



Enabling Legendary Discovery™

LEGEND MAX™

ELISA Kit with Pre-coated Plates



Human Angiopoietin-2

Cat. No. 440407

ELISA Kit for Accurate Quantitation of Human
Angiopoietin-2 in Serum, Plasma, Cell Culture
Supernatant, and Saliva

BioLegend, Inc.
biolegend.com

It is highly recommended that this manual be read in its entirety before using this product. Do not use this kit beyond the expiration date.

For Research Purposes Only. Not for use in diagnostic or therapeutic procedures. Purchase does not include or carry the right to resell or transfer this product either as a stand-alone product or as a component of another product. Any use of this product other than the permitted use without the express written authorization of BioLegend is strictly prohibited.



Table of Contents	Page
Introduction.....	2
Materials Provided.....	2
Materials to be Provided by the End-User.....	3
Storage Information.....	3
Health Hazard Warnings.....	3
Specimen Collection and Handling.....	4
Reagent and Sample Preparation.....	4
Assay Procedure.....	5
Assay Procedure Summary.....	7
Calculation of Results.....	8
Typical Data.....	8
Performance Characteristics.....	9
Specificity.....	9
Sensitivity.....	9
Recovery.....	9
Linearity.....	9
Intra-Assay Precision.....	9
Inter-Assay Precision.....	10
Biological Samples.....	10
Troubleshooting Guide.....	11
ELISA Plate Template.....	13

LEGEND MAX™ Human Angiopoietin-2 ELISA Kit

Introduction:

Human angiopoietin-2 is a member of the angiopoietin family of vascular growth factors and is a glycoprotein involved in angiogenesis and inflammation. It plays a vital role in embryonic and postnatal angiogenesis and is highly abundant in vascular endothelial cells. Angiopoietin-2, along with angiopoietin-1, are ligands for the receptor tyrosine kinase Tie2. It is also known to work synergistically with cytokines, such as VEGF, to promote tumor progression and associated angiogenesis. Studies have shown that it is a marker for endothelial activation. Due to its importance in inflammatory conditions, this analyte has a great potential for new disease therapies.

The BioLegend LEGEND MAX™ Human Angiopoietin-2 ELISA Kit is a Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) with a 96-well strip plate that is pre-coated with a mouse monoclonal anti-human angiopoietin-2 antibody. The detection antibody is a biotinylated mouse monoclonal anti-human angiopoietin-2 antibody. This kit is specifically designed for the accurate quantitation of human angiopoietin-2 in serum, plasma, cell culture supernatant, and saliva. This kit is analytically validated with ready-to-use reagents.

Materials Provided:

Description	Quantity	Volume (per bottle)	Part #
Anti-human Angiopoietin-2 Pre-coated 96-well Strip Microplate	1 plate		750003717
Human Angiopoietin-2 Detection Antibody	1 bottle	12 mL	750003719
Human Angiopoietin-2 Lyophilized Standard	1 vial	lyophilized	750002757
Avidin-HRP	1 bottle	12 mL	77897
Assay Buffer B	1 bottle	25 mL	79128
Wash Buffer (20X)	1 bottle	50 mL	78233
Substrate Solution F	1 bottle	12 mL	79132
Stop Solution	1 bottle	12 mL	79133
Plate Sealers	1 pack		78101

LEGEND MAX™ Human Angiopoietin-2 ELISA Kit

Materials to be Provided by the End-User:

- Microplate reader able to measure absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 1 µL to 1,000 µL
- Deionized water
- Wash bottle or automated microplate washer
- Log-Log graph paper or software for data analysis
- Tubes to prepare standard dilutions
- Timer
- Polypropylene vials
- Plate shaker

Storage Information:

Store unopened kit components between 2°C and 8°C. Do not use this kit beyond its expiration date.

