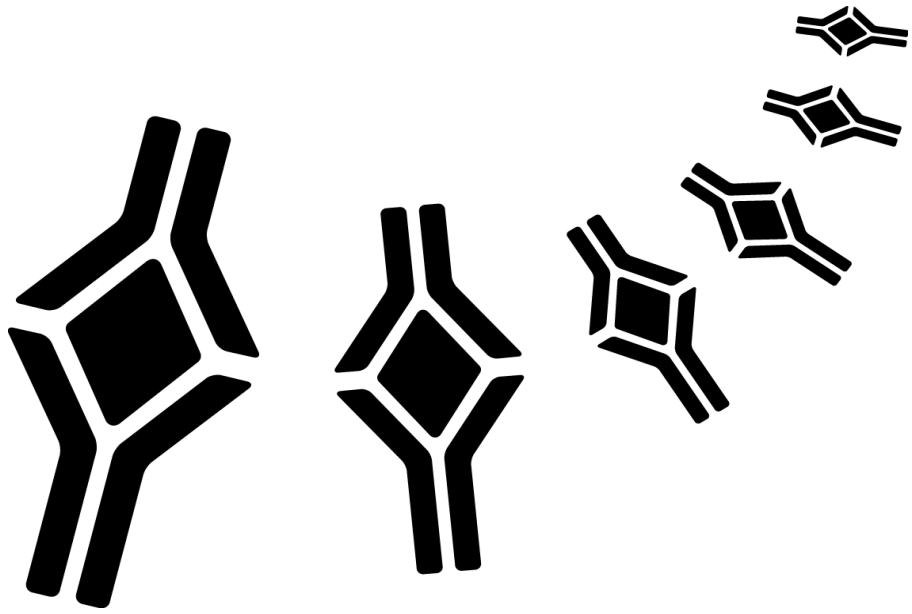


Human Angiopoietin-Like Protein 6 ELISA

Product Data Sheet

Cat. No.: RAG001R

For Research Use Only

VERSION 51 011211 46

CONTENTS

» **This kit is manufactured by:**
BioVendor – Laboratorní medicína a.s.

» **Use only the current version of Product Data Sheet enclosed with the kit!**

1. INTENDED USE

The Angiopoietin-Like Protein 6 (ANGPTL6) ELISA Kit 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. 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.

3. INTRODUCTION

Seven angiopoietin-like proteins (ANGPTLs) share the char-acteristic 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.

4. TEST 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 (Detector) 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. TECHNICAL HINTS

- It is recommended that all standards, QC sample 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 8-well strips, DO NOT let the strips DRY at any time during the assay.
- Keep Substrate Solution protected from light.
- The Stop Solution consists of phosphoric acid. Although diluted, the Stop Solution should be handled with gloves, eye protection and protective clothing.

6. REAGENT SUPPLIED

Kit Components	**Quantity**
1 plate coated with human ANGPTL6 Antibody	12 x 8-well strips
1 bottle Wash Buffer 10X	50 ml
1 bottle Diluent 5X	50 ml
1 bottle Detection Antibody	12 ml
1 vial Detector 100X (HRP Conjugated anti-rabbit IgG)	150 µl
1 vial human ANGPTL6 Standard (lyophilized)	200 ng
1 vial human ANGPTL6 QC sample (lyophilized)	
1 bottle Substrate Solution I (TMB)	6 ml
1 bottle Substrate Solution II (Peroxidase)	6 ml
1 bottle Stop Solution	12 ml
3 plate sealers (plastic film)	

7. MATERIALS REQUIRED BUT NOT SUPPLIED

- Microtiterplate reader at 450 nm, with the correction wavelength set at 540 nm or 570 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. PREPARATION 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.
- **Diluent 5X** has to be diluted with deionized water 1:5 before use (e.g. 50 ml Diluent 5X + 200 ml water) to obtain Diluent 1X.
- **Detector 100X (HRP Conjugated anti-rabbit IgG)** has to be diluted to the working concentration by adding 120 µl in 12 ml of Diluent 1X (1:100).

