Version 11-04/24

User's Manual

Human/Rat Angiotensin II ELISA Kit

REF **DEIA4485**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

Address: 45-1 Ramsey Road, Shirley, NY 11967, USA

Tel: 1-631-624-4882 (USA) 44-161-818-6441 (Europe) **Fax: 1-631-938-8221**

Email: info@creative-diagnostics.com **Web: www.creative-diagnostics.com**

PRODUCT INFORMATION

Intended Use

Human/Rat Angiotensin II ELISA Kit is to be used for the in vitro quantitative determination of Angiotensin II. This ELISA Kit is for research use only.

General Description

The Renin-Angiotensin system is essential for the control of blood pressure. Among the different peptides resulting from the proteolytic processing of angiotensinogen, the octapeptide Angiotensin II (AII) is the major hormone involved in the pathophysiology of hypertensive diseases as it mediates vasoconstrictor action. It is the target of choice allowing proper estimation of the Renin-Angiotensin system. The peptidic sequence for Angiotensin II is highly conserved across mammalian species, this is why our kit cross-reacts with all mammalian samples.

Principles of Testing

A specific monoclonal anti-Angiotensin II antibody is immobilised on a 96-well plate.

After immunological reaction with Angiotensin II and washing, the trapped molecule is covalently linked to the plate by the glutaraldehyde via amino groups.

After washing and denaturing treatment, Angiotensin II can react again with the acetylcholinesterase-labelled monoclonal antibody used as a Conjugate Solution.

The plate is then washed and Substrate Solution (Ellman' s Reagent) (enzymatic substrate for AChE and chromogen) is added to the wells. The AChE Conjugate Solution acts on the Substrate Solution (Ellman' s Reagent) to form a yellow compound.

The intensity of the colour, which is determined by spectrophotometry, is proportional to the amount of Conjugate Solution bound to the well and is proportional to the amount of Angiotensin II.

Reagents And Materials Provided

Antibody Coated Microtiter Strips (96 wells), ready to use

Conjugate Solution (1 vial), lyophilized

Angiotensin II standard (2 vials), lyophilized

Tween 20 (1 vial), liquid

Dilution Buffer (1 vial), lyophilized

Wash Solution Concentrate (1 vial), liquid

Substrate Solution (Ellman' s Reagent) (2 vials), lyophilized

Glutaraldehyde (1 vial), liquid

Borane Trimethylamine (2 vials), powder

Each kit contains sufficient reagents for 96 wells. This allows for the construction of one standard curve in duplicate and the assay of 32 samples in duplicate.

If you want to use the kit in two times, we provide one additional vial of Standard, one of Quality Control and one of Substrate Solution (Ellman' s Reagent).

Materials Required But Not Supplied

For sample preparation

Phenyl-cartridges 3 mL with 500 mg resin

Methanol

HCl

Ultra pure water

Inhibitor cocktail

For the assay

Precision micropipettes (20 to 1000 µL)

Spectrophotometer plate reader (405 or 414 nm filter)

Microplate washer (or washbottles)

Orbital microplate shaker

Multichannel pipette and disposable tips 30-300 µL

Ultra pure water

Polypropylene tubes

Water used to prepare all EIA reagents and buffers must be UltraPure (deionized & free from organic contaminant traces).

Otherwise, organic contamination can signifi cantly affect the enzymatic activity of the Conjugate Solution Acetylcholinesterase (AChE).

Do not use distilled water, HPLC-grade water or sterile water.

Storage

-20°C

Specimen Collection And Preparation

1. General precautions

All samples must be free from organic solvents prior to assay.

Samples should be assayed immediately after collection or should be stored at -20°C.

a. Culture media samples

Tissue culture supernatants may be assayed directly. If the Angiotensin II concentration in the medium is high

enough, the samples can be diluted with Dilution Buffer. The assay can be performed without any modifications.

When assaying less concentrated samples (when samples cannot be diluted with Dilution Buffer), dilute the Standard curve in the same culture medium as the one used in the experiment. This will ensure that the matrix for the standards is comparable to the samples.

We recommend that a standard curve be run first to ensure that the assay will perform in this particular medium.

b. Blood collection

Blood samples are collected in tubes kept on ice at 4°C and usually containing EDTA.

