Quantikine® ELISA

Mouse Fetuin A/AHSG Immunoassay

Catalog Number MFTA00

For the quantitative determination of mouse Fetuin A concentrations in cell culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Fetuin A, also known as AHSG (α2-Heremans-Schmid glycoprotein) is a member of the type 3 (plasma protein) subgroup of the Cystatin superfamily (1-5). This subgroup also includes Fetuin B, kininogen, and HPRG (histidine-proline-rich glycoprotein), most of which lack cysteine protease inhibitor activity (5). Fetuin A is a major bone matrix and plasma protein and a negative early response protein which is mainly produced by hepatocytes (1-8). It is found in the plasma as a 50-59 kDa, N- and O-glycosylated phosphoprotein with six intermolecular disulfide bonds between two N-terminal cystatin domains and a smaller C-terminal domain (1-4). The first cystatin domain is negatively charged and mediates binding of insoluble calcium phosphates, often called apatite (2, 4, 7). Fetuin A is the most abundant non-collagenous protein in bone (6, 7). It accumulates in the bone through strong binding to bone apatite, and inhibits new apatite formation (4, 6, 7). Mice deleted for Fetuin A have increased risk for vitamin D-induced ectopic calcification in blood vessels, but no obvious bone defects, presumably due to compensation by other bone matrix proteins (2, 9).

Fetuin A circulates as a component of calciprotein monomers (CPM), or calciprotein particles (CPP), both of which are important mineral chaperones that inhibit blood vessel calcification (4, 10-12). The majority of Fetuin A is present in CPM, which stabilize up to six molecules of calcium per molecule of Fetuin A and may be identical to 10 nm Posner clusters (10). Along with other negatively charged plasma proteins such as albumin, Fetuin A also coats and stabilizes CPP, which are larger calcium phosphate aggregates thought to be analogous to lipid-carrying lipoproteins (10-12). Scavenger receptor A (SR-AI/MSP1) in reticuloendothelial cells, such as marginal zone macrophages in the spleen and Kupffer cells in the liver, rapidly takes up CPP for clearance of calcium phosphates (12). Serum levels of Fetuin A-mineral complex correlate inversely with experimentally induced artery calcification in rats (13, 14). Similarly, human end stage renal failure patients on long-term hemodialysis with ectopic vascular calcification show significantly lower serum Fetuin A than healthy controls (9, 15). *In vitro*, these sera show impaired capacity to inhibit calcium phosphate precipitation that is corrected by addition of purified normal Fetuin A (9, 15).

Fetuin A is a negative acute-phase protein that shows significant (30-70%) decreases in the circulation during injury and infection (16-18). Early inflammatory cytokines, such as TNF-α, IFN-γ, IL-6 and IL-1β, downregulate liver production of Fetuin A (17, 18). However, Fetuin A is also protective during experimental endotoxemia or sepsis, possibly due to modulating macrophage production of HMGB1, TNF-α, and/or IFN-γ (18-20). Other functions for Fetuin A have been proposed due to its activity as a broad-specificity binding protein (4). Phosphorylated Fetuin A is reported to bind the insulin receptor, downregulate insulin signaling, and contribute to insulin resistance (21). Fetuin A is also reported to bind TLR4 and free fatty acids simultaneously, which may promote inflammation and downregulate insulin sensitivity when fatty acid concentrations are increased in type 2 diabetes (22). Roles for Fetuin A in cancer have also been proposed, both as a serum Ca^{2+}-dependent cell attachment factor for tumor cells, and as a TGF- and BMP-binding protein that interferes with signaling (4, 23).

The Quantikine Mouse Fetuin A Immunoassay is a 4.5 hour solid phase ELISA designed to measure mouse Fetuin A levels in cell culture supernates, serum, and plasma. It contains NS0-expressed recombinant mouse Fetuin A and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate the recombinant mouse Fetuin A accurately. Results obtained using natural mouse Fetuin A showed dose-response curves that were parallel to the standard curves obtained using the recombinant kit standards. These results indicate that this kit can be used to determine relative mass values for natural mouse Fetuin A.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse Fetuin A has been pre-coated onto a microplate. Standards, Control, and samples are pipetted into the wells and any mouse Fetuin A present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse Fetuin A is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of mouse Fetuin A bound in the initial step. The sample values are then read off the standard curve.