NOTE: *The diluted Detector is used within one hour of preparation.*

- **Substrate Solution I and II** have to be mixed together in equal volumes within 15 minutes of use.

NOTE: *Freshly prepare just before use the Substrate Solution and protect from light!*

- **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.

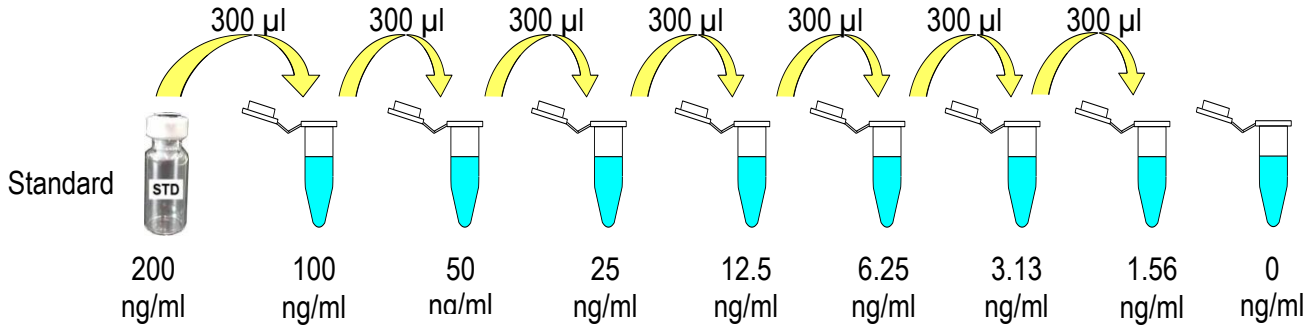
NOTE: *The reconstituted standard is aliquoted and stored at -20°C*

- Dilute the standard protein concentrate (STD) (**200 ng/ml**) in Diluent 1X. A seven-point standard curve using 2-fold serial dilutions in Diluent 1X is recommended.
- Suggested standard points are:
  100 , 50 , 25 , 12.5 , 6.25 , 3.13 , 1.56 and 0 ng/ml.

- **Human ANGPTL6 QC sample** has to be reconstituted with 1 ml of deionized water.
    - Refer to the Certificate of Analysis for current QC sample concentration. Mix the QC sample to ensure complete reconstitution and allow the QC sample to sit for a minimum of 15 minutes. The reconstituted QC sample is ready to use, do not dilute it.

Dilute further for the standard curve:

To obtain	Add	Into
100 ng/ml	300 µl of ANGPTL6 (200 ng/ml)	300 µl of Diluent 1X
50 ng/ml	300 µl of ANGPTL6 (100 ng/ml)	300 µl of Diluent 1X
25 ng/ml	300 µl of ANGPTL6 (50 ng/ml)	300 µl of Diluent 1X
12.5 ng/ml	300 µl of ANGPTL6 (25 ng/ml)	300 µl of Diluent 1X
6.25 ng/ml	300 µl of ANGPTL6 (12.5 ng/ml)	300 µl of Diluent 1X
3.13 ng/ml	300 µl of ANGPTL6 (6.25 ng/ml)	300 µl of Diluent 1X
1.56 ng/ml	300 µl of ANGPTL6 (3.13 ng/ml)	300 µl of Diluent 1X
0 ng/ml	300 µl of Diluent 1X	Empty tube



9. PREPARATION OF SAMPLES

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 Diluent 1X. Samples containing visible precipitates must be clarified before use.
NOTE: *As a starting point, 1/10 dilution of serum or plasma is recommended! If samples fall the outside range of assay, a lower or higher dilution may be required!*

10. ASSAY PROCEDURE

1. Determine the number of 8-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 8-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 Preparation of Reagents and 9. 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.
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 Detector **(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 mixed substrate solution.
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. 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.

11. CALCULATIONS

- Average the duplicate readings for each standard, QC 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 ANGPTL6 concentration (ng/ml) on the vertical (Y) axis (see **10.** TYPICAL DATA).
- Calculate the 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.

