Human APO-1/FAS ELISA Kit

Catalog Number
EK0462

For the quantitative determination of human Apoptosis inducing protein 1/ Fibroblast-associated (APO-1/FAS) concentrations in cell culture supernates, serum, and plasma.

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

For research use only. Not for use in diagnostic procedures.

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PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for APO-1/FAS has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any APO-1/FAS present is bound by the immobilized antibody. Following incubation unbound samples are removed during a wash step, and then a detection antibody specific for APO-1/FAS is added to the wells and binds to the combination of capture antibody-APO-1/FAS in sample. Following a wash to remove any unbound combination, and enzyme conjugate is added to the wells. Following incubation and wash steps a substrate is added. A coloured product is formed in proportion to the amount of APO-1/FAS 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 APO-1/FAS standard dilutions and APO-1/FAS sample concentration determined.

DETECTION RANGE

62.5 pg/ml - 4000 pg/ml

SENSITIVITY

The minimum detectable dose was 31pg/mL.

SPECIFICITY

This assay recognizes both natural and recombinant human APO-1/FAS. The factors listed below were prepared at 50ng/ml in Standard /sample Diluent and assayed for cross-reactivity and no significant cross-reactivity or interference was observed.

Table 1: Factors assayed for cross-reactivity

Recombinant human	**Recombinant mouse**	**Other proteins**
G-CSF	GM-CSF	b FGF acidic
GM-CSF	TNF-α□	b FGF basic
IL-1α□	MIP-1α	h PDGF
IL-1β□	MIP-1β	p PDGF
IL-2□	GM-CSF	h TGF-β1

PRECISION

The coefficient of variation of both intra-assay and inter-assay were less than 10%.

MATERIALS PROVIDED

1. Aluminium pouches with a Microwell Plate coated with monoclonal antibody to human APO-1/FAS (8×12)
2. 2 vials human APO-1/FAS Standard lyophilized, 4000 pg/ml upon reconstitution
3. 2 vials concentrated Biotin-Conjugate anti-human APO-1/FAS monoclonal antibody
4. 2 vials Streptavidin-HRP solution
5. 1 bottle Standard /sample Diluent
6. 1 bottle Biotin-Conjugate antibody Diluent
7. 1 bottle Streptavidin-HRP Diluent
8. 1 bottle Wash Buffer Concentrate 20x (PBS with 1% Tween-20)
9. 1 vial Substrate Solution
10. 1 vial Stop Solution
11. 4 pieces Adhesive Films
12. package insert

STORAGE

Table 2: Storage of the kit

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 – 8℃. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="9">Opened/ Reconstituted Reagents</td><td>Standard /sample Diluent</td><td rowspan="8">May be stored for up to 1 month at 2 – 8℃.**</td></tr>
<tr><td>Concentrated Biotin-Conjugate</td></tr>
<tr><td>Streptavidin-HRP solution</td></tr>
<tr><td>Biotin-Conjugate antibody Diluent</td></tr>
<tr><td>Streptavidin-HRP Diluent</td></tr>
<tr><td>Wash Buffer Concentrate 20x</td></tr>
<tr><td>Substrate Solution</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Standard</td><td>Aliquot and store for up to 1 month at ≤20℃.
Avoid repeated freeze-thaw cycles. Diluted standard shall not be reused.</td></tr>
</table>

	Microplate Wells	Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 – 8℃.**

**Provided this is within the expiration date of the kit.

THE REQUIRED ITEMS (not provided, but can help to buy):

1. Microplate reader (450nm).
2. Micro-pipette and tips: 0.5-10, 2-20, 20-200, 200-1000ul.
3. 37 ℃ incubator, double-distilled water or deionized water, coordinate paper, graduated cylinder.

PRECAUTIONS FOR USE

1. Store kit regents between 2℃and 8℃. After use all reagents should be immediately returned to cold storage(2℃ to 8℃).
2. Please perform simple centrifugation to collect the liquid before use.
3. To avoid cross contamination, please use disposable pipette tips.
4. The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material. Avoid contact of skin or mucous membranes with kit reagents or specimens. In the case of contact with skin or eyes wash immediately with water.
5. Use clean, dedicated reagent trays for dispensing the washing liquid, conjugate and substrate reagent. Mix all reagents and samples well before use.
6. After washing microtiter plate should be fully pat dried. Do not use absorbent paper directly into the enzyme reaction wells.
7. Do not mix or substitute reagents with those from other lots or other sources. Do not use kit reagents beyond expiration date on label.
8. Each sample, standard, blank and optional control samples should be assayed in duplicate or triplicate.
9. Adequate mixing is very important for good result. Use a mini-vortexer at the lowest frequency or Shake by hand at 10min interval when there is no vortexer.