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.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- 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.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Mouse Fetuin A Microplate	894364	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse Fetuin A.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Mouse Fetuin A Conjugate	894365	12 mL of a polyclonal antibody specific for mouse Fetuin A conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Mouse Fetuin A Standard	894366	200 ng of recombinant mouse Fetuin A in a buffered protein base with preservatives; lyophilized.	
Mouse Fetuin A Control	894367	Recombinant mouse Fetuin A in a buffered protein base with preservatives; lyophilized. The concentration range of mouse Fetuin A after reconstitution is shown on the vial label. The assay value of the Control should be within the range specified on the label.	
Assay Diluent RD1W	895038	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-26 Concentrate	895525	2 vials (21 mL/vial) of a concentrated buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895174	23 mL of diluted hydrochloric acid.	
Plate Sealers	N/A	4 adhesive strips.	

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

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 1000 mL graduated cylinders.
- Test tubes for dilution of standards and samples.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 20 minutes at 2000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*
Icteric samples are not suitable for use in this assay.

SAMPLE PREPARATION

Cell culture supernate samples require at least a 4-fold dilution. A suggested 4-fold dilution is 50 µL of sample + 150 µL of Calibrator Diluent RD5-26 (1X).

Serum and plasma samples require a 4000-fold dilution. A suggested 4000-fold dilution can be achieved by adding 10 µL of sample to 390 µL of Calibrator Diluent RD5-26(1X). Complete the 4000-fold dilution by adding 10 µL of the diluted sample to 990 µL of Calibrator Diluent RD5-26 (1X).

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Mouse Fetuin A Control - Reconstitute the Control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the Control undiluted.

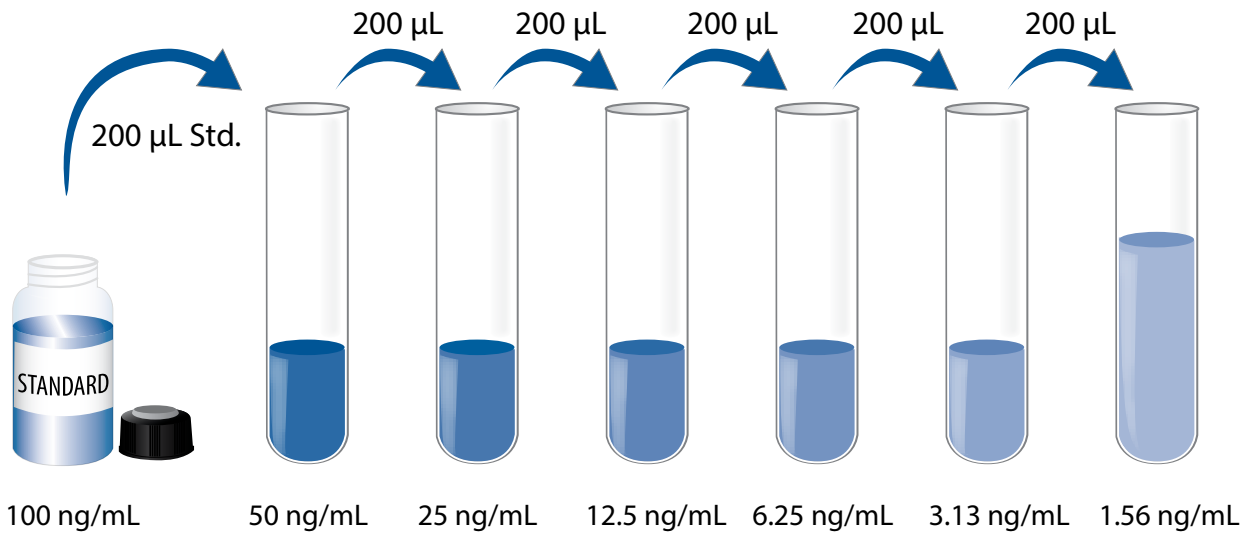
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 µL of the resultant mixture is required per well.

Calibrator Diluent RD5-26 (1X) - Add 20 mL of Calibrator Diluent RD5-26 Concentrate to 60 mL of deionized or distilled water to prepare 80 mL of Calibrator Diluent RD5-26 (1X).

