Introduction and Background

A. Overview

Angiogenin (Ang) also known as ribonuclease 5 is a protein that in humans is encoded by the ANG gene.[1] Angiogenin is a potent stimulator of new blood vessel formation. It hydrolyzes cellular tRNAs resulting in decreased protein synthesis and is similar to pancreatic ribonuclease.[2] Hooper et al. (2003) reviewed the evidence that angiogenins are involved in host defense and noted that inflammation provokes upregulated ANG mRNA expression in liver and an increase in detectable ANG protein in serum. Weremowicz et al. (1989, 1990) assigned the human angiogenin gene to chromosome 14q11.

B. Test Principle

Human ANG ELISA Kit was based on standard sandwich enzyme-linked immune-sorbent assay technology. Human ANG specific-specific polyclonal antibodies were precoated onto 96-well plates. The human specific detection polyclonal antibodies were biotinylated. The test samples and biotinylated detection antibodies were added to the wells subsequently and then followed by washing with PBS or TBS buffer. Avidin-Biotin-Peroxidase Complex was added and unbound conjugates were washed away with PBS or TBS buffer. HRP substrate TMB was used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the human ANG amount of sample captured in plate.

C. Notice for Application of Kit

1. Before using Kit, spin tubes and bring down all components to bottom of tube.
2. Duplicate well assay was recommended for both standard and sample testing.
3. Don't let 96-well plate dry, dry plate will inactivate active components on plate.
4. In order to avoid marginal effect of plate incubation due to temperature difference (reaction may be stronger in the marginal wells), it is suggested that the diluted ABC and TMB solution will be pre-warmed in 37℃ for 30 min before using.

D. Application

For quantitative detection of human ANG in sera, plasma, body fluids, tissue lysates or cell culture supernates.

Material and Method

A. List of component

1. Lyophilized recombinant human ANG standard: 5 ng/tube×2.
2. One 96-well plate precoated with anti- human ANG antibody.
3. Sample diluent buffer: 30 ml.
4. Biotinylated anti- human ANG antibody: 130 µl, dilution 1:100.
5. Antibody diluent buffer: 12 ml.
6. Avidin-Biotin-Peroxidase Complex (ABC): 130 µl, dilution 1:100.
7. ABC diluent buffer: 12 ml.
8. TMB color developing agent: 10 ml.
9. TMB stop solution: 10 ml.

B. Additional Required Materials But Not Provided

1. Microplate reader in standard size.
2. Automated plate washer.
3. Adjustable pipettes and pipette tips. Multichannel pipettes are recommended in the condition of large amount of samples in the detection.
4. Clean tubes and Eppendorf tubes.
5. Washing buffer (neutral PBS or TBS).

   Preparation of 0.01 M **TBS:** Add 1.2 g Tris, 8.5 g NaCl; 450 µl of purified acetic acid or 700 µl of concentrated hydrochloric acid to 1000 ml H_2O and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1 L.

   Preparation of 0.01 M **PBS:** Add 8.5 g sodium chloride, 1.4 g Na_2HPO_4 and 0.2 g NaH_2PO_4 to 1000 ml distilled water and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1 L.

C. Preparation of reagent

1. Reconstitution of the human ANG standard: ANG standard solution should be prepared no more than 2 hours prior to the experiment. Two tubes of ANG standard (5ng per tube) are included in each kit. Use one tube for each experiment.
   a. 10,000pg/ml of human ANG standard solution: Add 0.5 ml sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly.
   b. 5000pg/ml→156pg/ml of human ANG standard solutions: Label 6 Eppendorf tubes with 5000pg/ml, 2500pg/ml, 1250pg/ml, 625pg/ml, 313pg/ml, 156pg/ml, respectively. Aliquot 0.3 ml of the sample diluent buffer into each tube. Add 0.3 ml of the above 10, 000pg/ml ANG standard solution into 1st tube and mix. Transfer 0.3 ml from 1st tube to 2nd tube and mix. Transfer 0.3 ml from 2nd tube to 3rd tube and mix, and so on.

Note: The standard solutions are best used within 2 hours. The 5 ng/ml standard solution may be stored at

4℃ for up to 12 hours, or at -20℃ for up to 48 hours. Avoid repeated freeze-thaw cycles.

2. Preparation of biotinylated anti-human ANG antibody working solution: The solution should be prepared no more than 2 hours prior to the experiment.
   a. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
   b. Biotinylated anti-human ANG antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly.
3. Preparation of Avidin-Biotin-Peroxidase Complex (ABC) working solution: The solution should be prepared no more than 1 hour prior to the experiment.
   a. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
   b. Avidin-Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with the ABC dilution buffer and mixed thoroughly.

