apoptosis in response to Fas stimulation. For instance, Fas activation can lead to proliferation of human T cells (21) and fibroblasts (22) and sFasL can act as a chemoattractant for neutrophils (23). In human fibroblasts, sFasL stimulates phosphorylation of extracellular signal-regulated kinases (ERKS) 1 and 2, NF-κB activation, and interleukin-6 gene expression (24). In addition, Fas induces neurite growth via ERK activation and p35 upregulation (25).

The Quantikine® Human Fas Ligand/TNFSF6 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Fas Ligand in cell culture supernates, cell lysates, serum, and plasma. It contains CHO cell-expressed recombinant human Fas Ligand and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human 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 natural human Fas Ligand.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Fas Ligand has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Fas Ligand present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human 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 and color develops in proportion to the amount of Fas Ligand 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.
- 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.
- 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.

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
Human Fas Ligand Microplate	898947	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human 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.*
Human Fas Ligand Conjugate	892474	21 mL of a polyclonal antibody specific for human Fas Ligand conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human Fas Ligand Standard	892475	Recombinant human Fas Ligand in a buffer with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1S	895137	11 mL of a buffer with preservatives.	
Calibrator Diluent RD5K	895119	21 mL of a buffered protein base with preservatives.	
Cell Lysis Buffer 3	895366	21 mL of a concentrated buffered solution with preservatives. *Use diluted 1:5 in this assay. May turn yellow over time.*	
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	895032	6 mL of 2 N sulfuric 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.
- 100 mL and 500 mL graduated cylinders.
- PBS (for cell lysis)
- Test tubes for dilution of standards.
- Human Fas Ligand Controls (optional; R&D Systems®, Catalog # QC89).

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 and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Cell Lysates - Cells must be lysed prior to assay as directed in the Cell Lysis Procedure.

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 has not been validated for use in this assay.*

CELL LYSIS PROCEDURE

1. Centrifuge cells at 500 x g for 5 minutes and remove supernates. The supernate may be saved for assay in this kit.
2. Wash the cells twice in cold PBS.
3. Prepare Cell Lysis Buffer 3 (diluted 1:5)* as directed in the Reagent Preparation section. Add 1.0 mL of Cell Lysis Buffer 3 (diluted 1:5)* per 1.0 x 10^7 cells.
4. Vortex to resuspend the pellet and incubate at room temperature with gentle agitation for 30 minutes.
5. Centrifuge at 15,000 x g for 5 minutes to remove cellular debris, pipette off the cell lysate supernate, quantitate total protein, and assay immediately or aliquot and store at ≤ -70 °C.

   Note: *For cell lysate samples, quantitation of sample protein concentration using a total protein assay is recommended. The suggested range for total cell lysate protein added is 20-200 µg/well.*

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

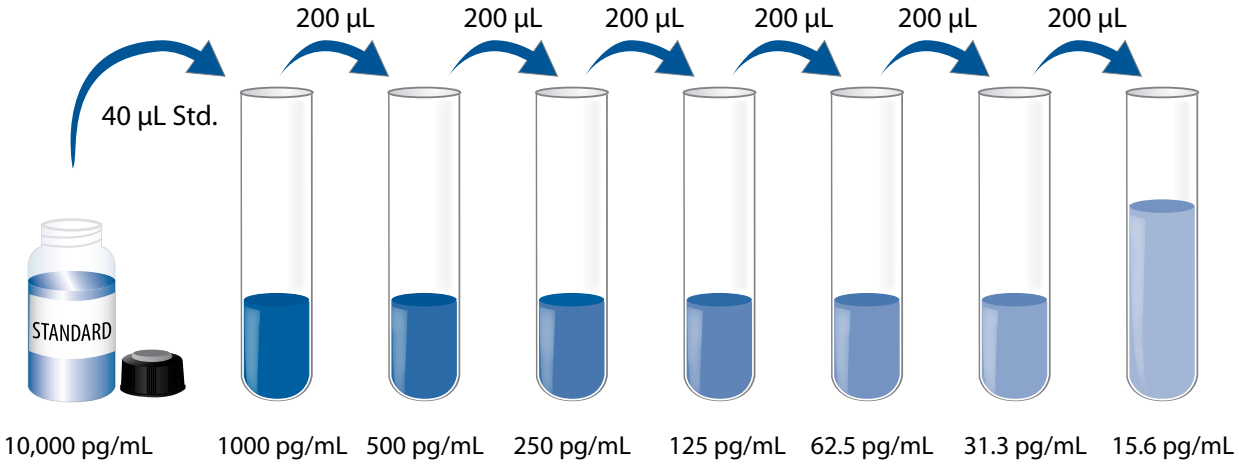
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Cell Lysis Buffer 3 (diluted 1:5) - Add 20 mL of Cell Lysis Buffer 3 to 80 mL deionized or distilled water to prepare 100 mL of Cell Lysis Buffer 3 (diluted 1:5). Add protease inhibitors according to the manufacturer's instructions.

