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Catalog No.: CKH119 **Lot No.:** TBD **Size:** 1 Plate (1 x 96 tests) **Expiration Date:** TBD

NOTE: this is a sample protocol which is subject to variation by Lot Number. Refer to the protocol inserted in your package for the current lot number specifications and expiration date or contact our technical support at tech@cellsciences.com

DESCRIPTION:

Angiostatin was observed initially as an angiogenesis inhibitor in serum and urine of mice bearing a Lewis Lung Carcinoma (3LL cells). Angiostatin is produced by the proteolytic cleavage of plasminogen by a serine protease from several human prostate carcinoma cell lines. The production of angiostatin by human pancreatic cancer cells can be inhibited by TGF-beta-1 in participation with plasminogen activator inhibitor 1 (PAI1). Angiostatin specifically inhibits endothelial cell proliferation. In an animal tumor model the factor produced by the primary tumor suppresses the growth of its remote metastases, which neovascularize and grow after tumor removal.

The Human Angiostatin ELISA Kit is an *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of human angiostatin in serum, plasma (heparin), and cell culture supernatants. This assay employs an antibody specific for human angiostatin coated on a 96-well plate. Standards and samples are pipetted into the wells and angiostatin present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human angiostatin antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of angiostatin bound. The stop solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

REAGENTS AND MATERIALS SUPPLIED WITH THE KIT:

Description	Quantity	Storage/Stability after Preparation
CKH119-A. Angiostatin Microplate	1 plate: 96 wells (12 strips x 8 wells) coated with Anti-Human Angiostatin	1 month at 4 °C *
CKH119-B. Wash Buffer Concentrate (20x)	25 mL	1 month at 4 °C
CKH119-C. Protein Standard (Recombinant Human Angiostatin)	2 vials (1 vial is enough to run each standard in duplicate.)	1 week at -80 °C
CKH119-D. Plate Covers	2 covers	
CKH119-E. Assay Diluent Concentrate (5x)	15 mL	1 month at 4 °C
CKH119-F. Detection Antibody (Biotinylated Anti-Human Angiostatin)	2 vials (each vial is enough to assay half microplate)	5 days at 4 °C
CKH119-G. HRP-Streptavidin Concentrate (800x)	200 µL	Do not store/reuse
CKH119-H. TMB One-Step Substrate Reagent	12 mL	N/A
CKH119-I. Stop Solution (0.2 M sulfuric acid)	8 mL	N/A

* Return unused wells to the pouch containing desiccant pack and reseal along the entire edge.

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STORAGE/STABILITY:

The entire kit may be stored at 2-8 °C for up to 6 months from date of shipment, or at -20 °C for up to 1 year from the date of shipment. For prepared reagent storage, see the table above. ***Avoid repeated freeze-thaw cycles.***

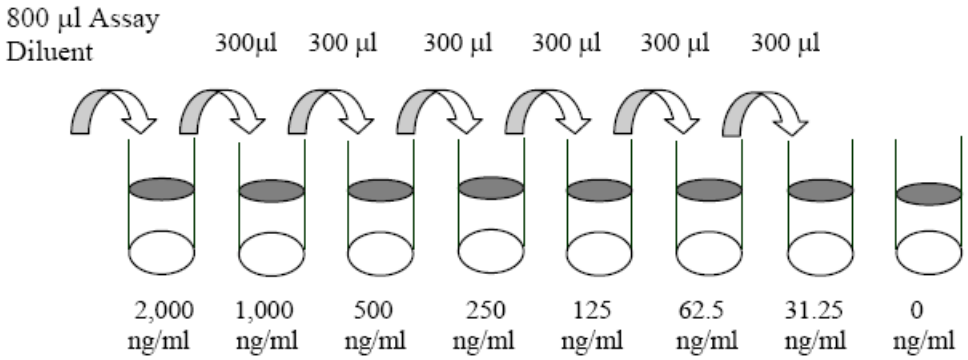
MATERIALS AND REAGENTS REQUIRED BUT NOT PROVIDED:

