Approval:

Title	**Instructions For Use – K-Postn Immunoassay**
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1.0	N/A

K-Postn Immunoassay

Instructions For Use

Catalogue Number PA017-RUO

RUO

Version 1.0 August 2023

INTENDED USE

For the quantitative determination of K-Postn in serum samples.

For Research Use Only. Not for use in diagnostic procedures.

BACKGROUND

Periostin is an extracellular matrix (ECM) protein which belongs to the fasciclin family (Ikeda-Iwabu *et al*., 2021). It has been shown to regulate bone mineral density and bone formation and is also reputed to represent a possible biomarker for fracture and osteoporosis (Zhu *et al*., 2021). In postmenopausal women, elevated levels of this protein are independently associated with increased fracture risk (Rousseau *et al*., 2014), and have been reported in individuals who have sustained an osteoporotic fracture (Varughese *et al*., 2018). Periostin, however, is expressed by a range of tissues, and has been implicated in various other morbidities including lung disease and oncogenesis (Chapurlat and Confavreux, 2016). Moreover, evidence is emerging of a potential role, within the context of bone health, for a cathepsin K generated fragment of periostin, namely K-Postn; a process that is restricted to the bone milieu (Garnero *et al*., 2017). Specifically, serum levels of K-Postn have been shown to be positively associated with incident fractures, in postmenopausal women, independently of various bone turnover markers, bone mineral density and the fracture risk assessment tool (FRAX) (Bonnet *et al*., 2017).

ASSAY PRINCIPLE

The K-Postn immunoassay has been designed to detect and quantify K-Postn within serum samples.

The plate is washed and then coated with biotinylated peptide which is incubated before washing to remove any unbound material. Standards, samples and primary antibody are then added and incubated. The primary antibody competitively binds to either the immobilised biotinylated peptide or soluble K-Postn fragments/non-biotinylated peptide (K-Postn Standard). Unbound primary antibody together with K-Postn fragments/non-biotinylated peptide are removed by a further wash step. A secondary horseradish peroxidase (HRP) conjugated antibody is then incubated on the plate and will bind to captured primary antibody. A further wash step is performed prior to the addition of a colorimetric substrate, which results in the formation of a blue coloured product in the presence of HRP. This enzymatic reaction is subsequently stopped by the addition of acidic stop solution to each test well (a yellow solution is formed). The colour intensity (absorbance) is read at 450 nm using a plate reader. The absorbance reading is inversely proportional to K-Postn concentration i.e., a high absorbance measurement denotes a low K-Postn concentration.

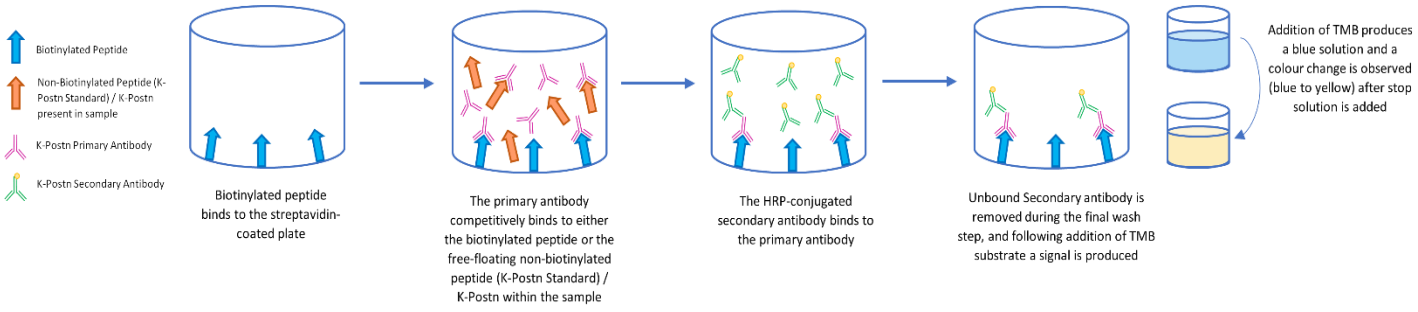


Figure 1. Procedure overview of the K-Postn Immunoassay

MATERIALS PROVIDED

Freezer Box Components	
K-Postn Biotinylated Peptide	1 vial containing 30 µL of Biotinylated peptide (concentration: 1.6 µg/mL) in *N,N*-dimethylformamide (DMF)
K-Postn Standard (Non-Biotinylated Peptide)	1 vial containing 30 µL of Non-Biotinylated peptide (concentration: 32 µg/mL) in *N,N*-dimethylformamide (DMF)
K-Postn Primary Antibody	1 vial containing 20 µL of K-Postn primary antibody (concentration: 41 µg/mL) in glycerol solution
K-Postn Secondary Antibody (HRP)	1 vial containing 30 µL of Goat Anti-Rabbit Antibody (HRP) (100 µg/mL) in glycerol solution

