ELISA Kit
Catalog # KHC1641

Human
Angiopoietin-2

www.invitrogen.com
Invitrogen Corporation
542 Flynn Road, Camarillo, CA 93012
Tel: 800-955-6288
E-mail: techsupport@invitrogen.com

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PURPOSE

The Invitrogen Human Angiopoietin-2 (Hu Ang-2) ELISA is to be used for the quantitative determination of Hu Ang-2 in serum, plasma, buffered solution and tissue culture medium. The assay will recognize both natural and recombinant Hu Ang-2.

INTRODUCTION

Angiopoietin-2 (Ang-2 and ANGPT2; accession number NP_001138) is a member of the angiopoietin protein family (angiopoietin-1, -2, -3, and -4 [human ortholog of mouse angiopoietin-3]) which plays an important role in the development and maintenance of blood vessels and the lymphatic system. The angiopoietins serve as ligands for the receptor tyrosine kinases Tie1 and Tie2. Members of the angiopoietin family are characterized by the presence of three domains: an N-terminal region lacking homology to any known structures, an alpha helical-rich coiled-coil segment similar to motifs found widely among proteins that have a propensity to aggregate, and a highly conserved C-terminal fibrinogen-like domain that mediates receptor interaction.

Angiopoietin-2 was identified in 1997, based on its homology (approximately 60%) with angiopoietin-1. The gene for human angiopoietin-2 maps to chromosome 8p23. The mature protein contains 496 amino acid residues. In addition, several N-terminal truncated forms arise due to alternative splicing, including Ang-2 443, expressed by primary endothelial cells and tumor tissues, and Ang-2A, Ang-2B, and Ang-2C, expressed by the testis.

Angiopoietin-2 is produced at sites of vascular remodeling, including blood vessels co-opted by tumors, the sprouting and regressing vessels of the ovary, the immature testis, and tissues affected by inflammatory processes. Stimuli that enhance angiopoietin-2 expression include leptin, angiotensin-2, TNF-α, VEGF, FGF-b, hypoxia, and HER2. Signaling pathways that involve PTEN/PI3 kinase/Akt and the transcription factors NF-κB and ETS1 have been implicated in the regulation of angiopoietin-2 expression.

The physiological roles of angiopoietin-2 are currently under investigation. Studies with knock-out mice indicate that angiopoietin-2 may not be important in embryonic vasculogenesis; however, the protein appears to play an important role in postnatal vascular remodeling and lymphatic patterning. Angiopoietin-2 knockout mice usually die within two weeks of birth. Transgenic mice overexpressing angiopoietin-2 suffer from disruption in blood vessel formation. Depending on context, angiopoietin-2 can act as a growth factor, a pro-apoptotic agent, an anti-apoptotic agent, and a chemotactic agent. In the absence of VEGF, angiopoietin-2 stimulates vascular regression and endothelial cell apoptosis. In the presence of VEGF, however, angiopoietin-2 appears to serve as a pro-angiogenic factor.

The signaling cascade arising from angiopoietin-2 stimulation is currently under investigation. With endothelial cells, angiopoietin-2 is not able to stimulate the autophosphorylation of the receptor tyrosine kinase Tie2. Angiopoietin-2 may instead antagonize the biological activity of angiopoietin-1 by acting as a high affinity endogenous inhibitor with these cells. In other cells, such as IBE cells (murine brain capillary cell line), with BaF3 cells (murine pro B cell line), and with fibroblasts ectopically expressing Tie2, angiopoietin-2 is able to directly

stimulate the autophosphorylation of Tie2. Studies with cells transfected with kinase-inactive downstream signaling molecules have revealed that PI3 kinase is involved in angiopoietic-2 stimulated chemotaxis, while c-Fyn and c-Fes are involved in capillary structuring. Angiopoietin-2 has been found to interact with the extracellular matrix. In glioma, angiopoietin-2 enhances tumor invasiveness by stimulating MMP-2 production in a pathway that involves integrin avb1, Focal Adhesion Kinase, p130(Cas), ERK1/2, and JNK.

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Read entire protocol before use.

PRINCIPLE OF THE METHOD

The Invitrogen Hu Ang-2 kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A monoclonal antibody specific to Hu Ang-2 has been coated onto the wells of the microtiter strips provided.

