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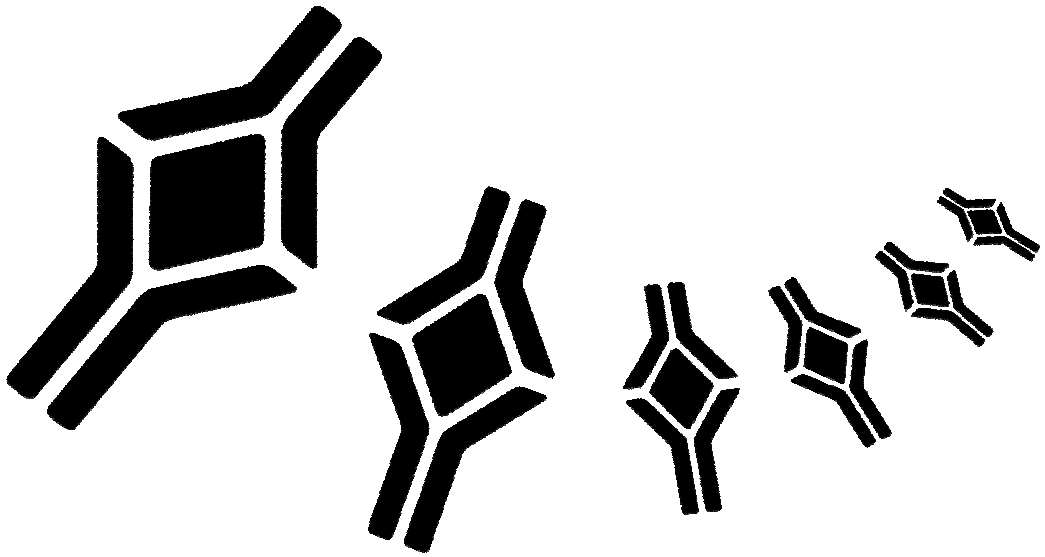


# HUMAN sFas Ligand ELISA

Product Data Sheet

Cat. No.: RBMS260/2

For Research Use Only

## CONTENTS



» **This kit is manufactured by:**
**BioVendor – Laboratorní medicína, a.s.**

» **Use only the current version of Product Data Sheet enclosed with the kit!**

# 1 INTENDED USE

The sFas Ligand ELISA is an enzyme-linked immunosorbent assay for quantitative detection of human sFas Ligand in cell culture supernatants, serum, plasma, and amniotic fluid. **The sFas Ligand ELISA is for in vitro diagnostic use. Not for use in therapeutic procedures.**

# 2 SUMMARY

Fas (APO-1, CD95) is a type I membrane protein that belongs to the TNF/nerve growth factor receptor family. Fas mediates apoptosis, the programmed cell death, when it is cross-linked with specific binding partners. The natural binding partner of Fas is its ligand, FasL, which is a 37 kDa type II-membrane protein that belongs to the TNF family which includes TNF, lymphotoxin, TNF-related apoptosis-inducing ligand (TRAIL), CD40 ligand, CD27 ligand, CD30 ligand, and OX40 ligand.

FasL is predominantly expressed on activated T-cells and NK cells, thus FasL-mediated cell death is involved in the T or NK cell-mediated cytotoxicity, some pathologic tissue damages, and the regulation of lymphocyte homeostasis.

FasL is also expressed in the testis, eye, and some malignant tumor cells, which has been proposed to contribute to their immune-privileged status.

A soluble form of FasL (sFasL) is naturally produced by metallo-proteinase-mediated processing. The soluble form resulting from this cleavage was shown to induce apoptosis in susceptible cells (8,31,9).

Several lines of evidence suggest that sFasL may be involved in the pathogenesis of tissue injury.

Circulating sFasL is elevated in the serum of patients with various diseases.

Markedly elevated levels of sFasL have been shown in TEN (Toxic Epidermal Necrolysis, Lyell's Syndrom) patients' sera (35). FasL furthermore turned out to be a sensor for DNA damage in skin cancer (14,33). Elevated expression levels of FasL have been measured in various proliferative disorders (12) and cancers like esophageal carcinomas (22) metastasizing colorectal tumors (16), hepatocellular carcinoma (20), multiple myeloma (4), sarcoma (15,18), Non-Hodgkin's lymphoma (10) and nasal lymphoma (26).

Liver dysfunction was shown to be paralleled by increased sFasL levels (19,30,27) as well as kidney damage (23,24,25).

