

ELISpot

Human Fas Ligand/TNFSF6

Catalog Number EL126

For the quantitative determination of the frequency of cells releasing human Fas Ligand.

This package insert must be read in its entirety before using this product.

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**FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

TABLE OF CONTENTS

Contents	Page
INTRODUCTION	2
PRINCIPLE OF THE ASSAY	3
ELISpot SCHEMATIC	4
LIMITATIONS OF THE PROCEDURE	5
PRECAUTIONS	5
MATERIALS PROVIDED.	5
STORAGE	5
OTHER SUPPLIES REQUIRED	6
TECHNICAL HINTS	6
REAGENT PREPARATION	6
SAMPLE PREPARATION	7
ASSAY PROCEDURE	7
CALCULATION OF RESULTS	8
REPRODUCIBILITY DATA.	8
TROUBLESHOOTING GUIDE	9
REFERENCES	10
ASSAY RECORD TEMPLATE	11

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INTRODUCTION

Fas ligand (FasL; also known as TNFSF6, CD178, and CD95L) is a 40 kDa type II transmembrane glycoprotein and member of the tumor necrosis factor (TNF) superfamily of proteins (1). FasL induces the apoptosis in cells expressing its receptor, Fas/Apo-1/CD95/TNFRSF6 (1). 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 lymphocyte mRNA (1). It is 281 amino acids in length and contains an 80 amino acid (aa) proline-rich cytoplasmic region, a 22 aa transmembrane region, and a 79 aa extracellular domain (ECD) (SwissProt #: P48023). The ECD contains three potential sites for N-linked glycosylation. Splicing variants produce two isoforms for FasL. Isoform 2 has an 11 aa substitution for residues 117 - 127 and a deletion of aa 128 - 281 found in isoform 1. Human FasL shares 77% aa sequence identity with mouse FasL and is constitutively expressed primarily in activated 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 homotrimer (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 region 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 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 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 cross-linking of sFasL homotrimers (20).

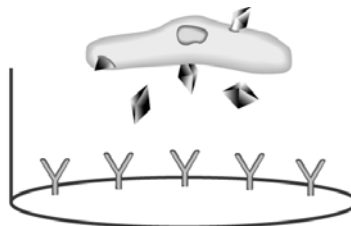
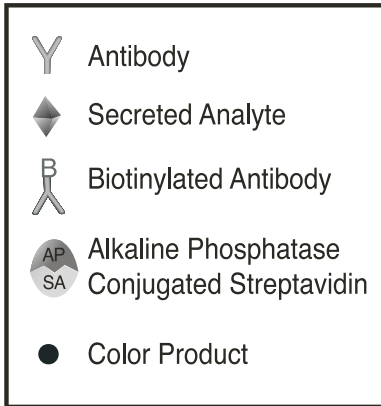
Certain types of cells do not undergo apoptosis in response to Fas stimulation. For example, Fas activation can lead to proliferation of human T cells (21) and fibroblasts (22), and sFasL can act as a chemoattractant for neutrophils (23). In human fibroblasts, sFasL stimulates phosphorylation of extracellular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

The human FasL ELISpot assay is designed for the detection of FasL secreting cells at the single cell level, and it can be used to quantitate the frequency of human FasL secreting cells. ELISpot assays are well suited for monitoring immune responses to various stimuli, treatments and therapies, and they have been used for the quantitation of antigen-specific CD4 and/or CD8 T cell responses. Other methods for the assessment of antigen-specific T cell responses, such as the chromium release assay with quantitation by limiting dilution, are tedious, and require previous *in vitro* expansion of T cells for several days. These assays typically are not suitable for measuring infrequent T cell responses that occur at less than 1 in 1000. ELISpot assays are highly reproducible and sensitive and can be used to measure responses with frequencies well below 1 in 100,000. ELISpot assays do not require prior *in vitro* expansion of T cells, and they are suitable for high-throughput analysis using only small volumes of primary cells. As such, ELISpot assays are useful tools for research in areas as diverse as antigen recognition, vaccine development, and the monitoring of various clinical trials.