Opened or Reconstituted Components	
Microplate wells	If not all microplate strips are used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal. Store between 2°C and 8°C for up to one month.
Standard	The remaining reconstituted standard stock solution can be aliquoted into polypropylene vials and stored at -70°C for up to one month. Avoid repeated freeze-thaw cycles.
Detection Antibody	Store opened reagents between 2°C and 8°C and use within one month.
Avidin-HRP	
Assay Buffer B	
Wash Buffer (20X)	
Substrate Solution F	
Stop Solution	

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled, or absorbed through the skin. Refer to the MSDS online at BioLegend's website for details (www.biolegend.com/msds).
2. Substrate Solution F is harmful if inhaled or ingested. Avoid skin, eye, and clothing contact.

LEGEND MAX™ Human Angiopoietin-2 ELISA Kit

3. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum, plasma, and other biological fluids in accordance with NCCLS regulations.
4. Stop Solution contains strong acid. *Wear eye, hand, and face protection.*
5. Before disposing of the plate, rinse it with an excess amount of tap water.

Specimen Collection and Handling:

Specimens should be clear and non-hemolyzed. If possible, unknown samples should be run at a number of dilutions to determine the optimal dilution factor that will ensure accurate quantitation.

Cell Culture Supernatant: If necessary, centrifuge all samples to remove debris prior to analysis. It is recommended that samples be stored at $< -70^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

Serum: Use a serum separator tube and allow clotting for at least 30 minutes, then centrifuge for 10 minutes at $1,000 \times g$. Remove serum layer and assay immediately or store serum samples at $< -70^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

Plasma: Collect blood samples in citrate, heparin, or EDTA containing tubes. Centrifuge for 10 minutes at $1,000 \times g$ within 30 minutes of collection. Assay immediately or store plasma samples at $< -70^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

Saliva: Collect saliva in a tube, centrifuge at $10,000 \times g$ for 5 minutes, and collect the aqueous layer. Assay immediately or store saliva samples at $< -20^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

Reagent and Sample Preparation:

Note: All reagents should be diluted immediately prior to use.

1. Dilute the 20X Wash Buffer to 1X with deionized water. For example, make 1 liter of 1X Wash Buffer by adding 50 mL of 20X Wash Buffer to 950 mL of deionized water. If crystals have formed in the 20X Wash Buffer, bring to room temperature and vortex until dissolved.
2. Reconstitute the Human Angiopoietin-2 Lyophilized Standard by adding the volume of Assay Buffer B to make the standard stock solution (refer to LEGEND MAX Kit Lot-Specific Certificate of Analysis). Allow the reconstituted standard to sit at room temperature for 15-20 minutes, then briefly vortex to mix completely.
3. Dilute serum and plasma samples 1:6 in Assay Buffer B before use. In general, cell culture supernatant and saliva samples are analyzed without

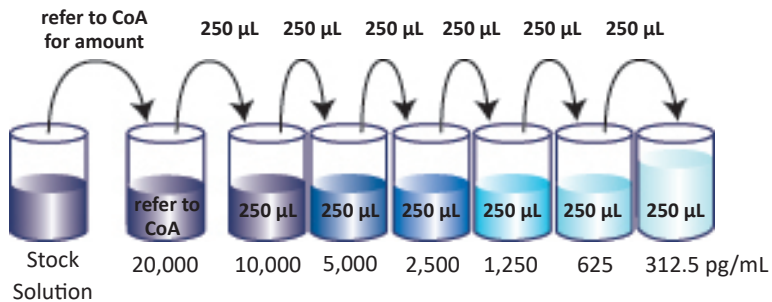
LEGEND MAX™ Human Angiopoietin-2 ELISA Kit

dilutions. However, if dilutions are required, use Assay Buffer B as the sample diluent.

Assay Procedure:

Note: Do not mix reagents from different kits or lots. Reagents and/or antibodies from different manufacturers should not be used with this kit. **Because angiopoietin-2 is present in saliva, it recommended to wear a mask while performing this assay.**

1. Bring all reagents to room temperature prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay.
2. If not all microplate strips will be used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal.
3. Prepare 500 μL of the 20,000 pg/mL top standard by diluting the appropriate amount of the standard stock solution in Assay Buffer B (refer to LEGEND MAX Kit Lot-Specific Certificate of Analysis). Perform six two-fold serial dilutions of the 20,000 pg/mL top standard in separate tubes using Assay Buffer B as the diluent. Thus, the human angiopoietin-2 standard concentrations in the tubes are 20,000 pg/mL, 10,000 pg/mL, 5,000 pg/mL, 2,500 pg/mL, 1,250 pg/mL, 625 pg/mL and 312.5 pg/mL, respectively. Assay Buffer B serves as the zero standard (0 pg/mL).