Human Fas Ligand Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Fas Ligand Standard with deionized or distilled water. This reconstitution produces a stock solution of 10,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 360 µL of Calibrator Diluent RD5K into the 1000 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 1000 pg/mL standard serves as the high standard. Calibrator Diluent RD5K 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, controls, and standards be assayed in duplicate.

1. Prepare all reagents, working standards, 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 100 µL of Assay Diluent RD1S 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.
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 Fas Ligand 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 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature on the benchtop. **Protect from light.**
9. 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.
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

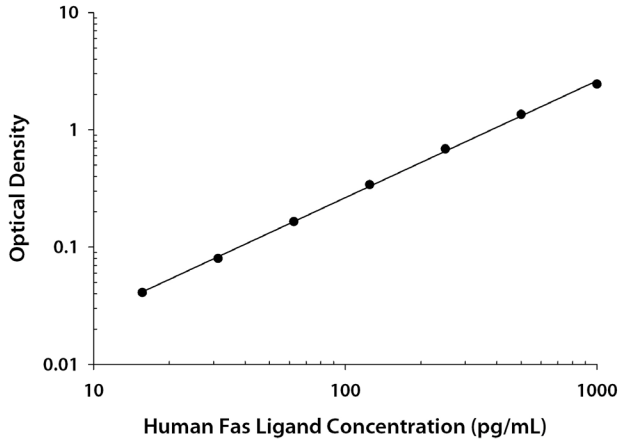
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a log/log 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 a log/log graph. The data may be linearized by plotting the log of the human Fas Ligand concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

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	0.042 0.043	0.043	—
15.6	0.083 0.084	0.084	0.041
31.3	0.123 0.123	0.123	0.080
62.5	0.207 0.209	0.208	0.165
125	0.383 0.387	0.385	0.342
250	0.726 0.733	0.730	0.687
500	1.358 1.437	1.398	1.355
1000	2.463 2.527	2.495	2.452

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)	92.1	240	587	98.6	244	576
Standard deviation	3.31	7.51	18.2	4.41	8.70	25.7
CV (%)	3.6	3.1	3.1	4.5	3.6	4.5

RECOVERY

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

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	98	93-103%
Serum (n=4)	101	96-103%
EDTA plasma (n=4)	103	94-111%
Heparin plasma (n=4)	96	92-101%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human Fas Ligand were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates (n=4)	Cell culture media (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	103	105	95	97	100
	Range (%)	102-104	103-109	84-101	91-103	98-104
1:4	Average % of Expected	105	109	104	103	97
	Range (%)	104-107	107-109	89-121	97-109	87-108
1:8	Average % of Expected	105	109	95	101	93
	Range (%)	100-107	108-110	83-105	96-111	84-108
1:16	Average % of Expected	105	110	90	93	88
	Range (%)	102-108	104-116	83-102	89-100	76-113