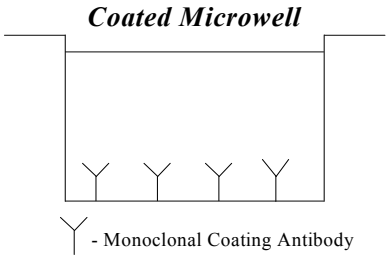
FasL is discussed to be involved in the pathogenesis of autoimmune diseases (28,29,2), especially the concentrations of sFasL are remarkably higher in the sera and synovial fluids of patients with severe rheumatoid arthritis as compared to normal controls (6,21).

Increased levels of soluble FasL in the serum of graft-versus-host-disease patients make it a good marker for treatment of the disease (1,11,32).

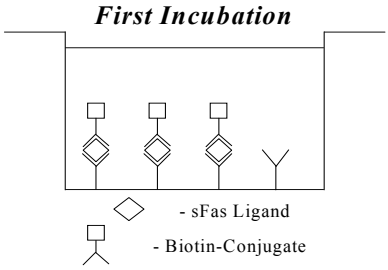
Levels of soluble Fas Ligand in bronchoalveolar lavage (BAL) fluid of humans with acute lung injury (ARDS) (17) and serum levels in congestive heart failure (34) and Multiple organ failure (7) were significantly higher than in healthy controls.
Cerebrospinal fluid from patients with severe brain injury contains high concentrations of FasL (3).
Elevations of serum FasL levels in hematological disorders (5) and HIV infections are furthermore described.

# 3 PRINCIPLES OF THE TEST

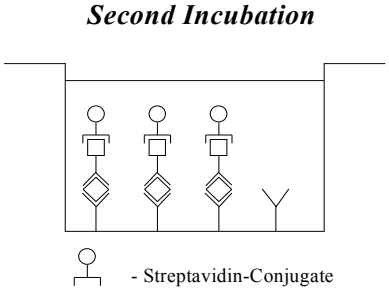
An anti-sFas Ligand monoclonal coating antibody is adsorbed onto microwells.



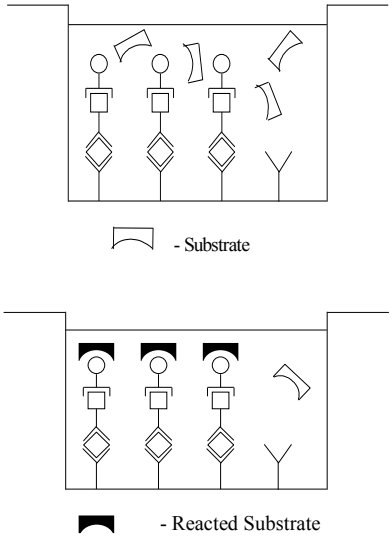

sFas Ligand present in the sample or standard binds to antibodies adsorbed to the microwells; a biotin-conjugated monoclonal anti-sFas Ligand antibody is added and binds to sFas Ligand captured by the first antibody.




Following incubation unbound biotin-conjugated anti-sFas Ligand is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-sFas Ligand. Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.




A coloured product is formed in proportion to the amount of sFasLigand present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450nm. A standard curve is prepared from seven sFas Ligand standard dilutions and sFas Ligand sample concentration determined.



# 4 REAGENTS PROVIDED

1 aluminium pouch with a **Antibody Coated Microtiter Strips** with Monoclonal Antibody (murine) to human sFas Ligand

1 vial (100 *µ*l) **Biotin-Conjugate** anti-sFas Ligand monoclonal (murine) antibody

2 vials s**Fas Ligand Standard**, lyophilized, 20 ng/ml upon reconstitution

1 vial (150 µl) **Streptavidin-HRP**

1 bottle (50 ml) **Wash Buffer Concentrate** 20x (PBS with 1% Tween 20)

1 vial (5 ml) **Assay Buffer Concentrate** 20x (PBS with 1% Tween 20 and 10% BSA)

1 vial (12 ml) **Sample Diluent** (buffered protein matrix)

1 vial (15 ml) **Substrate Solution**

1 vial (12 ml) **Stop Solution** (1M Phosphoric acid)

1 vial (0.4 ml each) **Blue-Dye, Green-Dye, Red-Dye**

4 adhesive **Plate Covers**

**Reagent Labels**

## 5 STORAGE INSTRUCTIONS

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on labels.

The expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

## 6 SPECIMEN COLLECTION

Cell culture supernatants, serum, plasma, and amniotic fluid are suitable for use in the assay. Remove the serum or plasma from the clot or cells, respectively, as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Samples must be stored frozen at -20°C to avoid loss of bioactive sFas Ligand. If samples are to be run within 24 hours, they may be stored at 2° to 8°C. Avoid repeated freeze-thaw cycles. Prior to assay, frozen sera or plasma should be brought to room temperature slowly and mixed gently and properly diluted with Sample Diluent.

For sample stability refer to 13.E.

# 7 MATERIALS REQUIRED BUT NOT PROVIDED

- 5 ml and 10 ml graduated pipettes

- 10 µl to 1,000 µl adjustable single channel micropipettes with disposable tips

- 50 µl to 300 µl adjustable multichannel micropipette with disposable tips

- Multichannel micropipette reservoir

- Beakers, flasks, cylinders necessary for preparation of reagents

- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)

- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)

- Glass-distilled or deionized water

- Statistical calculator with program to perform linear regression analysis

# 8 PRECAUTIONS FOR USE

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statements(s) for specific advice.

- Reagents are intended for in vitro diagnostic use and are not for use in therapeutic procedures.

- Do not mix or substitute reagents with those from other lots or other sources.

- Do not use kit reagents beyond expiration date on label.

- Do not expose kit reagents to strong light during storage or incubation.

- Do not pipette by mouth.

- Do not eat or smoke in areas where kit reagents or samples are handled.

- Avoid contact of skin or mucous membranes with kit reagents or specimens.

- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.

- Avoid contact of substrate solution with oxidizing agents and metal.

- Avoid splashing or generation of aerosols.

- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.

- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagents.

- Exposure to acids will inactivate the conjugate.

- Glass-distilled water or deionized water must be used for reagent preparation.

- Substrate solution must be at room temperature prior to use.

- Decontaminate and dispose specimens and all potentially contaminated materials as if they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.

- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0 % sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

# 9 PREPARATION OF REAGENTS

Prepare Wash Buffer (reagent A) and Assay Buffer (reagent B) before starting with the test procedure.

## A. Wash Buffer

If crystals have formed in the Wash Buffer Concentrate, warm it gently until they have completely dissolved.

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** into a clean 1,000 ml graduated cylinder. Bring final volume to 1,000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. The pH of the final solution should adjust to 7.4.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that the Wash Buffer is stable for 30 days. Wash Buffer may be prepared as needed according to the following table:

| Number of Strips | Wash Buffer Concentrate (ml) | Distilled Water (ml) |
|---|---|---|
| 1 - 6 | 25 | 475 |
| 1 - 12 | 50 | 950 |

## B. Assay Buffer

Mix the contents of the bottle well. Add contents of **Assay Buffer Concentrate** (5.0 ml) to 95 ml distilled or deionized water and mix gently to avoid foaming. Store at 2° to 8°C. Please note that the Assay Buffer is stable for 30 days. Assay Buffer may be prepared as needed according to the following table:

| Number of Strips | Assay Buffer Concentrate (ml) | Distilled Water (ml) |
|---|---|---|
| 1 - 6 | 2.5 | 47.5 |
| 1 - 12 | 5.0 | 95.0 |

## C. Preparation of Standard

Dissolve lyophilized **Standard** by addition of distilled water. Refer to the Certificate of Analysis for current volume of Distilled water needed for reconstitution of standard. Swirl gently to ensure complete solubilisation.

## D. Preparation of Biotin-Conjugate

Dilute the concentrated **Biotin-Conjugate** 1:100 just prior to use with **Assay Buffer** (reagent B) in a clean plastic tube. Mix the contents of the tube well.

Please note that the Biotin-Conjugate should be used within 30 minutes after dilution. The Biotin-Conjugate may be prepared as needed according to the following table:

| Number of Strips | Biotin-Conjugate (ml) | Assay Buffer (ml) |
|---|---|---|
| 1 - 6 | 0.03 | 2.97 |
| 1 - 12 | 0.06 | 5.94 |

## E. Preparation of Streptavidin-HRP

Make a 1:200 dilution of the concentrated **Streptavidin-HRP** solution with **Assay Buffer** as needed according to the following table:

| Number of Strips | Streptavidin-HRP (ml) | Assay Buffer (ml) |
|---|---|---|
| 1 - 6 | 0.03 | 5.97 |
| 1 - 12 | 0.06 | 11.94 |

## F. Addition of colour-giving reagents: Blue-Dye, Green-Dye, Red-Dye

This procedure is optional, does not in any way interfere with the test results, and is designed to help the customer with the performance of the test, but can also be omitted, just following the instruction booklet.

