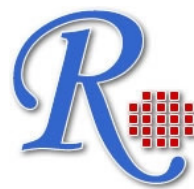


RayBio® Human Angiostatin ELISA Kit

**User Manual
(Revised Mar 1, 2012)**

**RayBio® Human Angiostatin
ELISA Kit Protocol**

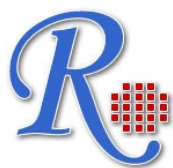
(Cat#: ELH-Angiostatin-001)



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**RayBio® Human Angiostatin
ELISA Kit Protocol**

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I. INTRODUCTION

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 type-1. 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 RayBio® Human Angiostatin ELISA (Enzyme-Linked Immunosorbent Assay) kit is an *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of human Angiostatin in serum, plasma (heparin), cell culture supernatants and urine. 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.

II. REAGENTS

1. Angiostatin Microplate (Item A): 96 wells (12 strips x 8 wells) coated with anti-human Angiostatin.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml of 20x concentrated solution.
3. Standards (Item C): 2 vials, recombinant human Angiostatin.

4. Assay Diluent (Item E): 15 ml of 5x concentrated buffered. For Standard/Sample (serum/plasma samples/cell culture medium/urine) diluent.
5. Detection Antibody Angiostatin (Item F): 2 vial of biotinylated anti-human Angiostatin (each vial is enough to assay half microplate).
6. HRP-Streptavidin Concentrate (Item G): 200 μ l of 800x concentrated HRP-conjugated streptavidin.
7. TMB One-Step Substrate Reagent (Item H): 12 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution.
8. Stop Solution (Item I): 8 ml of 0.2 M sulfuric acid.

III. STORAGE

May be stored for up to 6 months at 2° to 8°C from the date of shipment. Standard (recombinant protein) should be stored at -20 °C or -80 °C (recommended at -80 °C) after reconstitution. Opened Microplate Wells or reagents may be store for up to 1 month at 2° to 8°C. Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

Note: the kit can be used within one year if the whole kit is stored at -20 °C . Avoid repeated freeze-thaw cycles.

IV. ADDITIONAL MATERIALS REQUIRED

- 1 Microplate reader capable of measuring absorbance at 450 nm.
- 2 Precision pipettes to deliver 2 μ l to 1 ml volumes.
- 3 Adjustable 1-25 ml pipettes for reagent preparation.
- 4 100 ml and 1 liter graduated cylinders.
- 5 Absorbent paper.
- 6 Distilled or deionized water.
- 7 Log-log graph paper or computer and software for ELISA data analysis.
- 8 Tubes to prepare standard or sample dilutions.

V. REAGENT PREPARATION

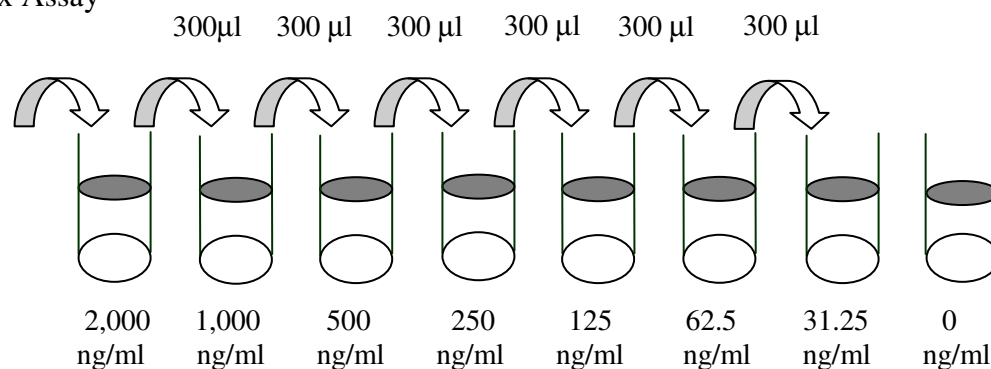
1. Bring all reagents and samples to room temperature (18 - 25°C) before use.
2. Sample dilution: If your samples need to be diluted, Assay Diluent A (Item D) should be used for dilution of serum/plasma samples. 1x Assay Diluent B (Item E) should be used for dilution of culture supernatants and urine.

Suggested dilution for normal serum/plasma: 3-30 fold*.

*Please note that levels of the target protein may vary between different specimens. Optimal dilution factors for each sample must be determined by the investigator.

3. Assay Diluent (Item E) should be diluted 5-fold with deionized or distilled water before use.
4. Preparation of standard: **Briefly spin the vial of Item C** and then add 800 μl 1x Assay Diluent (Item E, Assay Diluent should be diluted 5-fold with deionized or distilled water) into Item C vial to prepare a 2,000 ng/ml standard. **Dissolve the powder thoroughly by a gentle mix.** 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 before the next transfer. 1x Assay Diluent serves as the zero standard (0 ng/ml).

800 μl 1x Assay
Diluent



5. If the Wash Concentrate (20x) (Item 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.
6. Briefly spin the Detection Antibody vial (Item F) before use. Add 100 μ l of 1x Assay Diluent into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days). The detection antibody concentrate should be diluted 80-fold with 1x Assay Diluent and used in step 4 of Part VI Assay Procedure.
7. Briefly spin the HRP-Streptavidin concentrate vial (Item G) before use. HRP-Streptavidin concentrate should be diluted 800-fold with 1x Assay Diluent.



