

# Quantikine<sup>®</sup>

## Mouse Angiopoietin-like 3 Immunoassay

Catalog Number MANL30

**For the quantitative determination of Angiopoietin-like 3 (ANGPT-L3) concentrations in cell culture supernates, serum, and plasma.**

***This package insert must be read in its entirety before using this product.***

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**FOR RESEARCH USE ONLY.  
NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

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## INTRODUCTION

Angiopoietin-like 3 (ANGPT-L3) is a secreted glycoprotein that plays an important role in fatty acid metabolism. It is one of several molecules with structural similarity to the angiopoietins, which also contain an N-terminal coiled coil domain and a C-terminal fibrinogen-like domain (1 - 4). Mouse ANGPT-L3 shares 22% - 30% amino acid (aa) sequence identity with mouse ANGPT-L1, 2, 4, 6, and 7. It shares 77% aa sequence identity with human ANGPT-L3. ANGPT-L3 is expressed in the liver from early in development through adulthood (5, 6). It is found as 70 kDa, 50 kDa, and 32 kDa species and can form weakly associated non-covalent multimers *in vitro* (6). ANGPT-L3 directly inhibits lipoprotein lipase (LPL) and endothelial lipase, enzymes responsible for hydrolyzing circulating triglycerides and HDL phospholipids (7, 8). This activity requires a putative heparin-binding motif which is N-terminal to the coiled coil domain (9).

ANGPT-L3 is proteolytically cleaved in the liver by proprotein convertases (10). Full length ANGPT-L3 circulates in the plasma as do the separated N- and C-terminal fragments which contain the coiled coil domain and fibrinogen-like domains, respectively (9). Cleavage serves to activate ANGPT-L3, as the released N-terminal fragment is more potent than full length ANGPT-L3 at increasing plasma triglycerides and inhibiting endothelial lipase (9, 10). ANGPT-L3 does not bind the angiopoietin receptors Tie-1 or Tie-2, but its fibrinogen-like domain interacts with integrin  $\alpha_V\beta_3$  to induce endothelial cell adhesion, migration, and neovascularization (11). ANGPT-L3 also promotes the expansion of hematopoietic stem cells (12).

ANGPT-L3 promotes an increase in circulating triglyceride levels but does not alter VLDL or HDL secretion or uptake (7, 9, 13). ANGPT-L3 knockout mice are hypolipidemic and have elevated LPL activity (14). ANGPT-L3 expression *in vivo* is upregulated by liver X receptor (LXR) agonists and downregulated by insulin, leptin, and agonists of thyroid hormone receptor beta (TR $\beta$ ) or peroxisome proliferator-activated receptor beta (PPAR $\beta$ ) (15 - 18). Dysregulated ANGPT-L3 expression and elevated plasma triglyceride levels are characteristic of some strains of obese and diabetic mice (7, 13, 16). In humans, serum ANGPT-L3 levels are positively correlated with serum HDL-Cholesterol and adiponectin levels as well as with arterial intima-media thickness, an indicator for the progression of atherosclerosis (19, 20).

The Quantikine Mouse ANGPT-L3 immunoassay is a 4.5 hour solid phase ELISA designed to measure ANGPT-L3 in cell culture supernates, serum, and plasma. It contains Sf 21-expressed recombinant mouse ANGPT-L3 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural ANGPT-L3 showed linear curves that were parallel to the standard curves obtained using the Quantikine kit standards. These results indicate that the Quantikine Mouse ANGPT-L3 kit can be used to determine relative mass values for naturally occurring ANGPT-L3.

## **PRINCIPLE OF THE ASSAY**

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for ANGPT-L3 has been pre-coated onto a microplate. Standards, controls, and samples are pipetted into the wells and any ANGPT-L3 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for ANGPT-L3 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of ANGPT-L3 bound in the initial step. The color development is stopped and the intensity of the color is measured.

## **LIMITATIONS OF THE PROCEDURE**

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

## **TECHNICAL HINTS**

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- For best results, pipette reagents and samples into the center of each well.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.

## MATERIALS PROVIDED

**Mouse ANGPT-L3 Microplate** (Part 893718) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a rat monoclonal antibody against ANGPT-L3.

**Mouse ANGPT-L3 Conjugate** (Part 893719) - 12.5 mL of a polyclonal antibody against ANGPT-L3 conjugated to horseradish peroxidase with preservatives.

**Mouse ANGPT-L3 Standard** (Part 893720) - 2 vials (8 ng/vial) of recombinant mouse ANGPT-L3 in a buffered protein solution with preservatives; lyophilized.

