FluoBolt™-KLOTHO

METAL ENHANCED FLUORESCENCE IMMUNOASSAY for human α-KLOTHO

METAL ENHANCED FLUORESCENCE IMMUNOASSAY FOR
THE QUANTITATIVE DETERMINATION OF
α-KLOTHO IN HUMAN SERUM AND PLASMA

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CONTENT

1. METAL / PLASMON ENHANCED FLUORESCENCE 2

2. α-KLOTHO 2

3. CONTENT OF THE KIT 3

4. ADDITIONAL MATERIAL SUPPLIED WITH THE KIT 3

5. MATERIALS AND EQUIPMENT REQUIRED BUT NOT SUPPLIED 4

6. REAGENTS AND SAMPLE PREPARATION 4

7. ASSAY PROCEDURE 5

8. CALCULATION OF RESULTS 7

9. ASSAY CHARACTERISTICS 9

10. TECHNICAL HINTS 11

11. PRECAUTIONS 11

12. LITERATURE 12

13. NOTES 13

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1) METAL / PLASMON ENHANCED FLUORESCENCE

Metal Enhanced Fluorescence (MEF) offers the possibility to increase the analytical sensitivity of systems based on fluorescence detection dramatically. MEF is based on the fact that excitation light interacts with the electrons of metal nano-structures thus generating very high electromagnetic fields (Localised Surface Plasmons, LSPs) Therefore, such structures are also called "plasmonic structures" and the combination of (e.g. polymeric) support and structure is known as "plasmonic substrate”. These LSPs lead to an increase in emission output of fluorescent molecules (e.g. fluorescently labeled antibodies) when bound to surfaces with suitable nano-metal structures that enhances the signal dramatically. FIANOSTICS has developed a new plasmonic enhanced immunoassay platform in cooperation with Sony DADC BioSciences (now STRATEC Consumables since July 1st 2016), that allows up to 300 fold gains of sensitivity. This platform is fully compatible to standard laboratory methodology using 96 well microtiter plate format and assays based on this technology can be run on any standard fluorescence microplate reader. Its unique features enable fluorescence immunoassays with highest sensitivity and without washing steps.

2) α-KLOTHO

α-KLOTHO is a protein primarily expressed in kidney. It can be found either as a membrane bound or a secreted form. The membrane bound form consists of 1012 amino acids (aa), starting with a 56 aa long signalling sequence and followed by two glycosyl hydrolase 1 regions (position 57-506 and 515-953). Both glycosyl hydrolase 1 regions lack one essential Glu active site residue. Thus, it is inactive in vivo as a glycosidase although it belongs to the glycosyl hydrolase 1 family. KLOTHO’s secreted isoform, which predominates over the membrane bound form, consists of 549 amino acids (aa). It is

produced by alternative splicing and differs from the membrane bound form by aa 550 to 1012 missing.

α-KLOTHO is expressed in kidney, small intestine, placenta and prostate. The soluble peptide can be found in serum and cerebrospinal fluid. It may play a role in the calcium/ phosphorus homeostasis regulation by e.g. inhibiting active vitamin D synthesis. Further, it is also known as an anti-aging-hormone by extending life span by inhibiting insulin/ IGF1 signalling pathway, as experiments in mice showed. KLOTHO is a co-receptor of fibroblast growth factor 23 (FGF-23). Research has investigated association of altered serum KLOTHO levels with chronic kidney disease and failure, renal- and hepatocellular carcinomas, osteoporosis or cardiovascular diseases.

3) CONTENT OF THE KIT

ID	KIT COMPONENT	QUANTITY
HM	Anti-human KLOTHO antibody, pre-coated MEF-microtiter plate, packed in vacuum sealed aluminum bag	1 x 96 well
WP	Wash buffer concentrate 20x, natural cap	1 x 25 ml
HAF, HA3, HA5, HAA	Anti-human KLOTHO antibody, black flask, labeled with FITC, Cy3, Cy5 or AlexaFluor680	1 x 5 ml
HS	Standards 1-6, (400, 200, 100, 50, 25, 0 pmol/l), white caps, lyophilized	6 vials, 0.25 ml
HCA/B	Control A and B, yellow cap, lyophilized (for concentrations see label)	2 vials, 0.25 ml
HD	Sample diluent, natural cap, ready to use	1 x 10 ml