- Microplate reader capable of measuring absorbance at 450 nm
- Precision pipettes to deliver 2 µL to 1 mL volumes
- Adjustable 1-25 mL pipettes for reagent preparation
- 100 mL and 1 Liter graduated cylinders
- Absorbent paper
- Distilled or deionized water
- Log-log graph paper or computer and software for ELISA data analysis
- Tubes to prepare standard or sample dilutions

PREPARATION OF KIT REAGENTS:

1. Bring **all reagents and samples** to room temperature (18–25 °C) before use.
2. **Assay Diluent** (CKH119-E) should be diluted 5-fold with deionized or distilled water before use. *1x Assay Diluent may be stored 1 month at 4 °C.*
3. **Sample Dilution**: If necessary, 1x Assay Diluent (step 2 above) is used for dilution of serum/ plasma/ culture supernatant samples. Suggested dilution for normal serum/ plasma: 3-30 fold*.

   **note that levels of Angiostatin may vary between different samples. Optimal dilution factors for each sample must be determined by the investigator.*
4. **Standard Preparation**: Briefly spin the vial of CKH119-C and then add 800 µL 1x Assay Diluent (step 2 above) into CKH119-C vial to prepare a 2,000 ng/mL standard. Dissolve the powder thoroughly by gently mixing. Label 7 additional tubes for the standard dilution series. Pipette 300 µL 1x Assay Diluent into each tube. Use the 2,000 ng/mL standard to produce a dilution series (shown below). Mix each tube thoroughly by gently vortexing before the next transfer. 1x Assay Diluent serves as the zero standard (0 ng/mL). *Prepared Standard may be stored 1 week at -80 °C.*



5. **Wash Buffer**: If the Wash Concentrate (20x; CKH119-B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to yield 400 mL of 1x Wash Buffer. *1x Wash Buffer may be stored 1 month at 4 °C.*

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6. **Detection Antibody**: Briefly spin the Detection Antibody vial (CKH119-F) before use. Add 100 µL of 1x Assay Diluent into the vial to prepare the detection antibody concentrate. Pipette up and down to mix gently. The detection antibody concentrate should be diluted 80-fold with 1x Assay Diluent and used in step 5 of ELISA Method. *The prepared detection antibody concentrate may be stored 5 days at 4°C.*

7. **HRP-Streptavidin:** Briefly spin the HRP-Streptavidin concentrate vial (CKH119-G) and pipette up and down to mix gently before use, as precipitates may form during storage. HRP-Streptavidin concentrate should be diluted 800-fold with 1x Assay Diluent. *Do not store or reuse the diluted.*

   For example: Briefly spin the vial (CKH119-G) and pipette up and down to mix gently. Add 15 µL of HRP-Streptavidin concentrate into a tube with 11.985 mL 1x Assay Diluent to prepare 12 mL of an 800-fold diluted HRP-Streptavidin solution. Mix thoroughly. Make fresh on the day of use.

ELISA METHOD:

1. Bring all reagents and samples to room temperature (18-25 °C) before use. It is recommended that all standards and samples be run at least in duplicate.
2. Label removable 8-well strips as appropriate for your experiment. Return any unused wells to the pouch containing desiccant pack and reseal along the entire edge.
3. Add 100 µL of each standard (see **Preparation of Kit Reagents: Standard Preparation**) and sample into appropriate wells. Cover wells (CKH119-D Plate cover) and incubate for 2.5 hours at room temperature with gentle shaking.
4. Discard the solution and wash 4 times with 1x Wash Solution. Wash by filling each well with Wash Buffer (300 µL) using a multi-channel pipette 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.
5. Add 100 µL of 1x prepared biotinylated antibody (see **Preparation of Kit Reagents: Detection Antibody**) to each well. Incubate for 1 hour at room temperature with gentle shaking.
6. Discard the solution. Repeat the wash as in Step 4.
7. Add 100 µL of prepared Streptavidin solution (see **Preparation of Kit Reagents: HRP-Streptavidin**) to each well. Incubate for 45 minutes at room temperature with gentle shaking.
8. Discard the solution. Repeat the wash as in Step 4.
9. Add 100 µL of **TMB One-Step Substrate Reagent** (CKH119-H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.
10. Add 50 µL of **Stop Solution** (CKH119-I) to each well. Read at 450 nm immediately.

