

## EDI™ Human Fetuin-A ELISA Kit

### Enzyme Linked ImmunoSorbent Assay (ELISA) for the measurement of Human Fetuin-A Levels in Serum or Tissue Extract



#### INTENDED USE

This ELISA (enzyme-linked immunosorbent assay) kit is intended for the quantitative determination of human Fetuin-A, also known as alpha-2-HS glycoprotein (AHSG), in serum, plasma, cell culture supernatant, tissue extraction and urine. The measurement of Fetuin-A in serum or plasma aids in the diagnosis of some cancers and genetically inherited deficiencies of this serum protein. This Fetuin-A ELISA kit is for laboratory professional use.

#### SUMMARY OF PHYSIOLOGY

Fetuin-A, also known as alpha-2-HS glycoprotein, is a 59 kDa glycoprotein that consists of two amino-terminal cystatin domains and a smaller carboxyl-terminal domain. Fetuin-A is synthesized by the liver and secreted into blood stream, where its concentration in adult mammals ranges from 0.5 – 1.0 g/L. Fetuin-A occurs in high serum concentration during fetal life and involves in protease inhibitory activities and development-associated regulation of calcium metabolism and osteogenesis. It accumulates in bones and teeth as a major fraction of noncollagenous bone proteins. Biologically, studies have demonstrated that Fetuin-A is the major calcification inhibitor found in circulation, where it interferes with calcium salt precipitation. Recent study has indicated that Fetuin-A level drops in uremic patients on hemodialysis in comparison to normal healthy controls. The low Fetuin-A level in patients with chronic kidney failure strongly associates with a higher cardiovascular mortality. On the other hand, it is demonstrated that a higher than normal serum Fetuin-A in older population is associated with diabetes, which is independent from other markers of insulin resistance. Further, a higher Fetuin-A level may be an independent risk marker of patients with cardiovascular diseases.

#### ASSAY PRINCIPLE

This ELISA is designed, developed and produced for the quantitative measurement of human Fetuin-A in serum samples. The assay utilizes the two-site “sandwich” technique with two selected goat anti-human Fetuin-A polyclonal antibodies that bind to different epitopes of human Fetuin-A.

Assay standards, controls and prediluted patient serum samples containing human Fetuin-A are added to microtiter wells of microplate that was coated with a high affinity polyclonal goat anti-human Fetuin-A antibody. After the first incubation period, the antibody on the wall of microtiter well captures human Fetuin-A in the sample and unbound protein in each microtiter well is washed away. Then a horseradish peroxidase (HRP) conjugated polyclonal anti-human Fetuin-A antibody is added to each microtiter well and a “sandwich” of “capture antibody - human Fetuin-A - HRP conjugated tracer antibody” is formed. The unbound tracer antibody is removed in the subsequent washing step. HRP conjugated tracer antibody bound to the well is then incubated with a substrate solution in a timed reaction and then measured in a spectrophotometric microplate reader. The enzymatic activity of the tracer antibody bound to the Fetuin-A on the wall of the microtiter well is directly

proportional to the amount of Fetuin-A in the sample. A standard curve is generated

by plotting the absorbance versus the respective human Fetuin-A concentration for each standard on point-to-point or cubical scales. The concentration of human Fetuin-A in test samples is determined directly from this standard curve.

#### REAGENTS: Preparation and Storage

This test kit must be stored at 2 – 8°C upon receipt. For the expiration date of the kit refer to the label on the kit box. All components are stable at 2 – 8°C until this expiration date.

#### Prior to use, allow all reagents to come to room temperature.

Reagents from different kit lot numbers should not be combined or interchanged.