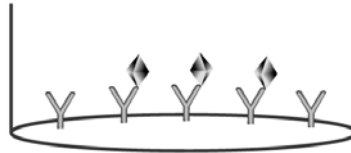
PRINCIPLE OF THE ASSAY

The enzyme-linked immunospot (ELISpot) assay was originally developed for the detection of individual B cells secreting antigen-specific antibodies (26, 27). This method has since been adapted for the detection of individual cells secreting specific cytokines or other antigens (28, 29). ELISpot assays employ the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique. A monoclonal antibody specific for human Fas Ligand has been pre-coated onto a PVDF (polyvinylidene difluoride)-backed microplate. Appropriately stimulated cells are pipetted into the wells and the microplate is placed into a humidified 37° C CO₂ incubator for a specified period of time. During this incubation period, the immobilized antibody in the immediate vicinity of the secreting cells binds secreted Fas Ligand. After washing away any cells and unbound substances, a biotinylated polyclonal antibody specific for human Fas Ligand is added to the wells. Following a wash to remove any unbound biotinylated antibody, alkaline-phosphatase conjugated to streptavidin is added. Unbound enzyme is subsequently removed by washing and a substrate solution (BCIP/NBT) is added. A blue-black colored precipitate forms at the sites of cytokine localization and appear as spots, with each individual spot representing an individual Fas Ligand secreting cell. The spots can be counted with an automated ELISpot reader system or manually using a stereomicroscope.

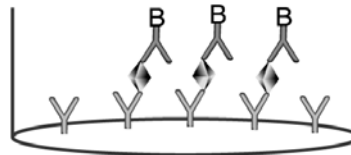
ELISpot SCHEMATIC



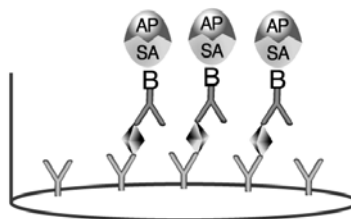
Incubate Fas Ligand-secreting cells in an antibody-coated well.



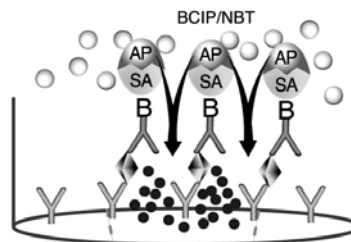
Remove cells by washing. Secreted Fas Ligand is captured by the immobilized antibody.



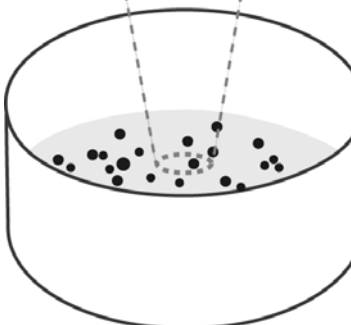
Incubate with biotinylated anti-Fas Ligand antibody.



Incubate with alkaline phosphatase conjugated streptavidin.



Add substrate and monitor the formation of colored spots.



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.
- Any variation in pipetting and washing techniques, incubation time or temperature, and kit age can cause variation in density of spots, intensity of specific staining and background level.

PRECAUTIONS

Some components of this kit contain sodium azide, which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

Do not use reagents from this kit with components from other R&D Systems ELISpot or ELISA kits and/or components manufactured by other vendors.

Do not remove the flexible plastic underdrain on the bottom of the microplate before or during incubation and development since it may damage the PVDF membrane filters. The underdrain cover may be removed only after completing the incubation with BCIP/NBT chromogen.

Although the toxicity of the chromogenic substrate BCIP/NBT is not currently known, wear gloves to avoid contact with skin. Follow local, state and federal regulations to dispose of used BCIP/NBT.

MATERIALS PROVIDED

Human Fas Ligand Microplate (Part 893816) - One 96-well PVDF-backed microplate coated with monoclonal antibody specific for human Fas Ligand.

Detection Antibody Concentrate (Part 893817) - 150 μ L of a 120X concentrated solution of biotinylated polyclonal antibody specific for human Fas Ligand with preservatives.

