Version 19-11/24

User's Manual

Human Vaspin ELISA Kit

REF **DEIA-NB24-24**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

The Human Vaspin ELISA Kit is intended to be used for quantitative measurement of human Vaspin in human serum for research purposes.

General Description

The Human Vaspin ELISA Kit is based on polyclonal rabbit antisera raised by genetic immunization of the rabbits. Vaspin also known as SerpinA12 is a serine protease inhibitor and consists of 395 amino acids forming 3 β-sheets and 9 α-helices. Molecular weight of Vaspin is about 45.2 kDa. It does not form multimeric aggregates or intra-molecular disulfide bridges and no binding proteins in human serum are known. The Vaspin gene is not only expressed by subcutaneous and visceral adipose but also by liver tissue, in the pancreas and in the human epidermis (granular keratinocytes / GK cells).

"Serum Vaspin levels were highest in the early morning before breakfast and fell to trough levels within 2 h after breakfast. Serum Vaspin levels also showed a preprandial rise and postprandial fall at lunch and dinner, although at lesser degrees than at breakfast. Intermeal Vaspin concentrations reached a nadir in the mid-afternoon and showed a nocturnal rise, with peak nighttime Vaspin levels being approximately 250 % of nadir levels. Unscheduled food ingestion after a prolonged fast significantly reduced serum Vaspin levels, suggesting that energy intake itself has a suppressive effect on serum Vaspin levels. The diurnal patterns of serum Vaspin concentrations were exactly reciprocal to that of insulin and of glucose".

A sexual dimorphism has been detected with higher Vaspin levels in girls increasing with age and pubertal stage. A preliminary investigation (n = 81) of Vaspin levels in healthy adult blood donors revealed higher Vaspin levels in women decreasing with increasing age. In this context, it is important to reflect, that oral contraceptives significantly increase serum Vaspin concentration.

Serum Vaspin concentration is independent of BMI but negatively associated with insulin sensitivity and obesity, thus "Vaspin was increased with worsening insulin resistance". If glucose metabolism / insulin sensitivity is improved by therapeutic intervention e.g. rosiglitazone, plasma Vaspin concentration decreases significantly. Interestingly lifestyle intervention results in increasing adiponectin concentrations as well as in improved insulin sensitivity but after a 10 month intervention Vaspin concentration remain unchanged. Thus, the mechanism regulating the Vaspin concentration in circulation is still unclear. Vaspin concentration might be even more influenced by glucose uptake than by body fat at least in pre-pubertal children.

In insulin resistance, diabetes as well as atherosclerosis inflammatory processes are involved and Vaspin might be a link between the endocrine and the immune system. To elucidate the role of Vaspin in inflammation its influence on TNF-α-stimulated production of reactive oxygen species was investigated in smooth muscle cells. Vaspin significantly decreased the TNF-α induced monocyte adhesion to SMCs as well as TNF-α induced intracellular signal cascade.

The diagnostic value of Vaspin remains unclear, conflicting results question its value as biomarker for visceral or total adipose tissue. As well as regarding insulin resistance while in children Vaspin might correlate with insulin sensitivity but in adults no correlation was found.

The Human Vaspin ELISA Kit is a tool for the further investigation and validation of Vaspin as a biomarker for the visceral adipose tissue, insulin sensitivity and glucose tolerance.

Principles of Testing

The Human Vaspin ELISA Kit is a so-called Sandwich-Assay. It utilizes specific and high affinity polyclonal antibodies for this protein. The Vaspin in the samples binds to the immobilized first antibody on the microtiter plate. In the following step, the biotinylated antibody binds in turn to Vaspin. After washing, Streptavidin-Peroxidase-Enzyme conjugate will be added, which will bind highly specific to the biotin and will catalyse the enzymatic reaction, which turns the colour of the substrate, quantitatively depending on the Vaspin level of the samples.