**Mouse ANGPT-L3 Control** (893721) - 2 vials of recombinant mouse ANGPT-L3 in a buffered protein base with preservatives; lyophilized. The concentration range of mouse ANGPT-L3 after reconstitution is shown on the vial label. The assay value of the Control should be within the range specified on the label.

**Assay Diluent RD1-21** (Part 895215) - 12.5 mL of a buffered protein solution with preservatives.

**Calibrator Diluent RD5-26 Concentrate** (Part 895525) - 21 mL of a buffered protein solution with preservatives.

**Wash Buffer Concentrate** (Part 895024) - 50 mL of a 25-fold concentrated solution of a buffered surfactant with preservatives.

**Color Reagent A** (Part 895000) - 12.5 mL of stabilized hydrogen peroxide.

**Color Reagent B** (Part 895001) - 12.5 mL of stabilized chromogen (tetramethylbenzidine).

**Stop Solution** (Part 895174) - 23 mL of a diluted hydrochloric acid solution.

**Plate Covers** - 4 adhesive strips.

## STORAGE

<b>Unopened Kit</b>	Store at 2 - 8° C. Do not use past kit expiration date.	
<b>Opened/ Reconstituted Reagents</b>	Diluted Wash Buffer	May be stored for up to 1 month at 2 - 8° C.*
	Stop Solution	
	Assay Diluent RD1-21	
	Calibrator Diluent RD5-26	
	Conjugate	
	Unmixed Color Reagent A	
	Unmixed Color Reagent B	Discard after use. Use a fresh Standard and Control for each assay.
	Standard	
	Control	
	Microplate Wells	Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*

\*Provided this is within the expiration date of the kit.

## OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of  $500 \pm 50$  rpm.
- **Polypropylene tubes.**

## PRECAUTION

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

## SAMPLE COLLECTION AND STORAGE

**Cell Culture Supernates** - Remove particulates by centrifugation and assay immediately or aliquot and store samples at  $\leq -20^{\circ}$  C. Avoid repeated freeze-thaw cycles.

**Serum** - Allow blood samples to clot for 2 hours at room temperature or overnight at  $2 - 8^{\circ}$  C before centrifuging. Centrifuge for 20 minutes at approximately  $2000 \times g$ . Remove serum and assay immediately or aliquot and store samples at  $\leq -20^{\circ}$  C. Avoid repeated freeze-thaw cycles.

**Plasma** - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 20 minutes at approximately  $2000 \times g$  within 30 minutes of collection. Assay immediately or aliquot and store samples at  $\leq -20^{\circ}$  C. Avoid repeated freeze-thaw cycles.

**Note:** *Do not use icteric samples. Citrate plasma has not been validated for use in this assay.*

## SAMPLE PREPARATION

Serum and plasma samples require a 100-fold dilution. A suggested 100-fold dilution is  $10 \mu\text{L}$  of sample +  $490 \mu\text{L}$  of Calibrator Diluent RD5-26 (1X) followed by  $70 \mu\text{L}$  of the diluted sample +  $70 \mu\text{L}$  of Calibrator Diluent RD5-26 (1X).

## REAGENT PREPARATION

Bring all reagents to room temperature before use.

**Mouse ANGPT-L3 Control** - Reconstitute the Control with 1.0 mL deionized or distilled water. Assay the Control undiluted.

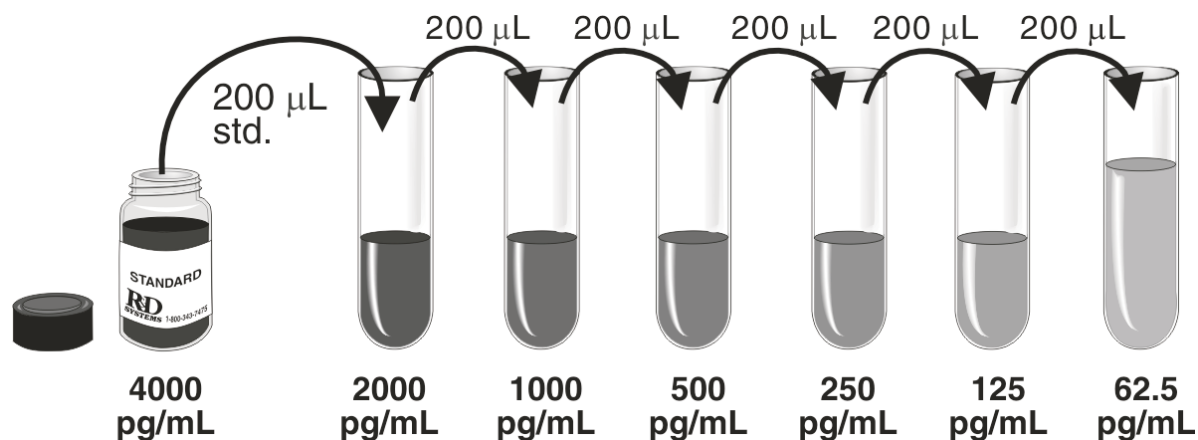
**Wash Buffer** - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 25 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 625 mL of Wash Buffer.

