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## MANUAL

### **ANGPTL3 (mouse/rat) Dual ELISA Kit**

*For research use only. Not for diagnostic use.*

Version 3 (05-May-2015)

**Cat. No. AG-45A-0015YEK-KI01**

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## 1. Intended Use

The ANGPTL3 (mouse/rat) Dual ELISA Kit is to be used for the *in vitro* quantitative determination of mouse or rat 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 mouse or rat ANGPTL3 in biological fluids. A polyclonal 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 biotinylated polyclonal antibody specific for ANGPTL3 (Detection Antibody). After removal of excess biotinylated antibody, HRP labeled streptavidin (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 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 mouse 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	(30 µl)	(DET)
1 vial HRP Labeled Streptavidin (lyophilized)	(2 µg)	(STREP-HRP)
1 vial mouse ANGPTL3 Standard (lyophilized)	(2 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 1:500 in ELISA Buffer 1X (4 µl DET + 2 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 200 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!

- **Mouse ANGPTL3 Standard (STD)** has to be reconstituted with 1 ml of deionized water.
  - This reconstitution produces a stock solution of 2 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) (**2 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:  
**1 , 0.5 , 0.25 , 0.125 , 0.063 , 0.031 , 0.016 and 0 ng/ml.**

**Dilute further for the standard curve:**

To obtain	Add	Into
<b>1 ng/ml</b>	300 µl of ANGPTL3 (2 ng/ml )	300 µl of ELISA Buffer 1X
<b>0.5 ng/ml</b>	300 µl of ANGPTL3 (1 ng/ml )	300 µl of ELISA Buffer 1X
<b>0.25 ng/ml</b>	300 µl of ANGPTL3 (0.5 ng/ml )	300 µl of ELISA Buffer 1X
<b>0.125 ng/ml</b>	300 µl of ANGPTL3 (0.25 ng/ml )	300 µl of ELISA Buffer 1X
<b>0.063 ng/ml</b>	300 µl of ANGPTL3 (0.125 ng/ml )	300 µl of ELISA Buffer 1X
<b>0.031 ng/ml</b>	300 µl of ANGPTL3 (0.063 ng/ml )	300 µl of ELISA Buffer 1X
<b>0.016 ng/ml</b>	300 µl of ANGPTL3 (0.031 ng/ml )	300 µl of ELISA Buffer 1X
<b>0 ng/ml</b>	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  $\leq -20^{\circ}\text{C}$  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  $\leq -20^{\circ}\text{C}$  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, 1/4,000 dilution of mouse samples and 1/400 dilution of rat samples are 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)

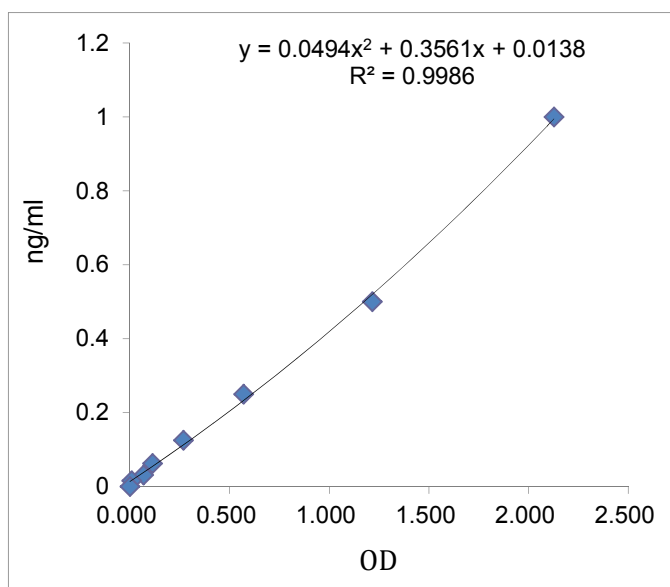
<input type="checkbox"/>	<p>1. Determine the number of 16-well strips needed for the assay and insert them in the frame for current use. The extra strips should be resealed in the foil pouch bag and stored at 4°C.</p> <p><b>NOTE:</b> Remaining 16-well strips coated with ANGPTL3 antibody when opened can be stored at 4°C for up to 1 month.</p>
<input type="checkbox"/>	<p>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 (<b>see 8.1. Preparation and Storage of Reagents and 8.2. Preparation of Samples</b>).</p>
<input type="checkbox"/>	<p>3. Cover the plate with plate sealer and incubate for <b>1 hour at 37°C</b>.</p>
<input type="checkbox"/>	<p>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.</p>
<input type="checkbox"/>	<p>5. Add 100 µl to each well of the Detection Antibody (<b>DET</b>). (<b>see 8.1. Preparation and Storage of Reagents</b>).</p>
<input type="checkbox"/>	<p>6. Cover the plate with plate sealer and incubate for <b>1 hour at 37°C</b>.</p>
<input type="checkbox"/>	<p>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.</p>
<input type="checkbox"/>	<p>8. Add 100 µl to each well of the diluted HRP Labeled Streptavidin (<b>STREP-HRP</b>) (<b>see 8.1. Preparation and Storage of Reagents</b>).</p>
<input type="checkbox"/>	<p>9. Cover the plate with plate sealer and incubate for <b>1 hour at 37°C</b>.</p>
<input type="checkbox"/>	<p>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.</p>
<input type="checkbox"/>	<p>11. Add 100 µl to each well of TMB Substrate Solution (<b>TMB</b>).</p>
<input type="checkbox"/>	<p>12. Allow the color reaction to develop <b>at room temperature (RT°C) in the dark for 10 minutes</b>.</p>
<input type="checkbox"/>	<p>13. Stop the reaction by adding 100 µl of Stop Solution (<b>STOP</b>). Tap the plate gently to ensure thorough mixing. The substrate reaction yields a blue solution that turns yellow when Stop Solution (<b>STOP</b>) is added.</p>
	<p><b>! CAUTION: CORROSIVE SOLUTION!</b></p>
<input type="checkbox"/>	<p>14. Measure the OD at 450 nm in an ELISA reader within 30 minutes.</p>

## 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 ANGPTL3 concentration (ng/ml) on the vertical (Y) axis (see **10. TYPICAL DATA**).
- Calculate the 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 mouse or rat ANGPTL3 in the samples.