10. Avoid microtiter plates drying during the operation.
11. Dilute samples at the appropriate multiple, and make the sample values fall within the standard curve. If samples generate values higher than the highest standard, dilute the samples and repeat the assay.
12. Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time and temperature, and kit age can cause variation in binding.
13. This method can effectively eliminate the interference of the soluble receptors, binding proteins and other factors in biological samples.

SAMPLE COLLECTION AND STORAGE

1. **Cell Culture Supernates** - Remove particulates by centrifugation.
2. **Serum** - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000 x g. Remove serum, avoid hemolysis and high blood lipid samples.
3. **Plasma** - Recommended EDTA as an anticoagulant in plasma. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection.
4. Assay immediately or aliquot and store samples at -20℃. Avoid repeated freeze-thaw cycles.
5. Dilute samples at the appropriate multiple (recommended to do pre-test to determine the dilution factor).
   Note: The normal human serum or plasma samples are suggested to make a 1:2 dilution.

REAGENT PREPARATION

1. Bring all reagents to room temperature before use.
2. **Wash Buffer** - Dilute 10mL of Wash Buffer Concentrate into deionized or distilled water to prepare 200mL of Wash Buffer. If crystals have formed in the concentrate Wash Buffer, warm to room temperature and mix gently until the crystals have completely dissolved.
3. **Standard** - Reconstitute the Standard with 1mL of Standard /sample Diluent. This reconstitution produces a stock solution of 8000 pg /mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

   Pipette 500μL of Standard/sample Diluent into the 2000 pg/mL tube and

the remaining tubes. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 4000 pg/mL standard serves as the high standard. The Standard/ sample Diluent serves as the zero standard (0 pg/mL).

If you do not run out of re-melting standard, store it at -20℃. Diluted standard shall not be reused.

4. Working solution of Biotin-Conjugate anti-human APO-1/FAS monoclonal antibody: Make a 1:100 dilution of the concentrated Biotin-Conjugate solution with the Biotin-Conjugate antibody Diluent in a clean plastic tube.

The working solution should be used within one day after dilution.

5. Working solution of Streptavidin-HRP: Make a 1:100 dilution of the concentrated Streptavidin-HRP solution with the Streptavidin-HRP Diluent in a clean plastic tube.

The working solution should be used within one day after dilution.

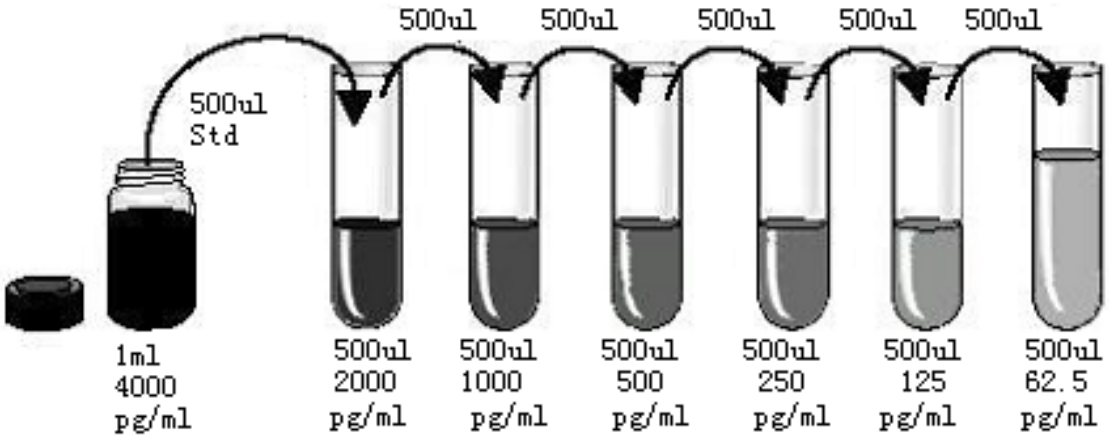

Figure 1: Preparation of APO-1/FAS standard dilutions

GENERAL ELISA PROTOCOL

1. Prepare all reagents and working standards as directed in the previous sections.
2. 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. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2-8℃ sealed tightly.
3. Add 100μL of Standard, control, or sample, per well. Cover with the

adhesive strip provided. Incubate for 1.5 hours at 37℃.