4. Wash the plate 4 times with at least 300 μL of 1X Wash Buffer per well and blot any residual buffer by firmly tapping the plate upside down on absorbent paper. All subsequent washes should be performed similarly.
5. Add 50 μL of Assay Buffer B to each well that will contain either standard dilutions or samples.
6. Add 50 μL of standard dilutions or samples to the appropriate wells.
7. Seal the plate with a plate sealer included in the kit and incubate the plate at room temperature with shaking for 2 hours.

LEGEND MAX™ Human Angiopoietin-2 ELISA Kit

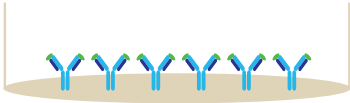
8. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.
9. Add 100 μ L of Human Angiopoietin-2 Detection Antibody to each well, seal the plate and incubate at room temperature with shaking for 1 hour.
10. Discard the contents of the plate into a sink, then wash the plate 4 times with 1X Wash Buffer as in step 4.
11. Add 100 μ L of Avidin-HRP to each well, seal the plate and incubate at room temperature for 30 minutes with shaking.
12. Discard the contents of the plate into a sink, then wash the plate 5 times with 1X Wash Buffer as in step 4. For this final wash, soak wells in 1X Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.
13. Add 100 μ L of Substrate Solution F to each well and incubate at room temperature for 25 minutes **in the dark**. * Wells containing human angiopoietin-2 should turn blue in color with an intensity proportional to its concentration. It is not necessary to seal the plate during this step.
14. Stop the reaction by adding 100 μ L of Stop Solution to each well. The solution color should change from blue to yellow.
15. Read absorbance at 450 nm within 15 minutes. If the reader is capable of reading at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

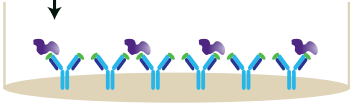
*** Optimal substrate incubation time depends on laboratory conditions and the optical linear ranges of ELISA plate readers.**

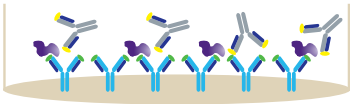
LEGEND MAX™ Human Angiopoietin-2 ELISA Kit

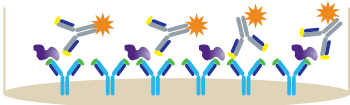
Assay Procedure Summary

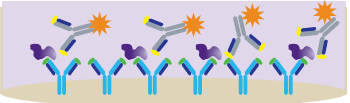
1. Wash 4 times
Add 50 μ L Assay Buffer B

