Product Information & ELISA Manual

Human FNDC4 ELISA Kit (Colorimetric)
NBP3-43459

Enzyme-linked Immunosorbent Assay
for quantitative detection.

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Novus kits are guaranteed for 6 months from date of receipt.

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1. Intended Use

The Human FNDC4 ELISA Kit (Colorimetric) is to be used for the *in vitro* quantitative determination of human FNDC4 in serum, plasma and cell culture supernatant. This ELISA Kit is for research use only.

2. Introduction

Fibronectin Type III Domain-containing Protein 4 (FNDC4) is an ortholog of FNDC5 / Irisin with 50% identity and 86% similarity compared to Irisin. FNDC4 as well as FNDC5 are extremely well conserved between species (1). The human FNDC4 gene is highly enriched in liver, brain tissue and adipocytes. The Fndc4 gene is upregulated in several mouse models of inflammation as well as in human inflammatory conditions such as inflammatory bowel disease. FNDC4 also inhibits lipogenesis *in vitro*, acts as an anti-inflammatory factor on macrophages and promotes fat browning in human visceral adipocytes by acting via its receptor ADGRF5 (also known as GPR116) (2, 3).

Recently, a new role of FNDC4 as a hepatokine has been published. Liver primarily controls the circulating levels of FNDC4 showing tight correlation with insulin sensitivity. Weight loss associated with improved insulin tolerance is accompanied by increased plasma FNDC4 concentrations in mice (4). In addition, a new orphan adhesion G protein-coupled receptor 116 (GPR116) has been identified as a receptor of FNDC4 in white adipose tissue (WAT), thereby establishing an endocrine FNDC4-GPR116 axis in the control of systemic glucose homeostasis. The FNDC4-GPR116 axis is impaired in diabetic patients and therapeutic injections of recombinant Fc-FNDC4 into pre-diabetic mice corrected pre-diabetic hyperglycemia.

FNDC4 is a factor with direct therapeutic potential in inflammatory bowel disease and possibly other inflammatory diseases and is a potential biomarker for different inflammatory, metabolic diseases as well as some cancers (5). FNDC4 is a potential biomarker of glioblastoma (6). Indeed FNDC4 promotes tumor proliferation in glioblastoma and glioblastoma patients with elevated FNDC4 expression show poor prognosis.

3. General References

(1) Irisin and the fibronectin type III domain-containing family: structure, signaling and role in female reproduction: M. Daudon, et al.; Reproduction. **164**, R1 (2022)

(2) FNDC4, a novel adipokine that reduces lipogenesis and promotes fat browning in human visceral adipocytes: G. Fruehbeck, et al.; Metabolism **108,** 154261 (2020)

(3) FNDC4 acts as an anti-inflammatory factor on macrophages and improves colitis in mice: M. Bosma, et al.; Nature Commun. **7,** 11314 (2016)

(4) Isolation and characterization of Orphan GPR116 mediates the insulin sensitizing effects of the hepatokine FNDC4 in adipose tissue: A. Georgiadi, et al.; Nature Commun. **12,** 2999 (2021)

(5) FNDC4 acts as an extracellular factor to promote the invasiveness of hepatocellular carcinoma partly via the PI3K/Akt signalling pathway: B. Wang, et al.; Cancer Med. **20,** 7242 (2021)

(6) Correlation of the prognostic value of FNDC4 in glioblastoma with macrophage polarization: H. Li, et al.; Cancer Cell Int. **22,** 273 (2022)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human FNDC4 in biological fluids. A polyclonal antibody specific for human FNDC4 has been precoated onto the 96-well microtiter plate. Standards and samples are pipetted into the wells for binding to the coated antibody. After extensive washing to remove unbound compounds, human FNDC4 is recognized by the addition of a biotinylated polyclonal antibody specific for human FNDC4 (Detection Antibody). After removal of excess biotinylated antibody, streptavidin-peroxidase (STREP-HRP) is added. Following a final washing, peroxidase activity is quantified using the substrate 3,3',5,5'-tetramethylbenzidine (TMB). The intensity of the color reaction is measured at 450 nm after acidification and is directly proportional to the concentration of human FNDC4 in the samples.