Since Angiotensin II is very unstable in biologic sample, we highly recommend using an inhibitor cocktail to prevent generation and/or degradation of Angiotensin II ex vivo.

We offer the inhibitor cocktail ready to use, quantity sufficient for 10 mL, 50 mL or 200 mL of blood. The samples are centrifuged at 3,000 g for 20 minutes at 4°C.

Samples should be immediately extracted or stored at -20°C until extraction.

Avoid thawing samples more than once.

2. Extraction protocol

a. Pre-wash phenyl cartridges with 1 mL of methanol, followed by 1 mL of water.

b. Pass 2 ml of plasma through the cartridge and then wash it with 1 mL of water.

c. Elute absorbed Angiotensin peptides with 0.5 mL of methanol.

d. Evaporate the methanol to dryness either by vacuum centrifugation or by evaporation under a stream of dry nitrogen.

e. Add 0.5 mL of Dilution Buffer, vortex and centrifuge at 3,000 g for 10 minutes at 4°C.

3. Recovery and calculation

To determine the recovery, the sample may be split into two equal aliquots and one spiked with a known amount of Angiotensin II (approximately equal to the expected amount in the sample).

The recovery will be determined after purification by comparing the concentration of the spiked and unspiked samples.

Either the original concentration of the sample or the recovery factor can be determined by solving the following equations simultaneously:

z = recovery factor X/a = original concentration of the unspiked sample in a volume known (a)

(X+Y)/b = concentration of spiked sample (pg/ml) after adding a known amount (Y) in a final volume (b)

The concentration of the unspiked and spiked samples determined by the ELISA are respectively equal to (X/a)z & [(X+Y)/b]z.

Example

Volume of the unspiked sample: a = 1 mL

Final volume of the spiked sample: b = 2 mL

Concentration determined by ELISA for the unspiked sample: (X/a)z = 8 pg/mL

Concentration determined by ELISA for the spiked sample: [(X+Y)/b]z = 16 pg/mL

Quantity of spike: Y = 30 pg in 1 mL

$Xz = 8 \Longleftrightarrow \Longleftrightarrow z = 8/X$

$[(X+30)/2]z = 16 \Longleftrightarrow \Longleftrightarrow [(X+30)]z = 32$

thus,

$[(X+30)]8/X = 32 \Longleftrightarrow \Longleftrightarrow X+30 = 4X \Longleftrightarrow \Longleftrightarrow 3X = 30 \Longleftrightarrow \Longleftrightarrow X = 10$

And

$Xz = 8 \Longleftrightarrow \Longleftrightarrow z = 0.8$

Note: To minimise calculations, the standard should be concentrated enough so that the addition of the standard does not alter the volume of the sample (a = b) to any great degree (i.e., the assumption is made that the volume is not changed by the addition of the standard).

Plate Preparation

1. **Plate preparation**

Prepare the Wash Buffer as indicated in the reagent preparation section.

Open the plate packet and select the sufficient strips for your assay and place the unused strips back in the packet.

Stability at +4°C: 1 month.

Rinse each well 5 times with Wash Buffer (300 µL/well).

Just before distributing the reagents and samples, remove the buffer from the wells by inverting the plate and shaking out the last drops on a paper towel.

2. **Plate set-up**

A plate set-up is suggested hereafter.

The contents of each well may be recorded on the template sheet provided at the end of this technical booklet.

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	S7	S3	*	*	*	*	*	*	*	*	*
B	B	S7	S3	*	*	*	*	*	*	*	*	*
C	B	S6	S2	*	*	*	*	*	*	*	*	*
D	NSB	S6	S2	*	*	*	*	*	*	*	*	*
E	NSB	S5	S1	*	*	*	*	*	*	*	*	*
F	NSB	S5	S1	*	*	*	*	*	*	*	*	*
G	S8	S4	QC	*	*	*	*	*	*	*	*	*
H	S8	S4	QC	*	*	*	*	*	*	*	*	*

B : Blank NSB : Non Specific Binding

S1-S8: Standards 1-8

* /OQ: Samples or Quality Controls

Reagent Preparation

Each kit contains sufficient reagents for 96 wells. This allows for the construction of one standard curve in duplicate and the assay of 36 samples in duplicate.