Alternatively, the dye solutions from the stocks provided (***Blue-Dye, Green-Dye, Red-Dye***) can be added to the reagents according to the following guidelines:

**1. Diluent:** Before sample dilution add the ***Blue-Dye*** at a dilution of 1:250 (see table below) to the diluent (1x) according to the test protocol. After addition of ***Blue-Dye***, proceed according to the instruction booklet.

| | |
|---|---|
| 5 ml Sample Diluent | 20 µl ***Blue-Dye*** |
| 12 ml Sample Diluent | 48 µl ***Blue-Dye*** |

**2. Biotin-Conjugate:** Before dilution of the concentrated conjugate, add the ***Green-Dye*** at a dilution of 1:100 (see table below) to the Assay Buffer used for the final conjugate dilution. Proceed after addition of ***Green-Dye*** according to the instruction booklet, preparation of Biotin-conjugate.

| | |
|---|---|
| 3 ml Assay Buffer | 30 µl ***Green-Dye*** |
| 6 ml Assay Buffer | 60 µl ***Green-Dye*** |
| 12 ml Assay Buffer | 120 µl ***Green-Dye*** |

**3. Streptavidin-HRP:** Before dilution of the concentrated Streptavidin-HRP; add the ***Red-Dye*** at a dilution of 1:250 (see table below) to the Assay Buffer used for the final Streptavidin-HRP dilution. Proceed after addition of ***Red-Dye*** according to the instruction booklet, preparation of Streptavidin-HRP.

| | |
|---|---|
| 6 ml Assay Buffer | 24 µl ***Red-Dye*** |
| 12 ml Assay Buffer | 48 µl ***Red-Dye*** |

a. Mix all reagents thoroughly without foaming before use.

b. Determine the number of Microwell Strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra **Microwell Strips coated with Monoclonal Antibody** (murine) to human sFas Ligand from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.

c. Wash the microwell strips twice with approximately 300 µl **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells.

   After the last wash, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.

d. Add 100 µl of **Sample Diluent** in duplicate to all standard wells. Prepare standard dilutions by pipetting 100 µl of reconstituted **sFas Ligand Standard** (refer to preparation of reagents), in duplicate, into well A1 and A2 (see Figure 1 and 2). Mix the contents by repeated aspiration and ejection and transfer 100 µl to well B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Continue this procedure five times, creating two rows of sFas Ligand standard dilutions ranging from 10 to 0.16 ng/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.

Figure 1. Preparation of sFas Ligand standard dilutions:

