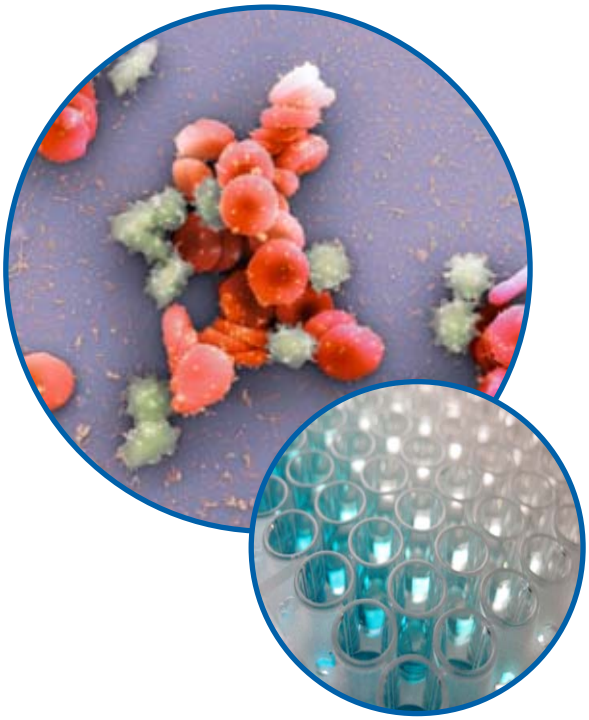


AdipoGen® LIFE SCIENCES

MANUAL

ANGPTL3 (human) ELISA Kit

For research use only. Not for diagnostic use.

Version 3 (15-June-2015)

Cat. No. AG-45A-0014YEK-KI01

Table of Contents

1. Intended Use

The ANGPTL3 (human) ELISA Kit is to be used for the *in vitro* quantitative determination of human ANGPTL3 in serum, plasma and cell culture supernatant. This ELISA Kit is for research use only.

2. Introduction

The angiopoietins are a family of growth factors that are specific for vascular endothelium. Conklin et al. (1) isolated a full-length cDNA encoding angiopoietin-like protein 3 (ANGPTL3) from a human fetal liver/spleen cDNA library. The deduced 460-amino acid ANGPTL3 protein has the characteristic structure of angiopoietins: a signal peptide, an extended helical domain predicted to form dimeric or trimeric coiled-coils, a short linker peptide, and a globular fibrinogen-like domain (FLD). Human ANGPTL3 shares 76% amino acid sequence identity with mouse Angptl3. Northern blot analysis of human tissues showed a preferential expression of 4 ANGPTL3 transcripts being 4.5, 3.0, 2.8, and 1.7 kb in liver. Camenisch et al. (2) determined showed that ANGPTL3 induced angiogenesis in the rat corneal assay. The FLD alone was sufficient to induce endothelial cell adhesion and in vivo angiogenesis. By microarray analysis, Zhang et al. (3) showed that mouse hematopoietic stem cell (HSC)-supportive fetal liver CD3-positive cells expressed Angptl2 and Angptl3. Long-term HSC expansion occurred when HSCs were cultured in the presence of Angptl2 and Angptl3 together with saturating levels of other growth factors, concluding that angiopoietin-like proteins can be potent stimulators of ex vivo expansion of HSCs. The KK obese mouse is moderately obese and has abnormally high levels of plasma insulin, glucose, and lipids. Koishi et al. (4) observed a mutant mouse strain named KK/San, which showed a hypolipidemia. By positional cloning, they discovered a genetic locus encoding a unique angiopoietin-like lipoprotein modulator was responsible for such hypolipidemia. It was found to be identical to angiopoietin-like protein-3, encoded by Angptl3, and had a highly conserved counterpart in humans. Overexpression of Angptl3 or intravenous injection of the purified protein in KK/San mice elicited an increase in circulating plasma lipid levels. These data suggested that Angptl3 regulates lipid metabolism in animals. The authors suggested the possibility that genetic variation in ANGPTL3 contributes to atherosclerosis, coronary artery disease, and diabetes mellitus. In vitro analysis of recombinant protein revealed that Angptl3 directly inhibits both endothelial lipase and lipoprotein lipase (LPL) activity (5, 6). Another line of evidence suggests that ANGPTL3 play an important role in regulation of HDL synthesis (7). The implication of ANGPTL3 in a number of metabolic dysfunctions suggests that ANGPTL3 is a novel predictor of these.

