



Fas Ligand ELISA Kit Cat. No. QIA27

Note that this user protocol is not lot-specific and is representative of the current specifications for this product. Please consult the vial label and the certificate of analysis for information on specific lots. Also note that shipping conditions may differ from storage conditions. Full details are available at www.calbiochem.com.

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Upon receipt of the kit, store **standards at -20°C**. All other components may be stored at 4°C. Conjugate 400X should not be stored at 4°C for more than 4 months. Do not expose reagents to excessive light. Let kit reagents and samples come to room temperature before use.

Intended Use

The Calbiochem® Fas Ligand ELISA is a non-isotopic immunoassay for the *in vitro* quantitation of human FasL protein in cell lysates, tissue culture medium, serum and plasma.

Background

Fas Ligand (FasL, CD95L) is a 40 kDa type II transmembrane protein of the TNF (TNF) family. Like other TNF family members, FasL is a homotrimeric molecule. The crystal structure of lymphotoxin complex with TNFR1 suggests by analogy that each FasL trimer binds three Fas molecules. Engagement of Fas by FasL on target cells triggers a cascade of subcellular events that ends in apoptosis. This cascade involves recruitment to Fas of a number of proteins sharing a homologous 'death domain'. A death-inducing signaling complex (DISC) is then formed at the intracellular region of stimulated Fas. Within the DISC, an adapter protein, FADD, mediates recruitment of death domain-containing members of the caspase (cysteine aspartate-specific protease) family. Oligomerization of caspase-8 drives its activation through self-cleavage. Caspase-8 then activates downstream effector caspases such as caspase-9 committing the cell to apoptosis.

The Fas/FasL system is one of the best-studied death systems. It plays an essential role in elimination of autoreactive lymphocytes and the deletion of activated T cells following an immune response. Fas/FasL mediates the killing of virus-infected cells and cancer cells by cytotoxic T cells and by natural killer cells. FasL is not only expressed in cells of the lymphoid/myeloid series, but it is also expressed by non-lymphoid cells where it contributes to immune privilege by inducing apoptosis in infiltrating proinflammatory immunocytes. The source of the death signal in Myc-induced apoptosis in fibroblasts is the specific autocrine interaction between Fas and FasL. In addition there is a relationship between DNA-damaging agents, p53, and the Fas/FasL pathway. Both FasL and Fas are induced after DNA damage, in part through a p53-dependent mechanism. It is believed that Myc acts to sensitize cells to a p53-induced Fas death signal.

Soluble FasL (26 kDa) generated by metalloprotease activity can kill Fas-positive activated T cells. The production of a soluble form of FasL by tumors might also be relevant *in vivo*. Soluble FasL has been detected in the sera of patients with lymphoid tumors and its functional capacity has been demonstrated in some instances. Soluble FasL is functionally active against cells that are highly sensitive to Fas-mediated apoptosis, even though it has less apoptosis-inducing activity than membrane bound FasL. It remains to be established whether tumor-derived soluble FasL could have systemic consequences by increasing apoptosis in tissues that are particularly prone to Fas-mediated apoptosis. Recombinant soluble FasL, which contains the almost complete extracellular region of FasL, inhibits apoptosis both of activated T cells and peripheral blood T cells induced by the membrane form of Fas. Thus, soluble FasL shows either agonist or antagonist activity, although the mechanism for the difference remains obscure.