Reagents And Materials Provided

1. **Microtiter plate:** ready for use, coated with rabbit-anti-hVaspin-antibody. Wells are separately breakable. (8x12) wells
2. **Standards (A-E):** lyophilized, (human Vaspin), concentrations are given on the QC-certificate. 5 x 750 µL
3. **Control Serum 1 (KS1):** lyophilized, (human serum), concentration is given on the QC-certificate. 1 x 250 µL
4. **Control Serum 2 (KS2):** lyophilized, (human serum), concentration is given on the QC-certificate. 1 x 250 µL
5. **Antibody Conjugate (AK):** ready for use, contains rabbit anti-hVaspin antibody, biotinylated. 1 x 12 mL
6. **Enzyme Conjugate (EK):** ready for use, contains horseradish-peroxidase conjugated to streptavidin. 1 x 12 mL
7. **Dilution Buffer (VP):** ready for use. Please shake before use! 1 x 120 mL
8. **Washing Buffer (WB):** 20-fold concentrated solution. 1 x 50 mL
9. **Substrate (S):** ready for use, horseradish-peroxidase-(HRP) substrate, stabilised H_2O_2 Tetramethylbencidine. 1 x 12 mL
10. **Stopping Solution (SL):** ready for use, 0.2 M sulfuric acid. 1 x 12 mL
11. **Sealing Tape:** for covering the microtiter plate. 3

Materials Required But Not Supplied

1. Distilled (Aqua destillata) or deionized water for dilution of the Washing Buffer WP (A. dest.), 950 mL.
2. Precision pipettes and multichannel pipettes with disposable plastic tips
3. Polyethylene PE/Polypropylene PP tubes for dilution of samples
4. Vortex-mixer
5. Microtiter plate shaker (350 rpm)
6. Microtiter plate washer (recommended)
7. Microplate reader ("ELISA-Reader") with filter for 450 and ≥590 nm

Storage

Store the kit at 2-8°C after receipt until its expiry date. The lyophilized reagents should be stored at –20 °C after

reconstitution. Avoid repeated thawing and freezing.

The shelf life of the components after initial opening is warranted for 4 weeks, store the unused strips and microtiter wells airtight together with the desiccant at 2-8°C in the clip-lock bag, use in the frame provided. The reconstituted components standards A-E and Control Sera KS1 and KS2 must be stored at –20°C (max. 4 weeks). For further use, thaw quickly but gently (avoid temperature increase above room temperature and avoid excessive vortexing). The 1:20 diluted Washing Buffer WB is 4 weeks stable at 2-8°C

Specimen Collection And Preparation

1. Sample type: Serum

2. Specimen collection

Use standard venipuncture for the blood sampling. Haemolytic reactions are to be avoided.

3. Required sample volume: 70 µL

4. Sample stability

Samples should be stored at - 20°C in firmly closable sample vials. Information of the long-term stability of Vaspin is not yet available. Freezing and thawing of samples should be minimized.

5. Interference

Triglyceride, bilirubin and hemolysate in the sample do not interfere to a concentration of 100 mg/mL and 100 µ g/mL or 5 mg/mL. However, the use of hemolytic, lipemic or icteric samples should be validated by the user.

6. Sample dilution

• Dilution: 1:4 with Dilution Buffer VP

• Pipette 210 µL Dilution Buffer VP in PE-/PP-Tubes (application of a multi-stepper is recommended in larger series), add 70 µL Serum (dilution factor 4). After mixing use 100 µL of this 1:4 diluted solution within 1 hour per determination in the assay.

• The excellent linearity of the assay system allows dilutions of 1:2 - 1:32

Reagent Preparation

Bring all reagents to room temperature (20 - 25°C) before use. Possible precipitations in the buffers have to be resolved before usage by mixing and / or warming. Reagents with different lot numbers cannot be mixed.

1. The Standards A – E is reconstituted with 750 µL Dilution Buffer VP. After resuspension, the standards are diluted according to a gradient - A (25 pg/mL), B (75 pg/mL), C (200 pg/mL), D (500 pg/mL), E (1000 pg/mL), which are prepared for immediate use.
2. The Control KS1 and KS2 are reconstituted with 250 µL Dilution Buffer VP. After reconstitution dilute the Control KS with the Dilution Buffer VP in the same ratio (1:4) as the sample.

Note: It is recommended to keep reconstituted reagents at room temperature for 15 minutes and then to mix them thoroughly but gently (no foam should result) with a Vortex mixer.

3. The required volume of Washing Buffer WP is prepared by 1:20 dilution of the provided 20-fold concentrate with Aqua dest.

