This short manual is an excerpt of:

For professional use only

ReFiNE® Dkk3 ELISA

For the in vitro determination of DKK3 in human urine

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Σ 96

+2 °C ±8 °C

IVD

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IMPORTANT NOTE

The following instructions serve as a short manual for the preparation and execution of the test. **It is important to note that this document does not replace the full instructions for use.**

1. INTENDED PURPOSE

ReFiNE® Dkk3 ELISA is an enzyme-linked immunosorbent assay (ELISA) intended for the quantitative determination of Dickkopf-related protein 3 (DKK3) in human urine samples. The assay is an *in vitro* diagnostic medical device and intended to be used by professional users in a laboratory environment. It can be performed manually or using an automated platform. The assay functions as an aid in the diagnosis of acute and/or chronically progressive renal impairment.

2. INTRODUCTION

Dickkopf-3 (DKK3), a member of a family of Wnt-pathway modulators[1], has been identified as a stress-induced, tubular epithelial cell-derived regulator of kidney fibrosis[2]. Genetic deletion, antibody-mediated blockade and tubular epithelial cell-specific abrogation of DKK3 reduced tubulointerstitial fibrosis in various mouse models of chronic kidney disease (CKD) and hence preserved kidney function[2]. Mechanistically, Dkk3 deficiency caused diminished canonical Wnt/ß-catenin signaling in tubular epithelial cells under pathological conditions, accompanied by amplified anti-fibrogenic inflammatory response within the injured kidney. DKK3 protein was detected in the urine of patients suffering from different types of CKD, correlating specifically and quantitatively with the magnitude of tubulointerstitial fibrosis in kidney biopsy specimens[2,3]. Tubulointerstitial fibrosis is the common pathological manifestation of etiologically different CKD that finally result in kidney failure. Thus, DKK3 in urine can be used as a diagnostic marker of progressive CKD. This was confirmed in a large patient cohort with various causes of CKD, where urinary DKK3 levels were independently associated with a significant decline in kidney function (glomerular filtration rate [GFR]) over the next 12 months, regardless of the cause of kidney injury[3]. Moreover, studies in subjects with clinically inapparent kidney damage revealed that even moderately increased levels of DKK3 in the urine – as a sign of ongoing tubular cell stress – signify increased risk for future acute kidney failure and progressive loss of kidney function thereafter[4]. The present enzyme-linked immune sorbent assay (ELISA) is intended for the quantitative determination of DKK3 in human urine. In this respect it serves as a specific measuring tool for "tubular stress" and thereby for the quantification of the risk of future kidney function loss resp. CKD progression[3-8].

3. MATERIALS PROVIDED

Cat. No.	Identifier	Kit components	Quanti-ty
Y1001	PLATE	Microtiter plate, pre-coated	12 x 8 wells
	FOL	Lightproof foil to cover the microtiter plate	3 x 1 piece
	CONJ	Conjugate, peroxidase-labeled, ready-to-use	1 x 14 ml
	STD	Standards, ready-to-use	1 x 6 vials
	CTRL 1	Control, ready-to-use (see product specification for range)	1 x 1 vial
	CTRL 2	Control, ready-to-use (see product specification for range)	1 x 1 vial
	SAMPLEBUF	Sample dilution buffer, ready-to-use	1 x 100 ml
K 0001.C.100	WASHBUF	Wash buffer concentrate, 10 x	2 x 100 ml
K 0002.15	SUB	Substrate (tetramethylbenzidine), ready-to-use	1 x 15 ml
K 0003.15	STOP	Stop solution, ready-to-use	1 x 15 ml

4. MATERIALS REQUIRED BUT NOT SUPPLIED

- Ultrapure water (Conductivity ≤ 0.055 µS/cm at 25 °C, particle size ≤ 0.2 µm)
- Calibrated precision pipettors and 10–1 000 µl single-use tips
- Absorbent paper
- Calibrated multi-channel pipets or repeater pipets and 10–1 000 µl single-use pipette tips
- Vortex mixer without specific requirements
- Centrifuge without specific requirements
- Standard single-use laboratory glass or plastic vials, cups, etc.
- Microtiter plate reader with 450 nm filters, optionally with reference wavelength (620 nm)

5. REAGENTS: STORAGE AND PREPARATION

- To run the assay more than once, ensure that reagents are stored at the conditions stated on the label. Prepare only the appropriate amount necessary for each run.