Principle of the Assay

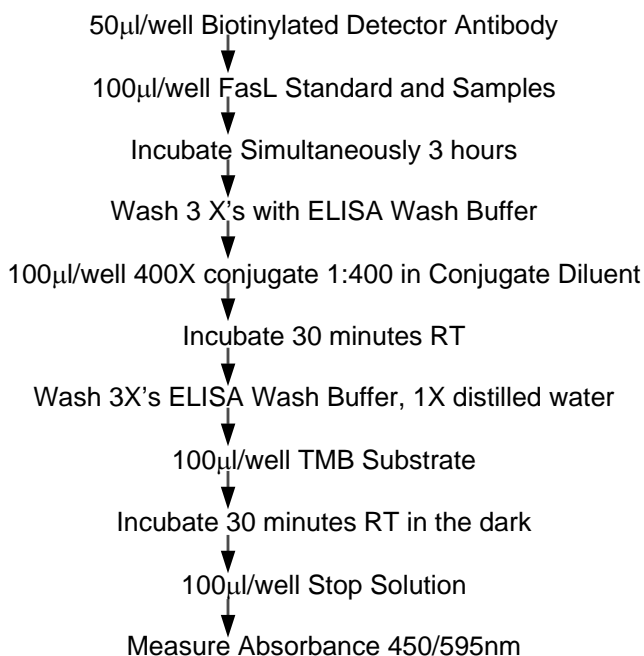
The Fas Ligand ELISA Kit is a “sandwich” enzyme immunoassay employing mouse monoclonal antibodies. A monoclonal antibody, specific for the human FasL protein, has been immobilized onto the surface of wells provided in the kit. The sample to be assayed and biotinylated detector monoclonal antibody are pipetted into the wells and allowed to incubate for three hours, during which time any FasL present binds to the capture and detecting antibodies. Unbound material is washed away and horseradish peroxidase-conjugated streptavidin is added, which binds to the detector antibody. The horseradish peroxidase catalyzes the conversion of the chromogenic substrate tetra-methylbenzidine (TMB) from a colorless solution to a blue solution (or yellow after the addition of stop reagent), the intensity of which is proportional to the amount of FasL protein in the sample. The colored reaction product is quantified using a spectrophotometer.

Quantitation is achieved by the construction of a standard curve using known concentrations of FasL (provided lyophilized). By comparing the absorbance obtained from a sample containing an unknown amount of FasL with that obtained from the standards, the concentration of FasL in the sample can be determined.

The Fas Ligand ELISA has the following characteristics:

- Can detect membrane bound FasL (Figures 4-6).
- Can detect soluble FasL (sFasL) in tissue culture supernatants (Figures 1, 4-6 and 8).
- Can detect sFasL in human sera (Figure 7).
- Has excellent sensitivity and precision (Figures 3 and 9).
- Has a short and simple format (see below).
- Has a 96 well format that can be easily adapted for high-throughput screening (Figure 1).

Summary of Procedure



Materials Provided

Standards should be assayed in duplicate. A FasL standard curve must be performed on the same plate and at the same time as the samples. The Fas Ligand ELISA provides sufficient reagents to run two sets of standard curves, and 41 samples (if assayed in duplicate all at once using one standard curve), or 34 samples (if assayed on two separate occasions using two standard curves).

- **Coated 96-Well Plate:** 96 removable wells coated with FasL monoclonal antibody.
- **FasL Standard:** two vials containing lyophilized FasL protein.
 - *Reconstituted standards should be discarded after one use.*
- **Detector Antibody:** biotinylated monoclonal anti-human FasL antibody.
- **400X Conjugate:** Streptavidin-Peroxidase Conjugate; 400-fold concentrated solution.
- **Conjugate Diluent:** buffer for dilution of 400X Conjugate.
- **TMB: Chromogenic Substrate**
- **Sample Diluent:** buffer used to dilute standards and samples.
- **20X Plate Wash Concentrate:** 20-fold concentrated solution of PBS and surfactant. Contains 2% chloroacetamide.
- **Antigen Extraction Agent (AEA):** use as directed in sample preparation section for the extraction of FasL from cell preparations.
- **Stop Solution:** 2.5 N sulfuric acid.
- **Plate Sealers:** to cover plates during incubations.

Materials Required But Not Provided

- 2-20 µl, 20-200 µl and 200-1000 µl precision pipettors with disposable tips.
- Automated plate washer, wash bottle or multichannel dispenser for washing.
- 1 liter graduated cylinder.
- Deionized or distilled H₂O.
- 0.2 µm syringe filter and syringe.

- Spectrophotometer capable of measuring absorbance in 96-well plates using dual wavelength of 450/595 nm or 450/540 nm. A single wavelength of 450 nm can also be used.
- Cell Resuspension Buffer: 50 mM Tris, containing 5 mM EDTA, 0.2 mM PMSF, 1 µg/ml pepstatin, and 0.5 µg/ml leupeptin adjusted to pH 8.0.