If you want to use the kit in two times, we provide one additional vial of Standard, one of Quality Control and one of Substrate Solution (Ellman' s Reagent).

All reagents need to be brought to room temperature (around 20°C) prior to the assay.

1. **Dilution Buffer**

Reconstitute the vial with 50 mL of UltraPure water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.

Stability at 4°C: 1 month.

2. **Angiotensin II standard (calibrated with the standard WHO 86/538)**

Reconstitute one Angiotensin II Standard vial with 1 mL of UltraPure water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.

The concentration of the first standard (S1) is 125 pg/mL.

Prepare seven polypropylene tubes (for the seven other standards S2 to S8) and add 500 µL of Dilution Buffer into each tube. Then prepare the seven standards by serial dilutions as follows:

Standard	Volume of Standard	Volume of Assay Buffer	Standard concentration pg/mL
S1	-	-	125
S2	500 µL of S1	500 µL	62.5
S3	500 µL of S2	500 µL	31.25
S4	500 µL of S3	500 µL	15.63
S5	500 µL of S4	500 µL	7.81
S6	500 µL of S5	500 µL	3.91
S7	500 µL of S6	500 µL	1.95
S8	500 µL of S7	500 µL	0.98

Stability at 4°C: 24 hours.

3. **Quality Control**

Reconstitute one vial with 1 mL of UltraPure Water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. Stability at 4°C: 24 hours.

4. **Conjugate Solution**

Reconstitute the vial with 10 mL of Dilution Buffer. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.

Stability at 4°C: 1 month.

5. **Glutaraldehyde**

Dilute 0,125 mL of concentrated Wash Buffer with 4,875 mL of UltraPure Water. Just before use add 100 µL of glutaraldehyde then mix thoroughly by gentle inversion.

Stability at 4°C: 24 hours.

6. **Borane trimethylamine**

Just before use, reconstitute one vial with 5 mL of 2N HCl/Methanol (50/50, v/v).

Vortex until complete dissolution. At this step, bubble formation could be observed.

Once reconstituted, Borane Trimethylamine **should be used immediately** as mentioned above.

7. **Substrate Solution**

Five minutes before use (development of the plate), reconstitute one vial of Substrate Solution (Ellman' s Reagent) with 49 mL of UltraPure water and 1 mL of concentrated Wash Buffer. The tube content should be thoroughly mixed.

Stability at 4°C and in the dark: 24 hours.

8. **Wash buffer**

Dilute 2 mL of concentrated Wash Buffer with 800 mL of UltraPure water.

Add 400 µL of Tween 20. Use a magnetic stirring bar to mix the content.

Stability at 4°C: 1 month.

Assay Procedure

It is recommended to perform the assays in duplicate and to follow the instructions hereafter.

1. **Plate preparation and Plate set-up** (refer to the section **Plate preparation**)
2. **Pipetting the reagents**

All samples and reagents must reach room temperature prior to performing the assay.

Use different tips to pipet the buffers, standards, samples, Conjugate Solution, antiserum and other reagents.

Before pipetting, equilibrate the pipette tips in each reagent.

Do not touch the liquid already in the well when expelling with the pipette tip.

3. **Dilution Buffer**

Dispense 100 µL to Non Specific Binding (NSB) wells.

4. **Angiotensin II Standard**

Dispense 100 µL of each of the eight standards (S8 to S1) in duplicate to appropriate wells.

Start with the lowest concentration standard (S8) and equilibrate the tip in the next higher standard before pipetting.

5. **Angiotensin II Quality Control and Samples**

Dispense 100 µL in duplicate to appropriate wells. Highly concentrated samples may be diluted in Dilution Buffer.

6. **Incubating and washing the plate**

Cover the plate with the cover sheet and incubate 1 hour at room temperature with gentle agitation.

7. **Pipetting of reagent**

a. Dispense 50 µL of Glutaraldehyde to each well (except Blank (Bk) wells) and incubate for 5 minutes at room temperature with gentle agitation.

b. Dispense 50 µL of Borane Trimethylamine to each well (except Blank (Bk) wells) and incubate for 5 minutes at room temperature with gentle agitation.

8. **Washing the plate**

a. Empty the plate by turning it over.

b. Rinse each well 5 times with Wash Buffer (300 µL/well).

c. At the end of the last washing step, remove the buffer from the wells by inverting.