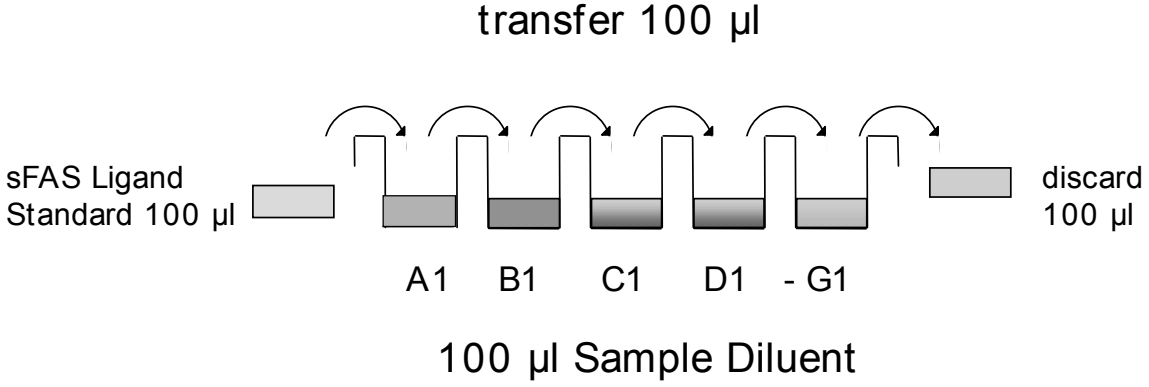



Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

| | **1** | **2** | **3** | **4** |
|---|---|---|---|---|
| **A** | Standard 1 (10 ng/ml) | Standard 1 (10 ng/ml) | Sample 1 | Sample 1 |
| **B** | Standard 2 (5 ng/ml) | Standard 2 (5 ng/ml) | Sample 2 | Sample 2 |
| **C** | Standard 3 (2.5 ng/ml) | Standard 3 (2.5 ng/ml) | Sample 3 | Sample 3 |
| **D** | Standard 4 (1.25 ng/ml) | Standard 4 (1.25 ng/ml) | Sample 4 | Sample 4 |
| **E** | Standard 5 (0.63 ng/ml) | Standard 5 (0.63 ng/ml) | Sample 5 | Sample 5 |
| **F** | Standard 6 (0.31 ng/ml) | Standard 6 (0.31 ng/ml) | Sample 6 | Sample 6 |
| **G** | Standard 7 (0.16 ng/ml) | Standard 7 (0.16 ng/ml) | Sample 7 | Sample 7 |
| **H** | Blank | Blank | Sample 8 | Sample 8 |

e. Add 100 µl of **Sample Diluent** in duplicate to the blank wells.
f. Add 50 µl of **Sample Diluent** to the sample wells.
g. Add 50 µl of each **Sample**, in duplicate, to the designated wells.

h. Prepare **Biotin-Conjugate** (refer to preparation of reagents).
i. Add 50 µl of diluted **Biotin-Conjugate** to all wells, including the blank wells.
j. Cover with a **Plate Cover** and incubate at room temperature (18° to 25°C) for 2 hours, if available on a rotator set at 100 rpm.
k. Remove Plate Cover and empty wells. Wash microwell strips 4 times according to point c. of the test protocol. Proceed immediately to the next step.
l. Prepare **Streptavidin-HRP** (refer to preparation of reagents).
m. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the blank wells.
n. Cover with a **Plate Cover** and incubate at room temperature (18º to 25ºC) for 1 hour, if available on a rotator set at 100 rpm.
o. Remove Plate Cover and empty wells. Wash microwell strips 4 times according to point c. of the test protocol. Proceed immediately to the next step.
p. Pipette 100 µl of **TMB Substrate Solution** to all wells, including the blank wells.
q. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 minutes. Avoid direct exposure to intense light.
**The colour development on the plate should be monitored and the substrate reaction stopped (see point r. of this protocol) before positive wells are no longer properly recordable.**
It is recommended to add the stop solution when the highest standard has developed a dark blue colour.
Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as an OD of 0.6 – 0.65 is reached.
r. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well, including the blank wells. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.
s. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the

blank wells. Determine the absorbance of both, the samples and the sFas Ligand standards.

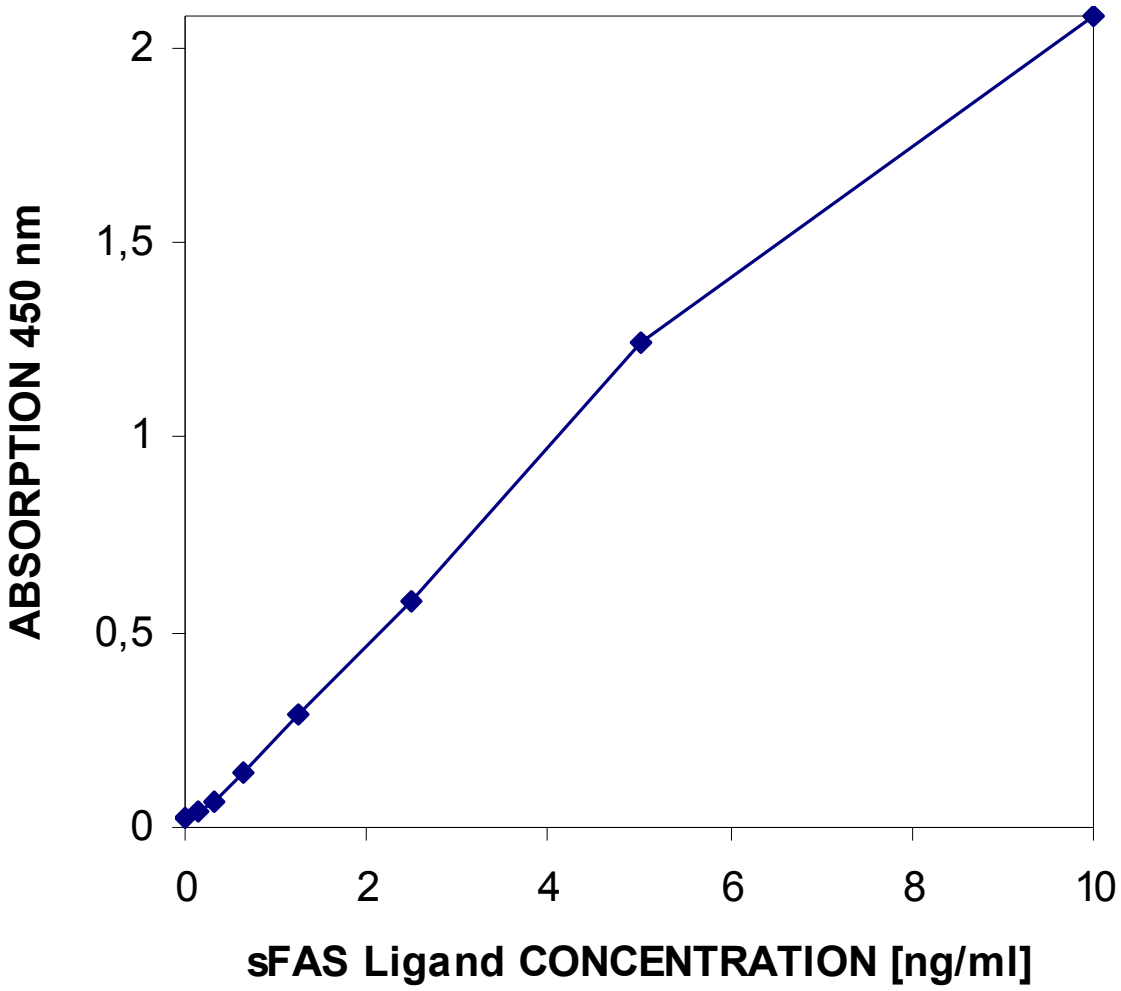
**Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.**