4) ADDITIONAL MATERIAL SUPPLIED WITH THE KIT

- 2 self-adhesive plastic films
- QC data sheet
- Protocol sheet

- Instruction manual for use
- 2 desiccant bags for plate storage

5) MATERIAL AND EQUIPMENT REQUIRED BUT NOT SUPPLIED

- Precision pipettes calibrated to deliver 10 µl, 20 µl, 50 µl, 200 µl, 500 µl and disposable tips
- Distilled or deionized water
- Plate washer, multichannel pipette or manifold dispenser for washing
- Refrigerator with 4°C (2-8°C)
- Fluorescence microplate reader
- Graph paper or software for calculation of results

6) REAGENTS AND SAMPLE PREPARATION

All reagents of the kit are stable at 4°C (2-8°C) until expiry date stated on the label of each reagent.

<u>Sample preparation:</u>
Collect venous blood samples by using standardized blood collection tubes for serum or plasma. We recommend performing plasma or serum separation by centrifugation as soon as possible, e.g. 10 min at 2000 x g, preferably at 4°C (2-8°C). The acquired plasma or serum samples should be measured as soon as possible. For longer storage aliquot samples and store at -25°C or lower. Do not freeze-thaw samples more than 4 times.

Lipemic or hemolyzed samples may give erroneous results. Samples should be mixed well before assaying.
For further information on sample stability contact us by e-mail at support@fianostics.at or by phone + 43/2622/27514.

Reagent preparation:
Add 250 µl of distilled or deionized water to the lyophilized HS (Standards) and HC (Controls). Leave at room temperature (18-26°C) for 15 min. Reconstituted HS and HC are stable at -25°C or lower until expiry date stated on the label. Reconstituted HS and HC can undergo 4 freeze-thaw cycles.

Bring WP (Wash buffer) concentrate (20x) to room temperature. Make sure that the solution is clear and without any salt precipitates before further dilution. Dilute the WP to working strength by adding the appropriate amount of distilled or deionized water, e.g. 25 ml of WP + 475 ml water, prior to use in the assay. Undiluted WP is stable at 4°C (2-8°C) until expiry date on the label. Diluted WP is stable at 4°C (2-8°C) up to one month. Only use diluted WP in the assay.

7) ASSAY PROCEDURE

All reagents and samples must be at room temperature (18-26°C) before use in the assay.

Mark position for standards, controls and samples on the protocol sheet. We recommend running samples and standards in duplicates.

Take the plasmonic enhanced microtiter plate out of the aluminum bag. Avoid touching the bottom of the plate with bare hands, because reading without washing is performed through the well bottom.

Seal all wells that **will not be used** in the following assay run with the accompanying adhesive film (cut to fit!).

In standard format, the kit is delivered with an AlexaFluor680 labeled detection antibody (HAA) because serum background fluorescence is minimal within this wavelength range. Therefore, if your reader is equipped with monochromatic

optics, please set Excitation/Emission to 679/702 nm or if you are using an optical filter-based reader, select a suitable filter pair (e.g. 670/720 nm). On request the kit can also be delivered with FITC, Cy3 or Cy5 (Ex/Em = 495/518 nm, 550/570 nm or 650/670 nm) labeled detection antibody.

1) Add 50 µl of the selected fluorescence labeled detection antibody (HAF or HA3 or HA5 or HAA) to all wells required. Swirl gently.

2) Add 10 µl of standard, control or sample to the wells according to the marked positions on the protocol sheet, swirl gently, cover tightly with the delivered adhesive film and incubate over night at room temperature (18-26°C) in the dark.

3a) If your reader allows bottom reading, read the plate without any further processing at the Ex/Em wavelength fitting to the delivered detection antibody (495/518 nm for HAF, 550/570 nm for HA3, 650/670 nm for HA5, 679/702 nm for HAA). Gain should be set to achieve at least 10000 fluorescence units (F.U.) between the signal of the 0 pmol/l and the 400 pmol/l KLOTHO standard. Samples with signals exceeding the signal of the highest standard must be re-run with an appropriate dilution using sample diluent (HD).

