Human uPTM3-DKD ELISA

Enzyme immunoassay for the quantitative determination of human unique Fetuin-A with specific post translational modification (PTM) in urine.

REF: 8103201

Research use only

.

Bio Preventive Medicine Corp.
6F-1., 6F-2., 6F-3., 6F-5., 6F-6. No.18, Taiyuan St., Zhubei City,
Hsinchu County 30265, Taiwan
Phone: +886 3 5601816

Version 4/Revised in Apr. 2023
Form: Doc. #:MA2E103-01-037 V.1

INTENDED RESEARCH USE

The Human uPTM3-DKD ELISA is a colorimetric immunoassay intended for quantitative measurement of unique Fetuin-A with specific post translational modification (PTM), namely as E103 in human urine. Because urine will vary with personal habits, such as the amount of water you drink, the value of E103 should be corrected by urine creatinine.

PRINCIPLE OF THE TEST

The Human uPTM3-DKD ELISA is a competitive immunoassay. In this assay, Standards or unknown urine samples are mixed with anti-E103 monoclonal antibody (mAb), and then incubated in a microplate pre-bounded with E103. The monoclonal antibody recognizes E103 in Standards or unknown samples under competition in microplate wells. After incubation, the Horse Radish Peroxide (HRP) conjugated secondary antibody is added, followed by an incubation with 3,3',5,5'-tetramethylbenzidine (TMB) substrate. After supplementation of stop solution, the relative reactivity is determined by absorbance measurement at 450 nanometers (nm) wavelength and plotted by comparison with a predetermined E103 standard curve.

WARNINGS AND PRECAUTIONS

- Do not reuse the microplate wells.
- Do not use reagents after expiration date.
- Improper temperature exposure during all storage and assay procedures can adversely affect results.
- Wear protective gloves during all assay procedures.
- Being light sensitive and skin irritative, TMB Substrate should avoid direct light exposure and skin contact during all storage and assay procedures.
- Avoid skin contact with Stop Solution which contains 0.5 N sulfuric acid. It may cause skin irritation and burns.
- Disposal of any discarded materials should be in accordance with local requirements and existing regulations for good laboratory practice.
- Reliable and reproducible results will be obtained when the assay procedure is practiced with a complete understanding of the package insert instructions and with adherence to good laboratory practice. The test results will be closely related to the operative skills of the end users.

SYMBOLS GLOSSARY

	Consult Instructions for Use		Do Not Reuse
	Temperature limitation		Date of manufacture
	Use By Date		Ready to use
	Batch Code		Manufacturer

CONTENTS

Sufficient for 96 determinations

COMPONENT	QUANTITY	SYMBOL
Coated Microplate with E103, READY TO USE	96 wells: 12 x 8-well strips	MICROPLATE
Standard, LYOPHILIZED Powder	2 vials	STANDARD
1000X mAb anti-E103	1 vial, 10 µL	mAb anti-E103
4000X HRP Conjugate	1 vial, 10 µL	CONJUGATE
Diluent, READY TO USE	1 vial, 50 mL	DILUENT
10X Wash Buffer	1 vial, 50 mL	10X WASH
TMB Substrate, READY TO USE	1 vial, 20 mL	TMB
Stop Solution, 0.5 N sulfuric acid, READY TO USE	1 vial, 20 mL	STOP
Control-H, LYOPHILIZED Powder	2 vials	CONTROL H
Control-L, LYOPHILIZED Powder	2 vials	CONTROL L

STORAGE AND STABILITY

- The shelf life of the human uPTM3-DKD ELISA kit is **12 months**, and the components in the reagents should be stored according to the recommendations in the table below.

