Instructions for use

Plasma Renin Activity (PRA) ELISA

REF   **MS E-5600R**

RUO

For research
use only –
Not for use
in diagnostic
procedures

Plasma Renin Activity (PRA) ELISA

INTENDED USE
For the quantitative determination of Plasma Renin Activity (PRA) in human plasma by an enzyme immunoassay.

PRINCIPLE OF THE TEST
This kit measures PRA and the results are expressed in term of mass of angiotensin-I (Ang-I) generated per volume of human plasma in unit time (ng/ml.h).

The blood sample is collected in a tube that contains EDTA. The plasma is separated and either stored frozen or kept at room temperature for immediate use, samples should not be chilled on ice or stored at temperatures between 0 and 10°C during collection or processing before adjustment of pH, this could lead to overestimation of renin activity. Before the start of immunoassay a protease inhibitor and the Generation buffer is added to the plasma sample, which will prevent Angiotensin-I (Ang-I) in plasma from degradation. The pH of the plasma sample should be around 6.0 after the addition of the supplied Generation buffer. The plasma sample is split in two and the fractions are incubated at 0–4°C (in ice bath) and 37°C respectively for 90 minutes or longer, to allow the generation of Ang-I by plasma renin at 37°C. Optionally, the pH can be adjusted to 6.5 or 7.4. Adjustment of pH is a critical step during the assay, acidification of plasma to pH 3.3 or lower for prolonged time with subsequent return to neutral pH causes irreversible activation of the renin (Derkx et al., 1987), on the other side incubation at pH higher than 8.0 can destroy renin. During the immunoassay incubation, another set of protease inhibitors are involved, which function to stop the new generation as well as degradation of Ang-I to smaller peptides.

The immunoassay of Ang-I is a competitive assay that uses two incubations, with a total assay incubation time of less than two hours. During the first incubation unlabelled Ang-I (present in the standards, controls and plasma samples) competes with biotinylated Ang-I to bind to the anti-Ang-I antibody. In the second incubation the labelled Streptavidin- HRP conjugate, binds to the immobilized Ang-I-Biotin. The washing and decanting procedures remove unbound materials. The colorimetric HRP substrate is added and, after stopping the color development reaction, the light absorbance (OD) is measured in a microplate reader. The absorbance values are inversely proportional to the concentration of Ang-I in the sample. A set of standards is used to plot a standard curve from which the concentrations of Ang-I in the samples and controls can be directly read.

PROCEDURAL CAUTIONS AND WARNINGS
1. Users should have a thorough understanding of this protocol for the successful use of this kit. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
2. Ang-I is presently not included in any external QC schemes. Therefore, each laboratory is suggested to establish its own internal QC materials and procedure for assessing the reliability of results.
3. When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
4. All kit reagents and specimens should be at room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and plasma specimens.
5. A standard curve must be established for every run. The kit controls should be included in every run and fall within established confidence limits.
6. Do not mix various lot numbers of kit components within a test and do not use any component beyond the expiration date printed on the label.
7. The substrate (TMB) solution is sensitive to light and should always be stored in dark bottles away from direct sunlight.
8. To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and controls.
9. Improper procedural techniques, imprecise pipetting, incomplete washing as well as improper reagent storage may be indicated when assay values for the controls do not reflect established ranges. The performance of this assay is markedly influenced by the correct execution of the washing procedure!

LIMITATIONS
1. This kit is specifically designed and validated for the determination of renin activity/Ang-I generation in EDTA plasma. Other sources of material should be validated before being applied.
2. The Ang-I level depends on multiple factors, including renin activity, renin substrate concentration, the plasma pH, temperature and selection of inhibitors. Therefore, only carefully prepared plasma samples are suitable for this test.
   Bacterial contaminations, repeated freeze and thaw cycles and dilution of plasma samples may affect the assay result.
3. The interpretation of the results should recognise the conditions that can affect renin secretion, such as sodium and potassium intake, posture, medications like diuretics, chlonidine, beta-blockers, estroprogestogens and peripheral vasodilators.
4. Do not use grossly haemolysed, lipaemic, icteric plasma, and any sample that was not handled properly according to the instruction.

SAFETY CAUTIONS AND WARNINGS

POTENTIAL BIOHAZARDOUS MATERIAL
All reagents in this kit should be considered a potential biohazard and handled with the same precautions as applied to any blood specimen. Human plasma samples should be handled as if capable of transmitting infections and in accordance with good laboratory practices.

CHEMICAL HAZARDS
Avoid contact with reagents containing PMSF and hydrogen peroxide. If contacted with any of these or other reagents in this kit, wash with plenty of water.

REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED
1. Disodium EDTA (2 mg/ml blood) collection tubes
2. Single and multi-channel pipettes and disposal tips
3. Distilled or deionized water
4. Disposable test tubes (glass or polypropylene)
5. Plate shaker
6. Microplate absorbance reader equipped with a 450 nm filter
7. 37°C incubator
8. Ice bath
9. 95% Ethanol

REAGENTS PROVIDED

1. AA E-0030   `WASH-CONC` `10x`   **Wash Buffer Concentrate –** Requires Preaparation X10

Contents:	Two bottles containing buffer with a non-ionic detergent and a non-mercury preservative.
Volume:	2x50 ml/bottle
Storage:	Refrigerate at 2-8°C
Stability:	12 months or as indicated on label.
Preparation:	Dilute 1:10 in distilled or deionized water before use. If one whole plate is to be used dilute 50 ml of the wash buffer concentrate in 450 ml of water.

2. MS E-5655   `SUBSTRATE`   **TMB Substrate** - Ready To Use.

Contents:	One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer.
Volume:	32 ml/vial
Storage:	Refrigerate at 2-8°C
Stability:	12 months or as indicated on label.

3. MS E-5680   `STOP-SOLN`   **Stopping Solution** - Ready To Use.

Contents:	One bottle containing 1M sulfuric acid.
Volume:	12 ml/bottle
Storage:	Refrigerate at 2-8°C
Stability:	12 months or as indicated on label.
Hazards identification:	

H290 May be corrosive to metals.
H314 Causes severe skin burns and eye damage.

4. **Standards** and **Controls**- Ready To Use.

Listed below are approximate concentrations, please refer to vial labels for exact concentrations:

Cat. no.	Symbol	Standard	Concentration*	Volume/Vial
MS E-5601	STANDARD A	Standard A	0 ng/ml	2.0 ml
MS E-5602	STANDARD B	Standard B	0.2 ng/ml	0.7 ml
MS E-5603	STANDARD C	Standard C	0.5 ng/ml	0.7 ml
MS E-5604	STANDARD D	Standard D	1.5 ng/ml	0.7 ml
MS E-5605	STANDARD E	Standard E	4 ng/ml	0.7 ml
MS E-5606	STANDARD F	Standard F	10 ng/ml	0.7 ml
MS E-5607	STANDARD G	Standard G	25 ng/ml	0.7 ml
MS E-5608	STANDARD H	Standard H	60 ng/ml	0.7 ml
MS E-5651	CONTROL 1	Control 1	Refer to vial labels for acceptable range!	0.7 ml
MS E-5652	CONTROL 2	Control 2		0.7 ml

* Approximate value - please refer to vial labels for exact concentrations.

Contents:	Synthetic angiotensin-I peptide in a protein-based buffer with a non-mercury preservative. The standards are calibrated against the World Health Organization reference reagent NIBSC code 86/536.
Storage:	Refrigerate at 2-8ºC
Stability:	12 months in unopened vials or as indicated on label.

5. MS E-5613   ASSAY-BUFF   **Assay Buffer** - Ready To Use.

Contents:	One bottle containing protein-based buffer with a non-mercury preservative.
Volume:	40 ml/bottle
Storage:	Refrigerate at 2-8ºC
Stability:	12 months or as indicated on label.

6. MS E-5631   ▥ 96   **Rabbit Anti-Ang-I Antibody Coated Microplate** - Ready To Use.

Contents:	Two 96 well pre-coated microplates in a resealable pouch with desiccant.
Storage:	Refrigerate at 2-8ºC
Stability:	12 months or as indicated on label.

7. MS E-5640   CONJUGATE-CONC 100x   **Streptavidin-Horseradish Peroxidase Conjugate Concentrate –** Requires Preparation X100

Contents:	Streptavidin-HRP conjugate in a protein-based buffer with a non-mercury preservative.
Volume:	0.5 ml/vial
Storage:	Refrigerate at 2-8ºC
Stability:	12 months or as indicated on label.
Preparation:	Dilute the conjugate concentrate 1:100 in assay buffer before use. The working conjugate solution is stable for 8 hours; discard the unused solution after this period.

8. MS E-5610   BIOTIN-AB   **Angiotensin-I-Biotin Conjugate** - Ready To Use.

Contents:	One bottle containing buffer, protease inhibitors, Angiotensin-I-Biotin conjugate and a non-mercury preservative.
Volume:	30 ml/bottle
Storage:	Refrigerate at 2-8°C
Stability:	12 months in unopened vial or as indicated on label.

