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Product Information

Angiotensin II EIA Kit
for serum, plasma, culture supernatant, and cell lysates

Catalog Number **RAB0010**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

The Angiotensin II Enzyme Immunoassay (EIA) Kit is an *in vitro* quantitative assay for detecting the Angiotensin II peptide based on the principle of a competitive enzyme immunoassay.

In this assay, a biotinylated Angiotensin II peptide is spiked into the samples and standards. The samples and standards are then added to the plate, where the biotinylated Angiotensin II peptide competes with endogenous (unlabeled) Angiotensin II for binding to the anti-Angiotensin II antibody. After a wash step, any bound biotinylated Angiotensin II then interacts with horseradish peroxidase (HRP)-streptavidin, which catalyzes a color development reaction. The intensity of the colorimetric signal is directly proportional to the amount of captured biotinylated Angiotensin II peptide and inversely proportional to the amount of endogenous Angiotensin II in the standard or samples. A standard curve of known concentration of Angiotensin II peptide can be established and the concentration of Angiotensin II peptide in the samples can be calculated accordingly.

Components

1. 96-well plate coated with secondary antibody (Item A) - RAB0010A: 96 wells (12 strips x 8 wells) coated with secondary antibody.
2. 20x Wash Buffer (Item B) - RABWASH3: 25 mL of 20x concentrated solution.
3. EIA Angiotensin II Peptide standard (Item C) - RAB0010C: 2 vials of Angiotensin II Peptide. 1 vial is enough to run each standard in duplicate.
4. Anti-Angiotensin II Detection Antibody, (Item N) - RAB0010F: 2 vials of anti-Angiotensin II.
5. Assay Diluent C (Item L): 30 mL diluent for standards, and serum or plasma.
6. EIA Angiostensin II 5x Assay Diluent B (Item E) - RABDIL10: 15 mL of 5x concentrated buffer. Diluent for standards, cell culture media, or other sample types, and HRP-Streptavidin.
7. Biotinylated Angiotensin II Peptide, (Item F) - RAB0010G: 2 vials of Biotinylated Angiotensin II Peptide, 1 vial is enough to assay the whole plate.
8. HRP-streptavidin (Item G) - RABHRP3: 600 µl 100x concentrated HRP-conjugated streptavidin.
9. Angiotensin II Positive Control Sample, Lyophilized (Item M) - RAB0010K: 1 vial of Positive Control.
10. TMB Substrate solution (Item H) - RABTMB2: 12 mL of 3,3′,5,5′- tetramethylbenzidine (TMB) in buffered solution.
11. Stop Solution (Item I) – RABSTOP3 8 mL of 0.2 M sulfuric acid.

Reagents and Equipment Required but Not Provided.

1. Microplate reader capable of measuring absorbance at 450 nm
2. Precision pipettes to deliver 2 µl 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. SigmaPlot® software (or other software which can perform four-parameter logistic regression models)
8. Tubes to prepare standard or sample dilutions
9. Orbital shaker
10. Aluminum foil

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
For sample and positive control dilutions, refer to Preparation, steps 6, 7, 8, and 10.

1. Keep kit reagents on ice during reagent preparation steps. Equilibrate plate to room temperature before opening the sealed pouch.

Note: **Assay Diluent C** should be used for dilution of samples, Item F, and Item C when testing **plasma or serum samples**. **1x Assay Diluent B** should be used for dilution of samples, Item F, and Item C when testing **cell culture media or other sample types**. **1x Assay Diluent B** is used to dilute Items N and G regardless of sample type.

2. 5x Assay Diluent B (Item E) should be diluted 5-fold with deionized or distilled water.

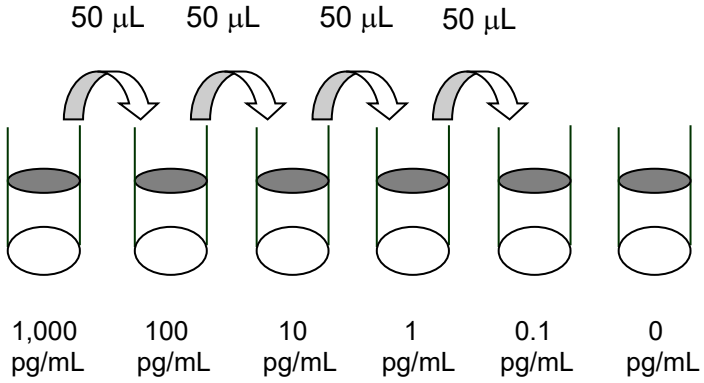
3. Briefly centrifuge the Anti-Angiotensin II Antibody vial (Item N) before use. Add 50 µL of 1x Assay Diluent B into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently.