5. Handling & Storage

- Reagent must be stored at 2-8°C when not in use.
- Plate and reagents should be at room temperature before use.
- Do not expose reagents to temperatures greater than 25°C.

6. Kit Components

• 1 vial FNDC4 Standard (lyophilized)	(100 ng)	(STD)
• 1 vial Detection Antibody	(30 µl)	(DET)
• 1 vial HRP Labeled Streptavidin (lyophilized)	(2 µg)	(STREP-HRP)
• 2 bottles Wash Buffer 10X	(2 x 30 ml)	(Wash Buffer 10X)
• 1 bottle ELISA Buffer 10X	(1 x 30 ml)	(ELISA Buffer 10X)
• 1 bottle TMB Substrate Solution	(12 ml)	(TMB)
• 1 bottle Stop Solution	(12 ml)	(STOP)
• 1 plate coated with FNDC4 Antibody	(6 x 16-well strips)	
• 2 plate Covers (plastic film)		
• 2 silica Gel Minibags		

7. Materials Required but *Not* Supplied

- Microtiter plate reader at 450 nm
- Calibrated precision single and multi-channel pipettes. Disposable pipette tips
- Deionized water
- Microtubes or equivalent for preparing dilutions
- Disposable plastic containers for preparing working buffers
- Plate washer: automated or manual
- Glass or plastic tubes for diluting and aliquoting standard

8. General ELISA Protocol

8.1. Preparation and Storage of Reagents

NOTE: *Prepare just the appropriate amount of the buffers necessary for the assay.*

- **Wash Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 50 ml Wash Buffer 10X + 450 ml water) to obtain Wash Buffer 1X.

- **ELISA Buffer 10X** has to be diluted with deionized water 1:10 before use (e.g. 20 ml ELISA Buffer 10X + 180 ml water) to obtain ELISA Buffer 1X.

- **Detection Antibody (DET)** has to be diluted to 1:500 in ELISA Buffer 1X (20 µl DET + 10 ml ELISA Buffer 1X).
  NOTE: *The diluted Detection Antibody is not stable and cannot be stored!*

- **HRP Labeled Streptavidin (STREP-HRP)** has to be reconstituted with 100 µl of ELISA Buffer 1X.
  - After reconstitution of STREP-HRP, prepare aliquots and store them at -20°C. **Avoid freeze/thaw cycles**.
  - Dilute the reconstituted STREP-HRP to the working concentration by adding 50 µl in 10 ml of ELISA Buffer 1X (1:200).

  NOTE: *The diluted STREP-HRP is not stable and cannot be stored!*

- **Human FNDC4 Standard (STD)** has to be reconstituted with 100µl of ELISA buffer 1x.
  - This reconstitution produces a stock solution of 1µg/ml. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes. Mix well prior to making dilutions.

  NOTE: *The reconstituted standard is aliquoted and stored at -20°C.*
  - Dilute the standard protein concentrate (STD) (**1 µg/ml**) in ELISA Buffer 1X. A seven-point standard curve using 2-fold serial dilutions in ELISA Buffer 1X is recommended.
  - Suggested standard points are:
    5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 and 0 ng/ml

Start with the dilution of the concentrate (STD):

To obtain	Add	Into
10 ng/ml	10µl of FNDC4 (STD) (1 µg/ml)	990 µl of ELISA Buffer 1X

Dilute further for the standard curve:

To obtain	Add	Into
5 ng/ml	300 µl of FNDC4 (10 ng/ml)	300 µl of ELISA Buffer 1X
2.5 ng/ml	300 µl of FNDC4 (5 ng/ml)	300 µl of ELISA Buffer 1X
1.25 ng/ml	300 µl of FNDC4 (2.5 ng/ml)	300 µl of ELISA Buffer 1X
0.625 ng/ml	300 µl of FNDC4 (1.25 ng/ml)	300 µl of ELISA Buffer 1X
0.312 ng/ml	300 µl of FNDC4 (0.625 ng/ml)	300 µl of ELISA Buffer 1X
0.156 ng/ml	300 µl of FNDC4 (0.312 ng/ml)	300 µl of ELISA Buffer 1X
0.078 ng/ml	300 µl of FNDC4 (0.156 ng/ml)	300 µl of ELISA Buffer 1X
0 ng/ml	300 µl of ELISA Buffer 1X	Empty tube