For example: Briefly spin the vial (Item G) and pipette up and down to mix gently . Add 15 μ l of HRP-Streptavidin concentrate into a tube with 12 ml 1x Assay Diluent to prepare a final 800 fold diluted HRP-Streptavidin solution (don't store the diluted solution for next day use). Mix well.

VI. ASSAY PROCEDURE:

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. Add 100 μ l of each standard (see Reagent Preparation step 2) and sample into appropriate wells. Cover well and incubate for 2.5 hours at room temperature or over night at 4°C with gentle shaking.

3. Discard the solution and wash 4 times with 1x Wash Solution. Wash by filling each well with Wash Buffer (300 μ l) 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.
4. Add 100 μ l of 1x prepared biotinylated antibody (Reagent Preparation step 6) to each well. Incubate for 1 hour at room temperature with gentle shaking.
5. Discard the solution. Repeat the wash as in step 3.
6. Add 100 μ l of prepared Streptavidin solution (see Reagent Preparation step 7) to each well. Incubate for 45 minutes at room temperature with gentle shaking.
7. Discard the solution. Repeat the wash as in step 3.
8. Add 100 μ l of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.
9. Add 50 μ l of Stop Solution (Item I) to each well. Read at 450 nm immediately.

VII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards as instructed.

2. Add 100 μ l standard or sample to each well.
Incubate 2.5 hours at room temperature or over night at 4°C.


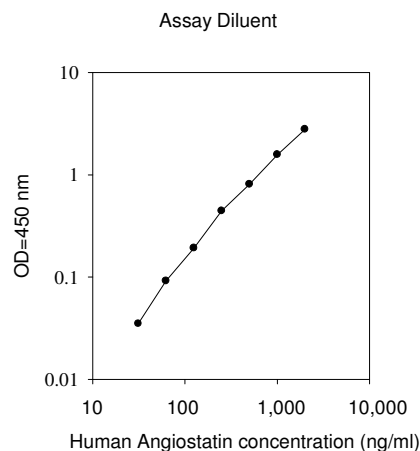
3. Add 100 μ l prepared biotin antibody to each well.
Incubate 1 hour at room temperature.
↓
4. Add 100 μ l prepared Streptavidin solution.
Incubate 45 minutes at room temperature.
↓
5. Add 100 μ l TMB One-Step Substrate Reagent to each well.
Incubate 30 minutes at room temperature.
↓
6. Add 50 μ l Stop Solution to each well.
Read at 450 nm immediately.

VIII. 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.

A. TYPICAL DATA

These standard curves are for demonstration only. A standard curve must be run with each assay.



B. SENSITIVITY

The minimum detectable dose of Angiostatin is typically less than 20 ng/ml.

C. RECOVERY

Recovery was determined by spiking various levels of human Angiostatin into human serum, plasma and cell culture media. 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

D. LINEARITY

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

E. REPRODUCIBILITY

Intra-Assay: CV<10%

Inter-Assay: CV<12%

IX. SPECIFICITY

Cross Reactivity: This ELISA kit shows no cross-reactivity with any of the cytokines tested (e.g., 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).

X. REFERENCES

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2. Dong Z et al. Macrophage-derived metalloelastase is responsible for the generation of angiostatin in Lewis lung carcinoma. *Cell* 88: 801-810 (1997);
3. Cao Y et al. Expression of angiostatin cDNA in a murine fibrosarcoma suppresses primary tumor growth and produces long-term dormancy of metastases. *Journal of Clinical Investigation* 101: 1055-1063 (1998);

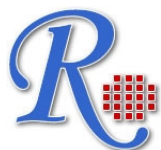
XI. TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Poor standard curve	<ol style="list-style-type: none"> 1. Inaccurate pipetting 2. Improper standard dilution 	<ol style="list-style-type: none"> 1. Check pipettes 2. Ensure a brief spin of Item C and dissolve the powder thoroughly by a gentle mix.
2. Low signal	<ol style="list-style-type: none"> 1. Too brief incubation times 2. Inadequate reagent volumes or improper dilution 	<ol style="list-style-type: none"> 1. Ensure sufficient incubation time; assay procedure step 2 may change to over night 2. Check pipettes and ensure correct preparation
3. Large CV	<ol style="list-style-type: none"> 1. Inaccurate pipetting 	<ol style="list-style-type: none"> 1. Check pipettes
4. High background	<ol style="list-style-type: none"> 1. Plate is insufficiently washed 2. Contaminated wash buffer 	<ol style="list-style-type: none"> 1. Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed. 2. Make fresh wash buffer
5. Low sensitivity	<ol style="list-style-type: none"> 1. Improper storage of the ELISA kit 2. Stop solution 	<ol style="list-style-type: none"> 1. Store your standard at $<-20^{\circ}\text{C}$ after reconstitution, others at 4°C. Keep substrate solution protected from light 2. Stop solution should be added to each well before measure

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