During the first incubation, standards of known Hu Ang-2 content, controls, and unknown samples are pipetted into the coated wells, followed by the addition of biotinylated second anti-Ang-2 antibody.

After washing, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a second incubation and washing to remove all the unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of Hu Ang-2 present in the original specimen.

REAGENTS PROVIDED

Note: *Store all reagents at 2 to 8°C.*

Reagent	***96 Test Kit***
Hu Angiopoietin-2 Standard, recombinant Hu Ang-2. Contains 0.1% sodium azide. Refer to vial label for quantity and reconstitution volume.	2 vials
Standard Diluent Buffer. Contains 0.1% sodium azide; 25 mL.	1 bottle
Hu Ang-2 Antibody Coated Wells. 96 Well Plate.	1 plate
Incubation Buffer. Contains 0.05% sodium azide; 12 mL.	1 bottle
Hu Ang-2 Biotin Conjugate, (Biotin-labeled anti-Ang-2). Contains 0.1% sodium azide; 6 mL.	1 bottle
Streptavidin-HRP (100X). Contains 3.3 mM thymol; 0.125 mL.	1 vial
Streptavidin-HRP Diluent. Contains 3.3 mM thymol; 25 mL.	1 bottle
Wash Buffer Concentrate (25X); 100 mL.	1 bottle
Stabilized Chromogen, *Tetramethylbenzidine (TMB);* 25 mL.	1 bottle
Stop Solution; 25 mL.	1 bottle
Plate Covers, adhesive strips.	3

Disposal Note: This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state and local regulations for disposal.

SUPPLIES REQUIRED BUT NOT PROVIDED

1. Microtiter plate reader capable of measurement at or near 450 nm.
2. Calibrated adjustable precision pipettes, preferably with disposable plastic tips. (A manifold multi-channel pipette is desirable for large assays.)
3. Distilled or deionized water.
4. Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.).
5. Data analysis and graphing software. Graph paper: linear (Cartesian), log-log, or semi-log, as desired.
6. Glass or plastic tubes for diluting and aliquoting standard.
7. Absorbent paper towels.
8. Calibrated beakers and graduated cylinders in various sizes.

PROCEDURAL NOTES/LAB QUALITY CONTROL

1. When not in use, kit components should be refrigerated. All reagents should be warmed to room temperature before use.
2. **Microtiter plates should be allowed to come to room temperature before opening the foil bags.** Once the desired number of strips has been removed, immediately reseal the bag and store at 2 to 8°C to maintain plate integrity.
3. Samples should be collected in pyrogen/endotoxin-free tubes.
4. Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
5. When possible, avoid use of badly hemolyzed or lipemic serum and plasma samples. If large amounts of particulate matter are present, centrifuge or filter prior to analysis.

6. It is recommended that all standards, controls and samples be run in duplicate.
7. Serum, plasma, or tissue culture sample(s) that measure >2000 pg/mL require additional dilution steps in their respective buffers.
8. When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
9. Cover or cap all reagents when not in use.
10. **Do not mix or interchange different reagent lots from various kit lots**.
11. Do not use reagents after the kit expiration date.
12. Read absorbances within 30 minutes of assay completion.
13. Any internal controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
14. All residual wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. ***Never*** insert absorbent paper directly into the wells.
15. Because *Stabilized Chromogen* is light sensitive, avoid prolonged exposure to light. Also avoid contact between *Stabilized Chromogen* and metal, or color may develop.

SAFETY

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

DIRECTIONS FOR WASHING

Incomplete washing will adversely affect the test outcome. All washing must be performed with the *Wash Buffer Concentrate (25X)* provided.

Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip (aspiration device) into the bottom of each well. Take care not to scratch the inside of the well.

After aspiration, fill the wells with at least 0.4 mL of diluted wash solution. Let soak for 15 to 30 seconds, then aspirate the liquid. Repeat as directed under **ASSAY METHOD**. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

Alternatively, the wash solution may be put into a squirt bottle. If a squirt bottle is used, flood the plate with wash buffer, completely filling all wells. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

If using an automated washer, the operating instructions for washing equipment should be carefully followed.

SAMPLE PREPARATION

Human serum and plasma require a 10-fold dilution in the *Standard Diluent Buffer*. For these samples, add 25 µL of the sample to a clean microfuge tube, followed by 225 µL of *Standard Diluent Buffer*. Tissue culture samples may be assayed neat. Mix well.