Assay Procedure

Note

1. Incubation at room temperature means: Incubation at 20 - 25°C. The Substrate S, stabilised H_2O_2-Tetramethylbencidine, is photosensitive–store and incubation in the dark.
2. When performing the assay, Blank, Standards A-E, Control Serum KS1/2 and the samples should be pipette as fast as possible (e.g. <15 minutes). To avoid distortions due to differences in incubation times, Antibody Conjugate AK, Enzyme Conjugate EK as well as the succeeding Substrate S should be added to the plate in the same order and in the same time interval as the samples. Stop Solution SL should be added to the plate in the same order as Substrate S. All determinations (Blank, Standards A-E, Control Serum KS1/2 and samples) should be assayed in duplicate. For optimal results, accurate pipetting and adherence to the protocol are recommended.
3. Shaking: The incubation steps should be performed at mean rotation frequency of a microtiter plate shaker. We recommend 350 rpm. Depending on the design of the shaker, the shaking frequency should be adjusted. Insufficient shaking may lead to inadequate mixing of the solutions and thereby to low optical densities, high variations and/ or false values, excessive shaking may result in high optical densities and/ or false values.
4. Washing: Proper washing is of basic importance for a secure, reliable and precise performance of the test. Incomplete washing is common and will adversely affect the test outcome. Possible consequences may be uncontrolled unspecific variations of measured optical densities, potentially leading to false results calculations of the examined samples. Effects like high background values or high variations may indicate washing problems. All washing must be performed with the provided Washing Buffer WP diluted to usage concentration. Washing volume per washing cycle and well must be 300 µL at least. The danger of handling with potentially infectious material must be taken into account.

   Automatic washing: When using an Automatic microtiter plate washer, the respective instructions fur use must be carefully followed. Device adjustments, e.g. for plate geometry and the provided washing parameters, must be performed. Dispensing and aspirating manifold must not scratch the inside well surface. Provisions must be made that the remaining fluid volume of every aspiration step is minimized. Following the last aspiration step of each washing cycle, this could be controlled, and possible remaining fluid could then be removed, by inverting the plate and repeatedly tapping it dry on non-fuzzy absorbent tissue.

   Manual washing: Washing Buffer may be dispensed via a multistepper device, a multichannel pipette, or a squirt bottle. The fluid may be removed by dynamical swinging out the microtiter plate over a basin. If aspirating devices are used, care has to be taken that the inside well surface is not scratched. Subsequent to every single washing step, the remaining fluid should be removed by inverting the plate and repeatedly tapping it dry on non-fuzzy absorbent tissue.

Assay Step

1. Set Standards A-E, test samples (1:4 diluted), Control Serum KS1/2 (1:4 diluted) wells on the pre-coated plate respectively, and then, records their positions. It is recommended to measure each standard and sample in duplicate.
2. Aliquot 100ul of Dilution Buffer VP (Blank), Standards A-E, Control Serum KS1/2 and test samples into wells.
3. Incubate: Seal the plate with a cover and incubate at 20-25°C, 350 rpm for 1 hour.

4. Wash: Aspirate the contents of the wells, and wash plate 5 times with 300 µL Washing Buffer WP. Do not let the wells dry completely at any time.
5. Add 100ul Antibody Conjugate AK into above wells. Add the solution at the bottom of each well without touching the sidewall, cover the plate and incubate at 20-25°C, 350 rpm for 1 hour.
6. Wash: Aspirate the contents of the wells, and wash plate 5 times with 300 µL Washing Buffer WP. Do not let the wells dry completely at any time.
7. Add 100ul Enzyme Conjugate EK into above wells. Add the solution at the bottom of each well without touching the sidewall, cover the plate and incubate at 20-25°C, 350 rpm for 1 hour.
8. Wash: Aspirate the contents of the wells, and wash plate 5 times with 300 µL Washing Buffer WP. Do not let the wells dry completely at any time.
9. Add 100ul of Substrate Solution S into each well, cover the plate and incubate at 20-25°C in dark for 30 minutes.
10. Stop: Add 100ul Stopping Solution SL into each well.
11. Measure the absorbance within 30 min at 450 nm, with ≥ 590 nm as reference wavelength.

Quality Control

Good laboratory practice requires that controls are included in each assay. A statistically significant number of controls should be assayed to establish mean values and acceptable ranges to assure proper performance. The test results are only valid if the test has been performed following the instructions. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable federal, state or local standards / laws. All standards and kit controls must be found within the acceptable ranges as stated on the QC Certificate. If the criteria are not met, the run is not valid and should be repeated. Each laboratory should use known samples as further controls.

Quality criteria

For the evaluation of the assay it is required that the absorbance values of the blank should be below 0.25, and the absorbance of Standard E should be above 0.8. Samples, which yield higher absorbance values than Standard E, should be re-tested with a higher dilution.

