Product Information & ELISA Manual

Human Angiopoietin-like Protein 6/ANGPTL6 ELISA Kit (Colorimetric) NBP3-43450

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 Angiopoietin-like Protein 6/ANGPTL6 ELISA Kit (Colorimetric) is to be used for the *in vitro* quantitative determination of human ANGPTL6 in serum, plasma and cell culture supernatant. This ELISA Kit is for research use only.

2. Introduction

Seven angiopoietin-like proteins (ANGPTLs) share the characteristic protein structure of the angiopoietin family (ANG), but differ in their inability to bind antiopoietin receptor, Tie-2. ANGPTL6 was originally named angiopoietin-related growth factor (AGF) having an N-terminal coiled-coil-like domain and a C-terminal fibrinogen-like domain, both of which are conserved in ANG (1). It is a circulating protein secreted by liver that induces angiogenesis by direct effect of epidermal ANGPTL6 on endothelial cells and proliferation of skin cells, and thereby promotes wound healing (1,2). Oike et al. (3) generated Angptl6 -/- mice, 80% of which died at about embryonic day 13. The surviving null mice developed marked obesity, lipid accumulation in skeletal muscle and liver, and insulin resistance accompanied by reduced energy expenditure relative to controls. Conversely, mice with constitutive overexpression of ANGPTL6 showed leanness and increased insulin sensitivity resulting from increased energy expenditure, and were also protected from high-fat diet-induced obesity, insulin resistance, and nonadipose tissue steatosis. Hepatic overexpression of ANGPTL6 by adenoviral transduction in mice fed a high-fat diet resulted in significant weight loss and increased insulin sensitivity. It was concluded that ANGPTL6 is a hepatocyte-derived circulating factor that counteracts obesity and obesity-related insulin resistance, meaning that ANGPTL6 may be a novel biomarker for metabolic diseases.

3. General References

(1) Angiopoietin-related growth factor (AGF) promotes epidermal proliferation, remodeling, and regeneration: Y. Oike, et al.; Proc. Natl. Acad. Sci. **100,** 9494 (2003)

(2) Angiopoietin-related growth factor (AGF) promotes angiogenesis: Y. Oike, et al.; Blood **103,** 3760 (2004)

(3) Angiopoietin-related growth factor antagonizes obesity and insulin resistance: Y. Oike, et al.; Nature Med. **11,** 400 (2005)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human ANGPTL6 in biological fluids. A monoclonal antibody specific for ANGPTL6 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, ANGPTL6 is recognized by the addition of a purified polyclonal antibody specific for ANGPTL6 (Detection Antibody). After removal of excess polyclonal antibody, HRP conjugated anti-rabbit IgG (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 ANGPTL6 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 plate coated with human ANGPTL6 Antibody	(6 x 16-well strips)	
2 bottles Wash Buffer 10X	(2 x 30 ml)	(Wash Buffer 10X)
2 bottles ELISA Buffer 10X	(2 x 30 ml)	(ELISA Buffer 10X)
1 vial Detection Antibody	(170µl)	(DET)
1 vial HRP 100X (HRP Conjugated anti-rabbit IgG)	(150 µl)	(HRP 100X)
1 vial human ANGPTL6 Standard (lyophilized)	(200 ng)	(STD)
1 bottle TMB Substrate Solution	(12 ml)	(TMB)
1 bottle Stop Solution	(12 ml)	(STOP)
2 plate sealers (plastic film)		
2 silica Gel Minibags		

7. Materials Required but *Not* Supplied

- Microtiterplate 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 1:65 in ELISA Buffer 1X (150 µl DET + 10 ml ELISA Buffer 1X).
  NOTE: *The diluted Detection Antibody is not stable and cannot be stored!*

- **HRP 100X (HRP Conjugated anti-rabbit IgG)** has to be diluted to the working concentration by adding 100 µl in 10 ml of ELISA Buffer 1X (1:100).
  NOTE: *The diluted HRP is used within one hour of preparation.*

- **Human ANGPTL6 Standard (STD)** has to be reconstituted with 1 ml of deionized water.

  - This reconstitution produces a stock solution of 200 ng/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) (**200 ng/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:

  100 , 50 , 25 , 12.5 , 6.25 , 3.13 , 1.56 and 0 ng/ml.