8.2. Sample Collection, Storage and Dilution

Serum : Use a serum separator tube. Let samples clot at room temperature for 30 minutes before centrifugation for 20 minutes at 1,000xg. Assay freshly prepared serum or store serum in aliquot at ≤ -20°C for later use. Avoid repeated freeze/thaw cycles.

Plasma : Collect plasma using heparin, EDTA, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. Assay freshly prepared plasma or store plasma sample in aliquot at ≤ -20°C for later use. Avoid repeated freeze/ thaw cycles.

Serum, Plasma or **Cell Culture Supernatant** have to be diluted in ELISA Buffer 1X. Samples containing visible precipitates must be clarified before use.

NOTE: *As a starting point, 1/2 dilution of serum or plasma is recommended! If sample values fall outside the detection range of the assay, a lower or higher dilution may be required!*

8.3. Assay Procedure (Checklist)

☐	1. Determine the number of 16-well strips needed for the assay and insert them in the frame for current use. The extra strips should be resealed in the foil pouch bag and stored at 4°C. ***NOTE:*** *Remaining 16-well strips coated with human FNDC4 antibody when opened can be stored at 4°C for up to 1 month.*
☐	2. Add 100 µl of the different standards into the appropriate wells in duplicate! At the same time, add 100 µl of diluted serum, plasma or cell culture supernatant samples in duplicate to the wells **(see 8.1. Preparation and Storage of Reagents and 8.2. Preparation of Samples)**.
☐	3. Cover the plate with plate sealer and incubate for **2 hours at room temperature.**
☐	4. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	5. Add 100 µl to each well of the Detection Antibody **(DET)**. **(see 8.1. Preparation and Storage of Reagents)**.
☐	6. Cover the plate with plate sealer and incubate for **1 hour at room temperature**.
☐	7. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	8. Add 100 µl to each well of the diluted HRP Labeled Streptavidin **(STREP-HRP) (see 8.1. Preparation and Storage of Reagents)**.
☐	9. Cover the plate with plate sealer and incubate for **30 min at room temperature**.
☐	10. Aspirate the coated wells and add 300 µl of Wash Buffer 1X using a multichannel pipette or auto-washer. Repeat the process for a total of five washes. After the last wash, complete removal of liquid is essential for good performance.
☐	11. Add 100 µl to each well of TMB Substrate Solution **(TMB)**.
☐	12. Allow the color reaction to develop **at room temperature (RT) in the dark for 10 - 15 minutes**.
☐	13. Stop the reaction by adding 100 µl of Stop Solution **(STOP)**. Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution **(STOP)** is added.
	! CAUTION: CORROSIVE SOLUTION!
☐	14. Measure the OD at 450 nm in an ELISA reader within 30 minutes.

9. Calculation of Results

- Average the duplicate readings for each standard, control and sample and subtract the average blank value (obtained with the 0 ng/ml point).
- Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the horizontal (X) axis vs. the corresponding FNDC4 concentration (ng/ml) on the vertical (Y) axis (see **10.** TYPICAL DATA).
- Calculate the FNDC4 concentrations of samples by interpolation of the regression curve formula in a form of a quadratic equation.
- If the test samples were diluted, multiply the interpolated values by the dilution factor to calculate the concentration of human FNDC4 in the samples.