3. General References

(1) Identification of a mammalian angiopoietin-related protein expressed specifically in liver: D. Conklin, et al.; Genomics **62,** 477 (1999)

(2) ANGPTL3 stimulates endothelial cell adhesion and migration via integrin alpha-v-beta-3 and induces blood vessel formation in vivo: G. Camenisch, et al.; J. Biol. Chem. **277,** 17281 (2002)

(3) Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells: C.C. Zhang, et al.; Nature Med. **12,** 240 (2006)

(4) Angptl3 regulates lipid metabolism in mice: R. Koishi, et al.; Nature Genet. **30,** 151 (2002)

(5) ANGPTL3 decreases very low density lipoprotein triglyceride clearance by inhibition of lipoprotein lipase: T. Shimizugawa, et al.; J. Biol. Chem. **277,** 33742 (2002)

(6) Angiopoietin-like protein3 regulates plasma HDL cholesterol through suppression of endothelial lipase: M. Shimamura, et al.; Arterioscler. Thromb. Vasc. Biol. **27,** 366 (2007)

(7) Hepatic proprotein convertases modulate HDL metabolism: W. Jin, et al.; Cell Metab. **6,** 129 (2007)

4. Assay Principle

This assay is a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human ANGPTL3 in biological fluids. A monoclonal antibody specific for ANGPTL3 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, ANGPTL3 is recognized by the addition of a purified polyclonal antibody specific for ANGPTL3 (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 ANGPTL3 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 ANGPTL3 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	(50 µl)	(DET)
1 vial HRP 100X (HRP Conjugated anti-rabbit IgG)	(150 µl)	(HRP 100X)
1 vial human ANGPTL3 Standard (lyophilized)	(20 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 1:250 in ELISA Buffer 1X
  (40 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 ANGPTL3 Standard (STD)** has to be reconstituted with 1 ml of deionized water.

  - This reconstitution produces a stock solution of 20 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) (**20 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:

    10 , 5 , 2.5 , 1.25 , 0.625 , 0.313 , 0.156 and 0 ng/ml.

Dilute further for the standard curve:

To obtain	Add	Into
10 ng/ml	300 µl of ANGPTL3 (20 ng/ml)	300 µl of ELISA Buffer 1X
5 ng/ml	300 µl of ANGPTL3 (10 ng/ml)	300 µl of ELISA Buffer 1X
2.5 ng/ml	300 µl of ANGPTL3 (5 ng/ml)	300 µl of ELISA Buffer 1X
1.25 ng/ml	300 µl of ANGPTL3 (2.5 ng/ml)	300 µl of ELISA Buffer 1X
0.625 ng/ml	300 µl of ANGPTL3 (1.25 ng/ml)	300 µl of ELISA Buffer 1X
0.313 ng/ml	300 µl of ANGPTL3 (0.625 ng/ml)	300 µl of ELISA Buffer 1X
0.156 ng/ml	300 µl of ANGPTL3 (0.313 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 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, 1/50 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 ANGPTL3 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 10 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 ANGPTL3 concentration (ng/ml) on the vertical (Y) axis (see **10.** TYPICAL DATA).
- Calculate the human ANGPTL3 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 ANGPTL3 in the samples.