Precautions and Recommendations

- **Store standards and 400X conjugate at –20°C; store all other components at 2°C - 8°C.** Do not expose reagents to excessive light.
- Let the kit sit at room temperature for 30 minutes before use. Best results will be obtained using reagents at room temperature.
- Wear disposable gloves and eye protection.
- Always use clean well-rinsed glassware. Soap residue may compromise assay performance.
- Use only the wells provided with the kit.
- Do not mix reagents from different kits.
- Do not mouth pipette or ingest any of the reagents.
- The buffers and reagents used in this kit contain anti-microbial and anti-fungal reagents. Care should be taken to prevent direct contact with these products.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Human samples may be contaminated with infectious agents. Do not ingest, expose to open wounds, or breathe aerosols. Dispose of samples properly.

Sample Preparation

Cell Lysate Protocol. Numerous extraction protocols can be used. The following protocol has been shown to work with a number of cell lines. It is provided as an example of a suitable extraction procedure, but should not be construed as necessarily being the method of choice. Users may wish to experiment with extraction procedures that work best in their hands.

1. For suspension cells, pellet by centrifugation, remove supernatant, resuspend with PBS and pellet by centrifugation. For attached cells, remove supernatant from cells (you may save the supernatant for testing in the ELISA). Wash cells once with PBS, harvest cells by scraping and gentle centrifugation.
2. Aspirate PBS leaving an intact cell pellet (at this point the cell pellet can be frozen at –80°C and lysed at a later date). We recommend for every 2.5×10^6 cells, resuspend the pellet in 100 µl of Resuspension Buffer (e.g. one T-75 flask with 10 mls of cells at 1×10^6 /ml would be resuspended with 400 µl Resuspension Buffer).
3. Add 20 µl of Antigen Extraction Agent (AEA provided) for every 100 µl of cell suspension.
4. Incubate 30 minutes on ice with occasional vortexing.
5. Transfer extracts to microcentrifuge tubes and centrifuge for 5 minutes at 500 x g at 4°C.
6. Aliquot cleared lysate to clean microfuge tubes. The sample should be aliquotted to avoid multiple freeze/thaws. These samples are now ready for analysis according to the instructions provided in the Detailed Protocol. Samples may be stored at –20°C for future testing in the Fas Ligand ELISA.
7. Samples found to contain greater than 5 ng/ml FasL (i.e., outside the range of the standard curve) must be diluted with Sample Diluent (provided), so that the FasL concentration falls within the range spanned by the standard curve, and assayed again.

Format for Biological Experiments Designed using 96-Well Tissue Culture Plates. This format is recommended for testing supernatant samples only. Grow cells in desired media and plate cells at desired concentration (100 μl /well) in a sterile 96-well tissue culture plate. The Fas Ligand ELISA is sensitive enough to detect FasL levels from 2.5×10^4 cells/well (see Figure 1). Treat cells with drug(s) of choice at the appropriate dose and time courses.

Culture Medium Protocol. For suspension cells: Pellet by centrifugation (1000 x g for 10 min, 4°C) and remove supernatant for testing. It is convenient to simply transfer the samples directly to the ELISA (100 μl /well). Samples may be stored at -20°C . For adherent cells: Remove tissue culture media, centrifuge tissue culture media (1000 x g for 10 min), and remove supernatant for testing. Samples may be store at -20°C .

Serum0 Protocol. Freshly drawn blood is incubated at room temperature for 30 min to allow clot to form. Loosen clot from the tube by ringing the side of the tube with a pasteur pipet. Incubate at 4°C for 30 minutes, allowing the clot to contract. The serum is separated by centrifugation at 10,000g for 10 min at 4°C .

