Immunoassay Kit

Catalog # KHA0011: 1 plate
KHA0012: 2 plates
KHA0011C: 5 plates

Human
SAA

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INTENDED USE

The BioSource Human Serum Amyloid A (Hu SAA) ELISA is to be used for the *in vitro* quantitative determination of Hu SAA in serum, plasma, buffered solution and tissue culture medium. The assay will recognize both natural and recombinant Hu SAA.

INTRODUCTION

Serum amyloid A (SAA) proteins comprise a family of small (12-14 kDa, 104-112 amino acid residues), differentially expressed proteins that are highly conserved among vertebrates. SAA proteins are involved in the acute phase responses; these are the immediate early host responses to inflammation. During the acute phase, circulating SAA levels are increased by 100-1000 fold, reaching concentrations of up to one milligram per milliliter. SAAs have also been implicated in several disease states including rheumatoid arthritis, atherosclerosis, AA amyloidosis, and coronary artery disease. Liver is the major site of SAA synthesis, although extrahepatic expression has also been reported.

The human SAA gene family maps to chromosome 11. In humans, four SAA genes and three protein products have been identified: human SAA1 and SAA2 are designated the acute phase SAA (A-SAA) isoforms; while, SAA4 is constitutively expressed, and SAA3 is a pseudogene. Mouse SAA gene family maps to chromosome 7. In mouse, five SAA genes and four protein products have been identified: mouse SAA1, SAA2, SAA3 are the acute phase isoforms, SAA5 is constitutively expressed, and SAA4 is a pseudogene. A-SAAs belong to a category of acute phase proteins that also includes C-reactive protein (CRP), haptoglobin, ceruloplasmin, complement components C3 and C4, a1-acid glycoprotein, a1-proteinase inhibitor, and fibrinogen. In

humans, both CRP and SAA are synthesized during the acute phase; whereas, in mouse, SAA is the predominant acute phase protein.

According to current models, severe physiological challenges such as tissue injury, microbial infection, surgical trauma, bone fracture, burns, and exhaustion, result in immediate inflammatory responses in the host. The role of the acute phase response is to counteract these challenges by preventing further pathogen entry, minimizing tissue damage, promoting repair, and restoring homeostasis. At the onset of the acute phase, macrophages and monocytes which are recruited to the site of inflammation, release the primary inflammatory mediators, IL-1 and TNF-α. These cytokines, in turn, signal the stromal cells to express secondary inflammatory mediators, IL-6, IL-8, and monocyte chemoattractant protein. Leukocytes attracted to the area produce additional cytokines. The cytokines produced at the site of injury, in particular IL-1 and IL-6, enter the plasma and provide stimuli for the liver to produce A-SAAs. Other cytokines that have been implicated in the regulation of SAA synthesis include LIF, CNTF, oncostatin M, cardiotropin, and possibly NGF. Analysis of SAA promoter regions reveals control by several transcription factors, including NF-κB, C/EBP, YY1, SAF/Sp1, and AP-2. AP-2 acts to repress transcription in non-liver tissues. NF-κB and C/EBP act synergistically to enhance transcription, while YY1 antagonizes the function of NF-κB and, therefore, serves to silence transcription.

Upon synthesis, SAA is released into the bloodstream where it immediately binds the HDL particles. Binding to HDL is known to protect the SAAs from degradation by proteolytic enzymes. There are a number of important homeostatic functions associated with the circulating SAA-HDL complexes; these functions have been

categorized as immune modulation, lipid transport, and anti-inflammatory. The circulating SAA is known to act as a chemoattractant and recruit additional monocytes, leukocytes, mast cells, and T lymphocytes to the site of inflammation. It also aides the tissue regeneration process by activating matrix metalloproteinases such as collagenase and strolemysin. Binding of SAA to HDL alters the reverse cholesterol transport function of HDL, allowing for the delivery of cholesterol for the site of repair. The SAA-HDL complex also serves to remove excess cholesterol released by the damaged tissue. The anti-inflammatory activities of circulating SAA include inhibition of lymphocyte cell function, inhibition of TNF-α- and IL-1-induced fevers, and inhibition of platelet aggregation. SAA is also known to bind neutrophils, abrogate the oxidative burst response, and prevent oxidative tissue damage.

Chronically elevated SAA levels are implicated in a group of protein misfolding disorders known as the amyloid A amyloidoses. These include reactive amyloidosis, caused by chronic inflammation or recurrent acute inflammation, familial Mediterranean fever, systemic AA amyloidosis, and visceral AA amyloidosis. Amyloid A amyloidoses are characterized by the deposition of insoluble plaques composed principally of proteolytically cleaved A-SAA. These plaques are primarily deposited in organ sites of inflammation and ultimately result in the degeneration of the affected organ. Despite the potential for causing fatal amyloidoses, SAAs are believed to play a protective role for the host. This hypothesis is supported by the observations that there is immediate, robust induction of SAAs in response to inflammatory signals and that SAAs are highly conserved through evolution.

