

## Human Angiopoietin-2 ELISA PROCEDURE SUMMARY

Total Assay Time - 225 min. (120+60+30+15)

Instruction Manual No. 100-180-APHM

	Allow all reagents to reach room temp.; arrange and label required # of strips. Dilute Sample Diluent (1:20) and Wash Buffer (1:100) with water. Dilute serum samples (0-1:5), HRP-conjugate (1:100) and Detection Ab (1:100) with 1x sample diluent. Do not dilute Standards.
<b>Step 1</b>	Pipet <b>100 ul</b> of pre-diluted <b>standards</b> and diluted samples (0-1:5) into appropriate wells. Mix gently, cover the plate and incubate for <b>120 min</b> at room temp
<b>Step 2</b>	Aspirate and <b>wash 3 times</b> with 1x wash solution. Dispense <b>100 ul of 1x Detection Antibody</b> to each well. Mix gently, cover the plate and incubate for <b>60 min</b> at room temp.
<b>Step 3</b>	Aspirate and <b>wash 3 times</b> with 1x wash solution. Dispense <b>100 ul of 1x HRP-streptavidin-conjugate</b> to each well. Mix gently, cover the plate and incubate for <b>30 min</b> at room temp.
<b>Step 4</b>	Aspirate and <b>wash 3 times</b> with 1x wash solution. Dispense <b>100 ul of TMB substrate Solution</b> . Mix gently, cover the plate and incubate for <b>15 min</b> at room temp. Blue color develops
<b>Step 5</b>	Pipette 100 ul stop solution into each well. Blue color turns yellow. Measure Absorbance at 450 nm.

## Human Serum Angiopoietin-2

### For Quantitative Determination of Angiopoietin-2 In Human Serum

CHECK LIST (Check each box after completing each of the above steps)

	Step 1	Step2	Step3	Step4	Step5
Start time					
End Time					

### KIT PROFILE

**Date kit opened** \_\_\_\_\_ **Technician:** \_\_\_\_\_

**Date used:** \_\_\_\_\_ **# Strips used** \_ **# Remaining** \_\_\_\_\_

**Date used:** \_\_\_\_\_ **# Strips used** \_ **# Remaining** \_\_\_\_\_

**Remarks** \_\_\_\_\_

\_\_\_\_\_



## Human Angiopoietin-2 ELISA KIT Cat. No. 100-180-APH

Kit Components, 96 tests	Cat #
Anti-Human ANG-2 coated strip plate (8 wells x 12 strips)	100-181
Human ANG-2 Standard A (0 ng/ml), 0.45 ml	100-182
Human ANG-2 Standard B (1 ng/ml), 0.45 ml	100-183
Human ANG-2 Standard C (2 ng/ml), 0.45 ml	100-184
Human ANG-2 Standard D (4 ng/ml), 0.45 ml	100-185
Human ANG-2 Standard E (8 ng/ml), 0.45 ml	100-186
Human ANG-2 Standard F (16 ng/ml), 0.45 ml	100-187
(100x) Anti-Human ANG-2 Detection Ab, 0.12 ml	100-188
(100x) Streptavidin-HRP Conjugate, 0.12ml	S-HRP100
(20x) Sample Diluent, 10ml	SD-20
Wash Buffer (100X), 10 ml	WB-100
TMB Substrate, 12 ml	80091
Stop solution, 12 ml	80101
Instruction Manual	100-180-APHM

### Introduction

Angiogenesis accounts for the formation of vasculature into previously avascular organs such as brain and kidney. Angiogenic activity in the adult is required during the normal tissue repair, and for the remodeling of the female reproductive organs (ovulation and placental development). Certain pathological conditions, such as tumor growth and diabetic retinopathy, also require angiogenesis. The genetic and molecular mechanism that influence angiogenesis has only recently begun to be studied and identified.

The angiopoietin (Ang) family of growth factors includes four members, all of which bind to the endothelial receptor tyrosine kinase Tie2. Two of the Angs, Ang-1 and Ang-4, activate the Tie2 receptor, whereas Ang-2 and Ang-3 inhibit Ang-1-induced Tie2 phosphorylation.

Angiopoietin-1 (Ang-1) is a secreted growth factor, which binds to and activates the Tie-2 receptor tyrosine kinase. This growth factor enhances endothelial cell survival and capillary morphogenesis, and also limits capillary permeability. Ang-2 binds the same receptor but does not activate it. Ang-2 may act as an antagonist for Ang-1 and Tie-2. Ang-2 disrupts capillary integrity, increasing vessel growth when VEGF levels are high, but causing vessel regression when VEGF levels are low. Angiopoietin and Tie genes are expressed in the mammalian the precursor of the adult kidney, where they may play a role in endothelial growth.