9. **Pipetting of reagent**

Dispense 100 µL of Angiotensin II Conjugate Solution to each well (except Blank (Bk) wells).

10. **Incubating the plate**

Cover the plate with a cover sheet and incubate overnight at 4°C.

11. **Developing and reading the plate**

a. Reconstitute Substrate Solution (Ellman' s Reagent) as mentioned in the **Reagent preparation** section.

b. Empty the plate by turning over. Rinse each well five times with 300 µL Wash Buffer. The 5th time,

slightlyshake the plate for 10 minutes on an orbital shaker.

c. Then rewash five times with 300 µL Wash Buffer. At the end of the last washing step, empty the plate and blot the plate on a paper towel to discard any trace of liquid.

d. Add 200µL of Substrate Solution (Ellman' s Reagent) to each well. Cover the plate with an aluminium sheet and incubate in the dark at room temperature. Optimal development is obtained using an orbital shaker.

e. Wipe the bottom of the plate with a paper towel, and make sure that no liquid has splashed outside the wells.

f. Read the plate at a wavelength between 405 and 414nm (yellow colour).

g. After addition of Substrate Solution (Ellman' s Reagent), the absorbance has to be checked periodically (every 30 minutes) until the maximum absorbance has reached a minimum of 0,5 A.U. blank subtracted.

Calculation

Make sure that your plate reader has subtracted the absorbance readings of the blank wells (absorbance of Substrate Solution (Ellman' s Reagent) alone) from the absorbance readings of the rest of the plate. If it is not the case, please do it.

a. Calculate the average absorbance for each NSB, standard and sample.

b. For each standard, plot the absorbance on y axis versus the concentration on x axis. Draw a best-fit line through the points.

c. To determine the concentration of your samples, find the absorbance value of each sample on the y axis.

d. Read the corresponding value on the x axis which is the concentration of your unknown sample.

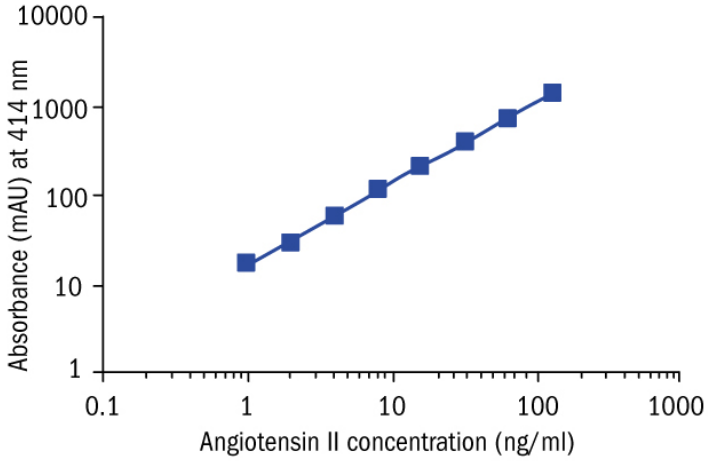
e. Samples with a concentration greater than 125 pg/mL should be re-assayed after dilution in Dilution Buffer.

f. Most plate readers are supplied with a curve-fitting software capable of graphing these data (4-parameter logistic fit 4PL). If you have this type of software, we recommend using it. Refer to it for further information.

Two vials of Quality Control are provided with this kit.

Your standard curve is validated only if the calculated concentration of the Quality Control obtained with the assay is +/- 25% of the expected concentration (see the label of QC vial).

Typical Standard Curve



Reference Values

Non-specific Binding <150 mAU

Limit of detection <5 pg/mL

Quality Control ±25% of the expected concentration (see the label on QC vial)