Main Box Components	
Nunc Immobilizer Plate	96-well streptavidin coated microtitre plate (pre-blocked) Cat# 436014
K-Postn Wash Buffer Concentrate	80 mL of a 10-fold concentration of buffered surfactant with preservatives
K-Postn Dilution Buffer Concentrate	6 mL of a 10-fold concentration of buffered surfactant with preservatives
TMB Substrate	12 mL of tetramethylbenzidine solution (ready to use)
Stop Solution	6 mL of 2 N sulfuric acid (ready to use)
Plate Sealers	4 adhesive strips

STORAGE

- Store the freezer box immediately upon receipt at -20°C
- Store the main box immediately upon receipt at 2-8°C
- Once opened, the remaining reagents should be disposed of and not returned to storage as product is single use only.

MATERIALS REQUIRED BUT NOT SUPPLIED

- Deionised or distilled water
- Calibrated pipettes and corresponding pipette tips
- Tubes and tube racks
- Clean containers for diluted wash buffers
- Graduated cylinder
- Vortex mixer
- Shaking Incubator (22°C, 100 rpm)
- Wash bottle or automated microplate washer
- Calibrated microtitre plate reader capable of reading at 450 nm
- Computer and computer software for data analysis

REAGENT PREPARATION (For full plate analysis)

- K-Postn Biotinylated Peptide
  - To be prepared prior to Step 5 of the assay procedure
  - Dilute 24 µL of K-Postn Biotinylated Peptide with 11,976 µL of K-Postn Dilution Buffer to prepare 12 mL of biotinylated peptide solution (500-fold dilution).

- K-Postn Standard (Non-Biotinylated Peptide)
  - To be prepared prior to Step 7 of the assay procedure
  - Standard curve preparation:
    - Label microtubes 1-9.
    - Pipette 288 µL of K-Postn Dilution Buffer into micro-tube 1.
    - Pipette 150 µL of K-Postn Dilution Buffer into micro-tubes 2-7.
    - Add 12 µL of the K-Postn Standard to micro-tube 1. Carry out a x2 dilution series by transferring 150 µL from micro-tube 1 to micro-tube 2. Mix each micro-tube thoroughly before transferring to next micro-tube. Repeat this process to create a calibration curve with the following concentrations: 1280, 640, 320, 160, 80, 40 and 20 ng/mL.
    - Micro-tube 8 is used as the zero standard (Maximum signal well). Pipette 150 µL of K-Postn Dilution Buffer into this micro-tube.
    - Micro-tube 9 is used for the non-specific binding (NSB) wells. Pipette 250 µL of K-Postn Dilution Buffer into this micro-tube.

- Serum Samples
  - To be prepared prior to Step 7 of the assay procedure
  - Dilute by at least the minimum required dilution with K-Postn Dilution Buffer i.e., a dilution of x50 is recommended, however, this should be optimised by the operator.

- K-Postn Primary Antibody
  - To be prepared prior to Step 7 of the assay procedure
  - Dilute 12 µL of K-Postn Primary Antibody with 5,988 µL of K-Postn Dilution Buffer to prepare 6 mL of primary antibody solution (500-fold dilution).

- K-Postn Secondary Antibody
  - To be prepared prior to Step 9 of the assay procedure

- Dilute 24 µL of secondary antibody with 11,976 µL of K-Postn Dilution Buffer to prepare 12 mL of secondary antibody solution (500-fold dilution)

ASSAY PROCEDURE

Volumes listed below are suitable for full plate analysis. Reduce if appropriate.