This kit has been configured for research use only and is not to be used in diagnostic procedures.

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Read entire protocol before use.

PRINCIPLE OF THE METHOD

The BioSource Hu SAA kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A highly purified monoclonal antibody against Hu SAA has been coated onto the wells of the microtiter strips provided.

During the first incubation, standards of known Hu SAA content, controls, and unknown samples are pipetted into the coated wells, followed by the addition of biotinylated second monoclonal antibody.

After washing, 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 SAA present in the original specimen.

REAGENTS PROVIDED

Note: *Store all reagents at 2 - 8°C.*

Reagent	*96 Test Kit*	*192 Test Kit*	*480 Test Kit*
Hu SAA Standard, recombinant Hu SAA1. Refer to vial label for quantity and reconstitution volume. Once reconstituted, aliquot and store at -80°C or below. Avoid repeated freeze-thaws.	2 vials	4 vials	10 vials
Standard Diluent Buffer. Contains 0.5 % ProClin® 300; 100 mL per bottle.	1 bottle	2 bottles	5 bottles
Hu SAA High and Low Control, recombinant Hu SAA1 in tissue culture matrix, lyophilized. Refer to vial label for reconstitution volume. Once reconstituted, aliquot and store at -80°C or below. Avoid repeated freeze-thaws.	2 vials	2 vials	4 vials
Hu SAA Antibody-Coated Wells, 96 wells per plate.	1 plate	2 plates	5 plates
Hu SAA Biotin Conjugate, (Biotin-labeled anti-Hu SAA). Contains 15 mM sodium azide; 6 mL per bottle.	1 bottle	2 bottles	5 bottles
Streptavidin-Peroxidase (HRP), (100x) concentrate. Contains 3.3 mM thymol; 0.125 mL per vial.	1 vial	2 vials	5 vials
Streptavidin-Peroxidase (HRP) Diluent. Contains 3.3 mM thymol; 25 mL per bottle.	1 bottle	1 bottle	3 bottles
Wash Buffer Concentrate (25x); 100 mL per bottle.	1 bottle	1 bottle	2 bottles
Stabilized Chromogen, Tetramethylbenzidine (TMB); 25 mL per bottle.	1 bottle	1 bottle	3 bottles
Stop Solution; 25 mL per bottle.	1 bottle	1 bottle	3 bottles
Plate Covers, adhesive strips.	3	6	15

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.

SUPPLIES REQUIRED BUT NOT PROVIDED

1. Microtiter plate reader capable of measurement at or near 450 nm.
2. Calibrated adjustable precision pipettes, preferably with disposable plastic tips. (A manifold multi-channel pipette is desirable for large assays.)
3. Distilled or deionized water.
4. Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.).
5. Data analysis and graphing software. Graph paper: linear (Cartesian), log-log, or semi-log, as desired.
6. Glass or plastic tubes for diluting and aliquoting standard.
7. Absorbent paper towels.
8. Calibrated beakers and graduated cylinders in various sizes.
9. 37°C incubator.

PROCEDURAL NOTES/LAB QUALITY CONTROL

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 - 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 serum and plasma samples. 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. Serum, plasma, or tissue culture sample(s) that measure >600 ng/mL require additional dilution steps in the *Standard Diluent Buffer*.
8. When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
9. Cover or cap all reagents when not in use.
10. **Do not mix or interchange different reagent lots from various kit lots**.
11. Do not use reagents after the kit expiration date.
12. Read absorbances within 30 minutes of assay completion.
13. The controls provided should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
14. 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.
15. Because *Stabilized Chromogen* is light sensitive, avoid prolonged exposure to light. Also avoid contact between *Stabilized Chromogen* and metal, or color may develop.

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.

DIRECTIONS FOR WASHING

Incomplete washing will adversely affect the test outcome. All washing must be performed with the *Wash Buffer* provided.

Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip (aspiration device) 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 solution. Let soak for 15 to 30 seconds, then aspirate the liquid. Repeat as directed under **ASSAY METHOD**. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

Alternatively, the wash solution may be put into a squirt bottle. If a squirt bottle is used, flood the plate with 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, the operating instructions for washing equipment should be carefully followed.

SAMPLE PREPARATION

Human serum and plasma require a 200-fold dilution in the *Standard Diluent Buffer*. For these samples, first prepare a 1:10 dilution by adding 10 µL of the sample to a clean microfuge tube, followed by 90 µL of *Standard Diluent Buffer*. Mix well. Next, prepare an additional 1:20 dilution by transferring 25 µL from the first tube into a second clean microfuge tube, followed by the addition of 475 µL of *Standard Diluent Buffer*. Mix well. Tissue culture samples should be analyzed neat.