3b) If your reader has no bottom read option or if you want to store the plate for documentation purposes, discard or aspirate the content of the wells and wash 3x with diluted wash buffer.

Use a minimum of 200 µl wash buffer per well. After the final wash, remove remaining fluid by strongly tapping the plate against a paper towel. Read the plate in top configuration without any further processing at the Ex/Em wavelength fitting to the chosen detection antibody (495/518 nm for HAF, 550/570 nm for HA3, 650/670 nm for HA5, 679/702 nm for HAA).

Hint: Quality of bottom reading (3a) may vary between microplate readers. For first time users we suggest performing the washing step and follow protocol 3b.

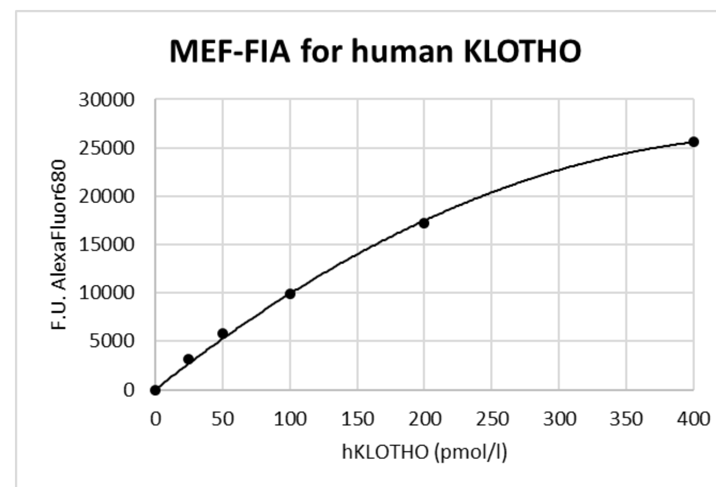
Gain should be set to achieve at least 10000 fluorescence units (F.U.) between the signals of the 0 pmol/l and the 400 pmol/l KLOTHO standard. Samples with signals exceeding the signal of the highest standard must be re-run with appropriate dilution using sample diluent (HD).

4) Store the plate with the 2 desiccant bags supplied at 4°C (2-8°C) in the aluminum bag. Unused wells are stable until expiry date stated on the label. Fluorescence signals of standards, controls and samples remain detectable for at least two months at the plate surface, depending on signal intensity achieved.

8) CALCULATION OF RESULTS

Subtract the fluorescence intensity of the 0 pmol/l standard from all other standards, samples and controls. Construct a calibration curve from the fluorescence units (F.U.) of the standards using commercially available software or graph paper. Read sample and control concentrations from this standard curve. Make sure to use appropriate curve fitting algorithm (e.g. linear or 4PL).

Example of a typical calibration curve:



The quality control (QC) protocol supplied with the kit shows the results of the final release QC for each kit lot at production date.

Fluorescence intensity obtained by customers may differ due to various influences and/or due to the normal decrease of signal intensity during shelf life.

However, this does not affect validity of results as long as the supplied kit controls read according to specifications (target ranges see labels).

9) ASSAY CHARACTERISTICS

Method	Metal Enhanced Direct Sandwich Fluorescence Immunoassay in 96-well plate format
Sample type	Serum, Plasma
Standard range	0 to 400 pmol/l (6 standards and 2 controls in a serum-based matrix)
Conversion factor	1 ng/ml = 16 pmol/l (MW: 62.1 kDa)
Sample volume	10 µl (undiluted sample) / well
Incubation time / temperature	overnight / room temperature (18-26°C)
Sensitivity	LOD (0 pmol/l + 3 SD): 2,5 pmol/l; LLOQ: 25 pmol/l
Specificity	This assay detects only α-KLOTHO and does not cross-react with β-KLOTHO. No interference of recombinant FGF-23 with the assay's signal up to a 100-fold molar excess was monitored.
Cross-reactivity	Human KLOTHO shares around 98-97% aa sequence with higher apes, 95-91% bovines, 91-89% pinnipeds and 87% mice. Cross-reactivity of this assay with other species than human has not been tested.