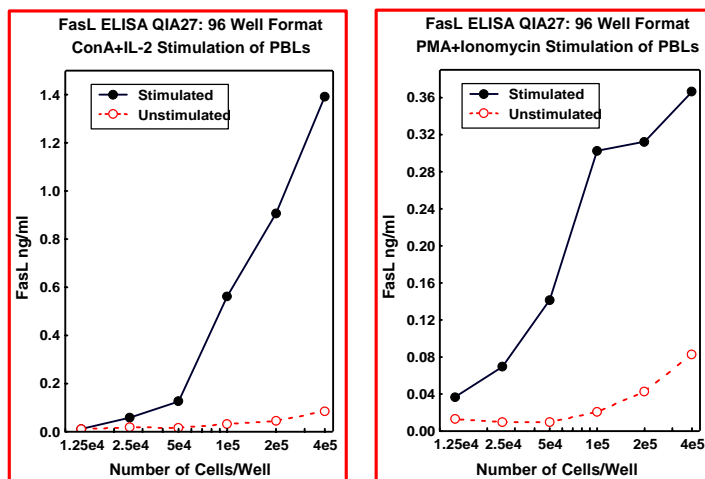


Figure 1. Sensitivity Using 96-Well Format. Fresh Peripheral Blood Lymphocytes (PBLs) were isolated and stimulated with either ConA (5 $\mu\text{g}/\text{ml}$)+IL-2 (10ng/ml) or PMA (10ng/ml)+ionomycin (500ng/ml) to induce soluble FasL expression. After stimulation the PBLs were immediately plated out at various cell densities into 96 plate wells. Cells were incubated at 37°C for 18 h. Upregulated supernatant was measured in the Fas Ligand ELISA. sFasL levels were detectable at cell concentrations of 2.5×10^4 cells/well and above.

Detailed Protocol

The Fas Ligand ELISA is provided with removable strips of wells so the assay can be carried out on two separate occasions. **Since conditions may vary, a standard curve must be determined each time the assay is performed.** Standards should be assayed in duplicate. Disposable pipette tips and reagent troughs should be used for all transfers to avoid cross-contamination of reagents or samples.

1. Remove the appropriate number of wells from the foil pouch and place them into the empty well holder. Return any unused wells to the foil pouch, reseal and store at 4°C . Let all other kit components sit at room temperature until used. *Best results will be obtained using reagents at room temperature.*
2. Prepare a working solution (1X) of Wash Buffer by adding 50 ml of the 20X concentrated solution (provided), to 950 ml of deionized water. Mix well.
3. Each time an assay is performed, reconstitute a Lyophilized Standard as described on the lyophilized FasL Standard vial label to give a concentration of 5 ng/ml. Let the reconstituted standard sit for 15 minutes at room temperature, with occasional swirling. Avoid excessive agitation of the standard. After reconstituting the FasL Standard it should be diluted with Sample Diluent. Obtain six tubes and label them 5, 2.5, 1.25, 0.625, 0.3125 and 0 ng/ml. Add 400 μl of Sample Diluent into each tube except the 5ng/ml tube (first tube) which gets "undiluted" reconstituted standard. Remove 800 μl from the original vial of lyophilized material and add it to the first tube. Remove 400 μl from the first tube (5ng/ml) and add it to the second tube (2.5 ng/ml) and mix gently. Repeat this procedure until you reach the fifth tube (0.3125 ng/ml). The last tube (0 ng/ml) should just be Sample Diluent. **Reconstituted standards should be discarded after one use.**
4. Prepare all samples (see pages 5 and 6). **A recommended starting dilution for all samples is a 1:3 dilution with sample diluent.**
5. Pipette 50 μl of the Detector Antibody into each well.
6. Add samples and each of the Fas Ligand standards (in duplicate) by pipetting 100 μl into appropriate wells using clean pipette tips for each sample.
7. Cover wells with a plate sealer and incubate at room temperature for 3 h.
8. Wash wells 3 times with 1X Wash Buffer making sure each well is filled completely.

9. Dilute a sufficient amount of the 400X Conjugate 1:400 in Conjugate Diluent to provide 100 μ l of 1X solution for each sample and standard well (For example: add 30 μ l to 11.97 ml of Conjugate Diluent), mix gently. **Filter with a 0.2 μ m syringe filter prior to use.**
10. Pipette 100 μ l of the 1X Conjugate into each well, cover with a plate sealer and incubate at room temperature for 30 min. Discard any unused 1X Conjugate.
11. Wash wells 3 times with 1X Wash Buffer making sure each well is filled completely.
12. **Flood entire plate with dH₂O.** Remove contents of wells by inverting over sink and tapping on paper towels.
13. Add 100 μ l of Substrate Solution to each well and incubate **in the dark** at room temperature for 30 min.
14. Add 100 μ l of Stop Solution to each well **in the same order** as the previously added Substrate Solution.
15. Measure absorbance in each well using a spectrophotometric plate reader. It is preferable to read at dual wavelengths of 450/595 nm (or 450/540 nm). A single wavelength of 450 nm can also be used. Wells must be read within 30 min of adding the Stop Solution.