## 11 CALCULATION OF RESULTS

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the sFas Ligand concentration on the abscissa. Draw a best fit curve through the points of the graph.
- To determine the concentration of circulating sFas Ligand for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding sFas Ligand concentration.
- **For samples which have been diluted according to the instructions given in this manual 1:2, the concentration has to be multiplied by the dilution factor (x2).**

  **Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low sFas Ligand levels. Such samples require further dilution of 1:4 - 1:8 with Sample Diluent in order to precisely quantitate the actual sFas Ligand level.**
- It is suggested that each testing facility establishes a control sample of known sFas Ligand concentration and runs this additional control with each assay. If the values obtained are not within the expected range of this control, the assay results may be invalid.
- A representative standard curve is shown in Figure 3. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

Figure 3. Representative standard curve for sFas Ligand ELISA. sFas Ligand was diluted in serial two-fold steps in Sample Diluent, symbols represent the mean of three parallel titrations.
Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.



Typical data using the sFas Ligand ELISA

Measuring wavelength: 450 nm
Reference wavelength: 620 nm

| Standard | sFas Ligand Concentration (ng/ml) | O.D. (450 nm) | O.D. Mean | C.V. (%) |
|---|---|---|---|---|
| 1 | 10 | 2.055 | 2.108 | 3.6 |
| | 10 | 2.161 | | |
| 2 | 5 | 1.227 | 1.269 | 4.7 |
| | 5 | 1.311 | | |
| 3 | 2.5 | 0.591 | 0.610 | 4.4 |
| | 2.5 | 0.629 | | |
| 4 | 1.25 | 0.31 | 0.318 | 3.6 |
| | 1.25 | 0.326 | | |
| 5 | 0.63 | 0.16 | 0.165 | 3.9 |
| | 0.63 | 0.169 | | |
| 6 | 0.31 | 0.096 | 0.0998 | 2.2 |
| | 0.31 | 0.099 | | |
| 7 | 0.16 | 0.076 | 0.072 | 8.9 |
| | 0.16 | 0.067 | | |
| Blank | 0 | 0.031 | 0.028 | |
| | 0 | 0.025 | | |

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. temperature effects). Furthermore shelf life of the kit may effect enzymatic activity and thus colour intensity. Values measured are still valid.

# 12 LIMITATIONS

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.

- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.

- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.

- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh Wash Buffer, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

- The use of immunotherapy has significantly increased the number of patients with human anti-mouse IgG antibody (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analysed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the Sample Diluent.

# 13 PERFORMANCE CHARACTERISTICS

## A. Sensitivity

The limit of detection of sFas Ligand defined as the analyte concentration resulting in an absorption significantly higher than that of the dilution medium (mean plus two standard deviations) was determined to be 0.07 ng/ml (mean of 6 independent assays).