## 10. Typical Data

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



Standard mANGPTL3 (ng/ml)	Optical Density (mean)
1	2.128
0.5	1.216
0.25	0.570
0.125	0.268
0.0625	0.113
0.0312	0.068
0.0156	0.009
0	0.000

**Figure:** Standard curve

## 11. Performance Characteristics

### A. Sensitivity (Limit of detection):

The lowest level of ANGPTL3 that can be detected by this assay is 15 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:** 0.016 ng/ml – 1 ng/ml

### C. Specificity:

This ELISA is specific for the measurement of natural and recombinant mouse or rat ANGPTL3. It does not cross-react mouse ANGPTL4, mouse adiponectin, mouse resistin, mouse RBP4, mouse vaspin, mouse Nampt, mouse clusterin, mouse GPX3, mouse progranulin, mouse IL-33, mouse leptin, mouse RELM- $\beta$ , human ANGPTL3, human ANGPTL4, human Nampt, rat Nampt.

### D. Intra-assay precision:

Six samples of known concentrations of mouse and rat ANGPTL3 were assayed in replicates 10 times to test precision within an assay.

Samples	Means (ng/ml)	SD	CV (%)	n
1	512.29	19.07	3.72	10
2	481.98	17.94	3.72	10
3	509.66	25.32	4.97	10
4	391.49	23.03	5.88	10
5	502.42	21.19	4.22	10
6	818.34	37.33	4.56	10

### E. Inter-assay precision:

Five samples of known concentrations of mouse and rat ANGPTL3 were assayed in 5 separate assays to test precision between assays.

Samples	Means (ng/ml)	SD	CV (%)	n
1	555.19	32.61	5.87	5
2	439.08	27.65	6.30	5
3	487.63	38.82	7.96	5
4	63.78	4.32	6.78	5
5	778.45	39.49	5.07	5

**F. Recovery:**

When samples (serum) are spiked with known concentrations of mouse and rat ANGPTL3, the recovery averages 100% (range from 90% to 110%).

Samples	Average recovery (%)	Range (%)
1	93.85	90-100
2	98.63	95-105
3	109.56	105-110

**G. Linearity:**

Different mouse and rat serum samples containing ANGPTL3 were diluted several fold (1/400 to 1/16,000) and the measured recoveries ranged from 91% to 106%.

Samples	Sample Dilution	Expected (ng/ml)	Observed (ng/ml)	% of Expected
<b>Mouse 1</b>	1 : 4,000	344.42	344.42	100
	1 : 8,000	172.21	169.07	98.18
	1 : 16,000	86.11	82.01	95.24
<b>Mouse 2</b>	1 : 4,000	442.20	442.20	100
	1 : 8,000	221.10	219.13	99.11
	1 : 16,000	110.55	105.95	95.84
<b>Rat 1</b>	1 : 400	100.14	100.14	100
	1 : 800	50.07	52.91	105.67
	1 : 1,600	25.04	22.92	91.56
<b>Rat 2</b>	1 : 400	67.21	67.21	100
	1 : 800	33.60	34.70	103.27
	1 : 1,600	16.80	15.81	94.07

**H. Expected values:**

ANGPTL3 levels range in mouse samples from **50 to > 1,000 ng/ml**.

ANGPTL3 levels range in rat samples from **10 to > 150 ng/ml**.

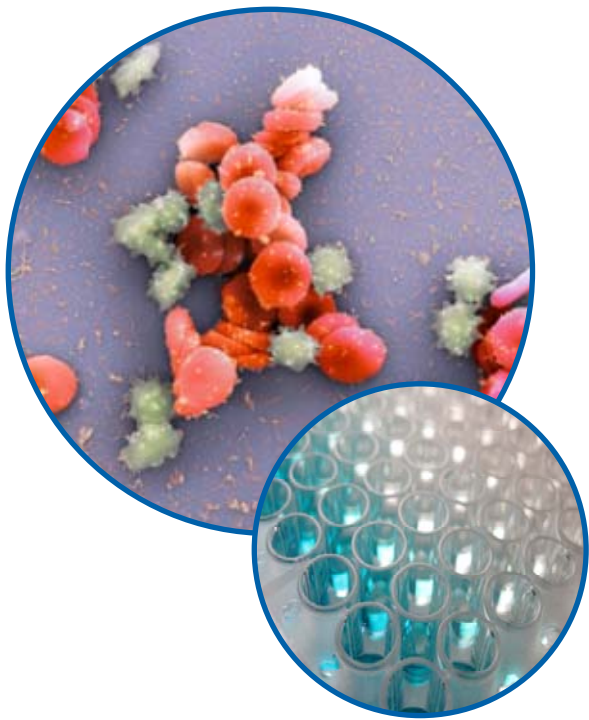
## 12. Technical Hints and Limitations

- 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



**Product Specific References:**

1. F. Quagliarini, et al.; PNAS **109**, 19751 (2012)

*For more References please visit [www.adipogen.com](http://www.adipogen.com)!*

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