10. Typical Data

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

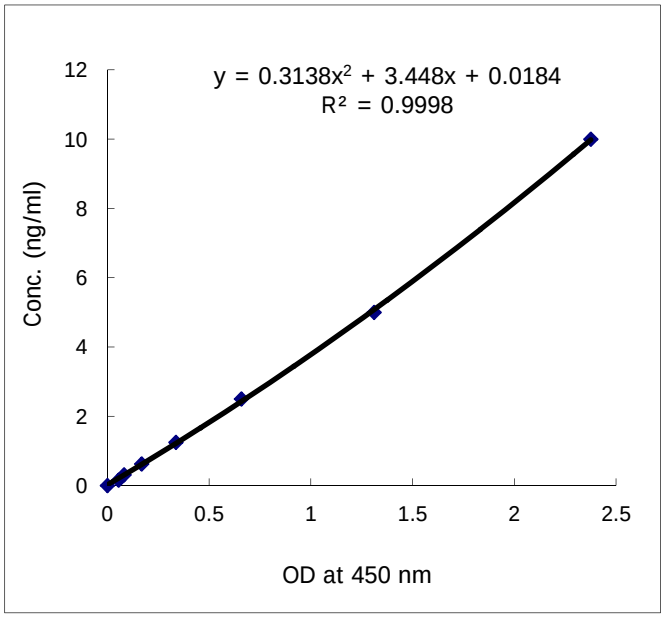


Figure: Standard curve

Standard hANGPTL3 (ng/ml)	Optical Density (mean)
10	2.376
5	1.311
2.5	0.659
1.25	0.337
0.625	0.169
0.313	0.082
0.156	0.056
0	0

11. Performance Characteristics

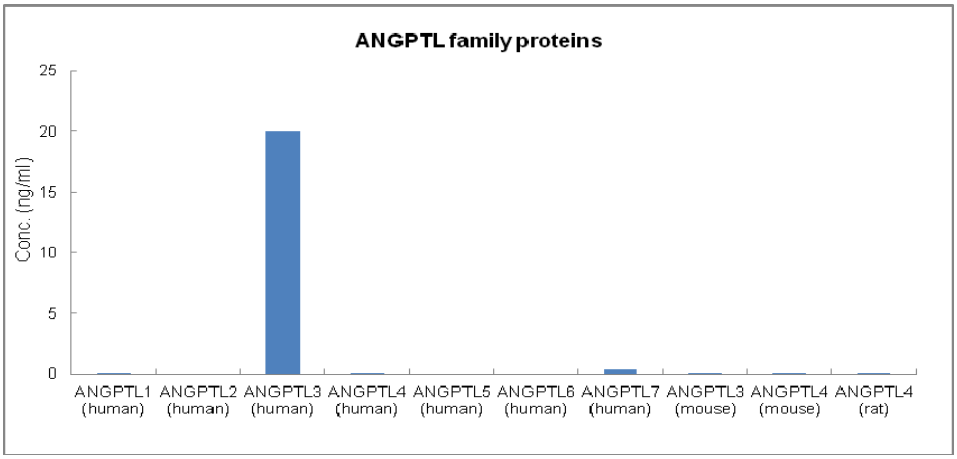
A. Sensitivity (Limit of detection):

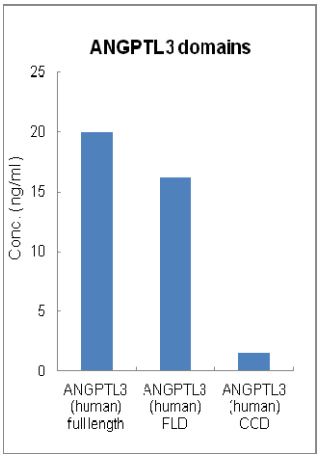
The lowest level of ANGPTL3 that can be detected by this assay is 75 pg/ml. ***NOTE:*** *The Limit of detection was measured by adding two standard deviations to the mean value of 50 zero standard.*

B. Assay range: 0.156 ng/ml – 10 ng/ml

C. Specificity:

This ELISA is specific for the measurement of natural and recombinant human ANGPTL3. It does not cross-react with human ANGPTL1, human ANGPTL2, human ANGPTL4, human ANGPTL5, human ANGPTL6, human ANGPTL7, human ANG1, human ANG2, human adiponectin, human resistin, human vaspin, human GPX3, human clusterin, human IL-33, human FABP4, human leptin, human RBP4, mouse ANGPTL3, mouse ANGPTL4, rat ANGPTL4.