## B. Reproducibility

### a. Intra-assay

Reproducibility within the assay was evaluated in three independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of sFas Ligand. Two standard curves were run on each plate. Data below show the mean sFas Ligand concentration and the coefficient of variation for each sample. The overall intra-assay coefficient of variation has been calculated to be 6.1%.

| Positive Sample | Experiment | sFas Ligand Concentration (ng/ml) | Coefficient of Variation (%) |
|---|---|---|---|
| 1 | 1 | 13.87 | 2.5 |
| | 2 | 13.25 | 7.9 |
| | 3 | 11.67 | 11.7 |
| 2 | 1 | 10.61 | 5.2 |
| | 2 | 10.57 | 4.9 |
| | 3 | 10.36 | 10.4 |
| 3 | 1 | 7.81 | 2.2 |
| | 2 | 7.46 | 5.3 |
| | 3 | 7.00 | 7.2 |
| 4 | 1 | 7.18 | 2.5 |
| | 2 | 6.34 | 3.6 |
| | 3 | 6.24 | 6.5 |
| 5 | 1 | 3.67 | 10.0 |
| | 2 | 3.61 | 2.0 |
| | 3 | 3.79 | 5.1 |
| 6 | 1 | 3.40 | 5.4 |
| | 2 | 3.59 | 9.1 |
| | 3 | 3.29 | 7.0 |
| 7 | 1 | 1.71 | 7.5 |
| | 2 | 1.75 | 3.6 |
| | 3 | 1.46 | 5.2 |
| 8 | 1 | 0.67 | 8.5 |
| | 2 | 0.81 | 1.2 |
| | 3 | 0.67 | 5.8 |

## b. Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in three independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of sFas Ligand Two standard curves were run on each plate. Data below show the mean sFas Ligand concentration and the coefficient of variation calculated on 18 determinations of each sample. The overall inter-assay coefficient of variation has been calculated to be 7 %.

| Sample | sFas Ligand Concentration (ng/ml) | Coefficient of Variation (%) |
|---|---|---|
| 1 | 12.92 | 8.8 |
| 2 | 10.51 | 1.3 |
| 3 | 7.42 | 5.5 |
| 4 | 6.59 | 7.8 |
| 5 | 3.69 | 2.4 |
| 6 | 3.43 | 4.4 |
| 7 | 1.64 | 9.6 |
| 8 | 0.72 | 11.5 |

## C. Spiking Recovery

The spiking recovery was evaluated by spiking of recombinant sFAS Ligand into four different sera. Recoveries were determined in three independent experiments with 6 replicates each. The amount of endogenous sFas Ligand in unspiked serum was substracted from the spike values. Recoveries ranged from 82 to 98% with an overall mean recovery of 85%.

## D. Dilution Linearity

Four serum samples with different levels of sFas Ligand were assayed at four serial two-fold dilutions with 4 replicates each. In the table below the per cent recovery of expected values is listed. Recoveries ranged from 97% to 119% with an overall mean recovery of 106 %.

| | | sFas Ligand Concentration (ng/ml) | | |
|---|---|---|---|---|
| Sample | Dilution | Expected Value | Observed Value | % Recovery of Exp. Value |
| 1 | 1:2 | - | 13.5 | - |
| | 1:4 | 6.8 | 7.3 | 107.5 |
| | 1:8 | 3.6 | 3.7 | 102.9 |
| | 1:16 | 1.9 | 2.0 | 106.3 |
| 2 | 1:2 | - | 11.3 | - |
| | 1:4 | 5.7 | 5.5 | 98.1 |
| | 1:8 | 2.8 | 2.9 | 105.5 |
| | 1:16 | 1.5 | 1.6 | 111.2 |
| 3 | 1:2 | - | 6.8 | - |
| | 1:4 | 3.4 | 4.0 | 119.2 |
| | 1:8 | 2.0 | 2.2 | 107.3 |
| | 1:16 | 1.1 | 1.1 | 101.4 |
| 4 | 1:2 | - | 6.5 | - |
| | 1:4 | 3.3 | 3.2 | 97.3 |
| | 1:8 | 1.6 | 1.7 | 106.6 |
| | 1:16 | 0.8 | 1.0 | 113.5 |

## E. Sample Stability

### a. Freeze-Thaw Stability

Aliquots of serum samples (unspiked or spiked) were stored at -20°C and thawed up to 5 times, and sFas Ligand levels determined. There was no significant loss of sFas Ligand by freezing and thawing up to 5 times.

