Innovative RESEARCH Results Driven Research

Human Angiotensin II ELISA Kit

Introduction

Angiotensin II, a main effector peptide in the renin-angiotensin system, acts as a growth promoting and angiogenic factor via type-1 angiotensin II receptors (1). Angiotensin II is suggested to be involved in the regulation of cell proliferation (2), angiogenesis (3), inflammation (4), and cancer (1).

Principal of the Assay

The Angiotensin II ELISA kit is designed for detection of human Angiotensin II in plasma, serum, and cell culture supernatants. This assay employs a quantitative sandwich enzyme immunoassay technique that measures Angiotensin II in less than 5 hours. A polyclonal antibody specific for Angiotensin II has been pre-coated onto a microplate. The Angiotensin II in standards and samples is sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for Angiotensin II, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Caution and Warning

- **Prepare all reagents (working diluent buffer, wash buffer, standards, biotinylated-antibody, and SP conjugate) as instructed, prior to running the assay.**
- **Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this protocol. However, the user should determine the optimal dilution factor.**
- **Spin down the SP conjugate vial and the biotinylated-antibody vial before opening and using contents.**
- This kit is for research use only.
- The kit should not be used beyond the expiration date.
- The Stop Solution is an acid solution.

Reagents

- **Angiotensin II Microplate:** A 96-well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against Angiotensin II.
- **Sealing Tapes:** Each kit contains 3 pre-cut, pressure-sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **Angiotensin II Standard:** Angiotensin II in a buffered protein base (8 ng, lyophilized).
- **Biotinylated Angiotensin II Antibody:** A 80-fold biotinylated polyclonal antibody against Angiotensin II (100 µl).
- **EIA Diluent Concentrate (10x)**: A 10-fold concentrated buffered protein base (20 ml).

- **Wash Buffer Concentrate (20x)**: A 20-fold concentrated buffered surfactant (30 ml, 2 bottles).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (80 µl).
- **Chromogen Substrate**: A ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).
- **Stop Solution**: A 0.5 N hydrochloric acid to stop the chromogen substrate reaction (12 ml).

Storage Condition

- Store components of the kit at 2-8^{0}C or -20^{0}C upon arrival up to the expiration date.
- Store SP Conjugate and Biotinylated Antibody at -20^{0}C
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8^{0}C
- Opened unused microplate wells may be returned to the foil pouch with the desiccant packs. Reseal along zip-seal. May be stored for up to 1 month in a vacuum desiccator.
- Diluent (1x) may be stored for up to 1 month at 2-8^{0}C.
- Store Standard at 2-8^{0}C before reconstituting with Diluent and at -20^{0}C after reconstituting with Diluent.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm
- Pipettes (1-20 µl, 20-200 µl, 200-1000µl and multiple channel)
- Deionized or distilled reagent grade water

Sample Collection, Preparation and Storage

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 2000 x g for 10 minutes and assay. Remove plasma and assay. The undiluted samples can be stored at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles. (The plasma samples need to contain o-phenanthroline 0.44 mM, EDTA 25 mM, p-hydroxy-mercuribenzoic acid 1mM and pepstatin A 0.12 mM)

  For low level of Angiotensin II, please use the extraction protocol as follows:
  Angiotensin II extraction protocol

  Buffer A: 1% trifluoroacetic acid (TFA, HPLC Grade) in H_2O
  Buffer B: 60% acetonitrile (HPLC Grade) in 1% TFA

  1. Acidify the sample with equal amount of Buffer A (1 ml sample: 1 ml Buffer A). Mix and centrifuge samples at 6,000 x g for 20 minutes at 4°C.
  2. Pack an extraction column using 200 mg of C18 resin. Pre-equilibrate the column with 1 ml of Buffer B once and then with 3 ml of Buffer A three times.
  3. Load the acidified plasma solution onto the pre-treated C18 column.
  4. Slowly wash the column with 3 ml of Buffer A twice.
  5. Elute the peptide slowly with 3 ml of Buffer B once and collect the eluant.
  6. Evaporate and dry the eluant in a freeze dryer or use a suitable substitute method.
  7. Keep the dried extract at -20°C and perform the assay as early as possible. Reconstitute the dried extract with 200 µl of MIX Diluent before the assay. Check sample pH with pH papers. If sample pH is below 6.5, neutralize the sample with 20 µl of 1M NaH_2PO_4. If the peptide value exceeds or does not fall in the range of detection, dilute or concentrate the sample accordingly.

- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2000 x g for 10 minutes. Remove serum and assay. The undiluted samples can be stored at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles. (The serum samples need to contain o-phenanthroline 0.44 mM, EDTA 25 mM, p-hydroxy-mercuribenzoic acid 1mM and pepstatin A 0.12 mM)
  For low level of Angiotensin II, please use the **extraction protocol** as described above.