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 (350μL) using a squirt bottle, manifold dispenser or auto-washer. 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.
5. Add 100 μL of the working solution of Biotin-Conjugate to each well. Cover with a new adhesive strip and incubate 1 hours at 37℃.
6. Repeat the aspiration/wash as in step 4.
7. Add 100 μL of the working solution of Streptavidin-HRP to each well. Cover with a new adhesive strip and incubate for 30 minutes at 37℃. Avoid placing the plate in direct light.
8. Repeat the aspiration/wash as in step 4.
9. Add 100 μL of Substrate Solution to each well. Incubate for 10-20 minutes at 37℃. Avoid placing the plate in direct light.
10. Add 100 μL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
11. Determine the optical density of each well immediately, using a microplate reader set to 450 nm.(optionally 630nm as the reference wave length;610-650nm is acceptable)

ASSAY PROCEDURE SUMMARY

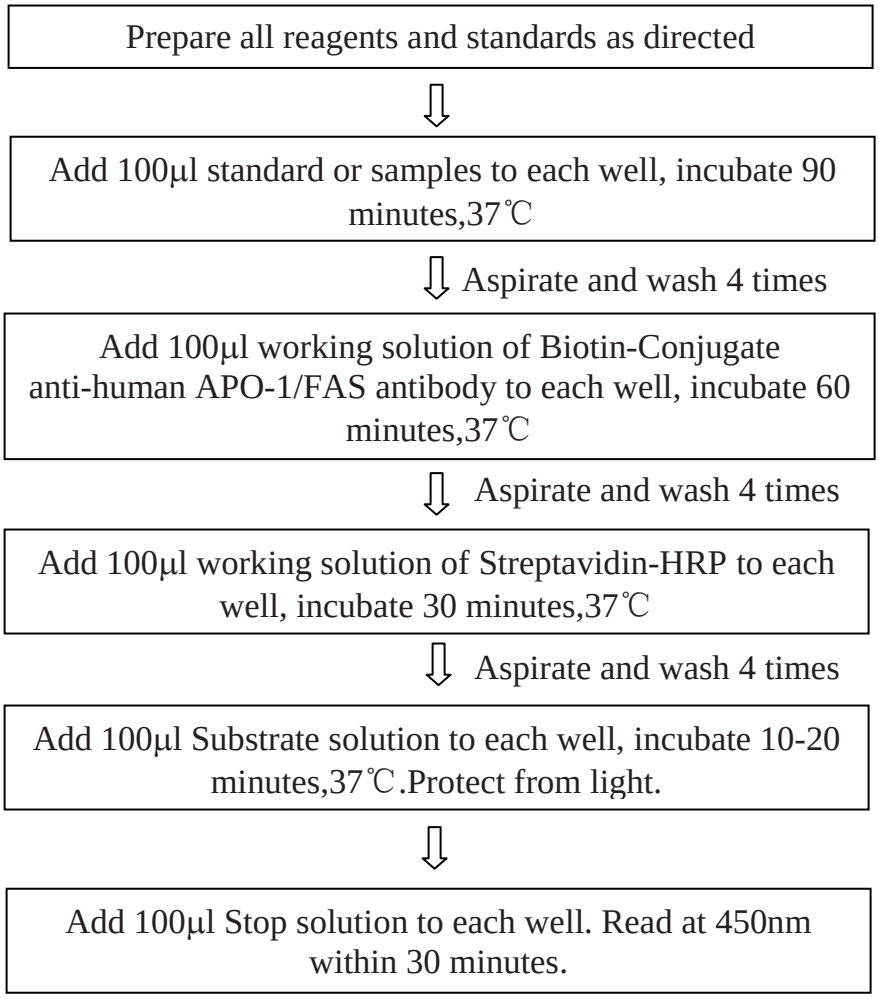

Figure 2: Assay procedure summary

TECHNICAL HINTS

1. When mixing or reconstituting protein solutions, always avoid foaming.
2. 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.
3. To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.

4. Substrate Solution should remain colorless until added to the plate. Stop Solution should be added to the plate in the same order as the Substrate Solution. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
5. A standard curve should be generated for each set of samples assayed. According to the content of tested factors in the sample, appropriate diluted or concentrated samples, it is best to do pre-experiment.

CALCULATION OF RESULTS

1. Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.
2. Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph.
3. The data may be linearized by plotting the log of the APO-1/FAS concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.
4. This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.