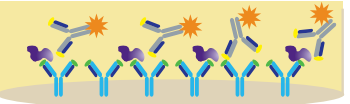

2. Add 50 μ L diluted standards or samples.
Incubate 2 hr, RT with shaking.


3. Wash 4 times
Add 100 μ L Detection Antibody.
Incubate 1 hr, RT with shaking.


4. Wash 4 times
Add 100 μ L Avidin-HRP.
Incubate 30 min, RT with shaking.


5. Wash 5 times.
Add 100 μ L Substrate Solution F.
Incubate 25 min*, RT, in the dark.


6. Add 100 μ L Stop Solution.


7. Read absorbance at 450 nm and 570 nm.

LEGEND MAX™ Human Angiopoietin-2 ELISA Kit

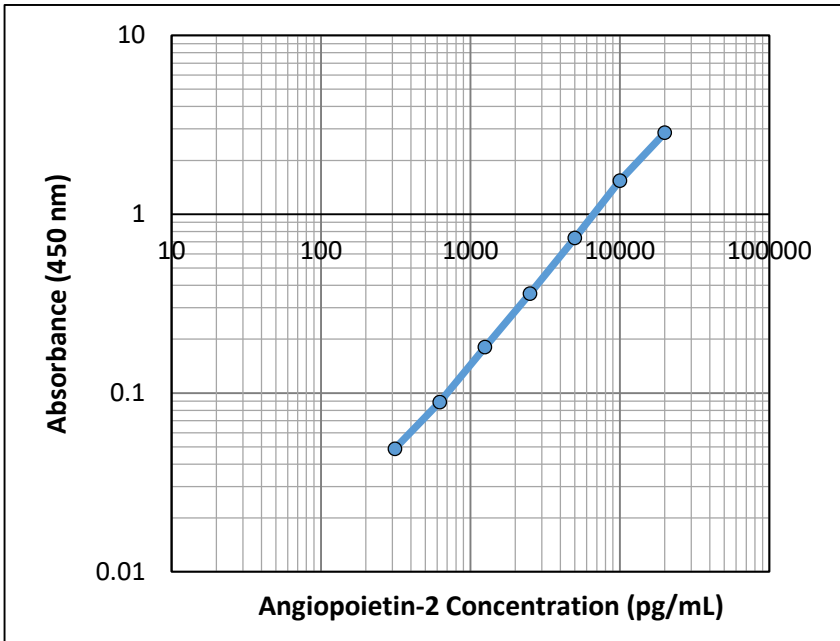
Calculation of Results:

The data can be best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm. If the appropriate software is not available, use log-log graph paper to determine sample concentrations. Determine the mean absorbance for each set of duplicate or triplicate standards, controls, and samples. Plot the standard curve on log-log graph paper with angiopoietin-2 concentration on the X-axis and absorbance on the Y-axis. Draw a best fit line through the standard points. To determine the unknown angiopoietin-2 concentrations, find the mean absorbance value of the unknown concentration on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the concentration.

If samples were diluted, multiply the concentration by the appropriate dilution factor. If a test sample's absorbance value falls outside the linear portion of the standard curve, the test sample needs to be re-analyzed at a higher (or lower) dilution, as appropriate.

Typical Data:

This standard curve was generated at BioLegend for demonstration purposes only. A standard curve must be run with each assay.



LEGEND MAX™ Human Angiopoietin-2 ELISA Kit

Performance Characteristics:

Specificity: No cross reactivity was observed when this kit was used to analyze VEGF-121, VEGF-165, FGF-146, Tie2, and ANGPT-1 at 50 ng/mL.

Sensitivity: The minimum detectable concentration of angiopoietin-2 is 55.8 pg/mL.

Recovery: Recombinant human angiopoietin-2 was spiked into serum, plasma, cell medium, and saliva at three different concentrations, then sample recovery was analyzed with the LEGEND MAX™ Human Angiopoietin-2 ELISA kit.

Sample Type	N	Recovery (%)
Serum	6	88.9
Citrate Plasma	3	78.4
EDTA Plasma	3	88.9
Heparin Plasma	3	98.1
Cell Culture Medium	2	115.3
Saliva	1	98.7

Linearity: Human serum and plasma (EDTA, heparin) samples were serially diluted two-fold to produce samples with concentrations within the dynamic range. Human citrate plasma samples were spiked with a high concentration of angiopoietin-2 then serially diluted. Cell culture supernatant from HUVEC cells (cultured for 5 days) was diluted four-fold prior to serial dilution. Saliva was diluted two-fold prior to serial dilution.

Sample Type	N	Linearity (%)
Serum	3	100.1
EDTA Plasma	3	99.0
Heparin Plasma	3	99.5
Citrate Plasma (spiked)	3	104.0
Cell Culture Supernatant (HUVEC)	1	103.2
Saliva	1	103.1

Intra-Assay Precision: Sixteen replicates of each of two samples containing different human angiopoietin-2 concentrations were tested in one assay.

Concentration	Sample 1	Sample 2
Number of Replicates	16	16
Mean Concentration (pg/mL)	2640.6	676.8
Standard Deviation	52.8	20.3
%CV	2.0	3.0

LEGEND MAX™ Human Angiopoietin-2 ELISA Kit

Inter-Assay Precision: Two samples containing different concentrations of human angiopoietin-2 were tested in ten independent assays.