REAGENT PREPARATION AND STORAGE

A. Reconstitution and Dilution of Hu SAA Standard

The Hu SAA Standard is calibrated to a highly purified *E. coli*-expressed recombinant protein.

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

1. Reconstitute standard to 600 ng/mL with *Standard Diluent Buffer*. Refer to the standard vial label for instructions. Label as 600 ng/mL Hu SAA. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Use the standard within 15 minutes of reconstitution or aliquot and store at -80°C.
2. Add 0.300 mL of *Standard Diluent Buffer* to each of 6 tubes labeled 300, 150, 75, 37.5, 18.8, and 9.4 ng/mL Hu SAA.
3. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

B. Dilution of Hu SAA Standard

Standard:	Add:	Into:
600 ng/mL	Prepare as described in Step 1.	
300 ng/mL	0.300 mL of the 600 ng/mL std.	0.300 mL of the Diluent Buffer
150 ng/mL	0.300 mL of the 300 ng/mL std.	0.300 mL of the Diluent Buffer
75 ng/mL	0.300 mL of the 150 ng/mL std.	0.300 mL of the Diluent Buffer
37.5 ng/mL	0.300 mL of the 75 ng/mL std.	0.300 mL of the Diluent Buffer
18.8 ng/mL	0.300 mL of the 37.5 ng/mL std.	0.300 mL of the Diluent Buffer
9.4 ng/mL	0.300 mL of the 18.8 ng/mL std.	0.300 mL of the Diluent Buffer
0 ng/mL	0.300 mL of the Diluent Buffer	An empty tube

Discard all remaining reconstituted and diluted standards after completing assay. Return the *Standard Diluent Buffer* to the refrigerator.

C. Storage and Final Dilution of Streptavidin-HRP

Please Note: The *Streptavidin-HRP* 100x concentrate is in 50% glycerol. This solution is viscous. To ensure accurate dilution, allow *Streptavidin-HRP* concentrate to reach room temperature. Gently mix. Pipette *Streptavidin-HRP* concentrate slowly. Remove excess concentrate solution from pipette tip by gently wiping with clean absorbent paper.

1. Within 1 hour of use, 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.

For Example:

# of 8-Well Strips	Volume of Streptavidin-HRP Concentrate	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

2. Return the unused *Streptavidin-HRP* concentrate to the refrigerator.

D. Dilution of Wash Buffer

Allow the 25x concentrate to reach room temperature and mix to ensure that any precipitated salts have redissolved. Dilute 1 volume of the 25x wash buffer concentrate 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.

Store both the concentrate and the Working Wash Buffer in the refrigerator. The diluted buffer should be used within 14 days.

ASSAY METHOD: PROCEDURE AND CALCULATIONS

Be sure to read the *Procedural Notes/Lab Quality Control* 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 and plasma samples 1:200 with *Standard Diluent Buffer*. (see **SAMPLE PREPARATION** section on page 13). Tissue culture samples should be assayed neat.
3. Add 100 µL of the *Standard Diluent Buffer* to zero wells. Well(s) reserved for chromogen blank should be left empty.
4. Add 100 µL of standards, controls, or samples (serum and plasma 1:200 prediluted) to the appropriate microtiter wells.
5. Pipette 50 µL of biotinylated anti-Hu SAA (*Biotin Conjugate*) solution into each well except the chromogen blank(s). Tap on the side of the plate for 30 seconds to mix.

6. Cover plate with *plate cover* and incubate for **2 hour at room temperature or 1 hour at 37°C**.
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). (Prepare the working dilution as described in **REAGENT PREPARATION AND STORAGE**, Section C.)
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**. ***Please 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. Plot on graph paper the absorbance of the standards against the standard concentration; optimally, the background absorbance may be subtracted from *all* data points, including standards, unknowns and controls, prior to plotting. Draw the best smooth curve through these points to construct the standard curve. If using curve fitting software, the four parameter algorithm provides the best curve fit.
16. Read the Hu SAA concentrations for unknown samples and controls from the standard curve plotted in step 15. **Multiply value(s) obtained for serum and plasma by 200 to correct for the overall 1:200 dilution.** Samples producing signals greater than that of the highest standard (600 ng/mL) should be further diluted in the *Standard Diluent Buffer* as described in step 2 and reanalyzed. Multiply the concentration found by the appropriate dilution factor.

TYPICAL DATA

The following data were obtained for the various standards over the range of 0 to 600 ng/mL Hu SAA.