- **Cell Culture Supernatants:** The cell culture media is advised to contain o-phenanthroline 0.44 mM, p-hydroxy-mercuribenzoic acid 1mM and pepstatin A 0.12 mM during the culture. After cell growth to ideal density, take out culture media and centrifuge cell culture media at 2000x g for 10 minutes to remove debris. Collect supernatants and add fresh o-phenanthroline 0.44 mM, EDTA 25 mM, p-hydroxy-mercuribenzoic acid 1mM and pepstatin A 0.12 mM to the media and assay. The undiluted samples can be stored at -20^{0}C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- **EIA Diluent Concentrate (10x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the EIA Diluent 1:10 with reagent grade water. Store for up to 1 month at 2-8^{0}C.
- **Standard Curve:** Reconstitute the 8 ng of Angiotensin II Standard with 4 ml of EIA Diluent to generate a standard solution of 2 ng/ml. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Dilute standard (2 ng/ml) 1:2 with EIA Diluent to produce solution of 1 ng/ml. Prepare duplicate or triplicate standard points by serially diluting the standard solution (1 ng/ml) 1:2 with equal volume of EIA Diluent to produce 0.5, 0.25, 0.125 and 0.063 ng/ml solutions. EIA Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20^{0}C.

Standard Point	Dilution	[ANGII] (ng/ml)
P1	Standard (2 ng/ml) + 1 part EIA Diluent	1.000
P2	1 part P1 + 1 part EIA Diluent	0.500
P3	1 part P2 + 1 part EIA Diluent	0.250
P4	1 part P3 + 1 part EIA Diluent	0.125
P5	1 part P4 + 1 part EIA Diluent	0.063
P6	EIA Diluent	0.000

- **Biotinylated Angiotensin II Antibody (80x):** Spin down the antibody briefly and dilute the desired amount of the antibody 1:80 with EIA Diluent. Any remaining solution should be frozen at -20^{0}C.
- **Wash Buffer Concentrate (20x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the Wash Buffer Concentrate 1:20 with reagent grade water.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 1:100 with EIA Diluent. Any remaining solution should be frozen at -20^{0}C.

Assay Procedure

- Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-30^{0}C).

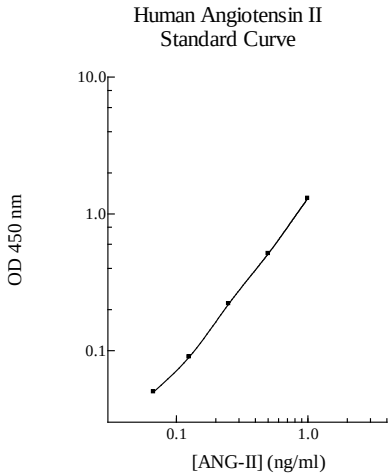
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 50 µl of Standard or sample per well. Cover wells with a sealing tape and incubate for two hours. Start the timer after the last sample addition.
- Wash five times with 200 µl of Wash Buffer manually. Invert the plate each time and decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid. If using a machine wash six times with 300 µl of Wash Buffer and then invert the plate, decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.
- Add 50 µl of Biotinylated Angiotensin II Antibody to each well and incubate for two hours.
- Wash the microplate as described above.
- Add 50 µl of Streptavidin-Peroxidase Conjugate per well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash the microplate as described above.
- Add 50 µl of Chromogen Substrate per well and incubate till the optimal color density develops, which usually takes about 20 minutes. Gently tap the plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
- Add 50 µl of Stop Solution to each well. The color will change from blue to yellow.
- Read the absorbance on a microplate reader at a wavelength of 450 nm **immediately**. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample.
- To generate Standard Curve, plot 4-parameter graph or semi-log graph using the Angiotensin II standard concentrations on the x-axis and the corresponding mean 450 nm absorbance on the y-axis. The best-fit line can be determined by regression analysis using log-log or four-parameter logistic curve-fit.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.



Performance Characteristics

- The minimum detectable dose of Angiotensin II is typically ~ 0.06 ng/ml.
- Intra-assay and inter-assay coefficients of variation were 5.0% and 7.1% respectively.

Cross-Reactivity

Species	% Cross Reactivity
Beagle	> 50%
Bovine	> 80%
Monkey	100%
Mouse	100%
Rat	100%
Swine	100%

	% Cross Reactivity
Angiotensin I	20%
Angiotensin III	30%

References

1. Ino K *et. al.* (2006) *Br J Cancer* 94(4):552-60
2. Fischer JW *et. al.* (2001) *Cardiovasc Res.* 51(4):784-91
3. Sarlos S (2003) *Am J Pathol.* 163(3): 879-87
4. Ogawa S (2006) *Hypertension* 47(4): 699-705

Version 1.7