Table 3:Typical data using the APO-1/FAS ELISA (Measuring wavelength:450nm,Reference wavelength:630nm)

Standard (pg/ml)	OD.	OD.	Average	Corrected
0	0.044	0.042	0.043	——
62.5	0.101	0.105	0.103	0.120
125	0.166	0.162	0.164	0.173
250	0.295	0.298	0.297	0.275
500	0.492	0.487	0.490	0.472
1000	0.881	0.874	0.878	0.828
2000	1.345	1.349	1.347	1.396
4000	1.957	1.962	1.960	1.951

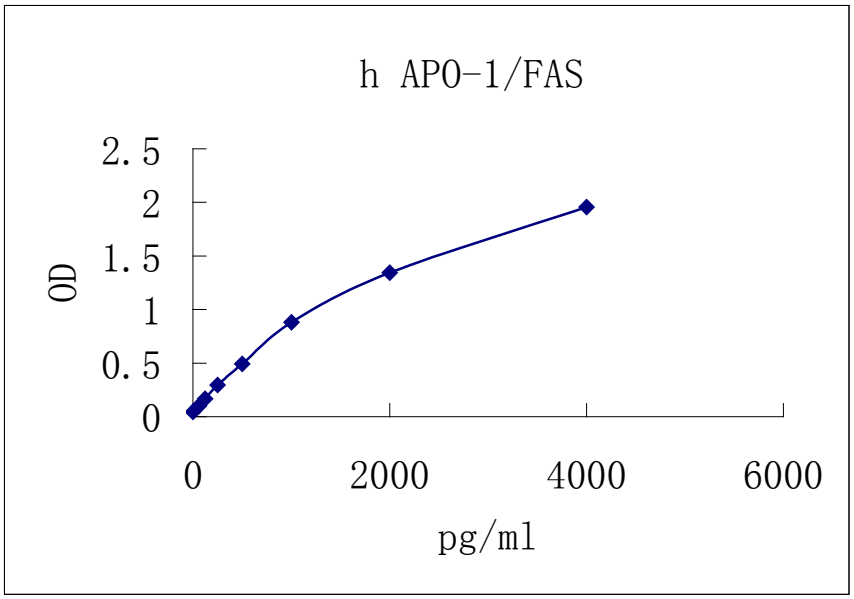

Figure 3:Representative standard curve for APO-1/FAS ELISA. APO-1/FAS was diluted in serial two-fold steps in Sample Diluent.

Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

BACKGROUND

Apoptosis inducing protein 1(Apo-1) also known as Fas (Fibroblast-associated), CD95, and TNFRSF6(325 amino acids, 45-48 kDa), was originally identified as a cell-surface protein that binds to monoclonal antibodies that are cytolytic for various human cell lines. Fas is expressed by a broad range of hematopoietic and non-hematopoietic cells including monocytes, neutrophils, activated lymphocytes and fibroblasts. Interaction of Apo on mature lymphocytes with its ligand (FasL) induces apoptosis and is thought to be important in peripheral tolerance. Fas-mediated death occurs much more rapidly than that triggered by TNFR1(Tumor necrosis factor receptor 1). Fas (APO-1 or CD95) is a cell-surface receptor that transduces apoptotic signals from Fasligand (FasL) (1, 2). It is a

glycoprotein with a mass estimated at 43 to 48 kDa (3, 4). Fas is a member of the Tumor Necrosis Factor Receptor Superfamily (TNFRSF), and it shares a cytoplasmic motif with TNF RI, referred to as the ‘death domain’, that binds cytoplasmic signaling molecules to trigger the cytoplasmic apoptotic signal (1, 2, 5). Fas is expressed to a large extent on activated T and B lymphocytes, and on malignant lymphoid cells. To a lesser extent, Fas is expressed on cells from liver, heart, kidney, ovaries, and on many other malignant cells. FasL, the physiological agonist for Fas, is also a transmembrane protein (1, 2, 5) with homology to the TNF family in its extracellular domain. FasL is expressed primarily by activated T lymphocytes and by cells of the small intestine and lung. Mice with mutations in either Fas or FasL exhibit accumulation of activated lymphocytes and classical autoimmune symptoms, suggesting that a major function of Fas-mediated apoptosis is the elimination of activated immune cells from the peripheral circulation (6). Similarly, humans with autoimmune lymphoproliferative syndrome have mutations in Fas (7, 8). Fas and FasL have been observed as soluble molecules in addition to their membraneassociated forms, suggesting additional complexity to regulation of this apoptotic mechanism (9 - 11). Soluble Fas (sFas) arises from alternatively spliced mRNA, leading to proteins with deletion or disruption of the single membrane-spanning domain (9, 10). Five alternatively spliced Fas mRNAs have been described (10), each protein detected in the supernate of cultures of peripheral blood mononuclear cells or certain tumor cell lines. Each sFas inhibited apoptosis induced by FasL (9, 10), and tumor-cell lines resistant to anti-Fas were shown to produce alternatively spliced Fas, thereby making them less sensitive to FasL. In addition, plasma Fas can arise by exfoliation of membrane vesicles, which also inhibit FasL-induced apoptosis (12). Serum Fas has been reported to be elevated in cancer patients (13 - 16), possibly originating in the tumor cell itself (17), and in autoimmune diseases (18, 19).

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