**Substrate Solution** - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100  $\mu$ L of the resultant mixture is required per well.

**Calibrator Diluent RD5-26 (1X)** - Dilute 20 mL of Calibrator Diluent RD5-26 Concentrate into 60 mL of deionized or distilled water to prepare 80 mL of Calibrator Diluent RD5-26 (1X).

**ANGPT-L3 Standard** - Reconstitute the Mouse ANGPT-L3 Standard with 2.0 mL of Calibrator Diluent RD5-26 (1X). Do not substitute other diluents. This reconstitution produces a stock solution of 4000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum 5 minutes with gentle agitation prior to making dilutions.

**Use polypropylene tubes.** Pipette 200  $\mu$ L of Calibrator Diluent RD5-26 (1X) into six polypropylene tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 4000 pg/mL standard serves as the high standard. Calibrator Diluent RD5-26 (1X) serves as the zero standard (0 pg/mL).



## ASSAY PROCEDURE

**Bring all reagents and samples to room temperature before use. It is recommended that all samples, control, and standards be assayed in duplicate.**

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, reseal.
3. Add 50  $\mu\text{L}$  of Assay Diluent RD1-21 to each well.
4. Add 50  $\mu\text{L}$  of Standard, Control, or sample\* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at  $500 \pm 50$  rpm. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with Wash Buffer (400  $\mu\text{L}$ ) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 100  $\mu\text{L}$  of Mouse ANGPT-L3 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 100  $\mu\text{L}$  of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 100  $\mu\text{L}$  of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

\*Serum/Plasma samples require dilution as directed in the Sample Preparation section.

## ASSAY PROCEDURE SUMMARY

1.  Bring all reagents to room temperature.  
 Prepare reagents and samples as instructed.  
 Return unused components to storage temperature as indicated in the instructions.
2.  Add 50  $\mu$ L Assay Diluent RD1-21 to each well.
3.  Add 50  $\mu$ L Standard, Control, or sample\* to each well.  
 Cover the plate and incubate for 2 hours at room temperature on the orbital shaker.
4.  Aspirate and wash each well five times.
5.  Add 100  $\mu$ L Conjugate to each well.  
 Cover the plate and incubate for 2 hours at room temperature on the orbital shaker.
6.  Aspirate and wash each well five times.
7.  Add 100  $\mu$ L Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
8.  Add 100  $\mu$ L Stop Solution to each well.
9.  Read Optical Density at 450 nm (correction wavelength set at 540 nm or 570 nm).

\*Serum/Plasma samples require dilution prior to assay as directed in the Sample Preparation section.

## CALCULATION OF RESULTS

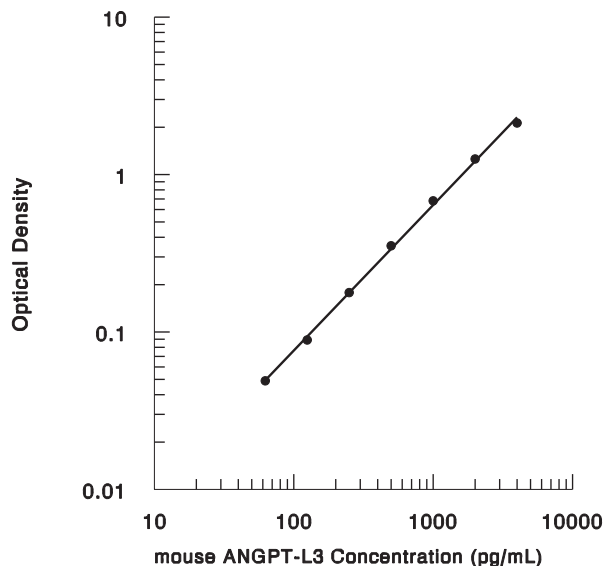
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the ANGPT-L3 concentrations versus the log of the O.D., and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

Since serum/plasma samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

## TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



pg/mL	O.D.	Average	Corrected
0	0.014 0.018 0.062	0.016	—
62.5	0.067 0.102	0.065	0.049
125	0.108 0.190	0.105	0.089
250	0.197 0.357	0.194	0.178
500	0.380 0.691	0.369	0.353
1000	0.702 1.230	0.697	0.681
2000	1.313 2.067	1.272	1.256
4000	2.220	2.144	2.128

## PRECISION

### Intra-assay Precision (Precision within an assay)

Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

### Inter-assay Precision (Precision between assays)

Three samples of known concentration were tested in forty separate assays to assess inter-assay precision.

