Human uPTM3-DKD ELISA

IVD CE

INTENDED USE

The Human uPTM3-DKD ELISA is a colorimetric immunoassay intended for quantitative measurement of unique Fetuin-A with specific post translational modification (PTM) in human urine and should be performed at qualified clinical laboratories by certified medical professionals, such as Medical Technologists. This product is for *in vitro* diagnostic use as an aid in risk assessment of renal complications in diabetic patients. Furthermore, the uPTM3-DKD test could also be applied to accurately differentiate progressive decliners of renal function in type 2 diabetes with microalbuminuria.

PRINCIPLE OF THE TEST

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

SYMBOLS GLOSSARY

IVD	In vitro diagnostic medical device	CE	CE marking
	Consult instructions for use	LOT	Batch code
	Storage temperature range		Use by (Expiration date)
	Manufacturer	EC REP	Authorized European representative
REF	Catalogue number	RTU	Ready to use

CONTENTS

Sufficient for 96 determinations

COMPONENT	QUANTITY	SYMBOL
Coated Microplate with unique PTM Fetuin-A (E103), READY TO USE	96 wells: 12 x 8-well strips	MICROPLATE
Calibrator, LYOPHILIZED Powder	2 vials	CALIBRATOR

1000X mAb anti-unique PTM Fetuin-A (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, 12 mL	TMB
Stop Solution, 0.5 N sulfuric acid, READY TO USE	1 vial, 12 mL	STOP

STORAGE AND STABILITY

COMPONENT	**STORAGE**
Coated Microplate	Stable at -20°C in plate pouch with desiccant until expiration date
Calibrator	Stable at -20°C until expiration date. Reconstituted Calibrators (5 µg/mL) are stable for one week at -20°C and repeated freeze-thaw cycles should be avoided.
1000X mAb anti-unique PTM Fetuin-A (E103)	Stable at -20°C until expiration date
4000X HRP Conjugate	Stable at -20°C until expiration date
Diluent	Stable at 4°C until expiration date
10X Wash Buffer	Stable at 4°C until expiration date. Prepared 1X Wash Buffer is stable for one week at room temperature.
TMB Substrate	Stable at 4°C until expiration date
Stop Solution	Stable at 4°C until expiration date

- Do not expose reagents to sun, heat, and moisture during storage and usage.
- Reagents are stable until the expiration date stated on the vials.
- Once opened, Diluent, 10X Wash Buffer, TMB Substrate and Stop Solution are stable for 4 weeks at 4°C, and Coated Microplate, 1000X mAb anti-unique PTM Fetuin-A (E103), and 4000X HRP Conjugate are stable for 4 weeks at -20°C.

REAGENTS PREPARATION

1. **Human unique PTM Fetuin-A (E103) Calibrators**

   Reconstitute the E103 Calibrator with 0.2 mL distilled or deionized water, sit for 10 minutes at room temperature until completely dissolved and mix gently. The reconstituted E103 Calibrator is now at a concentration of 5 µg/mL. Dilute the 5 µg/mL E103 Calibrator with Diluent by 5-fold, then mix gently to get a 1 µg/mL E103 Calibrator. Procedures for the serial dilution to generate all calibrators for establishing E103 calibration curve are shown in the following table and all calibrators must be prepared and mixed well immediately prior to use.

Calibrator	**Concentration (ng/mL)**	**Volume added to Diluent**	**Volume of Diluent**
1	500	200 µL 1 µg/mL Calibrator	200 µL
2	250	200 µL Calibrator 1	200 µL

3	125	200 µL Calibrator 2	200 µL
4	62.5	200 µL Calibrator 3	200 µL
5	31.25	200 µL Calibrator 4	200 µL
6	15.625	200 µL Calibrator 5	200 µL
7	7.813	200 µL Calibrator 6	200 µL
8	0	0 µL	200 µL

2. **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.

3. **1X mAb anti-unique PTM Fetuin-A (E103)**

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

4. **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.

MATERIALS REQUIRED BUT NOT PROVIDED

- Precision single micropipettes (1-10 µL, 20-100 µL, 100-200 µL, 200-1000 µL) and multi-channel pipettes (100 µL)
- 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
- Distilled or deionized water
- Adhesive plate seals
- Reagent reservoirs

WARNINGS AND PRECAUTIONS

- This test is for professional *in vitro* diagnostic use only.
- Do not use reagents after expiration date.
- Improper temperature exposure during all storage and assay procedures can adversely affect results.
- Do not reuse the microplate wells.
- 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 containing 0.5 N sulfuric acid. It may cause skin irritation and burns.

- Consider all clinical specimens potentially infectious.
- Disposal of any discarded materials should be in accordance with local requirements and existing regulations for good laboratory practice.

SPECIMEN COLLECTION AND HANDLING

- Morning urine samples must be collected in clean and dry containers. Avoid cross contamination.
- No additives or preservatives are necessary for integrity of urine samples.
- Urine samples are stored at -20°C until to be used. Avoid repeated freezing and thawing of urine samples.
- Before performing the assay, recover urine samples to room temperature. Centrifuge urine samples for 5 minutes at 1,000±20 x g. Take supernatant and assay immediately.
- Use Diluent for sample dilution if necessary.