Evaluation of Results

1. Average the duplicate absorbance values for each standard, including the zero, and all sample values.
2. On graph paper, plot the mean absorbance values for each of the standards on the Y axis, versus the concentration of each standard (ng/ml) on the X axis.
3. Determine the concentration of unknowns by interpolation from the standard curve. There are a variety of plate reader software packages available (Softmax, Molecular Devices Corporation, Menlo Park, CA; KinetiCalc, BioTek Instruments, Inc. Winooski, VT) for analysis of plate data, which simplifies this process.
4. For samples which have been diluted, the FasL concentration must be multiplied by the dilution factor (ie., if the sample was diluted five-fold, then the FasL value obtained from the standard curve must be multiplied by five).

Standard Curve

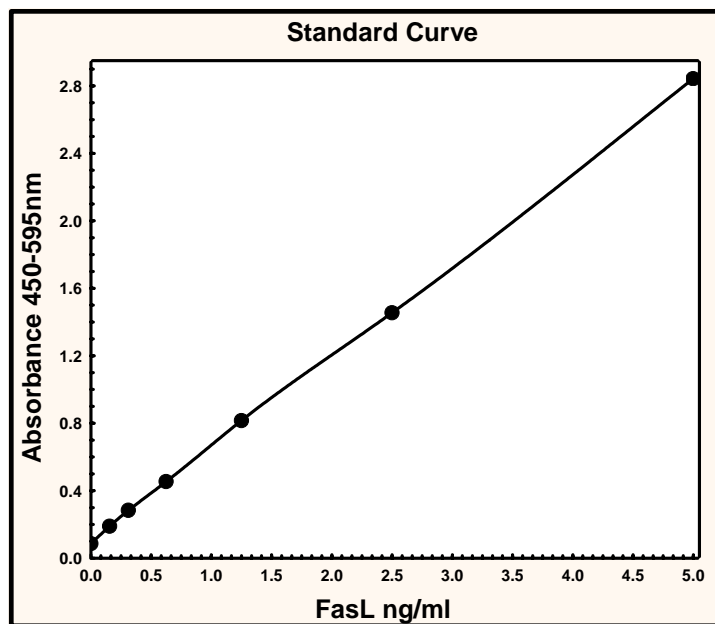


Figure 2. Standard Curve. The mean signal of each standard run in replicates of three in eight assays using two different lots of plates and two different lots of detector antibody. Recombinant FasL is used as the standard.

Assay Characteristics

Sensitivity: The assay can easily distinguish 0.02 ng/ml of FasL from zero (Figure 3).