Sample Preparation and Storage

Store samples to be assayed within 24 hours at 2-8℃. For long-term storage, aliquot and freeze samples at -20℃. Avoid repeated freeze-thaw cycles.

- **Cell culture supernate, tissue lysate or body fluids**: Remove particulates by centrifugation, analyze immediately or aliquot and store at -20℃.
- **Serum**: Allow the serum to clot in a serum separator tube (about 30 min) at room temperature. Centrifuge at approximately 1000 X g for 15 min. Analyze the serum immediately or aliquot and store frozen at -20℃.
- **Plasma**: Collect plasma using heparin, EDTA as an anticoagulant. Centrifuge for 30 min at 1000 x g within 30 min of collection. Analyze immediately or aliquot and store frozen at -20℃. Citrate is not recommended as the anticoagulant.

Sample Dilution Guideline

The user needs to estimate the concentration of the target protein in the sample and select a proper dilution factor so that the diluted target protein concentration falls near the middle of the linear regime in the standard curve. Dilute the sample using the provided diluent buffer. The following is a guideline for sample dilution. Several trials may be necessary in practice. **The sample must be well mixed with the diluents buffer.**

- High target protein concentration (100-1000 ng/ml). The working dilution is 1:100. i.e. Add 1 µl sample into 99 µl sample diluent buffer.

- Medium target protein concentration (1-100 ng/ml). The working dilution is 1:10. i.e. Add 10 µl sample into 90 µl sample diluent buffer.

- Low target protein concentration (156-10,000 pg/ml). The working dilution is 1:2. i.e. Add 50 µl sample to 50 µl sample diluent buffer.

- Very Low target protein concentration (≦ 156 pg/ml). No dilution necessary, or the working dilution is 1:2.

Reagent Preparation and Storage

1. Reconstitution of the human ANG standard: ANG standard solution should be prepared no more than 2 hours prior to the experiment. Two tubes of ANG standard (5ng per tube) are included in each kit. Use one tube for each experiment.
   a. 10,000 pg/ml of human ANG standard solution: Add 0.5 ml sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly.
   b. 5000 pg/ml→156 pg/ml of human ANG standard solutions: Label 6 Eppendorf tubes with 5000pg/ml, 2500pg/ml, 1250pg/ml, 625pg/ml, 313pg/ml, 156pg/ml, respectively. Aliquot 0.3 ml of the sample diluent buffer into each tube. Add 0.3 ml of the above 10, 000pg/ml ANG standard solution into 1st tube and mix. Transfer 0.3 ml from 1st tube to 2nd tube and mix. Transfer 0.3 ml from 2nd tube to 3rd tube and mix, and so on.

   Note: The standard solutions are best used within 2 hours. The 5 ng/ml standard solution may be stored at 4℃ for up to 12 hours, or at -20℃ for up to 48 hours. Avoid repeated freeze-thaw cycles.
2. Preparation of biotinylated anti-human ANG antibody working solution: The solution should be prepared no more than 2 hours prior to the experiment.
   a. The total volume should be: 0.1 ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
   b. Biotinylated anti-human ANG antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly.
3. Preparation of Avidin-Biotin-Peroxidase Complex (ABC) working solution: The solution should be prepared no more than 1 hour prior to the experiment.
   a. The total volume should be: 0.1 ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
   b. Avidin-Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with the ABC dilution buffer and mixed thoroughly.

D. Stability and storage

Store at 4℃ for frequent use, at -20℃ for infrequent use. Avoid multiple freeze-thaw cycles (Shipped with wet ice.)

E. Expiration

Four months at 4℃ and eight months at -20℃.

F. Protocol

The ABC working solution and TMB color developing agent must be kept warm at 37℃ for 30 min before use. When diluting samples and reagents, they must be mixed completely and evenly. Standard ANG detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of ANG amount in samples.