ASSAY PROCEDURE

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

1. Mix Calibrator 1-8 and each centrifuged urine sample with 1X mAb anti-E103 in a 1:1 ratio in microcentrifuge tubes (for triplicate tests, mixing 180 µL of each Calibrator/sample with 180 µL 1X mAb anti-E103 is recommended). Incubate for 2 hours at 25°C and 200 rpm on an orbital shaker.
2. Transfer 100 µL of each incubated mixture into assigned wells of E103 Coated Microplate, and incubate for 1.5 hours at 25°C and 200 rpm on an orbital shaker. Keep the microplate covered and level during all incubations.
3. After the incubation, discard the contents in the wells.
4. 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.
5. Add 100 µL 1X HRP Conjugate into each well. Incubate the microplate for 60 minutes at 25°C and 200 rpm on an orbital shaker.
6. After the incubation, discard the contents in the wells and wash the wells as described in Step 4.
7. Add 100 µL TMB Substrate into each well. Incubate in the dark for 30 minutes at room temperature.
8. Add 100 µL Stop Solution into each well. Mix by a brief shaking until the mixture become homogeneous.
9. Determine the absorbance at 450±10 nm within 30 minutes and calculate the results.

CALCULATION OF RESULTS

1. Use a 5- or 4- parameter logistic curve fit to establish a calibration curve. The concentration of unique PTM Fetuin-A in patient urine samples can be calculated from the calibration curve by interpolation. The range of this assay is 7.813 - 500 ng/mL.
2. Calculate the unique PTM Fetuin-A quantity with a urinary creatinine correction (ng/mg Cr).

LIMITATIONS OF THE PROCEDURE

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.

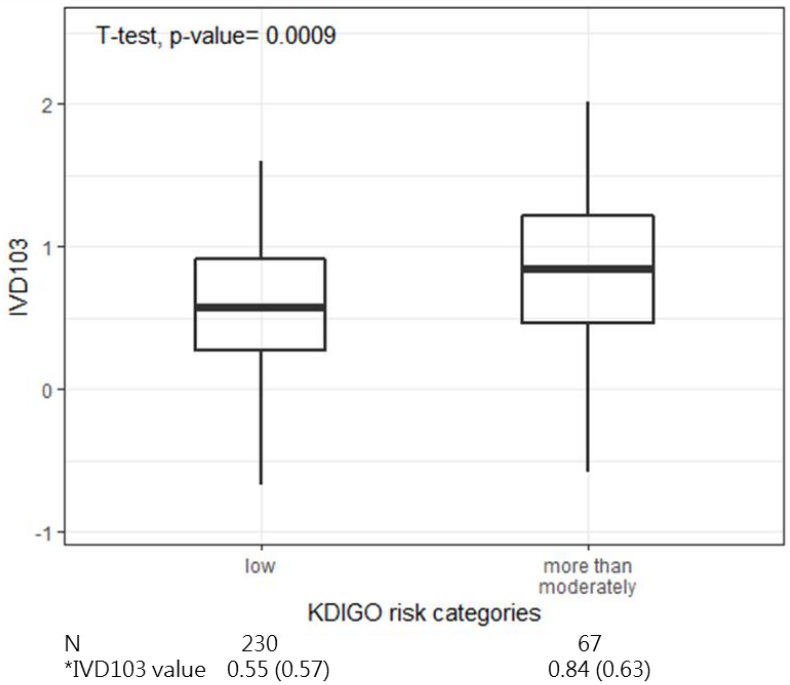
CLINICAL IMPLICATIONS

In addition to quantitatively measuring the concentration of unique PTM Fetuin-A in patient urine, the laboratory also needs to measure the creatinine concentration in the same patient to derive an unique PTM Fetuin-A to creatinine ratio. To meet the normal distribution assumption, the urinary unique PTM Fetuin-A to creatinine ratio was also presented under log-scale.

In our prospective cohort study, the unique PTM Fetuin-A levels of diabetic patients with more than moderately risk were significantly higher than those of patients with low risk (p-value= 0.0009). (Shown in Figure 1). The results showed a superior potential of baseline unique PTM Fetuin-A for predicting progressive decline of renal function compared with baseline UACR and eGFR. (Data not shown). Among patients with baseline microalbuminuria, we examined eGFR changes from baseline during a 1-year and a 2-year follow-up period. An eGFR slope < -3 mL/min/1.73 m^2/year was defined as progressive decline of renal function. Either a 1-year or a 2-year follow-up period and either using MDRD equation or CKD-EPI equation, all the sensitivity and negative predictive value (NPV) of predicting progressive decliner with the baseline unique PTM Fetuin-A level (below or above a cut-off) were more than 95% and 92%, respectively (Figure 2).