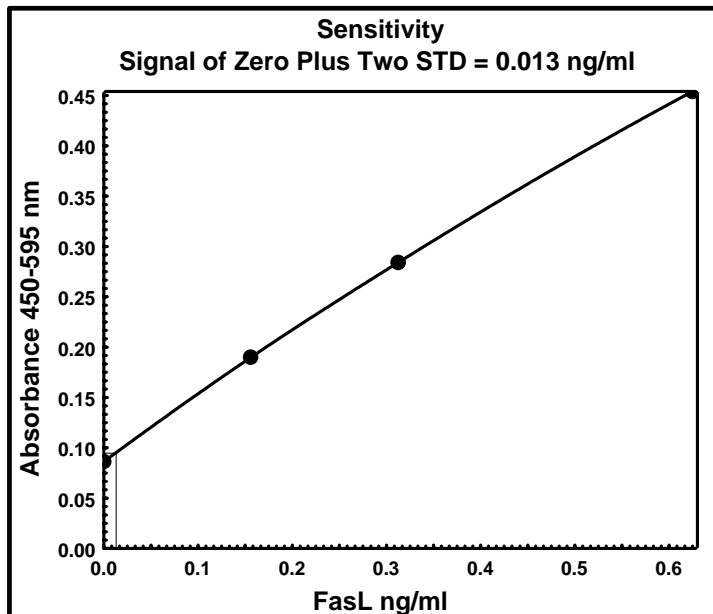


Figure 3. Sensitivity. The lower limit of detection (LLD), commonly used to define sensitivity, was measured by assaying four replicates of zero eight times using two different lots of plates and two different lots of detector antibody. The grand mean signal and pooled standard deviation of zero was calculated. The grand mean of each standard (run in replicates of three in the eight assays) was used for the standard curve (Figure 3), and the response, mean signal of zero plus two standard deviations, read in dose from the standard curve is the LLD; that is, the smallest dose that is not zero with 95% confidence.

Specificity

The Fas Ligand ELISA detects cellular and soluble FasL (Figures 4 and 5). When the FasL gene was transfected into the K562 both cellular and sFasL were detected by the assay in the transfected cell line but not in the non transfected paternal cell line (Figure 5).

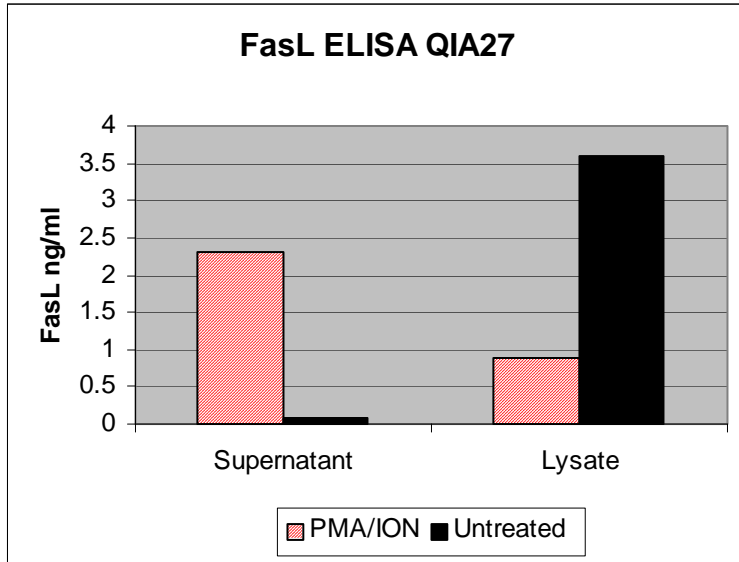


Figure 4. Levels of cellular FasL (lysates) and soluble FasL (supernatants). Human Peripheral Blood Lymphocytes were stimulated with PMA/Ionomycin. Results are expressed in concentration to demonstrate that the assay detects both forms of FasL.

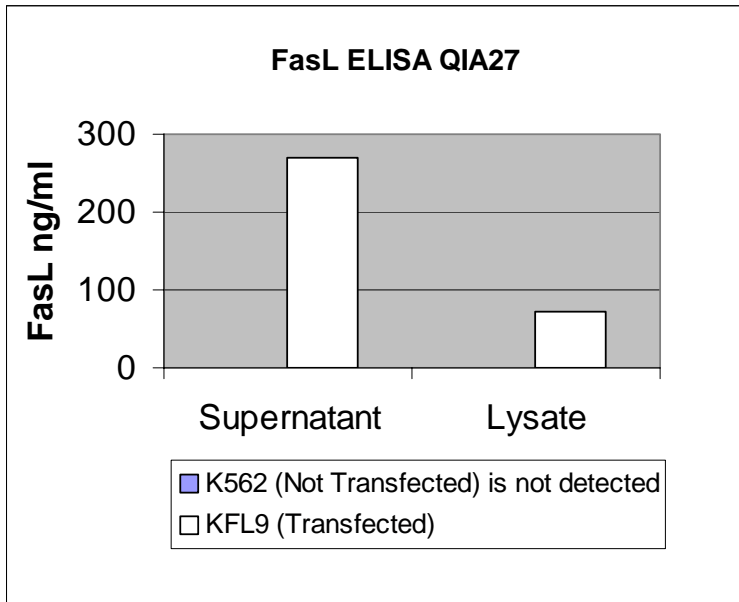


Figure 5. Levels of cellular FasL (lysates) and soluble FasL (supernatants) after transfection of the FasL gene. FasL is not detected in either the supernatant or lysate of the non-transfected parent cell line K562.