4. The antibody concentrate should then be diluted 100-fold with 1x Assay Diluent B. This is the anti-Angiotensin II antibody working solution, which will be used in Procedure, step 2.

Note: The following steps may be done during the antibody incubation procedure (Procedure, step 2).

5. Briefly centrifuge the vial of biotinylated Angiotensin II peptide (Item F) before use. Add the entire contents of of Item F to 10 mL of the appropriate 1x Assay Diluent (B or C). Pipette up and down to mix gently. The final concentration of biotinylated Angiotensin II will be 40 pg/mL. This is Working Stock of Item F and will be used as the diluent in Preparation, steps 6, 8, and 10.

Figure 1.
Dilution Series for Standards



6. Preparation of Standards: Label 6 microtubes with the following concentrations: 1,000 pg/mL, 100 pg/mL, 10 pg/mL, 1 pg/mL, 0.1 pg/mL, and 0 pg/mL. Pipette 450 µl of biotinylated Angiotensin II solution into each tube, except for the 1,000 pg/mL (leave this one empty).
Note: It is very important to make sure the concentration of biotinylated Angiotensin II is 20 pg/mL in all standards. To perform a 2-fold dilution of Item F for standards, add 2 mL of Working Stock Item F to 2 mL of the appropriate Assay Diluent. The final concentration of biotinylated Angiotensin II will be 20 pg/ml.

a. Briefly centrifuge the vial of standard Angiotensin II peptide (Item C). In the tube labeled 1,000 pg/mL, pipette 8 µl of Item C and 792 µl of 20 pg/mL biotinylated Angiotensin II solution (Preparation, step 5). This is the Angiotensin II stock solution (1,000 pg/mL Angiotensin II and 20 pg/mL biotinylated Angiotensin II). Mix thoroughly. This solution serves as the first standard.
b. To make the 100 pg/mL standard, pipette 50 µl of Angiotensin II stock solution into the tube labeled 100 pg/mL. Mix thoroughly.
c. Repeat this step with each successive concentration, preparing a dilution series (see Figure 1). Each time, use 450 µl of biotinylated Angiotensin II and 50 µl of the prior concentration until 0.1 pg/mL is reached. Mix each tube thoroughly before the next transfer.
d. The final tube (0 pg/mL Angiotensin II and 20 pg/mL biotinylated Angiotensin II) serves as the zero standard (or total binding).

7. Prepare a 10-fold dilution of Item F. To do this, add 2 µL of Item F to 18 µL of the appropriate 1x Assay Diluent (B or C). This solution will be used in steps 8 and 10.

8. Positive Control Preparation: Briefly centrifuge the positive control (Item M). To the tube of 100 µL of the prepared Positive Control Item M add 100 µL of Working Stock Item F. Also add 4 µL of 10-fold diluted Item F (prepared in step 7) to the tube. This is a 2-fold dilution of the positive control. Mix thoroughly. The positive control is a cell culture medium sample with an expected signal between 10–30% of total binding (70–90% competition) if diluted as described above. It may be diluted further if desired, but be sure the final concentration of biotinylated Angiotensin II is 20 pg/mL.

9. If Item B (20x Wash Concentrate) 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.

10. Sample Preparation: Use appropriate 1x Assay Diluent (B or C) plus biotinylated Angiotensin II to dilute samples, including serum/plasma, cell culture medium, and other sample types.
Notes: It is very important to make sure the final concentration of the biotinylated Angiotensin II is 20 pg/mL in every sample. To perform a 2-fold dilution of Item F for sample, add 125 µL of Working Stock Item F (see Preparation, step 5) to 125 µL of prepared sample. The final concentration of biotinylated Angiotensin II will be 20 pg/ml. For a higher dilution of the sample, dilute the sample with the appropriate Assay Diluent before performing above step.

    For example: to make a 4-fold dilution of sample, dilute sample 2-fold (62.5 µL of sample plus 62.5 µL of 1x Assay Diluent B or C). Mix together 125 µL of Working stock Item F (Preparation, step 5), 125 µL of the prepared sample; mix gently. The total volume is 250 µL, enough for duplicate wells on the microplate.

    Do not use Item F diluent from Preparation, step 6 for sample preparation.

    If undiluted samples are used, biotinylated Angiotensin II must be added to a final concentration of 20 pg/mL. For example, add 5 µL of 10-fold diluted Item F to 245 µL of sample.

11. Briefly centrifuge the HRP-Streptavidin vial (Item G) before use. The HRP-Streptavidin concentrate should be diluted 100-fold with 1x Assay Diluent B.