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

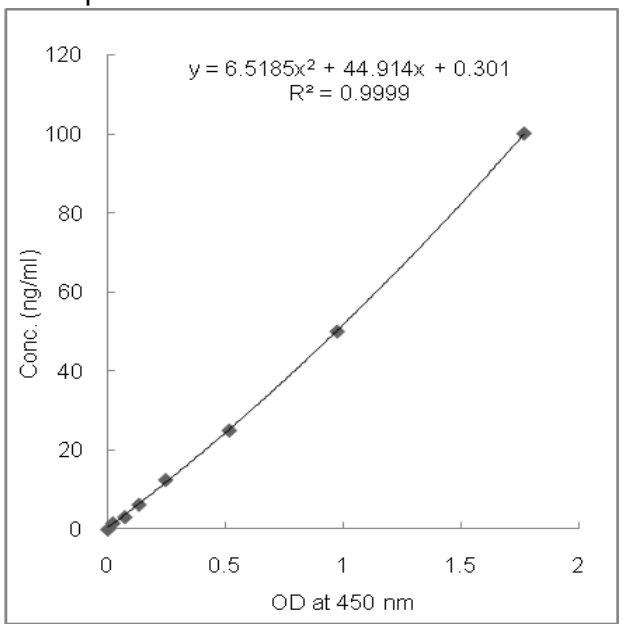


Figure: Standard curve

Standard hANGPTL6 (ng/ml)	Optical Density (mean)
100	1.766
50	0.973
25	0.515
12.5	0.245
6.25	0.132
3.13	0.072
1.56	0.021
0	0

12. PERFORMANCE CHARACTERISTICS

» Typical analytical data of BioVendor human ANGPTL6 ELISA, Clinical Range are presented in this chapter

- **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.*

- **Assay range**

1.56 ng/ml – 100 ng/ml

- **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.

- **Precision:**

Intra-assay (n =8)
Six samples of known concentrations of human ANGPTL6 were assayed in replicates 8 times to test precision within an assay.

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

Inter-assay (n = 8)
Six samples of known concentrations of human ANGPTL6 were assayed in 8 separate assays to test precision between assays.

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

- **Spinking Recovery:**

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

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

- **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%.

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

- **Expected values:**

ANGPTL6 levels range in plasma and serum from **50 to > 800 ng/ml** (from healthy donors).

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 detector 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. REFERENCES