- 1. Fetuin-A Antibody Coated Microplate (Cat. No. 30010)**  
One microplate with 12 x 8 strips (96 wells total) coated with antibody to human Fetuin-A. The plate is framed and sealed in a foil Ziploc bag with a desiccant. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.
- 2. Fetuin-A Tracer Antibody (Cat. No. 30009)**  
One vial containing 0.6 mL concentrated horseradish peroxidase (HRP) conjugated anti-human Fetuin-A tracer antibody in a stabilized protein matrix. This reagent must be diluted with Tracer Antibody Diluent before use. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.
- 3. Tracer Antibody Diluent (Cat. No. 30653)**  
One vial containing 12 mL ready to use Trizma Hydrochloride based buffer as supplied. It should be only used for tracer antibody dilution according to the assay procedures. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.
- 4. Fetuin-A Assay Buffer Concentrate (Cat. No. 10011)**  
One vial containing 11 mL of concentrated phosphate buffered saline based assay buffer with bovine serum albumin added. This concentrated assay buffer must be diluted 1:10 with distilled or deionized water (11 mL concentrate plus 99 mL distilled water) before use. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.
- 5. ELISA Wash Concentrate (Cat. No. 10010)**  
One bottle contains 30 mL of 30-fold concentrate. Before use the contents must be diluted with 870 mL of distilled water and mixed well. Upon dilution this yields a working wash solution containing a surfactant in phosphate buffered saline with a non-azide preservative. The diluted wash solution should be stored at room temperature and is stable until the expiration date on the kit box.

### 6. ELISA HRP Substrate (Cat. No. 10020)

One bottle contains 12 mL of tetramethylbenzidine (TMB) with hydrogen peroxide. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

### 7. ELISA Stop Solution (Cat. No. 10030)

One bottle contains 12 mL of 0.5 M sulfuric acid. This reagent should be stored at 2 – 8°C or room temperature and is stable until the expiration date on the kit box.

### 8. Fetuin-A Standards (Cat. No. 30001 – 30005)

Five vials each containing 0.5mL of human Fetuin-A in a liquid bovine serum based matrix with a non-azide preservative.

**Refer to vial for exact concentration for each standard.** All the standards should be stored at -20°C or below after the first use with up to 3 freeze cycles.

### 9. Fetuin-A Controls (Cat. No. 30007 – 30008)

Two vials each containing 0.5mL of human Fetuin-A in a liquid bovine serum based matrix with a non-azide preservative.

**Refer to vials for exact concentration range for each control.** Both controls should be stored at -20°C or below after the first use with up to 3 freeze cycles.

## SAFETY PRECAUTIONS

The reagents must be used in a clinical reference laboratory and are intended for in vitro diagnostic use by medical or laboratory professionals only. The source material for reagents containing bovine serum was derived in the contiguous 48 United States. It was obtained only from healthy donor animals maintained under veterinary supervision and found free of contagious diseases. Wear gloves while performing this assay and handle these reagents as if they are potentially infectious. Avoid contact with reagents containing TMB, hydrogen peroxide, or sulfuric acid. TMB may cause irritation to skin and mucous membranes and cause an allergic skin reaction. TMB is a suspected carcinogen. Sulfuric acid may cause severe irritation on contact with skin. Do not get in eyes, on skin, or on clothing. Do not ingest or inhale fumes. On contact, flush with copious amounts of water for at least 15 minutes. Use Good Laboratory Practices.

## MATERIALS REQUIRED BUT NOT PROVIDED

1. Precision single channel pipettes capable of delivering 10 µL, 25 µL, 100 µL, and 1000 µL.
2. Repeating dispenser suitable for delivering 100 µL.
3. Disposable pipette tips suitable for above volume dispensing.
4. Disposable 12 x 75 mm or 13 x 100 glass or plastic tubes.
5. Disposable plastic 100 mL and 1000 mL bottle with caps.
6. Aluminum foil.
7. Deionized or distilled water.
8. Plastic microtiter well cover or polyethylene film.
9. ELISA multichannel wash bottle or automatic (semi-automatic) washing system.
10. Spectrophotometric microplate reader capable of reading absorbance at 450 nm.