Biological Experiments:

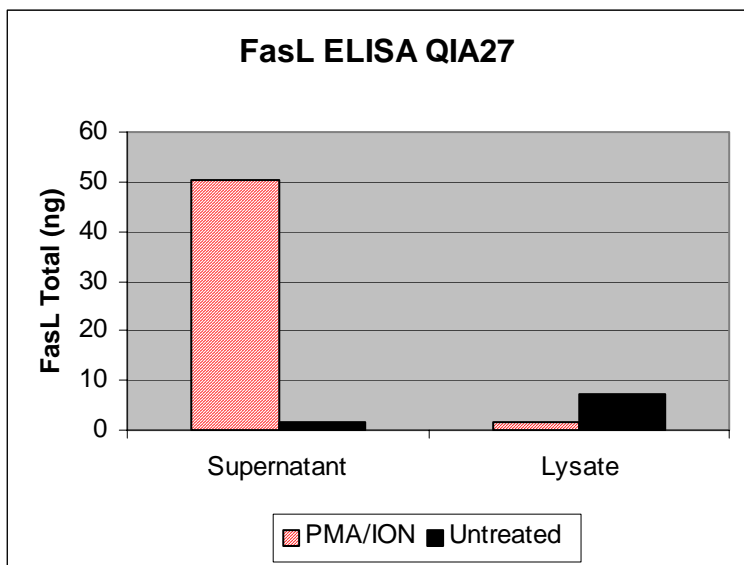


Figure 6. PMA and Ionomycin treatment of PBLs increased the levels of sFasL (supernatant) and decreased the levels of membrane bound FasL (lysates).

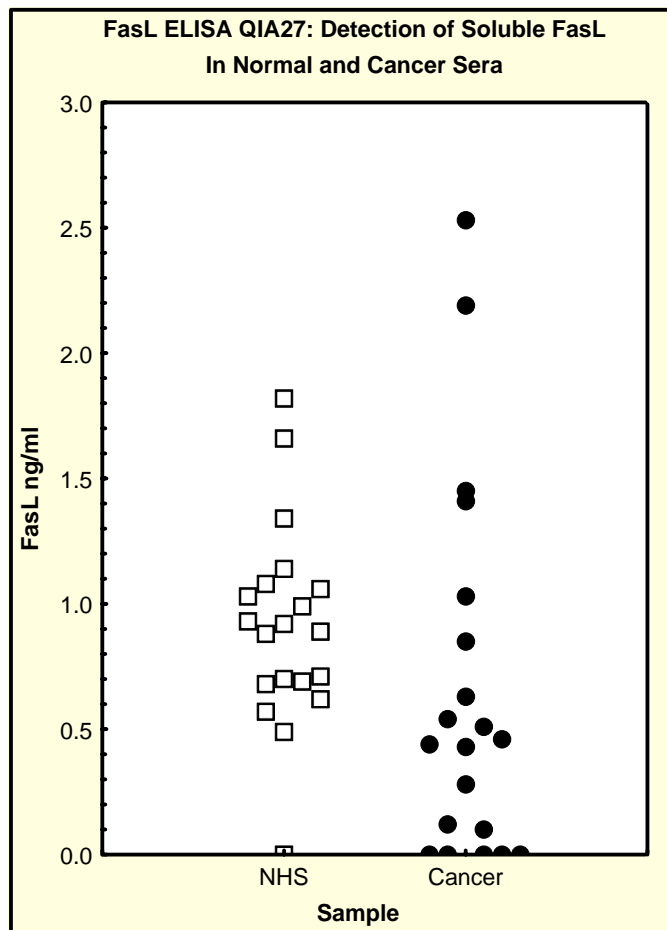


Figure 7. Detection of sFasL in Human Sera: Levels of sFasL detected by the assay are not significantly elevated in cancer sera samples tested compared to normal human sera (NHS).