Mouse Fetuin A Standard - Reconstitute the Mouse Fetuin A Standard with 2.0 mL of Calibrator Diluent RD5-26 (1X). This reconstitution produces a stock solution of 100 ng/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Pipette 200 µL of Calibrator Diluent RD5-26 (1X) into each tube. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Mouse Fetuin A Standard (100 ng/mL) serves as the high standard. Calibrator Diluent RD5-26 (1X) serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, Control, and samples be assayed in duplicate.

1. Prepare all reagents, working standards, Control, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL of Assay Diluent RD1W to each well.
4. Add 50 µL of Standard, Control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
5. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with Wash Buffer (400 µL) 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.
6. Add 100 µL of Mouse Fetuin A Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature on the benchtop. **Protect from light.**
9. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

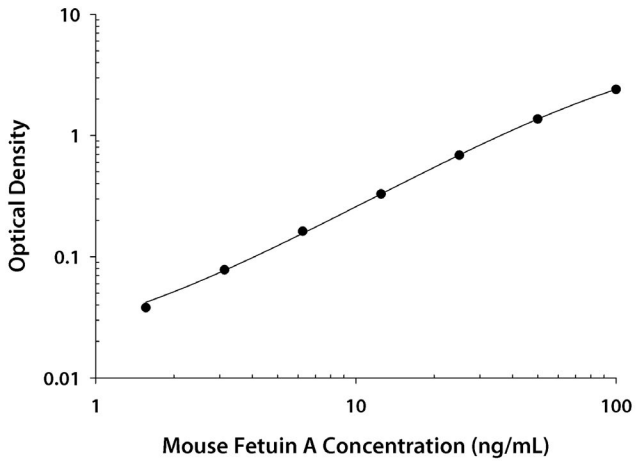
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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. The data may be linearized by plotting the log of the mouse Fetuin A 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.

Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



(ng/mL)	O.D.	Average	Corrected
0	0.020 0.022	0.021	—
1.56	0.059 0.059	0.059	0.038
3.13	0.098 0.100	0.099	0.078
6.25	0.181 0.185	0.183	0.162
12.5	0.349 0.351	0.350	0.329
25	0.699 0.716	0.708	0.687
50	1.374 1.406	1.390	1.369
100	2.399 2.447	2.423	2.402

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	6.4	16.1	47.0	6.0	16.0	47.0
Standard deviation	0.260	0.561	1.16	0.446	0.722	2.71
CV (%)	4.1	3.5	2.5	7.4	4.5	5.8

RECOVERY

The recovery of mouse Fetuin A spiked to three levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	105	100-111%

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of mouse Fetuin A were serially diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay. Samples were diluted prior to assay.

		Cell culture supernates (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	95	96	97	98
	Range (%)	91-99	93-99	94-100	84-114
1:4	Average % of Expected	93	96	100	103
	Range (%)	90-96	90-101	96-104	99-108
1:8	Average % of Expected	89	96	99	103
	Range (%)	83-95	87-99	96-104	98-106
1:16	Average % of Expected	90	95	96	102
	Range (%)	86-93	86-102	92-107	98-109

SENSITIVITY

Sixty-seven assays were evaluated and the minimum detectable dose (MDD) of mouse Fetuin A ranged from 0.042-0.337 ng/mL. The mean MDD was 0.123 ng/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-derived recombinant mouse Fetuin A produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of Fetuin A in this assay.

Sample Type	Mean (µg/mL)	Range (µg/mL)	Standard Deviation (µg/mL)
Serum (n=10)	248	216-300	29.4
EDTA Plasma (n=10)	216	159-259	32.0
Heparin plasma (n=10)	213	195-260	19.0

Cell Culture Supernates - Organs from mice were removed, rinsed in PBS, and kept on ice. The organs were then homogenized using a tissue homogenizer and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for 1 day. Aliquots of the cell culture supernates were removed and assayed for levels of natural Fetuin A.

Tissue Type	(ng/mL)
Liver	248
Spleen	47.1

SPECIFICITY

This assay recognizes natural and recombinant mouse Fetuin A.

The factors listed below were prepared at 1 µg/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 1 µg/mL in a mid-range recombinant mouse Fetuin A control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:

Cathepsin A
Cathepsin B
Cathepsin C
Cathepsin E
Cathepsin H
Cathepsin L (mature)
Cathepsin L (pro)
Cystatin A
Cystatin B
Cystatin C
Cystatin E/M
Cystatin F
Insulin R
Serpin A1
Serpin A5

Recombinant human:

Fetuin A
TLR-4

Recombinant rat:

Fetuin A

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