ELISA Kit

Catalog # KHS9501 (96 tests)

Human

APO-1/Fas/CD95

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Contents and Storage

Storage Store at 2 to 8°C.

Contents

Reagent	96 Test Kit
Hu APO-1/FAS Standard, recombinant Hu APO-1/Fas. Refer to vial label for quantity and reconstitution volume. Contains 0.1% sodium azide.	2 vials
Standard Diluent Buffer. Contains 0.1% sodium azide; 60 mL per bottle.	1 bottle
Hu APO-1/Fas ELISA Kit Antibody Coated Wells, 12 x 8 Well Strips	1 plate
Hu APO-1/Fas Biotin Conjugate (Biotin-labeled anti-hAPO-1/Fas). Contains 0.1% sodium azide; 6 mL per bottle.	1 bottle
Hu APO-1/FAS ELISA Kit Streptavidin-HRP (100X). Contains 3.3 mM thymol; 0.125 mL per vial.	1 vial
Streptavidin-HRP Diluent. Contains 3.3 mM thymol; 25 mL per bottle.	1 bottle
Wash Buffer Concentrate (25X); 100 mL per bottle.	1 bottle
Stabilized Chromogen, *Tetramethylbenzidine (TMB);* 25 mL per bottle.	1 bottle
Stop Solution; 25 mL per bottle.	1 bottle
Plate Covers, adhesive strips.	3

Disposal Note This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state and local regulations for disposal.

Safety All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

Introduction

Purpose

The Invitrogen Human APO-1/Fas (CD95) ELISA is to be used for the quantitative determination of Hu APO-1/Fas in human serum, buffered solution, or cell culture medium. The assay will recognize both natural and recombinant Hu APO-1/Fas.

For Research Use Only. CAUTION: Not for human or animal therapeutic or diagnostic use.

Principle of the Method

The Invitrogen Hu APO-1/Fas kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). An antibody specific for Hu APO-1/Fas has been coated onto the wells of the microtiter strips provided. Samples, including standards of known Hu APO-1/Fas content, control specimens, and unknowns, are pipetted into these wells, followed by the addition of a biotinylated second antibody.

During the first incubation, the Hu APO-1/Fas antigen binds simultaneously to the immobilized (capture) antibody on one site, and to the solution phase biotinylated antibody on a second site.

After removal of excess second antibody, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove all the unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of Hu APO-1/Fas present in the original specimen.

Methods

Materials Needed But Not Provided

- Microtiter plate reader (at or near 450 nm) with software
- Calibrated adjustable precision pipettes
- Distilled or deionized water
- Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.)
- Glass or plastic tubes for diluting solutions
- Absorbent paper towels
- Calibrated beakers and graduated cylinders

Procedural Notes

1. When not in use, kit components should be refrigerated. All reagents should be warmed to room temperature before use.
2. **Microtiter plates should be allowed to come to room temperature before opening the foil bags.** Once the desired number of strips has been removed, immediately reseal the bag and store at 2 to 8°C to maintain plate integrity.
3. Samples should be collected in pyrogen/endotoxin-free tubes.
4. Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
5. When possible, avoid use of badly hemolyzed or lipemic sera. If large amounts of particulate matter are present, centrifuge or filter prior to analysis.
6. It is recommended that all standards, controls and samples be run in duplicate.
7. When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
8. **Do not mix or interchange different reagent lots from various kit lots**.
9. Do not use reagents after the kit expiration date.
10. Absorbances should be read immediately, but can be read up to 2 hours after assay completion. For best results, keep plate covered in the dark.
11. In-house controls or kit controls, if provided, should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
12. All residual wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. ***Never*** insert absorbent paper directly into the wells.
13. Because Stabilized *Chromogen* is light sensitive, avoid prolonged exposure to light. Avoid contact between chromogen and metal, or color may develop.

Directions for Washing

- **Incomplete washing will adversely affect the test outcome**. All washing must be performed with the *Wash Buffer Concentrate (25X)* provided.
- Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip into the bottom of each well. Take care not to scratch the inside of the well. After aspiration, fill the wells with at least 0.4 mL of diluted *Wash Buffer*. Let soak for 15 to 30 seconds, then aspirate the liquid. Repeat as directed under Assay Procedure. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.
- Alternatively, the diluted *Wash Buffer* may be put into a squirt bottle. If a squirt bottle is used, flood the plate with the diluted *Wash Buffer*, completely filling all wells. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.
- If using an automated washer, follow the washing instructions carefully.