1. Aliquot 0.1ml per well of the 10,000pg/ml, 5000pg/ml, 2500pg/ml, 1250pg/ml, 625pg/ml, 313pg/ml, 156pg/ml human ANG standard solutions into the precoated 96-well plate. Add 0.1ml of the sample diluent buffer into the control well (Zero well). Add 0.1ml of each properly diluted sample of human sera, plasma, body fluids, tissue lysates or cell culture supernatants to each empty well. **See "Sample Dilution Guideline" above for details.** We recommend that each human ANG standard solution and each sample is measured in duplicate.
2. Seal the plate with the cover and incubate at 37℃ for 90 min.
3. Remove the cover, discard plate content, and blot the plate onto paper towels or other absorbent material. Do NOT let the wells completely dry at any time.
4. Add 0.1 ml of biotinylated anti-human ANG antibody working solution into each well and incubate the plate at 37℃ for 60 min.
5. Wash the plate three times with 0.01 M TBS or 0.01 M PBS, and each time let washing buffer stay in the wells for 1 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. **Plate Washing Method:** Discard the solution in the plate without touching the side walls. Blot the plate onto paper towels or other absorbent material. Soak each well with at least 0.3 ml PBS or TBS buffer for 1~2 minutes. Repeat this process two additional times for a total of THREE washes. Note: For automated washing, aspirate all wells and wash THREE times with PBS or TBS buffer, overfilling wells with PBS or TBS buffer. Blot the plate onto paper towels or other absorbent material.)
6. Add 0.1 ml of prepared ABC working solution into each well and incubate the plate at 37℃ for 30 min.
7. Wash plate 5 times with 0.01 M TBS or 0.01 M PBS, and each time let washing buffer stay in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. (See Step 5 for plate washing method).
8. Add 90 µl of prepared TMB color developing agent into each well and incubate plate at 25℃ in dark for 15-20 min (**Note:** For reference only, the optimal incubation time should be determined by end user. And the shades of blue can be seen in the wells with the four most concentrated human Activin A standard solutions; the other wells show no obvious color).
9. Add 0.1 ml of prepared TMB stop solution into each well. The color changes into yellow immediately.
10. Read the O.D. absorbance at 450nm in a microplate reader within 30 min after adding the stop solution. For calculation, (the relative $O.D._{450}$) = (the $O.D._{450}$ of each well) – (the $O.D._{450}$ of Zero well). The standard curve can be plotted as the relative $O.D._{450}$ of each standard solution (Y) vs. the respective concentration of the standard solution (X). The human ANG concentration of the samples can be interpolated from the standard curve.

Note: if the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

Summary

1. Add samples and standards and incubate the plate at 37℃ for 90 min. Do not wash.
2. Add biotinylated antibodies and incubate the plate at 37℃ for 60 min. Wash plate 3 times with 0.01 M TBS.
3. Add ABC working solution and incubate the plate at 37℃ for 30 min. Wash plate 5 times with 0.01 M TBS.
4. Add TMB color developing agent and incubate the plate at 37℃ for 25-30 min.
5. Add TMB stop solution and read.

G. Performance Characteristics

Typical result

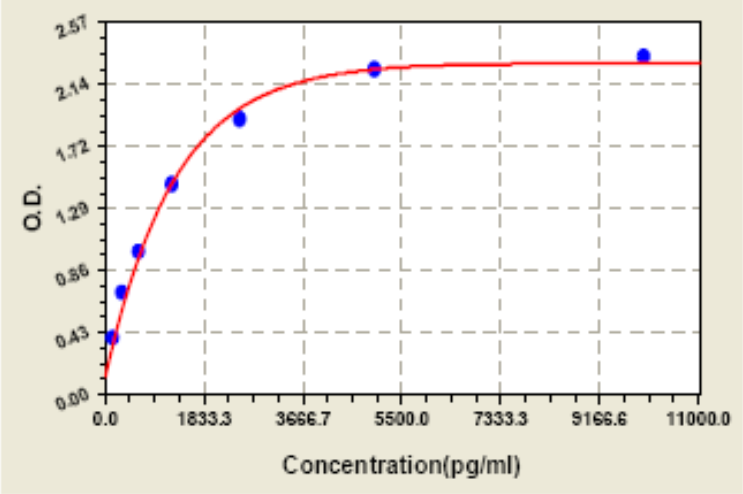
Typical Data Obtained from Human ANG

(TMB reaction incubate at 25℃ for 30 min)

Concentration	0.0pg/ml	156pg/ml	313pg/ml	625pg/ml	1250pg/ml	2500pg/ml	5000pg/ml	10,000pg/ml
O.D.	0.038	0.400	0.708	0.986	1.457	1.907	2.251	2.342

Typical Standard Curve

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



Range

78 pg/ml-5000 pg/ml

Sensitivity

< 12 pg/ml

Specificity

No detectable cross-reactivity with any other cytokine.

References

1. Weremowicz S, Fox EA, Morton CC, Vallee BL (1990). "Localization of the human angiogenin gene to chromosome band 14q11, proximal to the T cell receptor alpha/delta locus". *Am J Hum Genet* 47 (6): 973–81.
2. "Entrez Gene: ANG angiogenin, ribonuclease, RNase A family, 5". http://www.ncbi.nlm.nih.gov/sites/entrez?Db=gene&Cmd=ShowDetailView&TermToSearch=283.