- DKK3 is susceptible to proteolytic degradation. In order to avoid contamination by unprotected skin and saliva, **always** wear gloves and **avoid** talking (wear a mask if necessary) when working with samples, standards and controls. This is particularly important when transferring to open ELISA system analyzer (e.g. DYNEX TECHNOLOGIES DSX®) vials.
- For a fast and accurate test procedure, it is **strongly** recommended to prepare the standards, controls and diluted samples in microtiter tubes and to use an 8-channel pipette to transfer the standards, controls, and diluted samples to the microtiter plate during the test procedure.
- **Warm the sample dilution buffer** (SAMPLEBUF) in a water bath at 37 °C for at least 30 minutes before use. Then allow to cool to RT (20–30 °C).
- **Preparation of the wash buffer:** The **wash buffer concentrate (WASHBUF)** must be diluted with ultrapure water **1:10** before use (e.g., 100 ml WASHBUF + 900 ml ultrapure water), mix well. Due to the high salt content in the concentrate, crystals may form. Before dilution, the crystals have to be dissolved at room temperature (15–30 °C) or in a water bath at 37 °C. **WASHBUF stored at 2–8 °C** can be used until the indicated expiry date. **Wash buffer** (1:10 diluted WASHBUF) can be stored in a closed bottle at **2–8 °C for 1 month**.
- Take as many microtiter strips as needed from the kit. After opening the vacuum-tight aluminum packaging, store all unused microtiter strips covered with the supplied foil (FOL) and together with the desiccant bag in the closed aluminum packaging at **2–8 °C**. The strips are stable until expiry date stated on the label.
- Microtiter strips must be covered with foil during incubations.
- Substrate solution must be colorless before use.
- Avoid foaming when mixing reagents.
- All other test reagents are ready-to-use. Test reagents are stable until the indicated expiry date (see label) when stored at **2–8 °C**.
- The reagents must not be used after the expiration date indicated on the kit packaging.
- Do not mix reagents of the test kit with other lots. Furthermore, wells of different microtiter plates, even of the same lot, must not be combined and used for analysis.
- Do not interchange plugs and caps from different reagents.
- Quality controls must always be measured with each run.

6. SAMPLES: STORAGE AND PREPARATION

6.1 Sampling

Preferably, the central fraction of the morning urine is collected in a suitable container with a tight-fitting cap. The urine sample must be centrifuged and the supernatant transferred to a new sample tube before performing the test.

6.2 Sample storage

If the samples are not assayed immediately, the supernatant of the centrifuged urine samples must be stored at 2–8 °C and assayed within 3 days. For repeated measurements, it is recommended to aliquot the samples and to store them at -20 °C. Repeated freezing and thawing of samples is possible up to 5 times. Thawed samples must be mixed prior to dilution.

6.3 Sample preparation

Handle patient specimens as if capable of transmitting infectious agents. Prepare samples using normal laboratory techniques. Prior to analysis, the urine samples must be clarified by centrifugation; 10 minutes at approximately 370 x *g* (2 000 rpm).

6.4 Dilution of samples

The supernatant of the urine samples must be diluted 1:10 before performing the assay,

e.g. **25 µl** sample + **225 µl** SAMPLEBUF (sample buffer), mix well.

For testing in duplicates, pipet 2 x 100 µl of each prepared sample.

7. ASSAY PROCEDURE

7.1 Test principle

Sandwich ELISA

The ReFiNE® Dkk3 ELISA is a sandwich ELISA for the quantitative measurement of DKK3 in human urine.

The wells of the microtiter plate are coated with a monoclonal anti-human-DKK3 capture antibody. During the first incubation step, DKK3 present in the sample is bound to the immobilized capture antibody, forming the first antigen-antibody complex. After a washing step to remove all unbound sample components, a horseradish peroxidase (HRP)-labeled detection antibody is added. A capture antibody-

antigen-detection antibody-complex is formed. Unbound substances are removed by another washing step. After the addition of an enzymatic reaction substrate (tetramethylbenzidine, TMB), HRP catalyzes the oxidation of the colorless TMB into a blue, oxidized TMB. The reaction is stopped by adding an acidic stop solution and the color changes from blue to yellow. The intensity of the yellow color is directly proportional to the concentration of DKK3. Results are determined via a calibration curve.

7.2 Automated processing

For automated processing of the test, automat-specific adjustments must be made in the test procedure in order to meet technical requirements. For support and queries, please contact your supplier or Immundiagnostik AG.

Attention: DKK3 is susceptible to proteolytic degradation. In order to avoid contamination by unprotected skin and saliva, **always** wear gloves and **avoid** talking (wear a mask if necessary) when working with samples, standards and controls. This is particularly important when transferring to open ELISA system analyzer (e.g. DYNEX TECHNOLOGIES DSX®) vials.

7.3 Manual processing

Before use, allow all reagents and samples to reach room temperature (15–30 °C) and mix well.

Mark the positions for standards/samples/controls in the protocol sheet.