<table>
<tr><th rowspan="2">COMPONENT</th><th colspan="2">Open-vial</th></tr>
<tr><th>Expiry date</th><th>Temperature (°C)</th></tr>
<tr><td>Standard</td><td colspan="2">Reconstituted Standards (5 µg/mL) are stable for one week at -20°C and repeated freeze-thaw cycles should be avoided.</td></tr>
<tr><td>Control-H & Control-L</td><td colspan="2">Reconstituted Control-H & Control-L are stable for two week at -20°C and repeated freeze-thaw cycles should be avoided.</td></tr>
<tr><td>Coated Microplate</td><td rowspan="6">4 weeks (need to be within the expiry date of each component)</td><td>-10 ~ -30°C (Need to be placed in an aluminum foil bag containing desiccant)</td></tr>
<tr><td>1000X mAb anti-E103</td><td rowspan="2">-10 ~ -30°C</td></tr>
<tr><td>4000X HRP Conjugate</td></tr>
<tr><td>Diluent</td><td rowspan="3">2 ~ 8°C</td></tr>
<tr><td>10X Wash Buffer</td></tr>
<tr><td>TMB Substrate</td></tr>
<tr><td>Stop Solution</td><td></td></tr>
</table>

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision single micropipettes or multi-channel pipettes
- Microcentrifuge tubes and disposable tips
- Graduated cylinder 500 mL
- Vortex mixer and microcentrifuge
- Orbital shaker
- Plastic container for the preparation of reagents
- Microplate reader capable of endpoint measurement at 450±10 nm

- Adhesive plate seals
- ddH_2O

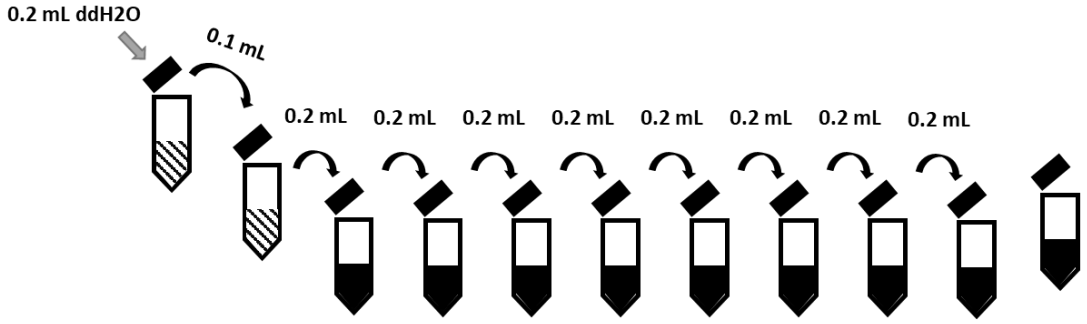
SPECIMEN COLLECTION AND HANDLING

- Morning urine samples must be collected in clean and dry containers. Avoid cross contamination.
- No additives or preservatives are required to preserve the integrity of urine samples.
- Urine samples can be temporarily stored at 20 ~ 25°C for 24 hours or 2 ~ 8°C for 72 hours before measurement.
- If sample isn't measured within the 24 to 72-hour period mentioned above, urine samples shall be stored at -10 ~ -30°C for up to four weeks. Avoid repeated freezing and thawing of the urine samples.
- Before performing the assay, please bring the sample temperature back to room temperature. Centrifuge urine samples for 5 minutes at 1,000±20 g. Take supernatant and assay immediately.
- Use Diluent for sample dilution if necessary.

REAGENTS PREPARATION

A. Prepare Standard Solution

Reconstitute the E103 *Standard* with 0.2 mL distilled or deionized water, sit for 10 minutes at room temperature until completely dissolved and mix gently. The reconstituted E103 Standard is now at a concentration of 5 µg/mL. Dilute the 5 µg/mL E103 Standard with *Diluent* by 5-fold, then mix gently to get a 1 µg/mL E103 Standard. Dilute the 1 µg/mL E103 Standard with *Diluent* by 2-fold, then mix gently to get a 500 ng/mL E103 Standard. Procedures for the serial dilution to generate Standards (Standard #1 ~ #7) for establishing E103 standard curve are shown in the following table and all Standards must be prepared and mixed well immediately prior to use. Please note that Standard #8 only constitutes 0.2 ml of Diluent.