ADI's Angiopoietin-2 ELISA kit is a highly sensitive sandwich type assay for the measurement of Angiopoietin-2 in serum. The assay can be adapted to measure Human Angiopoietin-2 in other biological fluids such as plasma, urine, culture medium etc.

### PERFORMANCE CHARACTERISTICS

**1. Detection limit-** Based on 4 replicate determinations of the zero standards, the minimum Angiopoietin-2 concentration detectable using this assay is ~300 pg/ml. The detection limit is defined as the value deviating by 2 SD from the zero standard.

**2. Expected Values:** A limited testing of 20 adult Human serum samples values of 0.5 - 11.77 ng/ml (average 2.55 ng/ml).

**3. Specificity:** This kit is specific to Angiopoietin-2 and does not show any significant reactivity to other Human serum proteins.

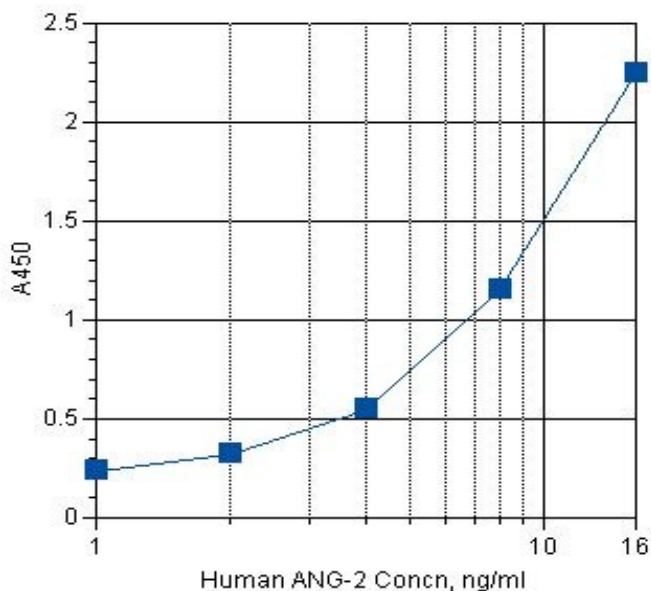
#### 4. Species Cross-reactivity

Cross-reactivity was tested with the following animal serum at no dilution: Monkey (132%), Rat (28%), Bovine (53%) and FBS (17%) serums had reactivity with Human ANG-2. Mouse, Hamster, Guinea Pig, Swine, Chicken, Sheep, Rabbit, and Goat all had less than 1% cross-reactivity.

## WORKSHEET OF TYPICAL ASSAY

Wells	Stds/samples	Mean A <sub>450</sub> nm	Calculated Concentration
A1, A2	<b>Standard A</b> (0 ng/ml)	0.15	
B1, B2	<b>Standard B</b> (1 ng/ml)	0.24	
C1, C2	<b>Standard C</b> (2 ng/ml)	0.32	
D1, D2	<b>Standard D</b> (4 ng/ml)	0.55	
E1, E2	<b>Standard E</b> (8 ng/ml)	1.15	
F1, F2	<b>Standard F</b> (16 ng/ml)	2.24	
G1, G2	<b>Sample 1</b>	1.75	(11.7 ng/ml)

**NOTE:** These data are for demonstration purpose only. A complete standard curve must be run in every assay to determine sample values. Each laboratory should determine their own normal reference values.



A typical assay Standard Curve (do not use this for calculating sample values)

## PRINCIPLE OF THE TEST

Human Angiotensin-2 ELISA kit is based on binding of Human Angiotensin-2 from samples to two antibodies, one immobilized on the microtiter well plates, and other bound to the enhancing protein Biotin, which then binds to streptavidin horseradish peroxidase conjugate. After a washing step, chromogenic substrate is added and colors developed. The enzymatic reaction (color) is directly proportional to the amount of Angiotensin-2 present in the sample. Adding stopping solution terminates the reaction. Absorbance is then measured on a microtiter well ELISA reader at 450 nm. and the concentration of Angiotensin-2 in samples and control is read off the standard curve.

## MATERIALS AND EQUIPMENT REQUIRED

Adjustable micropipet (5-1000ul) and multi-channel pipette with disposable plastic tips. Reagent troughs, plate washer (recommended) and ELISA plates Reader.

## PRECAUTIONS AND SAFETY INSTRUCTIONS

Standards, Sample Diluent, Detection Antibody and Streptavidin-HRP contain Proclin 300 (0.05%, v/v). Stop Solution contains 1% sulfuric acid. Follow good laboratory practices, and avoid ingestion or contact of any reagent with skin, eyes or mucous membranes. All reagents may be disposed of down a drain with copious amounts of water.

MSDS for TMB, sulfuric acid and Proclin 300, if not already on file, can be requested or obtained from the ADI website.