Sample	Intra-assay Precision			Inter-assay Precision		
	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	190	383	1346	197	418	1449
Standard deviation	10.9	23.6	79.1	17.4	27.1	90.5
CV (%)	5.7	6.2	5.9	8.9	6.5	6.2

## RECOVERY

The recovery of ANGPT-L3 spiked to levels throughout the range of the assay was evaluated.

Sample	Average % Recovery	Range
Cell culture supernates (n=4)	103	93 - 120%

## LINEARITY

To assess the linearity of the assay, samples containing high concentrations of ANGPT-L3 were serially diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture samples (n=2)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)
1:2	Average % of Expected	101	98	98	96
	Range (%)	100 - 101	96 - 99	93 - 102	91 - 99
1:4	Average % of Expected	103	98	98	95
	Range (%)	100 - 106	97 - 100	90 - 105	90 - 101
1:8	Average % of Expected	103	99	96	92
	Range (%)	97 - 110	96 - 102	88 - 103	87 - 99
1:16	Average % of Expected	91	97	96	92
	Range (%)	91 - 91	93 - 99	88 - 105	85 - 96

\*Samples were diluted prior to assay as directed in the Sample Preparation section.

## SENSITIVITY

Fifty two assays were evaluated and the minimum detectable dose (MDD) of ANGPT-L3 ranged from 1.64 - 9.62 pg/mL. The mean MDD was 4.29 pg/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

## CALIBRATION

This immunoassay is calibrated against a highly purified *Sf 21*-expressed recombinant mouse ANGPT-L3 produced at R&D Systems.

## SAMPLE VALUES

**Serum/Plasma** - Samples were evaluated for the presence of ANGPT-L3 in this assay.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum* (n=20)	244	115 - 435	78.7
EDTA plasma* (n=20)	311	137 - 486	89.6
Heparin plasma* (n=20)	234	139 - 343	52.0

\*Samples were diluted prior to assay as directed in the Sample Preparation section.

## Cell Culture Supernates -

Mouse liver tissue from one mouse was cut into 1 - 2 mm pieces and cultured in 100 mL of RPMI supplemented with 10% fetal bovine serum, 50  $\mu$ M  $\beta$ -mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin sulfate for 2 days. Cells were unstimulated or stimulated with 1 mg/mL of lipopolysaccharide for 2 days. The cell culture supernates were assayed for levels of mouse ANGPT-L3.

Mouse heart tissue from two mice was cut into 1 - 2 mm pieces and cultured in 100 mL of RPMI supplemented with 10% fetal bovine serum, 50  $\mu$ M  $\beta$ -mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin sulfate for 1 day. Cells were unstimulated or stimulated with 1 mg/mL of lipopolysaccharide for 1 day. The cell culture supernates were assayed for levels of mouse ANGPT-L3.

Mouse lung tissue from three mice was cut into 1 - 2 mm pieces and cultured in 100 mL of RPMI supplemented with 10% fetal bovine serum, 50  $\mu$ M  $\beta$ -mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin sulfate for 1 day. The cell culture supernates were assayed for levels of mouse ANGPT-L3.

Tissue Type	Observed Levels (pg/mL)
Liver, unstimulated	918
Liver, stimulated	1045
Heart, unstimulated	152
Heart, stimulated	131
Lung	232

## SPECIFICITY

This assay recognizes recombinant and natural mouse ANGPT-L3. The factors listed below were prepared at 50 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant mouse ANGPT-L3 control were assayed for interference. No significant cross-reactivity or interference was observed.

### Recombinant mouse:

Angiopoietin-3  
Angiopoietin-like 2  
Angiopoietin-like 4  
Angiopoietin-like 7

### Recombinant human:

Angiopoietin-like 3 (aa 17 - 460)  
Angiopoietin-like 3 (aa 17 - 220)  
LPL (aa 28 - 154)

Rat serum was not detectable in this assay.

This assay detects full-length and 27 kDa N-terminal cleaved mouse ANGPT-L3 but does not detect the 38 kDa C-terminal cleavage fragment.

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# PLATE LAYOUT

Use this plate layout as a record of standards and samples assayed.

1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
	<b>A</b>	<b>B</b>	<b>C</b>	<b>D</b>	<b>E</b>	<b>F</b>	<b>G</b>	<b>H</b>

# NOTES