10. Typical Data

The following data are obtained using the different concentrations of standard as described in this protocol:

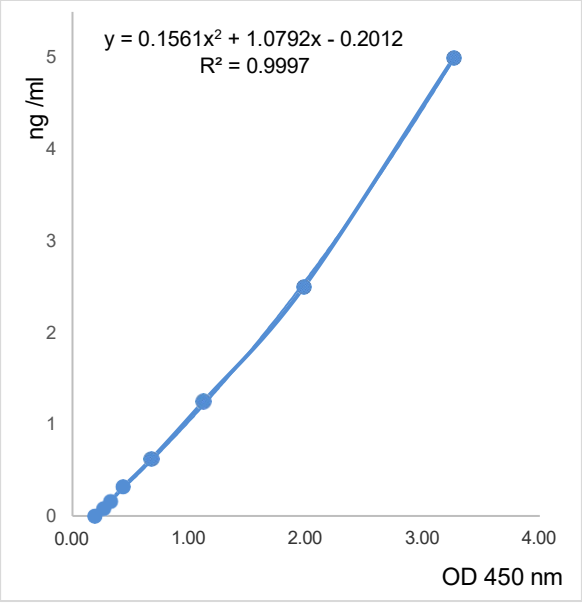

Figure: Standard curve

Standard FNDC4 (ng/ml)	Optical Density (mean)
5	3.266
2.5	1.982
1.25	1.123
0.625	0.678
0.312	0.432
0.156	0.326
0.078	0.268
0	0.194

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of human FNDC4 that can be detected by this assay is 40 pg/ml. ***NOTE:*** *The Limit of detection was measured by adding three standard deviations to the mean value of 50 zero standard.*

B. Assay range: 0.078 ng/ml – 5 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human FNDC4. It cross-reacts with mouse, rat, monkey and dog FNDC4. It does not cross-react with human, mouse or rat FNDC5 / Irisin.

D. Intra-assay precision:

Four samples of known concentrations of human FNDC4 were assayed in replicates 4 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
1	1.008	0.03	3.07	4
2	0.487	0.01	1.57	4
3	1.285	0.02	1.47	4
4	1.643	0.14	8.67	4

E. Inter-assay precision:

Four samples of known concentrations of human FNDC4 were assayed in 5 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
1	2.03	0.09	4.59	5
2	0.29	0.01	3.18	5
3	1.28	0.03	2.67	5
4	3.27	0.3	9.29	5

F. Recovery:

When samples (serum or plasma) are spiked with known concentrations of human FNDC4, the recovery averages 104% (range from 93% to 108%).

G. Linearity:

Different human serum samples containing human FNDC4 were diluted 1/2 and 1/4 and the measured recoveries ranged from 100% to 117%.

H. Expected values:

FNDC4 levels range in human plasma and serum from **<1ng to >5 ng/ml**.

12. Technical Hints and Limitations

- It is recommended that all standards, controls and samples be run in duplicate.
- Do not combine leftover reagents with those reserved for additional wells.
- Reagents from the kit with a volume less than 100 µl should be centrifuged.
- Residual wash liquid should be drained from the wells after last wash by tapping the plate on absorbent paper.
- Crystals could appear in the 10X solution due to high salt concentration in the stock solutions. Crystals are readily dissolved at room temperature or at 37°C before dilution of the buffer solutions.
- Once reagents have been added to the 16-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep TMB Substrate Solution (TMB) protected from light.
- The Stop Solution (STOP) consists of sulfuric acid. Although diluted, the Stop Solution (STOP) should be handled with gloves, eye protection and protective clothing.

13. Troubleshooting

PROBLEM	POSSIBLE CAUSES	SOLUTIONS
No signal or weak signal	Omission of key reagent	Check that all reagents have been added in the correct order.
	Washes too stringent	Use an automated plate washer if possible.
	Incubation times inadequate	Incubation times should be followed as indicated in the manual.
	Plate reader settings not optimal	Verify the wavelength and filter setting in the plate reader.
	Incorrect assay temperature	Use recommended incubation temperature. Bring substrates to room temperature before use.
High background	Concentration of STREP-HRP too high	Use recommended dilution factor.
	Inadequate washing	Ensure all wells are filling wash buffer and are aspirated completely.
Poor standard curve	Wells not completely aspirated	Completely aspirate wells between steps.
	Reagents poorly mixed	Be sure that reagents are thoroughly mixed.
Unexpected results	Omission of reagents	Be sure that reagents were prepared correctly and added in the correct order.
	Dilution error	Check pipetting technique and double-check calculations.

14. Notes