*** Allow the following reagents to warm up to room temperature before use: K-Postn Wash Buffer, K-Postn Dilution Buffer, TMB and Stop Solution***

1. Dilute 80 mL of K-Postn Wash Buffer Concentrate with 720 mL deionised water to prepare 800 mL of Wash Buffer. Mix thoroughly.
2. Dilute 5 mL of K-Postn Dilution Buffer Concentrate with 45 mL deionised water to prepare 50 mL of Dilution Buffer. Mix thoroughly.
3. Remove plate from the foil pouch.
4. Aspirate and wash each well using 300 µL/well Wash Buffer. Repeat the procedure twice for a total of 3 washes.
5. Add 100 µL of diluted Biotinylated Peptide into each well. Cover with the plate sealer and incubate plate at 22°C, under agitation for 1 hour at 100 rpm.
6. Aspirate and wash each well using 300 µL/well Wash Buffer. Repeat the procedure twice for a total of 3 washes.
7. Add 50 µL of prepared standards/samples/controls according to recommended plate layout (See below). Add 100 µL of K-Postn Dilution Buffer to NSB wells. Add 50 µL K-Postn Primary Antibody into each well, (**excluding NSB wells**). Cover with the plate sealer and incubate plate at 22°C, under agitation for 1 hour at 100 rpm.
8. Aspirate and wash each well using 300 µL/well Wash Buffer. Repeat the procedure twice for a total of 3 washes.
9. Add 100 µL of diluted K-Postn Secondary Antibody into each well. Cover with the plate sealer and incubate plate at 22°C, under agitation for 1 hour at 100 rpm.
10. Aspirate and wash each well using 300 µL/well Wash Buffer. Repeat the procedure twice for a total of 3 washes.
11. Add 100 µL of TMB Substrate solution into each well. Cover with the plate sealer and incubate at room temperature for 10 minutes. **Protect from light.**
12. Add 50 µL of Stop Solution to each well. The colour within the wells should change from blue to yellow.
13. Determine the optical density of each well within 10 minutes using a microplate reader set to 450nm.

RECOMMENDED PLATE LAYOUT

	1	2	3	4	5	6	7	8	9	10	11	12
A	**1280 ng/mL**		**NSB Wells**		Sample 8		Sample 16		Sample 24		Sample 32	
B	**640 ng/mL**		Sample 1		Sample 9		Sample 17		Sample 25		Sample 33	
C	**320 ng/mL**		Sample 2		Sample 10		Sample 18		Sample 26		Sample 34	
D	**160 ng/mL**		Sample 3		Sample 11		Sample 19		Sample 27		Sample 35	
E	**80 ng/mL**		Sample 4		Sample 12		Sample 20		Sample 28		Sample 36	
F	**40 ng/mL**		Sample 5		Sample 13		Sample 21		Sample 29		Sample 37	
G	**20 ng/mL**		Sample 6		Sample 14		Sample 22		Sample 30		Sample 38	
H	**Zero standard**		Sample 7		Sample 15		Sample 23		Sample 31		Sample 39	
	Standard Curve		NSB wells and serum samples									

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control and sample and subtract the NSB absorbance values. Construct a standard curve by reducing the data using computer software capable of generating a four-parameter logistic (4-PL) curve fit. If samples have been diluted, the concentration reported should be corrected for the dilution factor.

LIMITATIONS OF THE ASSAY

- For Research Use Only. Not for use in diagnostic procedures.
- The test is for single use only. Do not reuse.
- Store all components correctly.
- Use all unopened reagents before their expiry date.
- Do not mix or substitute reagents with those from other lots or sources.

- All procedures should be carried out in accordance with the protocol. Performing the assay outside of the prescribed time and temperature ranges may produce invalid results.
- This immunoassay has been optimised and validated for use with human serum only. For use with other sample types, the user should confirm sample characteristics and validate kit performance with the sample type they intend to analyse.

TECHNICAL HINTS

- It is recommended that all standards and samples be assayed in duplicate.
- To abrogate the matrix effect of serum, a **minimum required dilution** of x50 is recommended. Other suitable dilutions include x75, x100 and x150. Dilutions above x150 need further investigation to determine suitability in this assay.
- To avoid cross-contamination, change pipette tips between addition of each standard, sample, and reagent. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- We recommend the use of external controls for internal quality control.