Streptavidin-AP Concentrate A (Part 895358) - 150 μ L of a 120X concentrated solution of Streptavidin conjugated to Alkaline Phosphatase with preservatives.

Dilution Buffer 1 (Part 895307) - 12 mL of a buffer for diluting Detection Antibody Concentrate with preservatives.

Dilution Buffer 2 (Part 895354) - 12 mL of a buffer for diluting Streptavidin-AP Concentrate A with preservatives.

Wash Buffer Concentrate (Part 895308) - 50 mL of a 10X concentrated solution of a buffered surfactant with preservative.

BCIP/NBT Chromogen (Part 895867) - 12 mL of a stabilized mixture of 5-Bromo-4-Chloro-3' Indolyphosphate p-Toluidine Salt (BCIP) and Nitro Blue Tetrazolium Chloride (NBT).

Human Fas Ligand Positive Control (Part 893818) - 1 vial (8 ng) of recombinant human Fas Ligand; lyophilized.

STORAGE

Store the unopened kit at 2 - 8° C. Do not use beyond the kit expiration date. This kit is validated for single use only. Results obtained with opened/reconstituted reagents at a later date may not be reliable.

OTHER SUPPLIES REQUIRED

- Pipettes and pipette tips
- Deionized or distilled water
- Squirt bottle, manifold dispenser, or automated microplate washer
- 500 mL graduated cylinder
- 37° C CO₂ incubator
- Sterile culture media
- Dissection microscope or an automated ELISpot reader

TECHNICAL HINTS

- To minimize edge effect, place the microplate (bottom down) onto a piece of aluminum foil (about 4 x 6 inches). Add cells, cover the microplate with the lid and shape the foil around the edges of the microplate. The foil may be left on the microplate for the rest of the experimental procedure and removed after the BCIP/NBT has been washed off.
- Do not touch PVDF membrane filters with pipette tips when pipetting cells and reagents to avoid damage to the membrane.
- After completion of the experiment, do not dry the microplate at a temperature higher than 37° C since it may cause cracking of the PVDF membrane filters.
- The 96-well microplate provided in the kit is not sterile. However, due to the short incubation period and the presence of antibiotics in the culture media, microbial contamination has not been a problem during the ELISpot procedure.
- The kit is designed for single use only. The layout of the assay should be carefully planned to maximize the use of the plate and reagents provided.
- The controls listed are recommended for each ELISpot experiment.
 - Positive Control - Recombinant human Fas Ligand (provided).
 - Unstimulated/Negative Control - Use the same number of unstimulated cells as stimulated cells.
 - Background Control - Use sterile culture media.
 - Detection Antibody Control - Substitute phosphate buffered saline for Detection Antibody.

REAGENT PREPARATION

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare Wash Buffer, add 50 mL of Wash Buffer Concentrate to 450 mL of deionized water and mix well.

Human Fas Ligand Positive Control - Reconstitute the lyophilized human Fas Ligand with 250 µL of culture medium that is used to incubate cells.

Detection Antibody - Tap or vortex the vial to release reagent collected in the cap. Transfer 100 µL of Detection Antibody Concentrate into the vial labeled Dilution Buffer 1 and mix well.
For optimal performance, prepare Detection Antibody immediately before use.

Streptavidin-AP - Tap or vortex the vial to release reagent collected in the cap. Transfer 100 µL of Streptavidin-AP Concentrate A into the vial labeled Dilution Buffer 2 and mix well.
For optimal performance, prepare Streptavidin-AP immediately before use.

SAMPLE PREPARATION

The types of effector and responder cells used, method of cell separation, mode of stimulation, and length of incubation are to be determined by each investigator. R&D Systems cell selection products are suitable for the purification of effector and responder cells. For a complete product listing of human, mouse, and rat cell selection products, see the R&D Systems catalog or visit our website at www.RnDSystems.com.