## SPECIMEN COLLECTION AND HANDLING

Collect blood by venipuncture, allow clotting, and separating the serum by centrifugation at room temperature. Do not heat inactivate the serum. If sera can not be immediately assayed, these could be stored at -20°C for up to six months. Avoid repeated freezing and thawing of samples. No preservatives should be added to the serum. It is also possible to use plasma for testing.

## REAGENT PREPARATION

1. Dilute the 20x Sample Diluent 1:20 with water, e.g., 5ml diluent + 95ml water for a full assay plate. Prepare only the required amount; store at 2-8° C for up to 3-4 days.
2. Dilute the Wash Buffer 1:100 with water, e.g., 5ml of the stock + 495ml water. Store at room temperature for 1 week.
3. Dilute the Detection Ab (1:100) using the 1x Sample Diluent, e.g., 100 ul + 9.9 ml Sample Diluent for a full assay plate.
4. Dilute the Streptavidin HRP (1:100) using the 1x Sample Diluent, e.g., 100 ul + 9.9 ml Sample Diluent for a full assay plate.

## STORAGE AND STABILITY

The microtiter well plate and all other reagents, if unopened, are stable at 2-8°C until the expiration date printed on the label. Upon initial use of the kit components, remaining shelf life is 2 months with proper storage.

### TEST PROCEDURE ALLOW ALL REAGENTS TO REACH ROOM TEMPERATURE BEFORE USE.

1. **Do not dilute standards.** Dilute Human serum samples up to 1:10 using 1x Sample Diluent. Most normal samples will not have to be diluted for testing.

	Sample	Diluent	Total Volume	Dilution Factor
Step 1	20 ul	180ul	200 ul	1:10

2. Label or mark the microtiter well strips to be used on the plate.
3. Dispense 200-300 ul of wash buffer to all wells. Let stand for 5 to 15 minutes, then discard or aspirate the solution. The step should be done just before adding the samples, do not allow the wells to dry at any time during the assay.

**Note:** for ease of loading samples it is recommended that a second **uncoated** microwell plate should be used keeping diluted samples. This enables standards or samples to be transferred quickly to the ELISA plate using multichannel pipette.

4. Add 100ul standards and diluted samples into appropriate wells. Mix gently, and incubate at room temperature for 120 minutes.
5. Wash the wells with 3 times with 300 ul of 1x wash buffer.
6. Pipette 100 ul of 1x Anti-Human ANG-2 Detection Antibody into each well. Mix gently, and incubate for 60 minutes at room temperature. **Note:** the detection solution must be at room temperature.
7. Wash the wells with 3 times with 300 ul of 1x wash buffer.
8. Pipette 100 ul of 1x Streptavidin-HRP Conjugate into each well. Mix gently, and incubate for 30 minutes at room temperature. **Note:** the conjugate solution must be at room temperature.

9. Aspirate and wash the wells **3 times** with 1x wash buffer. We recommend using an automated ELISA plate washer for better consistency. Failure to wash the wells properly will lead to high blank or zero values. If washing manually, plate must be tapped over paper towel between washings to ensure proper washing.

10. Add **100 ul** of TMB Substrate into each well. Mix gently, and incubate for **15 minutes** at room temperature. Blue color develops. **Note:** TMB solution must be at room temperature.

11. Stop the reaction by adding **100 ul of stop** solution to **all wells**. Mix gently. Blue color turns yellow.

12. Measure the absorbance at **450 nm** using an ELISA reader. Color is stable for at least 30 min after stopping.

**NOTES:** Read instructions carefully before the assay. Do not allow reagents to dry on the wells. Careful aspiration of the washing solution is essential for good assay precision. Since timing of the incubation steps is important to the performance of the assay, pipet the samples without interruption and it should not exceed 5 minutes to avoid assay drift. If more than one plate is being used in one run, it is recommended to include a standard curve on each plate. The unused strips should be stored in a sealed bag at 2-8°C. Addition of the HRP substrate solution starts a kinetic reaction, which is terminated by dispensing the stopping solution. Therefore, keep the incubation time for each wells the same by adding the reagents in identical sequence. Plate readers measure absorbance vertically. Do not touch the bottom of the wells.

### DILUTION OF SAMPLES

Samples containing more than **16 ng/ml** Human Angiopoietin-2 should be further diluted and re-tested. The results obtained should be multiplied by the appropriate dilution factor.

### CALCULATION OF RESULTS

Calculate the mean absorbance for each duplicate. Subtract the absorbance of the zero standard from the mean absorbance values of standards and samples. Draw the standard curve on linear graph paper by plotting net absorbance values of standards against appropriate Angiopoietin-2 concentrations. Read off the Angiopoietin-2 concentrations of the control and patient samples. Multiply the values by the dilution factor of the samples. If samples were diluted 1:10 then the values must be multiplied by 10 with the results expressed as ng/ml.