## SPECIMEN COLLECTION

Only 10 µL of human serum or plasma is required for human Fetuin-A measurement. No special preparation of individual is necessary prior to specimen collection. Whole blood should be collected and must be allowed to clot for minimum 30 minutes at room temperature before the serum is separated by centrifugation (850 – 1500xg for 10 minutes). The serum should be separated from the clot within three hours of blood collection and transferred to a clean test tube. Serum samples may be stored at –20°C or below until measurement. Avoid more than three freeze-thaw cycles of specimen.

Twenty four hour urine sample is recommended to be used for the determination of urine Fetuin-A concentration. Spot urine from the

second morning urination may be used if strenuous physical activity shortly before sample collection has been ruled out and polyuric renal dysfunction is not present. Intra-individual day-to-day fluctuations in the concentration of urine proteins caused by diuresis may be reduced by relating to the urinary creatinine concentration.

For cell culture supernatant, tissue extracts, one should serially dilute test sample and measure multiple diluted samples for a more accurate Fetuin-A test result.

## ASSAY PROCEDURE

### 1. Patient Sample Preparation

Patient serum or plasma sample needs to be diluted 1:10,000 with assay buffer before being measured.

- (1) Label 2 test tubes (12x75 mm) with 1A and 1B.
- (2) Add 1 mL of assay buffer to each tube (both 1A and 1B).
- (3) Pipet 10 µL of patient serum or plasma sample to tube 1A and mix well (1:100 dilution).
- (4) Pipet 10 µL of diluted patient sample from 1A to tube 1B mix well (1:10,000 dilution).

*Note: It is recommended to use a precision/calibrated pipette and careful technique to perform the dilution in order to get precise results! We recommend using Eppendorf Repeat Pipette with 12.5 mL combitip for adding 1 mL assay buffer. 50 mL combitip is not recommended.*

Patient urine sample needs to be diluted 1:100 with assay buffer before being measured.

- (1) Label 1 test tube (12x75 mm) with 1.
- (2) Add 1 mL of assay buffer to tube.
- (3) Pipet 10 µL of patient urine sample to tube 1 and mix well (1:100 dilution).

*Note: If a higher than standard level 5 of Fetuin-A test result is obtained, a further dilution of urine sample (e.g. 1:500) should be measured for reporting a more accurate test result.*

### 2. Reagent Preparation

- (1) Prior to use allow all reagents to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.
- (2) Fetuin-A Assay Buffer Concentrate (Cat. 10011) and ELISA Wash Concentrate (Cat. 10010) must be diluted to working solution prior to use. Please see REAGENTS section for details.

### 3. Assay Procedure

- (1) Place a sufficient number of antibody coated microwell strips (Cat. 30010) in a holder to run human Fetuin-A standards, controls and unknown samples in duplicate.
- (2) Test Configuration

ROW	STRIP 1	STRIP 2	STRIP 3
A	STD 1	STD 5	SAMPLE 2
B	STD 1	STD 5	SAMPLE 2
C	STD 2	C 1	SAMPLE 3
D	STD 2	C 1	SAMPLE 3
E	STD 3	C 2	
F	STD 3	C 2	
G	STD 4	SAMPLE 1	
H	STD 4	SAMPLE 1	

- (3) Add 25 µL of standards (Cat. 30001-30005), controls (Cat. 30007-30008) and 1:10,000 diluted patient samples into the designated microwell. *Note: if urine sample is used, 1:100 diluted urine sample should be used.*
- (4) Add 100 µL of assay buffer to each well.
- (5) Mix gently and cover the plate with one plate sealer and also with aluminum foil to avoid exposure to light.
- (6) Incubate the plate at room temperature for 2 hours.