Preparation of Reagents

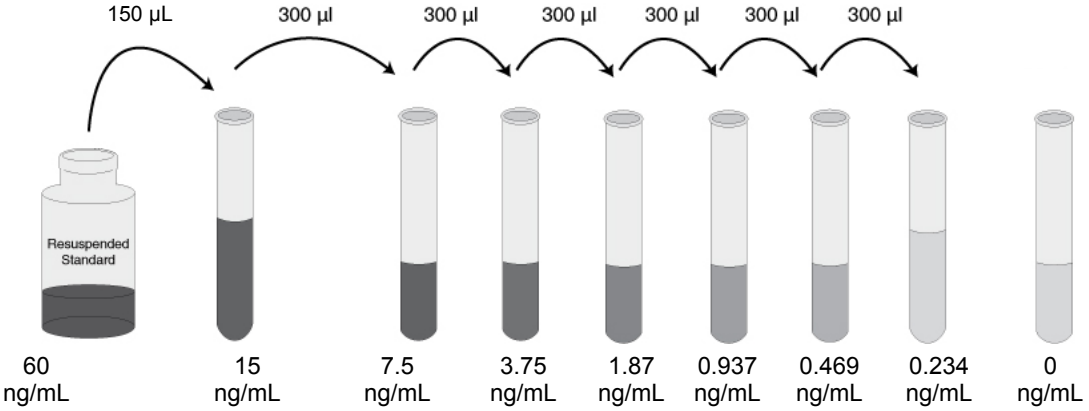
Dilution of Standard

Note: Either glass or plastic tubes may be used for standard dilutions.

1. Reconstitute standard to 60 ng/mL with *Standard Diluent Buffer*. Refer to standard vial label for instructions. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Use standard within 1 hour of reconstitution.
2. Add 0.150 mL of the reconstituted standard to a tube containing 0.450 mL *Standard Diluent Buffer*. Label as 15.0 ng/mL Hu APO-1/Fas. Mix.
3. Add 0.300 mL of *Standard Diluent Buffer* to each of 6 tubes labeled 7.50, 3.75, 1.87, 0.937, 0.469 and 0.234 ng/mL Hu APO-1/Fas.
4. Make serial dilutions of the standard as described in the following dilution diagram. Mix thoroughly between steps.

Note

Remaining reconstituted standard should be discarded. Return the *Standard Diluent Buffer* to the refrigerator.



Preparing SAV-HRP

Note: Prepare within 15 minutes of usage. The *Streptavidin-HRP (100X)* is in 50% glycerol, which is viscous. To ensure accurate dilution, allow *Streptavidin-HRP (100X)* to reach room temperature. Gently mix. Pipette *Streptavidin-HRP (100X*) slowly. Remove excess concentrate solution from pipette tip by gently wiping with clean absorbent paper.

1. Dilute 10 µL of this 100X concentrated solution with 1 mL of *Streptavidin-HRP Diluent* for each 8-well strip used in the assay. Label as Streptavidin-HRP Working Solution.
2. Return the unused *Streptavidin-HRP (100X)* to the refrigerator.

# of 8-Well Strips	Volume of Streptavidin-HRP (100X)	Volume of Diluent
2	20 µL solution	2 mL
4	40 µL solution	4 mL
6	60 µL solution	6 mL
8	80 µL solution	8 mL
10	100 µL solution	10 mL
12	120 µL solution	12 mL

Dilution of Wash Buffer

1. Allow the *Wash Buffer Concentrate (25X)* to reach room temperature and mix to ensure that any precipitated salts have redissolved. Dilute 1 volume of the *Wash Buffer Concentrate (25X)* with 24 volumes of deionized water (e.g., 50 mL may be diluted up to 1.25 liters, 100 mL may be diluted up to 2.5 liters). Label as Working Wash Buffer.
2. Store both the concentrate and the Working Wash Buffer in the refrigerator. The diluted buffer should be used within 14 days.

Assay Procedure

Be sure to read the *Procedural Notes* section before carrying out the assay.

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.

Note: A standard curve must be run with each assay.

1. Determine the number of 8-well strips needed for the assay. Insert these in the frame(s) for current use. (Re-bag extra strips and frame. Store these in the refrigerator for future use.)
2. Dilute serum samples 1:10 with *Standard Diluent Buffer* (e.g., add 20 µL of sample to 180 µL *Standard Diluent Buffer*). **Note**: Individual samples may require a greater or lesser dilution to fall within the range of the assay.
3. Add 100 µL of the *Standard Diluent Buffer* to the zero standard wells. Well(s) reserved for chromogen blank should be left empty.
4. Add 100 µL of standards to the appropriate microtiter wells. (See **Preparation of Reagents**.)
5. Pipette 50 µL of biotinylated anti-APO-1/Fas (*Biotin Conjugate*) solution into each well except the chromogen blank(s). Tap gently on the side of the plate to mix.
6. Cover plate with *plate cover* and incubate for **1 hour at room temperature**.
7. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **Directions for Washing**.
8. Add 100 µL Streptavidin-HRP Working Solution to each well except the chromogen blank(s). See **Preparation of Reagents**.
9. Cover plate with the *plate cover* and incubate for **30 minutes at room temperature**.
10. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **Directions for Washing**.
11. Add 100 µL of *Stabilized Chromogen* to each well. The liquid in the wells will begin to turn blue.
12. Incubate for **30 minutes at room temperature and in the dark**. ***Note*: Do not cover the plate with aluminum foil or metalized mylar**. The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceed the limits of the instrument. The O.D. values at 450 nm can only be read after the *Stop Solution* has been added to each well. If using a reader that records only to 2.0 O.D., stopping the assay after 20 to 25 minutes is suggested.
13. Add 100 µL of *Stop Solution* to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.
14. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µL each of *Stabilized Chromogen* and *Stop Solution*. Read the plate within 30 minutes after adding the *Stop Solution*.