In this prospective cohort study, the performance of uPTM3-DKD test could support the clinical utility that the uPTM3-DKD test as an aid for diagnosis risk of renal complications in diabetic patients. Furthermore, the uPTM3-DKD test also could be applied to accurately differentiate progressive decliners of renal function in type 2 diabetes with microalbuminuria.

Figure1. Distribution of IVD103 values of low risk and more than moderately risk patients



N	230	67
*IVD103 value	0.55 (0.57)	0.84 (0.63)

*Value wereshown as Mean(SD)

By eGFR(MDRD) slope

Statistics	1 year	2 year
Sensitivity	0.96	0.96
NPV	0.92	0.92

By eGFR(CKD-EPI) slope

Statistics	1 year	2 year
Sensitivity	0.95	1.00
NPV	0.92	1.00

Figure 2. The sensitivity and negative predictive value of predicting progressive decliner with the baseline IVD103 level among 55 patients with baseline microalbuminuria .

PERFORMANCE CHARACTERISTICS

Precision

Precision of the Human uPTM3-DKD ELISA was evaluated in a 5 day study. Using 3 human urine samples from known diabetic patients and 5 spiked human urine samples, the samples were tested twice a day in triplicates. Within-Run precision: Coefficient of variation (CV) was calculated for each of eight samples from the results of 3 determinations in each run. Between-Run precision: Coefficient of variation (CV) was calculated for each of eight samples from the results of 3 determinations each in 10 different runs.

The precision results are summarized in the following table:

Sample	Mean (ng/mL)	Within-Run (% CV)	Between-Run (% CV)
PS 1	9.03	13.30	11.34
PS 2	13.4	7.34	8.27
PS 3	42.8	6.72	8.43
SS 4	335	4.71	4.65
SS 5	193	4.03	3.01
SS 6	90.0	6.63	5.46
SS 7	24.2	7.77	10.92
SS 8	18.5	6.21	5.17

Measuring range

7.813 - 500 ng/mL

Linearity

The test was assessed and found to be linear from 1.769 to 500 ng/mL (R^2=0.99).

Detection limit

Limit of Blank (LoB) = 0.873 ng/mL

Limit of Detection (LoD) = 4.510 ng/mL

Limit of Quantitation (LoQ) = 18.983 ng/mL

Interference substances

Test Substance	High Test Conc.	Interference
Bilirubin	3.13 mg/L	No
Glybenclamide	76.90 nmol/L	No
Hemoglobin	3.91 mg/L	No

Ibuprofen	37.89 nmol/mL	No
Losartan	3.25 ng/mL	No
Metformin HCL	3.91 ng/mL	No
Simvastatin	3.34 mg/mL	No
Trichloromethiazide	12.50 mg/mL	No
Acetaminophen	46.88 mg/L	No
Ascorbic Acid	38.15 ng/mL	No
Creatinine	1500 mg/L	No
Glucose	7500 mg/L	No
Metronidazole	6.09 mg/L	No
Salicylic Acid	93.75 mg/L	No
Sodium Citrate	16.67 mg/L	No
Sodium oxalate	5.56 mg/L	No
Urea	12.21 mg/L	No
Uric Acid	200 mg/L	No
Urobilinogen	5.56 mg/L	No
Albumin	50 ug/mL	No

EXAMPLE OF CALIBRATION CURVE

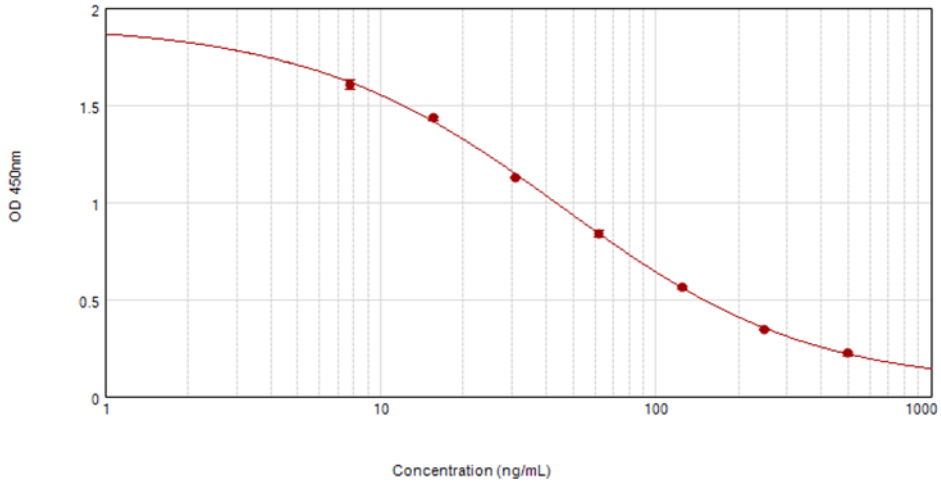


Figure 3: Calibration curve of the Human uPTM3-DKD ELISA (calibration range is 7.813 - 500 ng/mL).

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.

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