### b. Storage Stability

Aliquots of a serum sample (spiked or unspiked) were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the sFas Ligand level determined after 24 h. There was a loss of sFas Ligand immunoreactivity during storage at 37°C. Therefore higher temperatures during handling the serum samples should be avoided.

## F. Comparison of Serum and Plasma

From eight individuals, serum as well as EDTA and citrate, and heparin plasma obtained at the same time point were evaluated. sFas Ligand concentrations were not significantly different and therefore all these body fluids are suitable for the assay. It is nevertheless highly recommended to assure the uniformity of blood preparations.

## G. Specificity

The interference of circulating factors of the immune systems was evaluated by spiking these proteins at physiologically relevant concentrations into a serum sample. There was no detectable cross reactivity.

## H. Expected Values

There are no detectable sFas Ligand levels found in healthy blood donors.

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# 16 REAGENT PREPARATION SUMMARY

**A. Wash Buffer** Add **Wash Buffer Concentrate** 20 x (50 ml) to 950 ml distilled water

**B. Assay Buffer**

| Number of Strips | Assay Buffer Concentr. (ml) | Distilled Water (ml) |
|---|---|---|
| 1 - 6 | 2.5 | 47.5 |
| 1 - 12 | 5.0 | 95.0 |

**C. Standard** Reconstitute sFasL Standard in distilled water as stated on label of the standard vial.

**D. Biotin-Conjugate** Make 1:100 dilution according to the table.

| Number of Strips | Biotin-Conjugate (ml) | Assay Buffer (ml) |
|---|---|---|
| 1 - 6 | 0.03 | 2.97 |
| 1 - 12 | 0.06 | 5.94 |

**E. Streptavidin-HRP**

| Number of Strips | Streptavidin-HRP (ml) | Assay Buffer (ml) |
|---|---|---|
| 1 - 6 | 0.03 | 5.97 |
| 1 - 12 | 0.06 | 11.94 |

## 17 TEST PROTOCOL SUMMARY

- Wash microwell strips twice with **Wash Buffer**
- Add 100 µl **Sample Diluent**, in duplicate, to standard wells.
- Pipette 100 µl **reconstituted sFas Ligand Standard** into the first wells and create standard dilutions ranging from 10 to 0.16 ng/ml by transferring 100 µl from well to well. Discard 100 µl from the last wells
- Add 100 µl **Sample Diluent**, in duplicate, to the blank wells
- Add 50 µl **Sample Diluent** to sample wells.
- Add 50 µl **Sample**, in duplicate, to designated wells
- Prepare **Biotin-Conjugate**
- Add 50 µl of diluted **Biotin-Conjugate** to all wells
- Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C)
- Prepare **Streptavidin-HRP**
- Empty and wash microwell strips 4 times with **Wash Buffer**
- Add 100 µl diluted **Streptavidin-HRP** to all wells
- Cover microwell strips and incubate 1 hour at room temperature (18º to 25ºC)
- Empty and wash microwell strips 4 times with **Wash Buffer**
- Add 100 µl of **TMB Substrate Solution** to all wells including blank wells
- Incubate the microwell strips for about 10 minutes at room temperature (18°to 25°C).
- Add 100 µl **Stop Solution** to all wells including blank wells
- Blank microwell reader and measure colour intensity at 450 nm

**Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect, low sFas Ligand levels. Such samples require further dilution of 1:4 - 1:8 with Sample Diluent in order to precisely quantitate the actual sFas Ligand level.**

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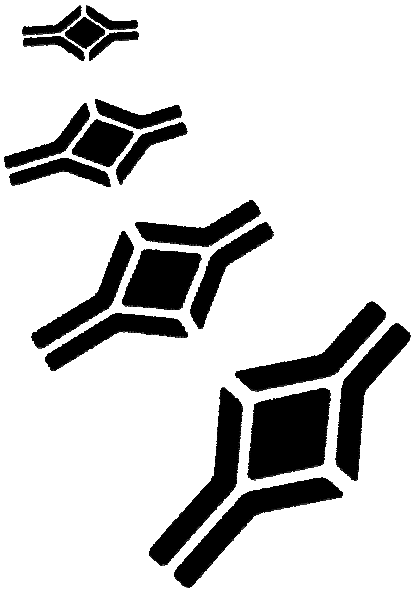


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