ASSAY PROCEDURE SUMMARY:

1. Prepare all reagents, samples and standards as instructed.
2. Add 100 µL standard or sample to each well. Incubate 2.5 hours at RT.
3. Add 100 µL prepared biotin antibody to each well. Incubate 1 hour at RT.
4. Add 100 µL prepared Streptavidin solution. Incubate 45 minutes at RT.
5. Add 100 µL TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at RT.
6. Add 50 µL Stop Solution to each well. Read at 450 nm immediately.

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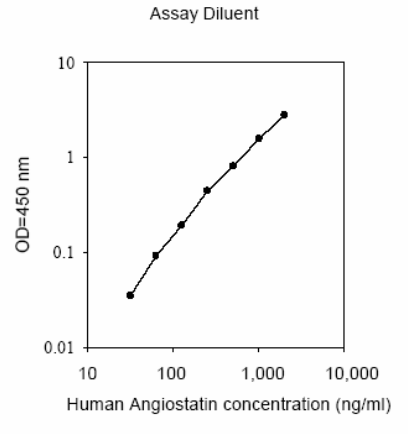
CALCULATION OF RESULTS:

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the average zero standard optical density. Plot the standard curve on log-log graph paper or using Sigma plot software, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points.

Typical Data:
This standard curve is for demonstration only. A standard curve must be run with each assay.



Sensitivity:
The minimum detectable dose of Human Angiostatin was determined to be 20,000 pg/mL.

Minimum detectable dose is defined as the analyte concentration resulting in an absorbance that is 2 standard deviations higher than that of the blank (diluent buffer).

Spiking & Recovery:
Recovery was determined by spiking various levels of Human Angiostatin into sample types listed. Mean recoveries are as follows:

Sample Type	Average % Recovery	Range (%)
Serum	92.45	81-101
Plasma	93.31	82-102
Cell culture media	95.43	83-104

Linearity:

Sample Type		Serum	Plasma	Cell Culture Media
1:2	Avg. % of Expected	91	92	93
	Range (%)	81-101	82-102	83-103
1:4	Avg. % of Expected	93	95	96
	Range (%)	82-102	83-103	85-104

REPRODUCIBILITY:

Intra-Assay CV%: < 10%

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Inter-Assay CV%: < 12%

SPECIFICITY:

Cross Reactivity: This ELISA kit shows no cross-reactivity with any of the cytokines tested (human Angiopoietin-1, B7-1, BMP-7, CD14, CD30, CD40, CD40 Ligand, CTLA-4, CXCL16, Dkk-4 , DR6, Endostatin, E-Selectin, Follistatin, HB-EGF, HVEM, ICAM-2, IGF-II, IL-10 Ra, IL-10 Rb, IL-18, IL-9 , IL-2 Ra, IL-2 Rb, IL-5 Ra, LAP, L-Selectin, M-CSF R, MMP-2, 3, 7, 8, 9, 10 and 12, PDGF-AB, SDF-1b, Tie-1, Tie-2, TIMP-3).

TROUBLESHOOTING GUIDE:

Problem	Cause	Solution
Poor standard curve	• Inaccurate pipetting • Improper standard dilution	• Check pipettes • Ensure a brief spin of Item C and dissolve the powder thoroughly by a gentle mix.
Low signal	• Improper preparation of standard and/or biotinylated antibody • Too brief incubation times • Inadequate reagent volumes or improper dilution	• Briefly spin down vials before opening. Dissolve the powder thoroughly. • Ensure sufficient incubation time; ELISA Method Step 2 may change to overnight. • Check pipettes and ensure correct preparation.
Large CV	• Inaccurate pipetting • Air bubbles in wells	• Check pipettes. • Remove bubbles in wells.
High background	• Plate is insufficiently washed • Contaminated wash buffer	• Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed. • Make fresh wash buffer.
Low sensitivity	• Improper storage of the ELISA Kit • Stop solution	• Store your standard at <-70°C after reconstitution, other reagents at 4°C. Keep substrate solution protected from light. • Stop solution should be added to each well before measure.

NOT FOR HUMAN USE. FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC OR THERAPEUTIC USE.

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