ASSAY PROCEDURE

Bring all reagents as needed to room temperature, except the Detection Antibody Concentrate and Dilution Buffer 1, which should remain at 2 - 8° C. All samples and controls should be assayed at least in duplicate. An Assay Record Template is provided at the back of this insert to record controls and samples assayed.

1. Fill all wells in the microplate with 200 μ L of sterile culture media and incubate for approximately 20 minutes at room temperature.
2. When cells are ready to be plated, aspirate the culture media from the wells. Immediately add 100 μ L of the appropriate cells or controls to each well (see Technical Hints for appropriate controls).
3. Incubate cells in a humidified 37° C CO₂ incubator. Optimal incubation time for each stimuli should be determined by the investigator. **Do not disturb the cells during the incubation period.**
4. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (250 - 300 μ L) 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.
Note: Adjust the height of the prongs of the manifold dispenser or autowasher to prevent damage to the membranes.
5. Add 100 μ L of diluted Detection Antibody into each well and incubate at **2 - 8° C** overnight.
6. Repeat step 4.
7. Add 100 μ L of diluted Streptavidin-AP into each well and incubate for 2 hours at room temperature.
8. Repeat step 4.
9. Add 100 μ L of BCIP/NBT Chromogen into each well and incubate for 1 hour at room temperature. **Protect from light.**
10. Discard the chromogen solution from the microplate and rinse the microplate with deionized water. Invert the microplate and tap to remove excess water. Remove the flexible plastic underdrain from the bottom of the microplate, wipe the bottom of the plate thoroughly with paper towels and dry completely either at room temperature (60 - 90 minutes) or 37° C (15 - 30 minutes).

CALCULATION OF RESULTS

The developed microplate can be analyzed by counting spots either manually using a dissection microscope or by using a specialized automated ELISpot reader. Specific spots are round and have a dark center with slightly fuzzy edges. Quantitation of results can be done, for example, by calculating the number of spot forming cells (SFC) per number of cells added into the well.

REPRODUCIBILITY DATA

Human PBMCs (1×10^5 cells/mL) were stimulated with 50 ng/mL of phorbol 12-myristate-13-acetate and 0.5 μ g/mL calcium ionomycin overnight at 37° C in a 5% CO₂ incubator. The sample was assayed in eight wells according to the procedure and analyzed with a dissection microscope.

Well	Number of Spots Counted
1	696
2	651
3	683
4	739
5	738
6	705
7	643
8	704

TROUBLESHOOTING GUIDE

Observation	Problem	Corrective Action
Following the incubation with BCIP/NBT chromogen and rinsing the microplate with deionized water, the dark-blue background color of filter membrane attenuates visualization and quantitation of spots.	Wet membrane	Microplates cannot be analyzed accurately until PVDF filter membranes are completely dry. Wait until membrane becomes dry, usually 15 - 30 minutes at 37° C or 60 - 90 minutes at room temperature.
The number of spots in the wells that contained the cells is high but their contrast as well as intensity of staining in the Positive Control wells is low.	Underdevelopment - perhaps as a result of using Streptavidin-AP and/or BCIP/NBT solutions that have not been adjusted to room temperature	Bring the temperature of the reagents to room temperature before adding to the wells.
The number of spots in the wells that contained cells is lower than expected whereas Positive Control wells turned black-blue.	Cell stimulation problem	Ensure that reagents used to stimulate the cytokine release from the cells retained their biological activity. One way to check is to perform immunocytochemistry on fixed cells after stimulation.
	Too few cells added to the wells	Increase the number of cells added per well.
Following incubation with BCIP/NBT and drying the microplate, the density of the spots makes it difficult to quantify them.	Too many cells were added to the wells	Make dilutions of cells (<i>i.e.</i> , 1×10^6 , 5×10^5 , 1×10^5 , 5×10^4 , 1×10^4 cells per well) to determine the optimal number of cells that will result in formation of distinct spots.

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ASSAY RECORD TEMPLATE

Use this template as a record of samples and controls run in the assay.

1								
2								
3								
4								
5								
6								
7								
8								
9								
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11								
12								
	A	B	C	D	E	F	G	H