9. MS E-5614　　　PMSF　　　**PMSF – Requires Preparation** - Ready To Use.

Contents:　One bottle containing phenylmethylsulfonyl fluoride (PMSF).
Storage:　Refrigerate at 2-8°C
Stability:　12 months or as indicated on label.
Preparation:　Reconstitute by adding 0.5 ml of 95% ethanol to the bottle and vortex for two minutes to completely dissolve the PMSF. Refrigerate after first use, vortex again to re-dissolve contents. Do not keep the bottle open unnecessarily.

10. MS E-5515　　BUFF　　　**Generation Buffer** - Ready To Use.

Contents:　Buffer and non-toxic antibiotic.

Volume:　5 ml/bottle

Storage:　Refrigerate at 2-8°C

Stability:　12 months or as indicated on label

SPECIMEN COLLECTION AND STORAGE

A minimum of 0.5 ml of plasma is required per duplicate determination. Appropriate sample collection is essential to the accurate determination of angiotensin-I. The generation and degradation of angiotensin-I can be minimized by the following recommended collection procedure:

1. Collect 2 ml of blood into an EDTA venipunture tube or syringe.
2. Centrifuge blood for 15 minutes at 5000 rpm at room temperature.
3. Transfer plasma sample to a test tube at room temperature.
4. If samples are to be assayed now proceed to the Angiotensin-I generation procedure, otherwise freeze samples immediately at -20°C or less. Avoid freezing and thawing samples more than once.

ANGIOTENSIN-I GENERATION PROCEDURE

1. If a freshly drawn plasma sample is being used proceed to step 2.
   If frozen plasma samples are being used thaw them as follows. Quickly bring frozen plasma samples to room temperature by placing the tubes in a container with room temperature water.
2. Transfer 0.5 ml of the plasma sample into a test tube.
3. Add 5 µl of the PMSF solution to the 0.5 ml of plasma sample (1:100 ratio). Vortex the tube to mix thoroughly.
4. Add 50 µl of the generation buffer to the treated sample from step 3 (1:10 ratio). Vortex the tube again to mix thoroughly.
5. Divide the treated sample from step 4 equally into two aliquots by transferring 0.25 ml into two test tubes. Incubate one aliquot for 90 minutes or longer (do not exceed 180 minutes) at 37°C, place the second aliquot on an ice bath (0°C). Be sure to record the incubation time used for the aliquots as this is used for calculations.
6. At the end of the incubation period place the 37°C aliquot on the ice-bath for 5 minutes to cool it down quickly.
7. Bring both aliquots to room temperature by placing in a bath with room temperature water for 5–10 minutes (do not exceed 10 minutes).

ASSAY PROCEDURE

1.	Allow all kit components to reach room temperature. Remove the required number of well strips and assemble into the plate frame.
2.	Pipette **50 µl** of each **standard, control and treated plasma sample** (both 37°C and 0°C aliquots) into correspondingly labelled wells in duplicate.
3.	Pipette **100 µl** of the **angiotensin-I-biotin conjugate** into each well *(the use of a multichannel pipette is recommended).*
4.	**Incubate** on a plate shaker (~200 rpm) for **60 minutes** at room temperature.
5	**Wash** the wells **5 times** each time with **300 µl/well** of **diluted wash buffer**. After washing tap the plate firmly against absorbent paper to remove any residual liquid *(the use of an automatic strip washer is strongly recommended).* *The performance of this assay is markedly influenced by the correct execution of the washing procedure!*
6.	Pipette **150 µl** of the **streptavidin-HRP conjugate working solution** into each well *(the use of a multichannel pipette is recommended).*
7.	**Incubate** on a plate shaker (~200 rpm) for **30 minutes** at room temperature.
8.	**Wash** the wells **5 times** with the same procedure as in step 5.
9.	Pipette **150 µl** of the **TMB substrate** into each well *(the use of a multichannel pipette is recommended).* **Incubate** on a plate shaker (~200 rpm) for **10 to 15 minutes** at room temperature.
10.	Add **50 µl** of **stopping solution** to each well and mix thoroughly by gently tapping the plate.
11.	Measure the absorbance at 450 nm in all wells with a microplate reader between 0–20 minutes after addition of the stopping solution.