REAGENT PREPARATION AND STORAGE

A. Reconstitution and Dilution of Hu Angiopoietin-2 Standard

The *Hu Angiopoietin-2 Standard* is prepared from a highly purified NS0-expressed recombinant protein.

Note: Either glass or plastic tubes may be used for standard dilutions.

1. Reconstitute standard to 20,000 pg/mL with *Standard Diluent Buffer*. Refer to the standard vial label for instructions. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Use the standard within 15 minutes of reconstitution.
2. Add 0.1 mL of the reconstituted standard to a tube containing 0.9 mL *Standard Diluent Buffer*. Label as 2000 pg/mL Hu Ang-2. Mix.
3. Add 0.300 mL of *Standard Diluent Buffer* to each of 6 tubes labeled 1000, 500, 250, 125, 62.5, and 31.2 pg/mL Hu Ang-2.
4. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

B. Dilution of Hu Angiopoietin-2 Standard

Standard:	Add:	Into:
2000 pg/mL	Prepare as described in Step 2.	
1000 pg/mL	0.300 mL of the 2000 pg/mL std.	0.300 mL of the Diluent Buffer
500 pg/mL	0.300 mL of the 1000 pg/mL std.	0.300 mL of the Diluent Buffer
250 pg/mL	0.300 mL of the 500 pg/mL std.	0.300 mL of the Diluent Buffer
125 pg/mL	0.300 mL of the 250 pg/mL std.	0.300 mL of the Diluent Buffer
62.5 pg/mL	0.300 mL of the 125 pg/mL std.	0.300 mL of the Diluent Buffer
31.2 pg/mL	0.300 mL of the 62.5 pg/mL std.	0.300 mL of the Diluent Buffer
0 pg/mL	0.300 mL of the Diluent Buffer	An empty tube

Discard all remaining reconstituted and diluted standards after completing assay. Return the *Standard Diluent Buffer* to the refrigerator.

C. Storage and Final Dilution of Streptavidin-HRP (100X)

Please Note: The *Streptavidin-HRP (100X)* is in 50% glycerol. This solution is viscous. To ensure accurate dilution, allow *Streptavidin-HRP (100X)* to reach room temperature. Gently mix. Pipette *Streptavidin-HRP (100X)* slowly. Remove excess concentrate solution from pipette tip by gently wiping with clean absorbent paper.

1. Within 15 minutes of use, dilute 10 µL of this 100X concentrated solution with 1 mL of *Streptavidin-HRP Diluent* for each 8-well strip used in the assay. Label as Streptavidin-HRP Working Solution.

For Example:

# of 8-Well Strips	Volume of Streptavidin-HRP (100X)	Volume of Diluent
2	20 µL solution	2 mL
4	40 µL solution	4 mL
6	60 µL solution	6 mL
8	80 µL solution	8 mL
10	100 µL solution	10 mL
12	120 µL solution	12 mL

2. Return the unused *Streptavidin-HRP (100X)* to the refrigerator.

D. Dilution of Wash Buffer

Allow the *Wash Buffer Concentrate (25X)* to reach room temperature and mix to ensure that any precipitated salts have redissolved. Dilute 1 volume of the *Wash Buffer Concentrate (25X)* with 24 volumes of deionized water (e.g., 50 mL may be diluted up to 1.25 liters, 100 mL may be diluted up to 2.5 liters). Label as Working Wash Buffer.

Store both the concentrate and the Working Wash Buffer in the refrigerator. The diluted buffer should be used within 14 days.

ASSAY METHOD: PROCEDURE AND CALCULATIONS

Be sure to read the *Procedural Notes/Lab Quality Control* section before carrying out the assay.

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.

Note: A standard curve must be run with each assay.

1. Determine the number of 8-well strips needed for the assay. Insert these in the frame(s) for current use. (Re-bag extra strips and frame. Store these in the refrigerator for future use.)
2. Dilute serum and plasma samples 1:10 with *Standard Diluent Buffer*. Tissue culture samples may be assayed neat. (see **SAMPLE PREPARATION** section on page 12).
3. Add 25 µL of the *Incubation Buffer* to each well.
4. Add 100 µL of the *Standard Diluent Buffer* to the zero standard wells. Well(s) reserved for chromogen blank should be left empty.
5. Add 100 µL of standards, controls, or samples (serum and plasma prediluted) to the appropriate microtiter wells.