Conc. (ng/ml) / Content in tube	Standards										
	-	-	-	#1	#2	#3	#4	#5	#6	#7	#8
	5000	1000	500	250	125	62.5	31.25	15.625	7.813	3.906	0
ddH_2O (ml)	0.2	X	X	X	X	X	X	X	X	X	X
Diluent (ml)	X	0.4	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

2 x serial dilution

B. Control-H、Control-L

Reconstitute the E103 *Controls* with 0.2 mL distilled or deionized water, sit for 10 minutes at room temperature until completely dissolved and mix gently.

C. 1X Wash Buffer

Recover *10X Wash Buffer* to room temperature prior to use until all the salt crystals are dissolved. Calculate the required amount of 1X Wash Buffer for each assay. For each microplate, mix 50 mL *10X Wash Buffer* with 450 mL distilled or deionized water. Mix uniformly but gently.

D. 1X mAb anti-E103

Calculate the required amount of 1X mAb anti- E103 for each assay, and mix *1000X mAb anti-E103* with *Diluent* according to the amount required. For each microplate, mix 8 µL *1000X mAb anti-E103* with 8 mL *Diluent*. Mix uniformly but gently.

E. 1X HRP Conjugate

Calculate the required amount of 1X HRP Conjugate for each assay, and mix *4000X HRP Conjugate* with *Diluent* according to the amount required. For each microplate, mix 3 µL *4000X HRP Conjugate* with 12 mL Diluent. Mix uniformly but gently.

ASSAY PROCEDURE

Prepare enough microplate modules for all Standards, CTL, and urine samples and secure them in a holder. Recover all reagents to room temperature prior to use.

(1) Respectively, add 50 µL of Standard solution #1-#8 (in duplicate) and urine samples into each well of *E103 Coated Microplate*.
(2) Add 50 µL of 1X mAb anti-E103 into each well with either Standard solution (#1-#8) or urine samples already added.
(3) Incubate for 2 hours at 25°C and 200 rpm on an orbital shaker.
(4) After the incubation, discard the contents in the wells.
(5) Wash each well with 300-400 µL 1X Wash Buffer. Discard the contents and sharply strike the wells on absorbent paper to remove residual liquid. Wash for a total of 4 times.
(6) Add 100 µL 1X HRP Conjugate into each well. Incubate the microplate for 30 minutes at 25°C and 200 rpm on an orbital shaker.
(7) After the incubation, discard the contents in the wells and wash the wells as described in Step 4 and 5.
(8) Add 100 µL *TMB Substrate* into each well. Incubate in the dark for 30 minutes at room temperature.
(9) Add 100 µL *Stop Solution* into each well. Mix by a brief shaking until the mixture become homogeneous.
(10) Determine the absorbance at 450±10 nm within 30 minutes and calculate the results.

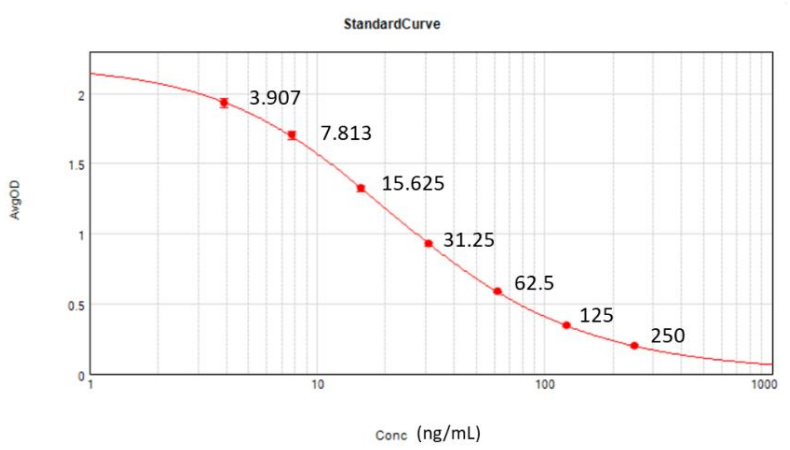
DATA ANALYSIS

A. Calculation

(1) The standard curve of this assay fits to a sigmoidal 4- or 5- parameter logistic equation. The results of curve-fitting can be obtained with any computer program having a 4- or 5- parameter logistic function.

(2) Graph the standard curve by plotting the OD.450 values on the Y-axis with linear scale against the standard concentrations on the X-axis with logarithm scale.