Calculation

1. Calculate the mean absorbance value for the blank from the duplicated determination (well A1/A2).
2. Subtract the mean absorbance of the blank from the mean absorbances of all other samples and standards.
3. Plot the standard concentrations on the x-axis versus the mean value of the absorbance of the standards on the y-axis.
4. Recommendation: Calculation of the standard curve should be done by using a computer program, because the curve is in general (without respective transformation) not ideally described by linear regression. A higher-grade polynomial, or four parametric logistic (4-PL) curve fit or non-linear regression are usually suitable for the evaluation (as might be spline or point-to-point alignment in individual cases).
5. The Vaspin concentration in ng/mL (or pg/mL, according the chosen unit for the standards) of the samples can be calculated by multiplication with the respective dilution factor.

Exemplary calculation of Vaspin concentrations

Sample dilution: 1:4

Measured extinction of your sample 0.56

Measured extinction of the blank 0.03

Your measurement program will calculate the Vaspin concentration of the diluted sample automatically by using the difference of sample and blank (0.03) for the calculation.

In this exemplary case the following equation is solved by the program to calculate the Vaspin concentration in the sample:

$0.53 = 0.0233x^3 - 0.0871x^2 + 0.1482x - 0.0523$

$x = 0.1735$

If the dilution factor (1:4) is taken into account, the Vaspin concentration of the undiluted sample is

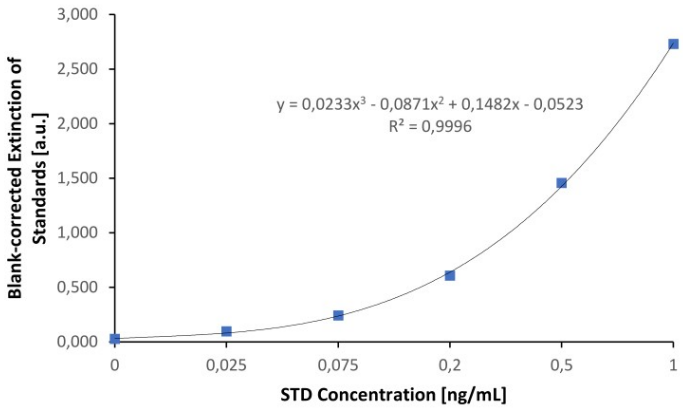
$0.1735 \times 4 = 0.694$ ng/mL

Typical Standard Curve

The following data is for demonstration only and cannot be used in place of data generation at the time of assay.

	Blank	A	B	C	D	E
ng/mL	0	0.025	0.075	0.20	0.50	1.0
$OD_{(450\text{-}620\ nm)}$	0.027	0.096	0.241	0.608	1.456	2.729

The exemplary shown standard curve in Figure below cannot be used for calculation of your test results. You have to establish a standard curve for each test you conduct!



Precision

Intra-Assay Variance

Three exemplary serum samples were diluted and measured 20 times in one assay.

	Sample 1	Sample 2	Sample 3
Mean [ng/mL]	0.226	1.192	1.536
SD	0.006	0.045	0.02
CV [%]	2.85	3.81	1.42
n	20	20	20

Inter-Assay Variance

Serum samples were diluted as recommended and Vaspin concentration was measured in various independent tests.

	Sample 1	Sample 2	Sample 3
Mean [ng/mL]	0.189	0.823	2.885
SD	0.016	0.037	0.137
CV [%]	8.55	4.52	4.75
n	9	9	9

Sensitivity

We measured the blank (dilution buffer only) 16 times in one assay. The resulting standard deviation was used for calculating the concentration which can be differentiated from the blank. Thus, lowest amount of Vaspin detectable is 4 pg/mL.