Standard Hu SAA (ng/mL)	Optical Density (450 nm)
0	0.065
	0.062
9.4	0.148
	0.152
18.8	0.188
	0.209
37.5	0.334
	0.410
75	0.617
	0.650
150	1.241
	1.370
300	2.331
	2.325
600	3.421
	3.251

LIMITATIONS OF THE PROCEDURE

Do not extrapolate the standard curve beyond the 600 ng/mL standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute samples with concentrations exceeding the linear portion of the standard curve with the *Standard Diluent Buffer*; reanalyze these and multiply results by the appropriate dilution factor.

The influence of various drugs and the use of biological fluids in place of tissue culture media have not been thoroughly investigated. The rate of degradation of native Hu SAA 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.

This kit is for research use only.
Not for human therapeutic or diagnostic use.

PERFORMANCE CHARACTERISTICS

SENSITIVITY

The minimum detectable dose of Hu SAA is <4 ng/mL. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times, and calculating the corresponding concentration.

PRECISION

1. Intra-Assay Precision

Samples of known Hu SAA concentration were assayed in replicates of 12 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (ng/mL)	61.7	203.6	585.8
SD	4.6	9.3	36.6
%CV	7.4	4.6	6.2

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision

Samples were assayed 36 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (ng/mL)	61.3	198.4	598.8
SD	4.8	14.7	42.0
%CV	7.8	7.4	7.0

SD = Standard Deviation
CV = Coefficient of Variation

LINEARITY OF DILUTION

Human serum, EDTA plasma, citrate plasma, heparin plasma, and tissue culture medium spiked with recombinant Hu SAA were serially diluted in *Standard Diluent Buffer* over the range of the assay. Linear regression analysis of samples versus the expected concentration yielded average correlation coefficients of 0.996 for serum, 0.973 for EDTA plasma, 0.995 for citrate plasma, 0.997 for heparin plasma, and 0.999 for tissue culture medium.

RECOVERY

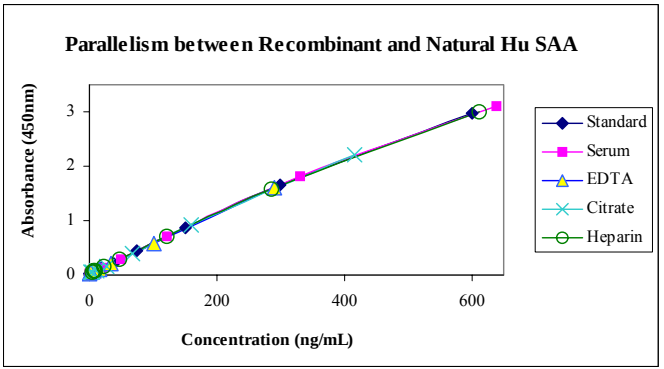
The recoveries of recombinant Hu SAA added to human serum, EDTA plasma, citrate plasma, heparin plasma, and tissue culture medium containing 10% fetal bovine serum or 1% calf serum were measured with the BioSource Hu SAA ELISA.

Sample Type	Average % Recovery
Serum*	114
EDTA plasma*	111
Citrate plasma*	108
Heparin Plasma*	84
RPMI+10% fetal bovine serum	102
DMEM + 1% calf serum	89

* Serum and plasma were pre-diluted 200-fold as described in sample preparation procedure.

PARALLELISM

Random human serum, EDTA plasma, citrate plasma, and heparin plasma samples were serially diluted in the *Standard Diluent Buffer* and analyzed as described in **ASSAY METHOD**. The optical density of each dilution was plotted against the Hu SAA standard curve. Parallelism was demonstrated by the figure below and indicated that the standard accurately reflects the Hu SAA content in natural samples.



SPECIFICITY

Buffered solutions of a panel of recombinant human, mouse, and rat (rHu, rMs, and rRt, respectively) proteins ranging in concentrations from 5 to 10 µg/mL were assayed with the BioSource Hu SAA kit. Cross-reactivity results were as follows:

Analyte	% Cross-reactivity	Analyte	% Cross-reactivity
rHu CRP	0	rRt IL-1α	0
rHu Haptoglobin	0	rRt IL-2	0
rHu IL-1α	0	rRt GRO	0
rHu IL-2	0	rRt VEGF	0
rHu IL-3	0	rMs CRP	0
rHu IL-6	0	rMs IL-1α	0
rHu IL-7	0	rMs IL-13	0
rHu IL-13	0	rMs KC	0
rHu PDGF-BB	0	rMs SCF	0
rHu GRO-α	0	rMs Eotaxin	0
rHu IFN-γ	0	rMs TNF-α	0
rHu SCF	0	rMs VEGF	0
rHu TNF-α	0	rMs SAA1	1
rHu VEGF	0	rMs SAA2	0
rRt CRP	0		

HIGH DOSE HOOK EFFECT

No hook effect was observed with concentrations up to 50 µg/mL.

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Human SAA Assay Summary