(3) The concentration of unique PTM Fetuin-A in patient urine samples can be obtained by inputting the OD.450 value and inversing fitted standard curve.



B. Quality criteria of assay

The quality control (Control-H and Control-L) should be included in each experiment for quality assurance. The suggested reference range of quality control is as following:

Name	E103 value (ng/mL)
Control-H	82.78 ~ 144.35
Control-L	11.55 ~ 30.87

C. Application

All concentration of E103 need to be urinary creatinine-corrected for use. The steps are as following:

(1) E103/Ucr = the value of urinary E103 (reported in ng/mL) dividing the value of urinary creatinine (reported in mg/mL).

(2) IVD103 = Log_{10} (E103/Ucr), which is the version under logarithmic scale.

DETECTION CAPABILITY

Limit of Blank (LoB) = 0.216 ng/mL;
Limit of Detection (LoD) = 1.901 ng/mL;
Limit of Quantitation (LoQ) = 5.428 ng/mL

LINEARITY AND MEASURING RANGE

The test was assessed and found to be linear from 4.90 to 255.39 ng/mL and measuring range is 5.428 to 250 ng/mL.

PRECISION

Repeatability

Samples were tested in triplicate. Reagents from three different lots were used each day for 20 days. Variation of within-day, day-to-day, and lot-to-lot were under 15%.

Sample	Mean conc. of E103 (ng/mL)	Within-run (%CV)	Run-to-run (%CV)	Day-to-day (%CV)
Sample A	11.32	8.12	8.45	6.69
Sample B	32.09	7.57	11.63	0
Sample C	40.46	4.06	9.1	0
Sample D	59.39	5.22	3.42	4.45
Sample E	130.38	5.2	4.31	4.6
Sample F	172.92	5.51	9.17	0

Reproducibility

Samples were tested in triplicate with two runs each day. Tests were performed in three independent laboratories for 5 days. Variation of within-run, run-to-run, day-to-day, and site-to-site were under 15%.

Sample	Mean conc. of E103 (ng/mL)	Within-run (%CV)	Run-to-run (%CV)	Day-to-day (%CV)	Site-to-Site (%CV)
Sample a	8.72	12.38	9.13	16.95	10
Sample b	26.83	8.98	6.64	13.74	13.91
Sample c	39.71	8.6	5.56	12.55	15.17
Sample d	67.26	6.34	5.04	10.62	11.27
Sample e	142.57	5.66	5.11	8.33	11.05
Sample f	167.38	6.13	4.91	9.01	10.81

INTERFERENCE

Potential interference	**The highest non-interference concentration (mg/L)**
Acetaminophen	600
Ascorbic Acid	90
Albumin	1500
Direct bilirubin	1800
Creatinine	3750
Enalapril	187.5
Glucose	60000
Glibenclamide	6
Hemoglobin	375
Ibuprofen	750
Losartan	300
Metformin HCL	20000
Metronidazole	120
Salicylic Acid	37.5
Simvastatin	45

Potential interference	The highest non-interference concentration (mg/L)
Sodium Citrate	150
Sodium oxalate	750
Trichloromethiazide	75
Urea	6250
Uric Acid	375
Urobilinogen	37.5

REFERENCES

(1) Jin-Shuen Chen et al. ERA-EDTA, 2011; June 23-26. Czech Republic.
(2) Tzu-Ling Tseng et al. The Endocrine Society 95th Annual Meeting & Expo, 2013; June 15-18. USA.
(3) Tzu-Ling Tseng et al. ASN Kidney Week, 2016; November 15–20. USA.
(4) Chuang, LM et al. (2020) DNlite-IVD103, A novel urinary test, predicts progressive eGFR decline in type 2 diabetes with microalbuminuria. Kidney International Reports, Volume 5, issue 3, supplement, S264, March 01. https://doi.org/10.1016/j.ekir.2020.02.685

Bio Preventive Medicine Corp.
6F-1., 6F-2., 6F-3., 6F-5., 6F-6. No.18, Taiyuan St., Zhubei City,
Hsinchu County 30265, Taiwan
Phone: +886 3 5601816