Storage/Stability
Standard, Biotinylated Angiotensin II peptide, and Positive Control should be stored at –20 °C or –70 °C (recommended at –70 °C) after arrival. Avoid multiple freeze-thaws.

The remaining kit components may be stored at –20 °C. Opened microplate wells and antibody (Item N) may be stored for up to 1 month at 2–8 °C. Return unused wells to the pouch containing desiccant pack and reseal along entire edge.

Procedure

1. Keep kit reagents on ice during reagent preparation steps. It is recommended that all standards and samples be run at least in duplicate.

2. Add 100 µL of anti-Angiotensin II antibody (see Preparation, step 3) to each well. Incubate for 1.5 hours at room temperature with gentle shaking (1–2 cycles/sec) or incubate overnight at 4 °C.

3. Discard the solution and wash wells 4 times with 1x Wash Buffer (200–300 µL each). Washing may be done with a multichannel pipette or an automated plate washer. Complete removal of liquid at each step is essential to good assay 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 µL of each standard (see Preparation, step 6), positive control (see Preparation, step 8), and sample (see Preparation, step 10) into appropriate wells. Be sure to include a blank well (Assay Diluent only). Cover wells and incubate for 2.5 hours at room temperature with gentle shaking (1–2 cycles/sec) or overnight at 4 °C.

5. Discard the solution and wash 4 times as directed in step 3

6. Add 100 µL of prepared HRP-Streptavidin solution (see Preparation, step 11) to each well. Incubate for 45 minutes with gentle shaking at room temperature. It is recommended that the incubation time should not be shorter or longer than 45 minutes.

7. Discard the solution and wash 4 times as directed in step 3.

8. Add 100 µL of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking (1–2 cycles/sec).

9. Add 50 µL of Stop Solution (Item I) to each well. Read absorbances at 450 nm immediately.

Results

Calculations

Calculate the mean absorbance for each set of duplicate standards, controls and samples, and subtract the blank optical density. Plot the standard curve using SigmaPlot software (or other software which can perform four-parameter logistic regression models), with standard concentration on the x-axis and percentage of absorbance (see calculation below) on the y-axis. Draw the best-fit straight line through the standard points.

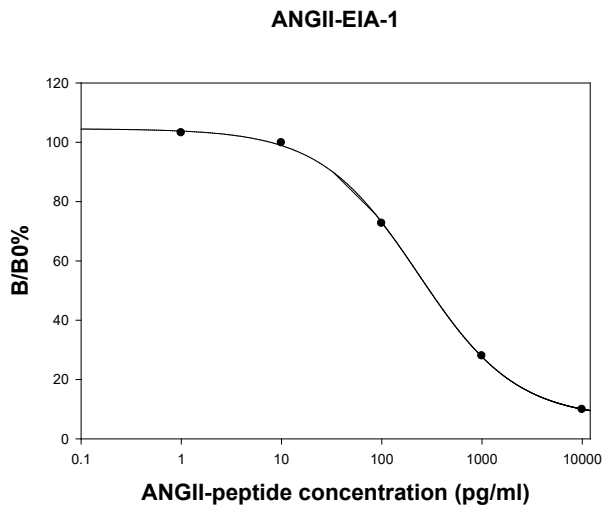
Percentage absorbance =

$$(B - \text{blank OD})/(B_o - \text{blank OD})$$

where:
B = OD of sample or standard and
B_o = OD of zero standard (total binding)

Typical Data

Standard curve(s) is for demonstration only. Standard curve(s) must be run with each assay.



Product Profile
Sensitivity: The minimum detectable concentration of Angiotensin II is 2.62 pg/mL.

Detection Range: 0.1–1,000 pg/mL

Reproducibility:
Intra-Assay: CV <10%
Inter-Assay: CV <15%

Specificity
Cross Reactivity: This kit shows no cross-reactivity with any of the cytokines tested: Ghrelin, Nesfatin, NPY, and APC.

Reference
1. Skurk, T. et al., Angiotensin II and its metabolites stimulate PAI-1 protein release from human adipocytes in primary culture. Hypertension, **37(5)**, 1336–40 (2001).

Appendix
Troubleshooting Guide

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standard dilution	Ensure a brief spin of Item C and dissolve the powder thoroughly with gentle mixing.
Low signal	Too brief incubation times	Ensure sufficient incubation time; Procedure, step 2 may change to over night
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Large CV	Inaccurate pipetting	Check pipettes
High background	Plate is insufficiently washed	Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Make fresh wash buffer
Low sensitivity	Improper storage of the EIA kit	Store the standard at <–20 °C after reconstitution, others at 4 °C. Keep substrate solution protected from light
	Stop solution	Stop solution should be added to each well before measurement.

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