- (7) Prepare Tracer Antibody Working Solution by 1:21 fold dilution of the Fetuin-A Tracer Antibody (Cat. 30009) with the Tracer Antibody Diluent (Cat. 30653). For each strip, it is required to mix 1 mL of Tracer Antibody Diluent with 50  $\mu$ L of Fetuin-A Tracer Antibody in a clean test tube.
- (8) Remove the aluminum foil and the plate sealer. Aspirate the contents of each well. Wash each well 5 times by dispensing 350  $\mu$ L of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
- (9) Add 100  $\mu$ L of above diluted tracer antibody working solution to each of the wells.
- (10) Cover the plate with one plate sealer and also with aluminum foil to avoid exposure to light.
- (11) Incubate the plate at room temperature for 30 minutes.
- (12) Remove the aluminum foil and the plate sealer. Aspirate the contents of each well. Wash each well 5 times by dispensing 350  $\mu$ L of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.
- (13) Add 100  $\mu$ L of ELISA HRP Substrate (Cat. 10020) into each of the wells.
- (14) Cover the plate with aluminum foil to avoid exposure to light.
- (15) Incubate the plate at room temperature for 20 minutes
- (16) Remove the aluminum foil. Add 100  $\mu$ L of ELISA Stop Solution (Cat. 10030) into each of the wells. Mix gently.
- (17) Read the absorbance at 450 nm within 10 minutes in a microplate reader

*NOTE: to reduce the background, one can set the instrument to dual wavelength measurement at 450 nm with background wavelength correction set at 595 nm, 620 nm or 630 nm.*

The human serum or plasma Fetuin-A concentrations for the controls and 1:10,000 diluted samples are read directly from the standard curve using their respective corrected absorbance.

#### EXAMPLE DATA AND STANDARD CURVE

A typical absorbance data and the resulting standard curve from human Fetuin-A ELISA are represented. **This curve should not be used in lieu of standard curve run with each assay.**

Well I.D.	OD 450 nm Absorbance			Results ng/mL
	Readings	Average	Corrected	
0	0.097	0.098	0.000	
ng/mL	0.099			
12.5	0.173	0.179	0.081	
ng/mL	0.185			
38	0.364	0.371	0.273	
ng/mL	0.379			
120	0.876	0.913	0.815	
ng/mL	0.949			
370	2.298	2.312	2.214	
ng/mL	2.325			
Control 1	0.471	0.484	0.386	55.1 ng/mL
	0.498			
Control 2	1.697	1.690	1.592	258.9 ng/mL
	1.700			

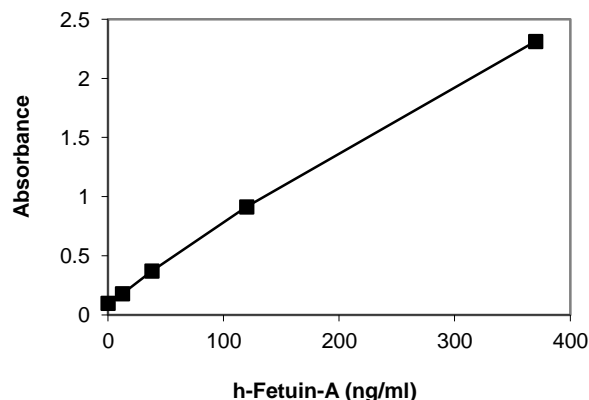
#### PROCEDURAL NOTES

1. It is recommended that all standards, controls and unknown samples be assayed in duplicate. The average absorbance reading of each duplicate should be used for data reduction and the calculation of results.
2. Keep light sensitive reagents in the original amber bottles.
3. Store any unused antibody coated strips in the foil Ziploc bag with desiccant to protect from moisture.
4. Careful technique and use of properly calibrated pipetting devices are necessary to ensure reproducibility of the test.
5. Incubation times or temperatures other than those stated in this insert may affect the results.
6. Avoid air bubbles in the microwell as this could result in lower binding efficiency and higher CV% of duplicate reading.
7. All reagents should be mixed gently and thoroughly prior to use. Avoid foaming.

#### INTERPRETATION OF RESULTS

1. Calculate the average absorbance for each pair of duplicate test results.
2. Subtract the average absorbance of the STD 1 (0 ng/mL) from the average absorbance of all other readings to obtain corrected absorbance.
3. The standard curve is generated by the corrected absorbance of all standard levels on the ordinate against the standard concentration on the abscissa using point-to-point or log-log paper. Appropriate computer assisted data reduction programs may also be used for the calculation of results.
4. We recommend a linear ordinate for optical density and a linear abscissa for concentration.