15. Use a curve fitting software to generate the standard curve. A four parameter algorithm provides the best standard curve fit.
16. Read the concentrations for unknown samples and controls from the standard curve. **Multiply value(s) obtained for sample(s) by 10 to correct for the 1:10 dilution in step 2.** (Samples producing signals greater than that of the highest standard should be further diluted in *Standard Diluent Buffer* and reanalyzed, multiplying the concentration found by the appropriate dilution factor.)

Typical Data (Example)

The following data were obtained for the various standards over the range of 0 to 15 ng/mL Hu APO-1/Fas.

Standard Hu APO-1 (ng/mL)	Optical Density (450 nm)
15	2.47
7.5	1.43
3.75	0.69
1.87	0.378
0.937	0.225
0.469	0.142
0.234	0.102
0	0.067

Performance Characteristics

Sensitivity

The minimum detectable dose of Hu APO-1/Fas is < 20 pg/mL. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times.

Precision

1. Intra-Assay Precision

Samples of known Hu APO-1/Fas concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	3.6	6.2	11.4
SD	0.2	0.3	0.5
%CV	5.6	4.8	4.4

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision

Samples were assayed 48 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	3.4	5.6	12.3
SD	0.2	0.5	1.1
%CV	5.9	8.9	8.9

SD = Standard Deviation
CV = Coefficient of Variation

Linearity of Dilution

Human serum containing 14 ng/mL of measured Hu APO-1/Fas was serially diluted in *Standard Diluent Buffer* over the range of the assay. Linear regression analysis of samples versus the expected concentration yielded a correlation coefficient of 0.99.

Recovery

The recovery of Hu APO-1/Fas added to human serum averaged 87%. The recovery of Hu APO-1/Fas added to tissue culture medium containing 1% fetal bovine serum averaged 100%, while the recovery of Hu APO-1/Fas added to tissue culture medium containing 10% fetal bovine serum averaged 106%.

Specificity

Buffered solutions of a panel of substances at 45 ng/mL were assayed with the Invitrogen Hu APO-1/Fas kit. The following substances were tested and found to have no cross-reactivity: human IL-4, IL-5, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, sIL-2R, sIL-6R, sICAM-1, sVCAM-1, IFN-γ, TNF-α; mouse IL-2, IL-3, TNF-α; rat IL-1β, IFN-γ, TNF-α.

Expected Values

A limited number (n = 10) of sera from apparently normal individuals were evaluated with the Invitrogen Hu APO-1/Fas kit. The values ranged from 3.3 to 7.1 ng/mL. The mean value was 6.2 ng/mL.

Limitations of the Procedure

Do not extrapolate the standard curve beyond the top standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute all samples above the top standard point with *Standard Diluent Buffer*; reanalyze these and multiply results by the appropriate dilution factor.

The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of serum samples have not been thoroughly investigated. The rate of degradation of native Hu APO-1/Fas in various matrices has not been investigated. The immunoassay literature contains frequent references to aberrant signals seen with some sera, attributed to heterophilic antibodies. Though such samples have not been seen to date, the possibility of this occurrence cannot be excluded.