6. Pipette 50 µL of biotinylated *Hu Ang-2 Biotin Conjugate* solution into each well except the chromogen blank(s). Tap on the side of the plate for 30 seconds to mix.
7. Cover plate with *plate cover* and incubate for **2 hours at room temperature**.
8. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
9. Add 100 µL Streptavidin-HRP Working Solution to each well except the chromogen blank(s). (Prepare the working dilution as described in **REAGENT PREPARATION AND STORAGE**, Section C.)
10. Cover plate with the *plate cover* and incubate for **30 minutes at room temperature**.
11. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
12. Add 100 µL of *Stabilized Chromogen* to each well. The liquid in the wells will begin to turn blue.
13. Incubate for **30 minutes at room temperature and in the dark**. ***Please Note*: Do not cover the plate with aluminum foil or metalized mylar**. The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceed the limits of the instrument. The O.D. values at 450 nm can only be read after the *Stop Solution* has been added to each well. If using a reader that records only to 2.0 O.D., stopping the assay after 20 to 25 minutes is suggested.

13. Add 100 µL of *Stop Solution* to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.
14. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µL each of *Stabilized Chromogen* and *Stop Solution*. Read the plate within 30 minutes after adding the *Stop Solution*.
15. Plot on graph paper the absorbance of the standards against the standard concentration. (Optimally, the background absorbance may be subtracted from *all* data points, including standards, unknowns and controls, prior to plotting.) Draw the best smooth curve through these points to construct the standard curve. If using curve fitting software, the four parameter algorithm provides the best curve fit.
16. Read the Hu Ang-2 concentrations for unknown samples and controls from the standard curve plotted in step 15. **Multiply value(s) obtained for serum and plasma by 10 to correct for the overall 1:10 dilution in step 2.** Samples producing signals greater than that of the highest standard (2000 pg/mL) should be further diluted in the *Standard Diluent Buffer* as described in step 2 and reanalyzed. Multiply the concentration found by the appropriate dilution factor.

TYPICAL DATA

The following data were obtained for the various standards over the range of 0 to 2000 pg/mL Hu Ang-2.

Standard Hu Ang-2 (pg/mL)	Optical Density (450 nm)
0	0.049
	0.039
31.2	0.100
	0.090
62.5	0.145
	0.133
125	0.231
	0.227
250	0.476
	0.449
500	0.910
	0.934
1000	1.803
	1.731
2000	3.105
	3.168

LIMITATIONS OF THE PROCEDURE

Do not extrapolate the standard curve beyond the 2000 pg/mL standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute samples with concentrations exceeding the linear portion of the Standard Curve with the appropriate diluent buffer; reanalyze these and multiply results by the appropriate dilution factor.

The influence of various drugs and the use of biological fluids in place of tissue culture media have not been thoroughly investigated. The rate of degradation of native Hu Ang-2 in various matrices has not been investigated. The immunoassay literature contains frequent references to aberrant signals seen with some sera, attributed to heterophilic antibodies. Though such samples have not been seen to date, the possibility of this occurrence cannot be excluded.

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PERFORMANCE CHARACTERISTICS

SENSITIVITY

The minimum detectable dose of Hu Ang-2 is <6 pg/mL. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times, and calculating the corresponding concentration.

PRECISION

1. Intra-Assay Precision

Samples of known Hu Ang-2 concentration were assayed in replicates of 16 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	131.5	544.7	1480.4
SD	2.8	25.5	69.7
%CV	2.1	4.7	4.7

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision

Samples were assayed 48 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	129.1	527.8	1542.8
SD	5.6	34.1	135.7
%CV	4.4	6.5	8.8

SD = Standard Deviation
CV = Coefficient of Variation

LINEARITY OF DILUTION

Human serum, EDTA plasma, citrate plasma, heparin plasma, and tissue culture sample containing natural Hu Ang-2 were serially diluted in *Standard Diluent Buffer* over the range of the assay. Linear regression analysis of samples versus the expected concentration yielded average correlation coefficients of 0.998 for serum and 1.000 for EDTA plasma, citrate plasma, heparin plasma, and tissue culture medium.