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#### EXPECTED VALUES

Seventy normal adult sera were measured with this human Fetuin-A ELISA. The ninety-five percentile normal range was found to be 0.35 to 0.95 g/L with a mean value of 0.57 g/L and a standard deviation of 0.13 g/L.

The 10,000-fold dilution factor must be added to each sample for the original sample Fetuin-A concentration. For example, a 1/10,000 fold diluted sample value is 24.3 ng/mL directly from the standard curve, the original sample Fetuin-A concentration should be

$$24.3 \text{ ng/mL} \times 10,000 = 243000 \text{ ng/mL} = 0.243 \text{ g/L}$$

## LIMITATION OF THE PROCEDURE

- The lowest concentration of human Fetuin-A directly measurable is 5.0 ng/mL (assay analytical sensitivity). After back calculation for the 1/10,000 fold dilution of patient serum sample, the assay measures the lowest serum Fetuin-A concentration at 50 µg/mL of original serum sample.
- Since there is no Gold Standard concentration available for human Fetuin-A measurement, the values of assay standards were established by diluting a highly purified recombinant human Fetuin-A in a protein matrix.
- For unknown sample value read directly from the assay that is greater than 350 ng/mL, it is recommended to measure a further diluted sample for more accurate measurement.
- If there is not a microplate reader in your laboratory being able to read beyond 2.0 at OD 450 nm, one can just run an assay without the standard level 6 from the standard set.
- Bacterial or fungal contamination of serum specimens or reagents, or cross-contamination between reagents may cause erroneous results.
- Water deionized with polyester resins may inactivate the horseradish peroxidase enzyme.

## QUALITY CONTROL

To assure the validity of the results each assay should include adequate controls with known Fetuin-A levels. We recommend that all assays include laboratory's own Fetuin-A controls in addition to those provided with this kit.

## PERFORMANCE CHARACTERISTICS

### Sensitivity

The analytical sensitivity of the human Fetuin-A ELISA as determined by the 95% confidence limit on 20 duplicate determination of zero standard is 5.0 ng/mL.

### Linearity

Two human serum samples were diluted with assay buffer and assayed. The results in the value of ng/mL are as follows:

#	DILUTION	OBSERVED VALUE	EXPECTED VALUE	RECOVERY %
1	1:10,000	21.9		
	1:20,000	11.9	11.0	108
	1:40,000	5.3	5.5	96
	1:80,000	2.9	2.7	107
	1:160,000	1.6	1.4	114
2	1:10,000	192		
	1:20,000	99.9	96	104
	1:40,000	45.2	48	94
	1:80,000	22.2	24	93
	1:160,000	13.4	12	112

### Precision

The intra-assay precision is validated by measuring two samples in a single assay with 20 replicate determinations.

Mean Fetuin-A Value (ng/mL)	CV (%)
33.6	5.5
121.1	4.8

The inter-assay precision is validated by measuring two samples in duplicate in 12 individual assays.

Mean Fetuin-A Value (ng/mL)	CV (%)
32.4	6.8
123.7	5.7

## Recovery

Two patient samples were spiked with various amounts of human Fetuin-A and assayed. The results in the value of ng/mL are as follows:

#	Orig. Value	Amount Spiked	Observed Value	Expected Value	Recovery %
1	33.6	21	25.1	27.3	92
		63	44.4	48.3	92
		200	120.1	116.8	103
2	121.1	21	68.9	71.1	97
		63	88.6	92.1	96
		200	157.1	160.6	98

## WARRANTY

This product is warranted to perform as described in its labeling and literature when used in accordance with all instructions. Epitope Diagnostics, Inc. DISCLAIMS ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, and in no event shall Epitope Diagnostics, Inc. be liable for consequential damages. Replacement of the product or refund of the purchase price is the exclusive remedy for the purchaser. This warranty gives you specific legal rights and you may have other rights, which vary from state to state.

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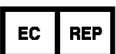
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