Detection Range

0.98-125 pg/ml

Detection Limit

0.5-1.5 pg/ml

Specificity

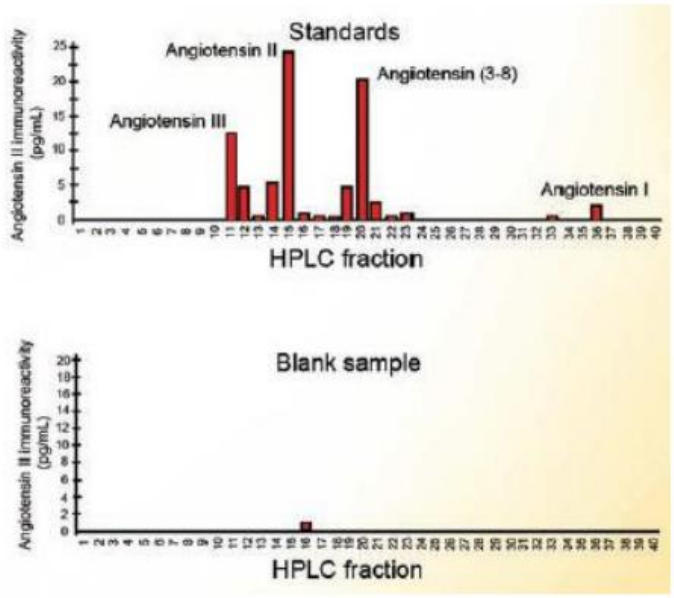
1. **Cross-reactivity**

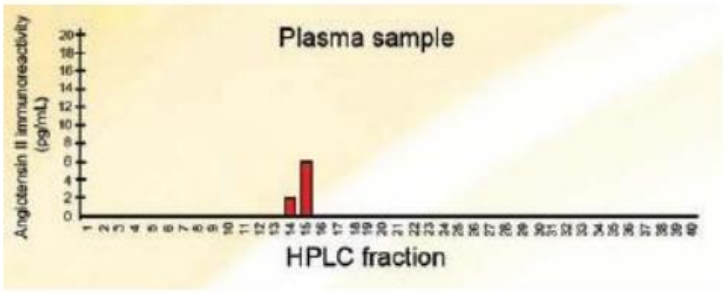
The peptidic sequence for Angiotensin II is highly conserved across mammalian species, this is why our kit cross-reacts with all mammalian samples.

Angiotensin II	100 %
Angiotensin I	4 %
Angiotensin III	36 %
Angiotensin 3-8	33 %
Angiotensin 1-7	<0.01 %

2. **Specifity**

Comparison of HPLC profiles of Angiotensin standards, a blank sample and a plasma sample.





Reproducibility

pg/mL	Intra-assay coefficient of variation (%)	Inter-assay coefficient of variation (%)
100	7	7
20	2	5
5	6	10
2	10	14.5

Precautions

Users are recommended to read all instructions for use before starting work.

1. Each time a new pipette tip is used, aspirate a sample or reagent and expel it back into the same vessel. Repeat this operation two or three times before distribution in order to equilibrate the pipette tip.
2. For research laboratory use only.
3. Not for human diagnostic use.
4. Do not pipet liquids by mouth.

5. Do not use kit components beyond the expiration date.
6. Do not eat, drink or smoke in area in which kit reagents are handled.
7. Avoid splashing.
8. The total reagents contain less than 100 µg of sodium azide.
9. Flush the drains thoroughly to prevent the production of explosive metal azides.
10. **Borane-trimethylamine is highly toxic. Handle this reagent with care.**
11. Wearing gloves, laboratory coat and glasses is recommended when assaying kit materials and samples.
12. **Temperature: Unless otherwise specified, all the experiments are done at room temperature (RT), that is around 20°C. Working at 25°C or more affects the assay and decreases its efficiency.**

Possible Problems and Interference Elimination

1. **Absorbance values are too low:**

   a. incubation in wrong conditions (time or temperature),

   b. reading time too short,

   c. Conjugate Solution, Glutaraldehyde, Wash Buffer or Borane Trimethylamine have not been dispensed,

   d. organic contamination of water.

2. **NSB value too high:**

   a. contamination of NSB wells with Angiotensin II standard,

   b. inefficient washing,

   c. Borane Trimethylamine has been dispensed not on time or not at all.

3. **High dispersion of duplicates:**

   a. poor pipetting technique

   b. irregular plate washing.

4. **If a plate is accidentally dropped after dispatch of the Substrate Solution (Ellman' s Reagent) or if it needs to be revealed again:**

   a. one only needs to wash the plate, add fresh Ellman' s Reagent and proceed with a new development.

   b. Otherwise, the plate can be stored at 4°C with wash buffer in wells while waiting for technical advice from the CD.