Appendix

Troubleshooting Guide

Elevated background	*Cause:* Insufficient washing and/or draining of wells after washing. Solution containing either biotin or SAV-HRP can elevate the background if residual is left in the well. *Solution:* Wash according to the protocol. Verify the function of automated plate washer. At the end of each washing step, invert plate on absorbent tissue on countertop and allow to completely drain, tapping forcefully if necessary to remove residual fluid. *Cause:* Contamination of substrate solution with metal ions or oxidizing reagents. *Solution:* Use distilled/deionized water for dilution of wash buffer and use plastic equipment. DO NOT COVER plate with foil. *Cause:* Contamination of pipette, dispensing reservoir or substrate solution with SAV-HRP conjugate. *Solution:* Do not use chromogen that appears blue prior to dispensing onto the plate. Obtain new vial of chromogen. *Cause:* Incubation time is too long or incubation temperature is too high. *Solution:* Reduce incubation time and/or temperature.
Elevated sample/ standard ODs	*Cause:* Incorrect dilution of standard stock solution; intermediary dilutions not followed correctly. *Solution*: Follow the protocol instructions regarding the dilution of the standard. *Cause:* Incorrect dilution of the Streptavidin-HRP Working Solution. *Solution:* Warm solution of Streptavidin-HRP (100X) to room temperature, draw up slowly and wipe tip with kim-wipe to remove excess. Dilute ONLY in SAV diluent provided. *Cause*: Incubation times extended. *Solution:* Follow incubation times outlined in protocol. *Cause:* Incubations carried out at 37°C when RT is dictated. *Solution:* Perform incubations at RT (= 25 ± 2°C) when instructed in the protocol.
Poor standard curve	*Cause:* Improper preparation of standard stock solution. *Solution:* Dilute lyophilized standard as directed by the vial label only with the standard diluent buffer or in a diluent that most closely matches the matrix of your sample. *Cause:* Reagents (lyophilized standard, standard diluent buffer, etc.) from different kits, either different cytokine or different lot number, were substituted. *Solution:* NEVER substitute any components from another kit. *Cause*: Errors in pipetting the standard or subsequent steps. *Solution*: Always dispense into wells quickly and in the same order. Do not touch the pipette tip on the individual microwells when dispensing. Use calibrated pipettes and the appropriate tips for that device.

Weak/no color develops	*Cause:* Reagents not at RT (25 ± 2°C) at start of assay. *Solution:* Allow ALL reagents to warm to RT prior to commencing assay. *Cause:* Incorrect storage of components, e.g., not stored at 2 to 8°C. *Solution:* Store all components exactly as directed in protocol and on labels. *Cause:* Working SAV-HRP solution made up longer than 15 minutes before use in assay. *Solution:* Use the diluted SAV-HRP within 15 minutes of dilution. *Cause:* TMB solution lost activity. *Solution 1:* The TMB solution should be clear before it is dispensed into the wells of the microtiter plate. An intense aqua blue color indicates that the product is contaminated. Please contact Technical Support if this problem is noted. To avoid contamination, we recommend that the quantity required for an assay be dispensed into a disposable trough for pipetting. Any TMB solution left in the trough should be discarded. *Solution 2:* Avoid contact of the TMB solution with items containing metal ions. *Cause:* Attempt to measure analyte in a matrix for which the ELISA assay has not been optimized. *Solution:* Please contact Technical Support for advice when using non-validated sample types. *Cause:* Wells have been scratched with pipette tip or washing tips. *Solution:* Use caution when dispensing and aspirating into and out of microwells.
Poor Precision	*Cause:* Errors in pipetting the standards, samples or subsequent steps. *Solution:* Always dispense into wells quickly and in the same order. Do not touch the pipette tip on the individual microwells when dispensing. Use calibrated pipettes and the appropriate tips for that device. Check for any leaks in the pipette tip. *Cause:* Repetitive use of tips for several samples or different reagents. *Solution:* Use fresh tips for each sample or reagent transfer. *Cause:* Wells have been scratched with pipette tip or washing tips. *Solution:* Use caution when dispensing and aspirating into and out of microwells.

Technical Support

Contact Us

For more troubleshooting tips, information, or assistance, please call, email, or go online to www.invitrogen.com/ELISA.

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References

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2. Lanier, L. et al. (1994) *J. Immunology* 153:2417-2428.
3. Nagata, S. and Goldstein, P. (1995) *Science* 267:1449-1456.

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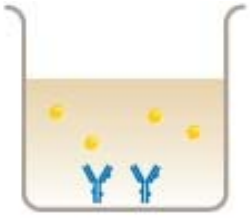
Explanation of symbols

Symbol	Description	Symbol	Description
REF	Catalogue Number	LOT	Batch code
RUO	Research Use Only	IVD	*In vitro* diagnostic medical device
	Use by		Temperature limitation
	Manufacturer	EC REP	European Community authorised representative
[-]	Without, does not contain	[+]	With, contains
	Protect from light		Consult accompanying documents
	Directs the user to consult instructions for use (IFU), accompanying the product.		

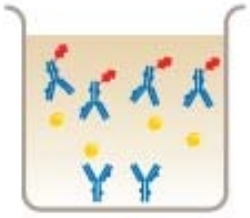
Hu APO-1/Fas Assay Summary

Add 100 µL of Standards,
controls and samples
(dilute samples 1:10 with standard diluent buffer)



Add 50 µL of Biotin Conjugate
Incubate for 1 hr at RT

↓ aspirate and wash 4x



Incubate 100 µL of Streptavidin-HRP
Working Solution for 30 minutes at RT

↓ aspirate and wash 4x

Incubate 100 µL of Stabilized Chromogen
for 30 minutes at RT

↓

Add 100 µL of Stop Solution
Read at 450 nm

Total time: 2 hr