Linearity of Dilution

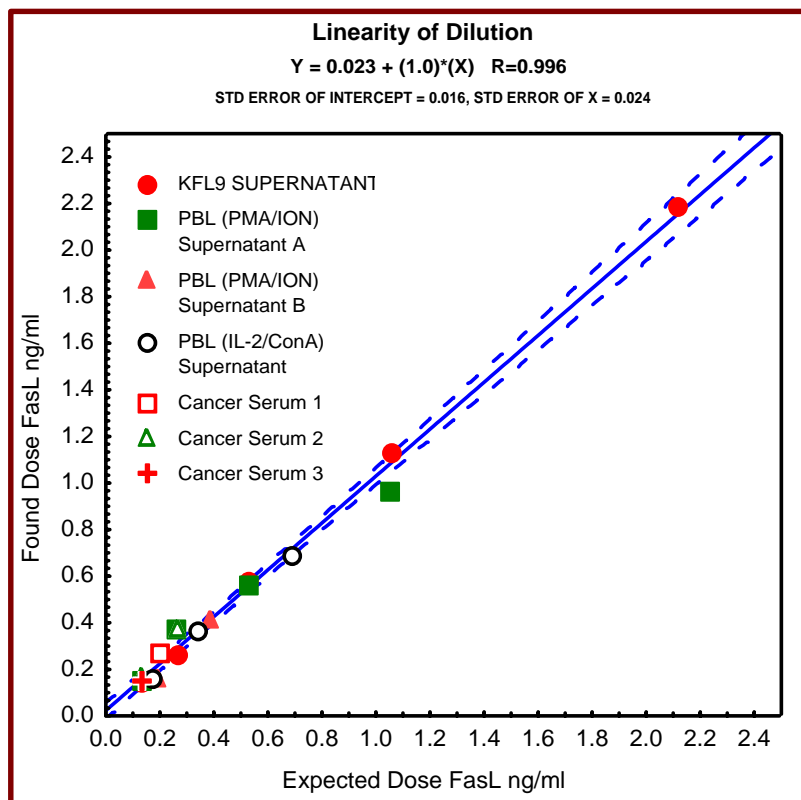


Figure 8. Parallelism. The study tested linearity of dilution with seven positive samples. The dilutions were run in the Fas Ligand ELISA and the "found" doses were plotted against the "expected" doses to determine linearity of dilution. The slope is not significantly different from one and the intercept is not significantly different from zero. These studies are consistent with the absence of cross-reacting and matrix effects such as pH, salts, and endogenous binders that interfere with the reagents used in the assay.

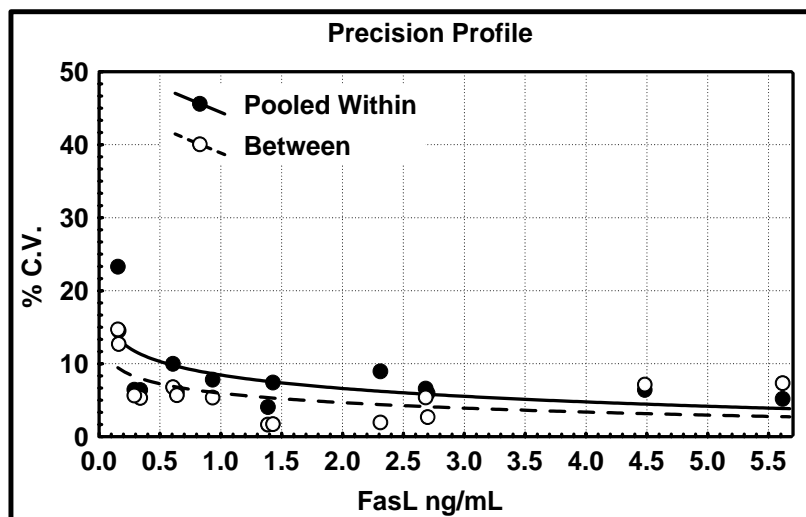


Figure 9. Precision. The pooled coefficients of variation (according to the formula of Henry et. al., 1974) and between assay coefficients of variation are plotted against FasL levels. The pooled data were collected from samples run four times using two different lots of plates and two different lots of detector antibody in replicates of three.

Reagent Stability

All of the reagents included with the Fas Ligand ELISA have been tested for stability.

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