Dilute further for the standard curve:

To obtain	Add	Into
100 ng/ml	300 µl of ANGPTL6 (200 ng/ml)	300 µl of ELISA Buffer 1X
50 ng/ml	300 µl of ANGPTL6 (100 ng/ml)	300 µl of ELISA Buffer 1X
25 ng/ml	300 µl of ANGPTL6 (50 ng/ml)	300 µl of ELISA Buffer 1X
12.5 ng/ml	300 µl of ANGPTL6 (25 ng/ml)	300 µl of ELISA Buffer 1X
6.25 ng/ml	300 µl of ANGPTL6 (12.5 ng/ml)	300 µl of ELISA Buffer 1X
3.13 ng/ml	300 µl of ANGPTL6 (6.25 ng/ml)	300 µl of ELISA Buffer 1X
1.56 ng/ml	300 µl of ANGPTL6 (3.13 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, 1/5 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 ANGPTL6 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 **1 hour at 37°C.**
☐	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 three 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 37°C**.
☐	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 three 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 **(see 8.1. Preparation and Storage of Reagents)**.
☐	9. Cover the plate with plate sealer and incubate for **1 hour at 37°C**.
☐	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°C) in the dark for 30 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 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 human ANGPTL6 concentration (ng/ml) on the vertical (Y) axis (see **10.** TYPICAL DATA).
- Calculate the human ANGPTL6 concentrations of samples by interpolation of the regression curve formula as shown above 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 ANGPTL6 in the samples.

10. Typical Data

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

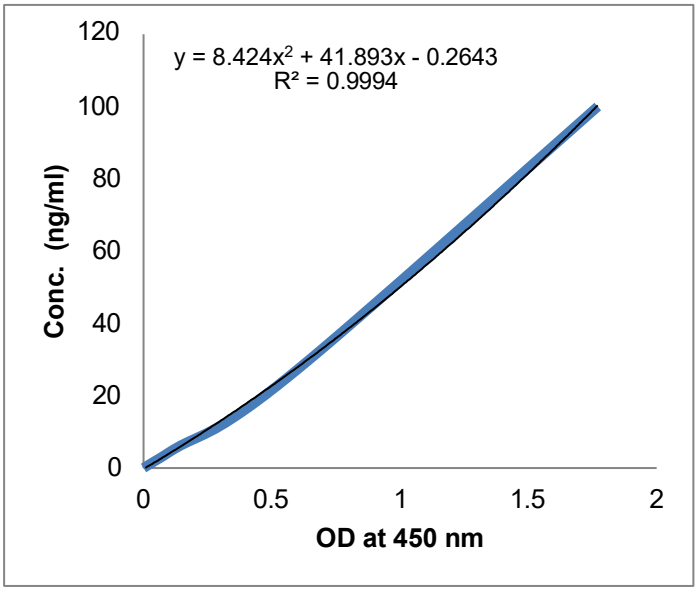

Figure: Standard curve

Standard hANGPTL6 (ng/ml)	Optical Density (mean)
100	1.7705
50	0.974
25	0.5605
12.5	0.319
6.25	0.145
3.13	0.0725
1.56	0.0355
0	0

11. Performance Characteristics

A. Sensitivity (Limit of detection):

The lowest level of ANGPTL6 that can be detected by this assay is 1.2 ng/ml. ***NOTE:*** *The Limit of detection was measured by adding two standard deviations to the mean value of 50 zero standard.*

B. Assay range: 1.56 ng/ml – 100 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human ANGPTL6. It does not cross-react with human adiponectin, human resistin, human RBP4, human RELM-β, human FABP4, human Nampt, human ANG1, human ANG2, human clusterin, human progranulin, human GPX3, human vaspin, human ANGPTL3, human ANGPTL4, human ANGPTL7.

D. Intra-assay precision:

Six samples of known concentrations of human ANGPTL6 were assayed in replicates 8 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
1	178.45	5.88	3.30	8
2	662.79	11.25	1.70	8
3	584.63	9.88	1.69	8
4	397.97	12.92	3.25	8
5	739.63	13.15	1.78	8
6	546.35	8.27	1.51	8

E. Inter-assay precision:

Six samples of known concentrations of human ANGPTL6 were assayed in 8 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
1	179.27	6.03	3.36	8
2	610.74	52.20	8.55	8
3	245.16	10.44	4.26	8
4	747.29	48.71	6.52	8
5	397.97	12.92	3.25	8
6	561.28	45.45	8.10	8

F. Recovery:

When samples (serum) are spiked with known concentrations of human ANGPTL6, the recovery averages 96% (range from 85% to 105%).

Samples	Average recovery (%)	Range (%)
1	88.77	85-95
2	101.17	95-105
3	100.43	95-105

G. Linearity:

Different human serum samples containing ANGPTL6 were diluted several fold (1/10 to 1/40) and the measured recoveries ranged from 95% to 102%.

Samples	Sample Dilution	Expected (ng/ml)	Observed (ng/ml)	% of Expected
1	1 : 10	756.83	756.83	100
	1 : 20	378.42	362.26	95.73
	1 : 40	189.21	190.49	100.68
2	1 : 10	499.35	499.35	100
	1 : 20	249.67	245.52	98.34
	1 : 40	124.84	125.66	100.66
3	1 : 10	888.90	888.90	100
	1 : 20	444.45	448.17	100.84
	1 : 40	222.23	220.54	99.24

H. Expected values:

ANGPTL6 levels range in plasma and serum from **30 to >800 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 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