RECOVERY

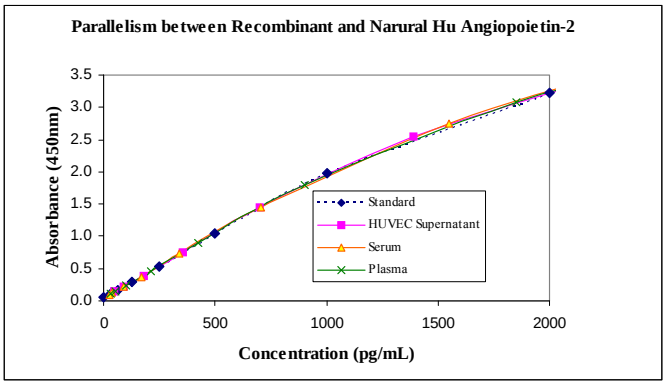
The recoveries of recombinant Hu Ang-2 added to human serum, EDTA plasma, citrate plasma, heparin plasma, and tissue culture medium were measured on the Invitrogen Hu Ang-2 ELISA.

Sample Type	Average % Recovery	Range
Serum*	101	92 – 111%
EDTA plasma*	99	94 – 103%
Citrate plasma*	108	100 – 119%
Heparin Plasma*	103	94 – 115%
EBM-2 + 2% fetal bovine serumº	119	113 - 127
RPMI + 10% calf serumº	95	93 – 96%
DMEM + 10% calf serumº	98	94 – 102%

*Serum and plasma were pre-diluted 10-fold as described in sample preparation procedure.
^Background levels of ANG-2 present in normal fetal bovine and calf serum were subtracted from their respective spiked matrix.
ºRecoveries in DMEM and RPMI supplemented with 10% fetal bovine serum were 138 and 142%, respectively.

PARALLELISM

Supernatants from human umbilical vein endothelial cells (HUVEC) as well as normal human serum and plasma samples were serially diluted in the *Standard Diluent Buffer*. The optical density of each dilution was plotted against the Hu Ang-2 standard curve. Parallelism was demonstrated by the figure below and indicated that the standard accurately reflects the Hu Ang-2 content in natural samples.



SPECIFICITY

Buffered solutions of a panel of substances ranging in concentrations from 10,000 to 50,000 pg/mL were assayed with the Invitrogen Hu Ang-2 kit and found to have no cross-reactivity: Human Angiopoietin-1, Angiopoietin-4, GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, and TNF-α; Mouse Angiopoietin-3, EGF, FGF-b, G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, PDGF-BB, TNF-α, and VEGF; Rat GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, and TNF-α; Monkey IL-8, MCP-1, MIP-1α, MIP-1β, and RANTES.

Random, normal serum samples from various species were also evaluated with the Invitrogen Hu Ang-2 kit. No cross-reactivity was observed with goat, hamster, mouse, and rat serum samples. There was moderate cross-reactivity with rabbit and swine, and significant cross-reactivity with fetal bovine, calf, horse, and monkey serum samples.

HIGH DOSE HOOK EFFECT

No hook effect was observed with concentrations up to 1 µg/mL.

EXPECTED VALUES

Fifty random serum and plasma samples and HUVEC cultured in EBM-2 medium, supplemented with growth factors and 2% fetal bovine serum were grown to 20-100% confluence and supernatants were evaluated for Ang-2 levels with the Invitrogen kit.

Sample	Range (pg/mL)	Average (pg/mL)
Serum (n=20)	621 - 2,236	1,444
EDTA plasma (n=10)	677 – 2,923	1,877
Citrate plasma (n=9)	950 – 2,709	1,806
Heparin plasma (n=10)	936 – 1,632	1,135
HUVEC Supernatants (n=6)	1,030 – 14,934	5,302

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Explanation of symbols

Symbol	Description	Symbol	Description
REF	Catalogue Number	LOT	Batch code
RUO	Research Use Only	IVD	*In vitro* diagnostic medical device
	Use by		Temperature limitation
	Manufacturer	EC REP	European Community authorised representative
[-]	Without, does not contain	[+]	With, contains
	Protect from light		Consult accompanying documents
	Directs the user to consult instructions for use (IFU), accompanying the product.		

Human Angiopoietin-2 Assay Summary