Concentration	Sample 1	Sample 2
Number of Assays	10	10
Mean Concentration (pg/mL)	3144.6	682.7
Standard Deviation	427.8	40.2
%CV	13.6	5.9

Biological Samples: Human serum and plasma (citrate, EDTA, and heparin) samples were diluted six-fold and tested using the LEGEND MAX™ Human Angiopoietin-2 ELISA kit.

	Serum	Citrate Plasma	EDTA Plasma	Heparin Plasma
N	9	3	6	6
Min (pg/mL)	3400.4	3470.0	3556.4	3965.5
Max (pg/mL)	10039.2	8615.1	7696.4	9374.5
Mean (pg/mL)	5071.1	6204.5	5515.5	5735.3

Troubleshooting Guide:

Problem	Probable Cause	Solution
High Background	Background wells were contaminated	Avoid cross-well contamination by using the provided plate sealers. Use multichannel pipettes and change tips between pipetting samples and reagents.
	Insufficient washes	Increase number of washes. Increase soaking time between washes prior to addition of substrate solution.
	TMB Substrate Solution was contaminated	TMB Substrate Solution should be clear and colorless prior to addition to wells. Use a clean container prior to pipetting substrate solution into wells.
No or poor signal	Detection Antibody, Avidin-HRP or Substrate solution were NOT added	Rerun the assay and follow the protocol.
	Wrong reagent or reagents were added in wrong sequential order	
	Insufficient plate agitation	The plate should be agitated during all incubation steps using a plate shaker at a speed where solutions in wells are within constant motion without splashing.
	The wash buffer contains Sodium Azide (NaN ₃)	Avoid Sodium Azide contamination in the wash buffer as it inhibits HRP activity.
	Incubations were done at an inappropriate temperature, timing or without agitation	Rerun the assay and follow the protocol.
Low or poor standard curve signal	The standard was incorrectly reconstituted or diluted	Adjust the calculations and follow the protocol.
	Standard was inappropriately stored	Store the reconstituted standard stock solution in polypropylene vials at -70°C. Avoid repeated freeze-thaw cycles.
	Reagents added to wells with incorrect concentrations	Check for pipetting errors and the correct reagent volume.

LEGEND MAX™ Human Angiopoietin-2 ELISA Kit

Problem	Probable Cause	Solution
Signal is high, standard curves have saturated signal	Standard reconstituted with less volume than required	Reconstitute new lyophilized standard with the correct volume of solution recommended in the protocol.
	Standards/samples, detection antibody, Avidin-HRP or substrate solution were incubated for too long	Rerun the assay and follow the protocol.
Sample readings are out of range	Samples contain no or below detectable levels of the analyte	If samples are below detectable levels, it may be possible to use a larger sample volume. Contact technical support for appropriate protocol modifications.
	Samples contain analyte concentrations greater than highest standard point	Samples may require dilution and analysis.
High variation in samples and/or standards	Multichannel pipette errors	Confirm that pipette calibrations are accurate.
	Plate washing was not adequate or uniform	Ensure pipette tips are tightly secured. Ensure uniformity in all wash steps.
	Non-homogenous samples	Thoroughly mix samples before assaying.
	Samples may have high particulate matter	Remove particulate matter by centrifugation.
	Cross-well contamination	Do not reuse plate sealers. Always change tips for reagent additions. Ensure that pipette tips do not touch the reagents on the plate.

ELISA Plate Template												
	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

LEGEND MAX™ Human Angiopoietin-2 ELISA Kit



Legend Max™ Kits are manufactured by BioLegend Inc.
8999 BioLegend Way
San Diego, CA 92121
Tel: 1.858.768.5800
Tel: US & Canada Toll-Free: 1.877.Bio.Legend (1.877.246.5343)
Fax: 1.877.455.9587
Email: info@biolegend.com
Biolegend.com

For a complete list of world-wide BioLegend offices and distributors,
please visit our website at: biolegend.com

440407_R02