SENSITIVITY

Twenty assays were evaluated and the minimum detectable dose (MDD) of human Fas Ligand ranged from 1.36-5.10 pg/mL. The mean MDD was 3.21 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 CHO cell-expressed recombinant human Fas Ligand produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human Fas Ligand 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)	124	50.1-181	30.0
EDTA Plasma (n=30)	119	53.1-182	27.9
Heparin Plasma (n=30)	118	46.9-179	28.8

Cell Culture Supernates:
Human peripheral blood lymphocytes (PBLs) were cultured in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 10 µg/mL PHA. Aliquots of the cell culture supernates were removed on days 1 and 5 and assayed for levels of human Fas Ligand.

Condition	Day 1 (pg/mL)	Day 5 (pg/mL)
Unstimulated	ND	164
Stimulated	309	839

ND=Non-detectable

Cell Lysates - White blood cells from fresh whole blood were isolated over Ficoll, washed twice in PBS and lysed (1 x 10^7 cells) in Cell Lysis Buffer 3 (diluted 1:5). 175 µg of cell lysate was assayed for levels of human Fas Ligand and measured 725 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human Fas Ligand.

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 Fas Ligand control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

4-1BB
APRIL
BLyS
CD27 Ligand
CD30 Ligand
CD40 Ligand
DcR3
Fas
GITR Ligand
LIGHT
LT-α1/β2
LT-α2/β1
OX40 Ligand
TNF-α
TNF-β
TRAIL
TRANCE
TWEAK
VEGI

Recombinant mouse:

CD27 Ligand
CD30 Ligand
Fas
Fas Ligand
LT-α1/β2
LT-α2/β1
OX40 Ligand
TNF-α
TNF-α (truncated)
TRANCE

Other recombinants:

porcine TNF-α
rat TNF-α

REFERENCES

1. Takahashi, T. *et al.* (1994) Int. Immunol. **6**:1567.
2. Hohlbaum, A.M. *et al.* (2002) Clin. Immunol. **103**:1.
3. Griffith, T.S. *et al.* (1995) Science **270**:1189.
4. Bellgrau, D. *et al.* (1995) Nature **377**:630.
5. Xerri, L. *et al.* (1997) Mol. Pathol. **50**:87.
6. Hill, L.L. *et al.* (1999) Science **285**:898.
7. Mizutani, Y. *et al.* (1997) Cancer **79**:1180.
8. Friesen, C. *et al.* (1999) Leukemia **13**:1854.
9. Tanaka, M. *et al.* (1995) EMBO J. **14**:1129.
10. Kayagaki, N. *et al.* (1995) J. Exp. Med. **182**:1777.
11. Powell, W.C. *et al.* (1999) Curr. Biol. **9**:1441.
12. Matsuno, H. *et al.* (2001) J. Rheumatol. **28**:22.
13. Vargo-Gorgola, T. *et al.* (2002) Arch. Biochem. Biophys. **408**:155.
14. Tanaka, M. *et al.* (1998) Nat. Med. **4**:31.
15. Serrao, K.L. *et al.* (2001) Am. J. Physiol. Lung Cell Mol. Physiol. **280**:L298.
16. Kim, J.M. *et al.* (2000) Scand. J. Gastroenterol. **35**:40.
17. Schneider, P. *et al.* (1998) J. Exp. Med. **187**:1205.
18. Mogi, M. *et al.* (2001) Lab. Invest. **81**:177.
19. Suda, T. *et al.* (1997) J. Exp. Med. **186**:2045.
20. Holler, N. *et al.* (2003) Mol. Cell. Biol. **23**:1428.
21. Alderson, M.R. (1993) J. Exp. Med. **178**:2231.
22. Aggarwal, B.B. *et al.* (1995) FEBS Lett. **364**:5.
23. Seino, K-I. *et al.* (1998) J. Immunol. **161**:4484.
24. Ahn, J-H. *et al.* (2001) J. Biol. Chem. **276**:47100.
25. Desbarats, J. *et al.* (2003) Nature Cell Biol. **5**:118.