Precision:

Intra-assay: 4 samples of known concentrations were tested 3 times within 1 assay run
CVs ranged from 1-11.0%

Inter-assay: 4 samples of known concentrations were tested in duplicates within 3 different assay runs
CVs ranged from 6-10%

Spike/Recovery:

The recovery of KLOTHO in serum was evaluated by adding known amounts of human recombinant KLOTHO to 4 different human serum samples. Mean recovery was 82% (69-101%).

Mean recovery in Citrat- and Heparin-Plasma was 75% (n=4) each and 88% in EDTA-Plasma (n=4).

Linearity:

3 human serum samples were spiked with recombinant KLOTHO and diluted 1+1 and 1+2 with the sample diluent (HD) supplied with the kit. Mean linearity was 69% (see table below).

	Measured (pmol/l)		
Dilution	**Sample #1**	**Sample #2**	**Sample #3**
1+0	292	239	209
1+1	168	182	166
1+2	152	115	106
	Expected (pmol/l)		
1+1	146	120	104
1+2	97	80	70
	Linearity (%)		
1+1	87%	66%	63%
1+2	64%	69%	66%

Specificity:

Analyte Specificity:

This assay detects human α-KLOTHO. Addition of recombinant FGF-23, which is considered to be binding to KLOTHO, to the standards supplied with this kit did not reduce signal intensity. Measurement of β-KLOTHO is not possible with this kit. The presence of β-KLOTHO does not interfere with the detection of α-KLOTHO.

Species Specificity:

Human KLOTHO shares around 98-97% aa sequence with higher apes (e.g. orangutan or chimpanzee), 95-91% bovines (e.g. cattle or

yak), 91-89% pinnipeds (e.g. walrus or monk seal) and 87% mice. Reactivity of this assay with other species than human has not been tested. So, using this assay for KLOTHO measurements in serum or plasma of species with high sequence homology may be possible but must be evaluated by the user. FIANOSTICS does not take responsibility for functionality of the assay in non-human samples.

10) TECHNICAL HINTS

- Do not mix or substitute reagents with those from other lots or sources.
- Do not mix stoppers and caps from different reagents or use reagents between lots.
- Do not use reagents beyond expiration date.
- Protect reagents from direct sunlight.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Avoid foaming when mixing reagents

11) PRECAUTIONS

- All test components of human source were tested against HIV-Ab and HBsAg and were found negative. Nevertheless, they should be handled and disposed as if they were infectious.
- Liquid reagents contain ≤0.1% Proclin 300 as preservative. Proclin 300 is not toxic in concentrations used in this kit. It may cause allergic skin reactions – avoid contact with skin, eyes or mucous membranes.
- Do not pipette by mouth.
- Do not eat, drink, smoke or apply cosmetics where reagents are used.
- Wear gloves, protective glasses and lab jacket while performing this assay.

12) LITERATURE

- **Single step, direct fluorescence immunoassays based on metal enhanced fluorescence (MEF-FIA) applicable as micro plate-, array-, multiplexing- or point of care-format.** Hawa G et al., Anal Biochem. 2018;549:39-44.

- **The Prognostic Role of Klotho in Patients with Chronic Kidney Disease: A Systematic Review and Meta-analysis.** Liu QF, Yu LX, Feng JH, Sun Q, Li SS, Ye JM. Dis Markers. 2019 Jun; 2019:6468729.

- **Klotho plays a critical role in clear cell renal cell carcinoma progression and clinical outcome.** Kim JH, Hwang KH, Lkhagvadorj S, Jung JH, Chung HC, Park KS, Kong ID, Eom M, Cha SK. Korean J Physiol Pharmacol. 2016 May; 20(3):297-304.

- **The Biological Role of Klotho Protein in the Development of Cardiovascular Diseases.** Olejnik A, Franczak A, Krzywonos-Zawadzka A, Kałużna-Oleksy M, Bil-Lula I. Biomed Res Int. 2018 Dec; 2018:5171945.

- **Klotho and chronic kidney disease.** Hu MC, Kuro-o M, Moe OW. Contrib Nephrol. 2013; 180:47-63.

- **Pathophysiological implications of fibroblast growth factor-23 and Klotho and their potential role as clinical biomarkers.** Donate-Correa J, Muros de Fuentes M, Mora-Fernández C, Navarro-González JF. Clin Chem. 2014 Jul; 60(7):933-40.

13) NOTES

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