Remove the required microtiter strips from the kit. Store all unused strips covered with the supplied foil (FOL) together with the desiccant bag in the closed aluminum packaging at 2–8 °C.

Attention: DKK3 is susceptible to proteolytic degradation. In order to avoid contamination by unprotected skin and saliva, **always** wear gloves and **avoid** talking (wear a mask if necessary) when working with samples, standards and controls to avoid contamination by unprotected skin and saliva.

For a fast and accurate test procedure, it is **strongly** recommended to prepare the standards, controls and diluted samples in microtiter tubes and to use an 8-channel pipette to transfer the standards, controls, and diluted samples to the microtiter plate during the test procedure.

To ensure a reliable result, pipetting of standards/controls/diluted samples **in multiple values is recommended**.

1.	Add each **100 µl standards/controls/ diluted samples** into the respective wells.
2.	Cover the strips with the enclosed foil (FOL) and incubate for **30 min at room temperature (15–30 °C)**.
3.	Discard the content of each well and wash **4 times** with **250 µl wash buffer**. After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.*
4.	Add **100 µl conjugate** (CONJ) into each well.
5.	Cover the strips with the enclosed foil (FOL) and incubate for **30 min at room temperature (15–30 °C)**.
6.	Discard the content of each well and wash **4 times** with **250 µl wash buffer**. After the final washing step, remove residual wash buffer by firmly tapping the plate on absorbent paper.*
7.	Add **100 µl substrate** (SUB) into each well.
8.	Incubate for **10–20 min**** **at room temperature (15–30 °C)** in the **dark**.
9.	Add **100 µl stop solution** (STOP) into each well and mix well.
10.	Determine **absorption immediately** with an ELISA reader at **450 nm** against 620 nm as a reference. If no reference wave-length is available, read only at 450 nm.

* To achieve best results, i.e., the maximum ratio between specific and background signal, careful washing is essential (steps 3 and 6). It its crucial to remove the wash solution completely. For that purpose, tap the plate firmly on several layers of absorbent tissue. Automated washers must be verified according to results obtained by manual washing.

** The intensity of the color change is temperature-sensitive. We recommend observing the color change and stopping the reaction upon good differentiation. Experience has shown that it can be stopped after 15 minutes at the latest.

8. WARNINGS AND PRECAUTIONS

- Urine samples must be centrifuged after collection and the supernatant must be stored at 2–8 °C and analyzed within 3 days.
- The assay is to be carried out exclusively according to the working instructions enclosed with the kit.

- The kit components contain sodium azide or ProClin™ 300 to protect against bacterial contamination. Sodium azide as well as ProClin™ 300 are toxic and may cause allergic reactions in contact with skin. **Warning:** May cause an allergic skin reaction (H317). Wear protective gloves/ protective clothing/ eye protection/ face protection while using the kit components. **IF ON SKIN:** Wash with plenty of water. If skin irritation or rash occurs: Get medical advice/ attention. Substrates for enzymatic color reactions are also described as toxic. Any contact with skin or mucous membranes should be avoided.
- The 10x Wash Buffer Concentrate (WASHBUF) contains detergents which may cause severe irritation upon contact with the eyes. **Warning:** Causes severe eye irritation (H319). **IN CASE OF CONTACT WITH EYES:** Rinse cautiously with water for several minutes. If possible, remove contact lenses. Continue rinsing. If eye irritation persists: Seek medical advice/attention.
- The stop solution consists of diluted sulphuric acid (H_2SO_4). H_2SO_4 is a strong acid and must be used with care even in diluted form. H_2SO_4 causes burns on contact with the skin. It should therefore be handled with protective gloves, protective clothing and safety goggles. In case of contact with the acid, the burnt area must be rinsed immediately with plenty of water. Do not inhale vapors and avoid inhalation. May be corrosive to metals (H290). Keep only in original container.
- In the event of warranty claims, the rejected material must be returned to the manufacturer, Immundiagnostik AG, accompanied by a written explanatory statement within 14 days.
- Serious incidents are to be reported to Immundiagnostik AG and the national regulatory authorities.
- This kit has been manufactured and placed on the market in accordance with Regulation (EU) 2017/746 (IVDR).

9. LITERATURE

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10. SYMBOL EXPLANATION

LOT	Lot number	REF	Catalogue number
IVD	*In Vitro* Diagnostic Medical Device	→REF	To be used with
	Manufacturer	Σ n	Contains sufficient for <n> tests
	Temperature limitation		Use by
S	Consult product specification data sheet	i	Consult instructions for use
CE	European Conformity	UDI	Unique Device Identification
	Corrosive	!	Warning: For associated hazard statements please see chapter PRECAUTIONS