D. Intra-assay precision:

Six samples of known concentrations of human ANGPTL3 were assayed in replicates 6 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
1	81.69	0.96	1.17	6
2	111.58	2.59	2.32	6
3	108.06	1.07	0.99	6
4	145.16	2.19	1.51	6
5	130.98	3.00	2.29	6
6	76.31	1.18	1.55	6

E. Inter-assay precision:

Six samples of known concentrations of human ANGPTL3 were assayed in 6 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
1	81.54	3.49	4.29	6
2	109.32	6.52	5.96	6
3	145.20	9.61	6.62	6
4	129.35	10.75	8.31	6
5	126.42	10.54	8.33	6
6	75.04	5.82	7.75	6

F. Recovery:

When samples (serum) are spiked with known concentrations of human ANGPTL3, the recovery averages 89% (range from 80 to 105%).

Samples	Average recovery (%)	Range (%)
1	86.86	80-100
2	90.01	85-105
3	89.43	85-105

G. Linearity:

Different human serum samples containing ANGPTL3 were diluted several fold (1/50 to 1/200) and the measured recoveries ranged from 80% to 105%.

Samples	Sample Dilution	Expected (ng/ml)	Observed (ng/ml)	% of Expected
1	1 : 50	136.80	136.80	100
	1 : 100	68.40	63.07	92.22
	1 : 200	34.20	29.27	85.60
2	1 : 50	141.23	141.23	100
	1 : 100	70.62	69.59	98.54
	1 : 200	35.31	33.72	95.51
3	1 : 50	83.23	83.23	100
	1 : 100	41.61	39.68	95.35
	1 : 200	20.81	19.44	93.44

H. Expected values:

ANGPTL3 levels range in plasma and serum from **20 to 150 ng/ml** (from healthy donors).

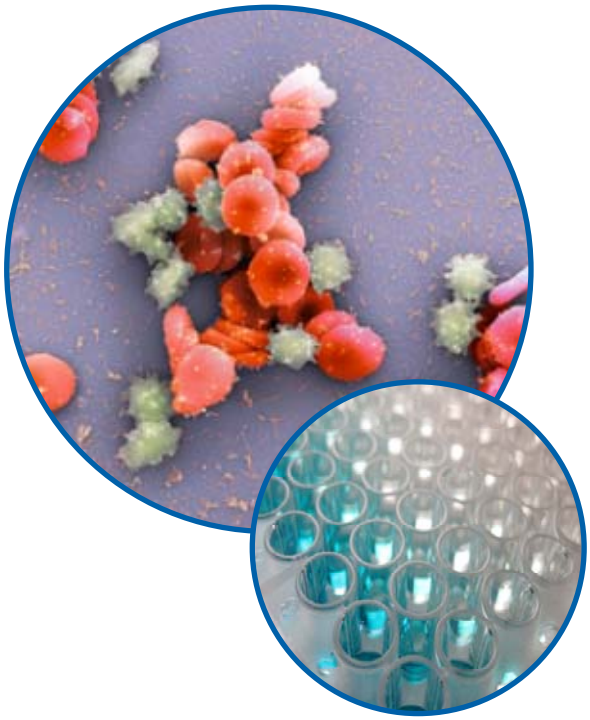
12. Technical Hints and Limitations

- 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



Product Specific References:

1. Exercise training restores the endothelial response to vascular growth factors in patients with stable coronary artery disease: E.B. Beck, et al.; Eur. J. Prev. Cardiol. **19,** 412 (2012)
2. Atypical angiopoietin-like protein that regulates ANGPTL3: F. Quagliarini, et al.; PNAS **109,** 19751 (2012)
3. Clinical characteristics and plasma lipids in subjects with familial combined hypolipidemia: a pooled analysis: I. Minicocci, et al.; J. Lipid Res. **54,** 3481 (2013)
4. Identification of adipokine clusters related to parameters of fat mass, insulin sensitivity and inflammation: G. Flehmig, et al.; PLos One **9,** e99785 (2014)

For more References please visit www.adipogen.com!

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