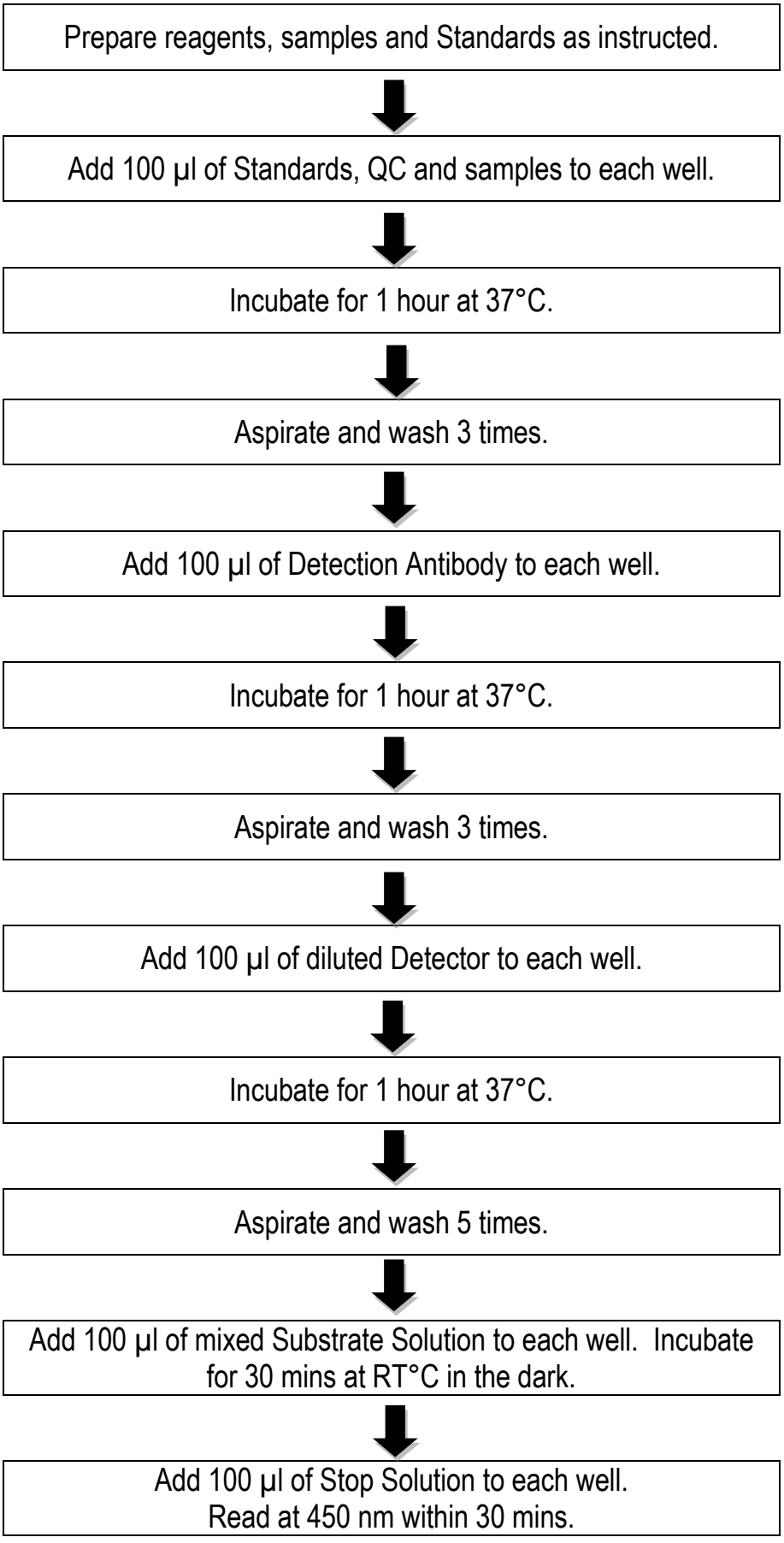
References to ANGPTL6:

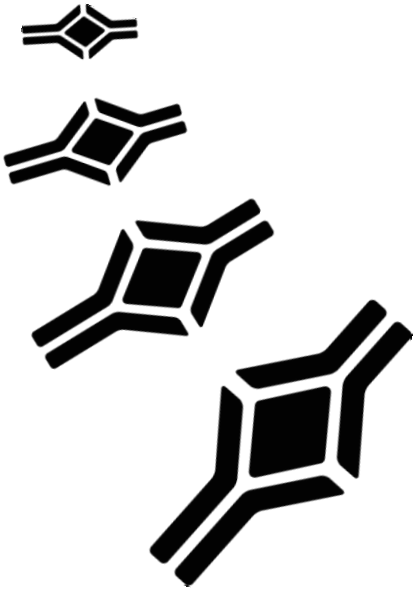
1. Angiopoietin-related growth factor (AGF) promotes epidermal proliferation, remodeling, and regeneration: Y. Oike, et al.; Proc. Natl. Acad. Sci. 100, 9494 (2003)
2. Angiopoietin-related growth factor (AGF) promotes angiogenesis: Y. Oike, et al.; Blood 103, 3760 (2004)
3. Angiopoietin-related growth factor antagonizes obesity and insulin resistance: Y. Oike, et al.; Nature Med. 11, 400 (2005)

References to this product:

1. H. Stepan, et al.; Am. J. Hypertens. 22, 314 (2009)
2. T. Ebert, et al.; Metabolism 58, 547 (2009)
3. J. Namkung, et al.; Metabolism 60, 564 (2010)

Assay Procedure Summary





Gentaur Molecular Products
Voortstraat 49
1910 Kampenhout, Belgium
http://www.gentaur-worldwide.com