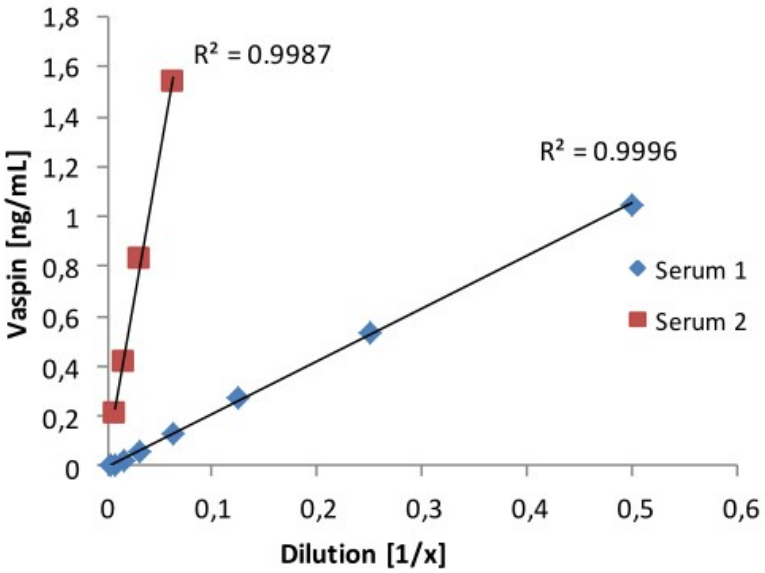
Specificity

Cross reactivity with related proteins. Proteins were diluted in dilution buffer and applied to the testsystem with the indicated analytic concentration.

Protein	Concentration used	Concentration measured	Relative Cross reactivity [%]
Alpha-1 antichymotrypsin Serpin A3	220 µg/mL	0.000055 µg/mL	0.000025
Alpha-1-antitrypsin Serpin A1	2 mg/mL	0.000000022 mg/mL	0.0000011
Thyroxine-binding globulin Serpin A7	30 µg/mL	0.00003 µg/mL	0.000206667
Adiponectin	20 µg/mL	0.00003 µg/mL	0.00015
Leptin	50 ng/mL	0.003 ng/mL	0.006

Linearity

The linearity of serum dilutions is over a very wide range excellent. Two serum samples with high and low Vaspin content were serial diluted and dilution was measured by Vaspin ELISA. Results are shown in Figure below. It was possible to quantify Vaspin down to 4 pg/mL. In both cases dilution resulted in linear decrease of concentration.



Recovery

For evaluation of disturbing substances in serum samples as well as assessing correctness we enriched several human serum samples with two different amounts of recombinant Vaspin. For control purposes same amounts of Vaspin were added to buffer and relative recovery in serum samples was calculated based on the value found in buffer.

	Recombinant Vaspin in Buffer	Sample 1 Recovery [%]	Sample 2 Recovery [%]	Sample 8 Recovery [%]	Sample9 Recovery [%]	Sample 4 Recovery [%]	Sample 5 Recovery [%]	Sample 17 Recovery [%]	Sample 18 Recovery [%]
Endogenous Vaspin content [ng/mL]		0.233	0.1055	0.2595	0.517	0.118	0.744	1.457	0.2895
Serum enriched with 50 pg/Ml [ng/mL]	0.052	0.271 95	0.139 88	0.302 97	0.588 103	0.151 89	0.943 118	1.601 106	0.345 101
Serum enriched with 500 pg/mL [ng/mL]	0.408	0.591 87	0.455 89	0.705 99	1.013 102	0.432 77	1.066 79	1.915 95	0.695 92

Recovery range was 77 – 102 % for 500 pg/mL and 88 – 118 % for 50 pg/mL enrichment, on avarage 97 % of the added recombinant material was found.

Interferences

Interference of physiologically appearing substances with Vaspin was investigated. Serum samples have been enriched with different concentrations of possibly interfering substances and the amount of Vaspin was measured and compared with the Vaspin concentration in the sample without any enrichment. None of the tested substances interfered significantly with the Vaspin measurement.

	Triglyceride (100 mg/mL)	Bilirubin (100 µg/mL)	Hemoglobin (5 mg/mL)
Sample 1	106	112	114
Sample 2	93	107	91
Sample 3	96	123	91

Precautions

1. For research and professional use only. The Creative Diagnostics kit is suitable only for in vitro use and not for internal use in humans and animals.
2. Follow strictly the test protocol. Use the valid version of the package insert provided with the kit. Be sure that everything has been understood. Creative Diagnostics will not be held responsible for any loss or damage (except as required by statute) howsoever caused, arising out of noncompliance with the instructions provided.
3. Do not use obviously damaged or microbial contaminated or spilled material.
4. This kit contains material of human and/or animal origin. Therefore all components and patient´s specimens should be treated as potentially infectious.
5. Appropriate precautions and good laboratory practices must be used in the storage, handling and disposal of the kit reagents. The disposal of the kit components must be made according to the local regulations.
6. Following components contain human serum: Control Sera KS1 / KS2, Standards A-E

   Source human serum for the Controls provided in this kit was tested and found non-reactive for Hepatitis-B surface antigen (HBsAg), Hepatitis C virus (HCV), and Human Immunodeficiency Virus 1 and 2 (HIV). No known methods can offer total security of the absence of infectious agents; therefore all components and patient´s specimens should be treated as potentially infectious.