PRECAUTIONS

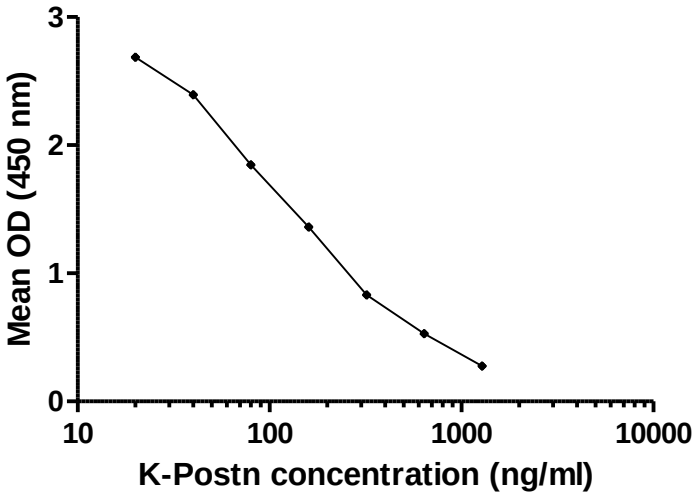
- Wear personal protective equipment including gloves, clothing, eye and face protection. Wash hands after handling reagents.
- K-Postn Biotinylated Peptide and K-Postn Standard contain *N,N*-dimethylformamide (DMF) which is a solvent that is considered harmful, flammable and an irritant. Wear gloves and protective clothing when handling and avoid breathing fumes.
- Stop Solution is an acidic solution.
- The buffers and antibodies within the kit are classified as irritants as they contain preservatives which may cause an allergic skin reaction and harm to the environment.
- Refer to Product Safety Data Sheet (SDS) for full breakdown of hazards and handling procedures.

PERFORMANCE CHARACTERISTICS

Typical Results:

This standard curve is provided for illustrative purposes only.

A standard curve should be generated for each set of samples analysed.



The range of the standard curve of this immunoassay is 20 – 1280 ng/mL. It is essential the final measurement falls within the linear range of the standard curve. Sample readings above or below the linear range should be concentrated or diluted.

Limit of Detection:

The lowest concentration of K-Postn giving an absorbance reading greater than two standard deviations (SD) above the mean zero (blank matrix) reading (n=32) was determined to be 18.11 ng/mL.

Precision:

Intra-Assay Coefficient of Variation (CV) was determined by measurement of twenty-four replicates of three diluted serum samples (high, medium and low) on one analytical assay run.

Intra-Assay CVs (within assay precision) = 12.27%

Inter-Assay Coefficient of Variation (CV) was determined by measurement of duplicates of eight diluted serum samples on six analytical assay runs performed over four days.

Inter-Assay CV (between assay precision) = 15.13%

Analytical Recovery:

To assess the recovery of the assay, K-Postn (32 µg/mL) was spiked into 10 serum samples, before dilution between x50 - x150, and measurement on the assay. Observed K-Postn concentrations were then compared to baseline K-Postn in each sample.

Average recovery = 103.8%

Analytical Linearity:

To assess the linearity of the assay, 10 serum samples, spiked with K-Postn (32 µg/mL), were diluted x50, before being serially diluted and measured on the assay.

Dilution Factor	**Average % Expected value**
X2	99.15
X4	99.00
X8	99.44
X16	103.86
Overall Average	100.35

Specificity / Interference:

To assess specificity of the K-Postn immunoassay, serum samples were spiked with potentially interfering substances listed in the table below. No significant difference was observed between baseline serum levels of K-Postn, and levels upon spiking with each substance.

Substance	Concentration tested	Average Recovery (%)
Cathepsin K	220 pg/mL	97.26
Periostin	87 pg/mL	92.80
Bilirubin, unconjugated	4.0 µg/mL	114.18
Cholesterol	4.0 µg/mL	109.49
Haemoglobin	100 µg/mL	100.08
Bilirubin, conjugated	4.0 µg/mL	101.67
Human Serum Albumin	400 µg/mL	93.07
IgM	19.8 µg/mL	94.91
DMSO	1.0 % v/v	100.92
Water	1.0 % v/v	92.59

DISPOSAL

Serum samples and used kit components are potentially biohazardous. Dispose appropriately in line with local clinical waste guidelines.

TROUBLESHOOTING

For help or advice please contact ProAxsis Ltd on +44 (0) 2890 730444 or info@proaxsis.com

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SYMBOLS USED

Symbol	Meaning
	**Research Use Only**
	**Catalogue Number**
	**Lot Number**
	**Consult Instructions**
	**Manufactured By**
	**Expiry Date**
	**Storage Upper Limit of Temperature**
	**Storage Temperature Limitations**
	**Single use only. Do not re-use**

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