CALCULATIONS

PERFORMANCE CHARACTERISTICS
1. Using immunoassay software, choose either a 4-parameter or 5-parameter curve fitting method for calculating results.
2. If a sample reads more than 60 ng/ml then dilute the sample (that has undergone the angiotensin-I generation procedure) with Standard A at a dilution of no more than 1:10 and rerun the sample. The result obtained should be multiplied by the dilution factor.
   Note: Samples must be diluted only after they have undergone the angiotensin-I generation procedure; do not dilute any samples before performing the angiotensin-I generation procedure.
3. Calculate the plasma renin activity (PRA) in each sample using the following equation:

$$PRA = \left\{ \frac{[\text{Ang-I (37°C)}] - [\text{Ang-I (0°C)}]}{\text{Time (hrs)}} \right\} \times 1.11$$

Where time (hrs) is the incubation time used during the generation step.
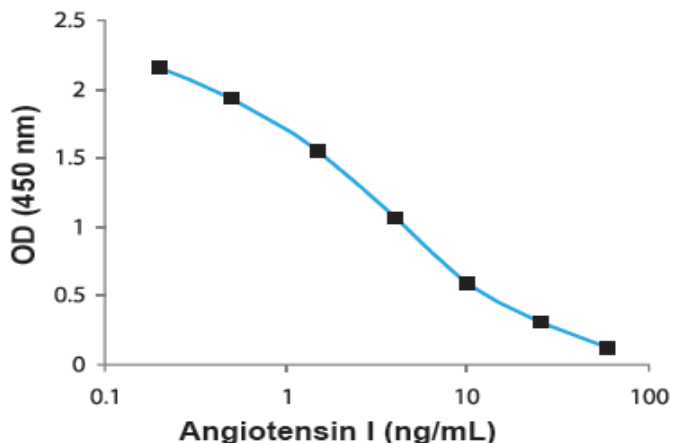
TYPICAL TABULATED DATA

Sample data only. Do not use to calculate results

Standard	Mean OD (450 nm)	Ang-I (ng/ml)
A	2.303	0
B	2.156	0.2
C	1.937	0.5
D	1.552	1.5
E	1.066	4
F	0.591	10
G	0.311	25
H	0.122	60

TYPICAL STANDARD CURVE
Sample curve only, do not use to calculate results.

PERFORMANCE CHARACTERISTICS

SENSITIVITY
The limit of detection (LoD) was determined from the analysis of 40 samples of the blank and a low value sample and it was
calculated as follows:

$$LoD = \mu B + 1.645\sigma B + 1.645\sigma S,$$

where σB and σS are the standard deviation of the blank and low value sample and μB is the mean value of the blank.

LoD = 0.14 ng/mL of Angiotensin I

SPECIFICITY (CROSS-REACTIVITY)
The following compounds were tested for cross-reactivity using the Abraham method with angiotensin-I cross reacting at 100%:

Antigen	Sequence	% Cross-Reactivity
Angiotensin-I	DRVYIHPFHL	100
Angiotensin 1-9	DRVYIHPFH	0.015
Angiotensin-II	DRVYIHPF	<0.001
Angiotensin-III	RVYIHPF	<0.001
Angiotensin 1-5	DRVYI	<0.001
Renin Substrate human	DRVYIHPFHLVIHN	0.001

RECOVERY

Spiked samples were prepared by adding defined amounts of angiotensin-I to three plasma samples. The results (in ng/ml) are tabulated below:

Sample	Observed Result	Expected Result	Recovery %
1.Unspiked	0.86	-	-
+0.48	1.43	1.34	107
+1.92	2.82	2.78	101
+5.77	6.47	6.63	98
+11.53	10.58	12.40	85
1.Unspiked	2.84	-	-
+0.48	3.30	3.32	99
+1.92	5.34	4.77	112
+5.77	8.84	8.61	103
+11.53	13.08	14.38	91
1.Unspiked	9.45	-	-
+0.48	9.92	9.93	100
+1.92	11.35	11.37	100
+5.77	13.82	15.22	91
+11.53	17.62	20.99	84

LINEARITY

Three plasma samples were diluted with Standard A. The results (in ng/ml) are tabulated below:

Sample	Obs. Result	Exp. Result	Recovery %
1	10.96	-	-
1:2	5.739	5.48	105
1:4	2.718	2.74	99
1:8	1.423	1.37	104
1:16	0.776	0.685	113
2	15.798	-	-
1:2	8.273	7.899	105
1:4	3.934	3.950	100
1:8	1.948	1.975	99
1:16	1.146	0.987	116
3	30.7	-	-
1:2	16.142	15.350	105
1:4	7.477	7.675	97
1:8	3.542	3.838	92
1:16	1.574	1.919	82

INTERFERENCE

Interference testing was per formed according to CLSI guideline EP7-A2. Plasma samples with varying levels of angiotensin-I were spiked with potential interfering substances at recommended levels and analyzed. Results were compared to the same plasma samples with no extra substances added to calculate the % interference.

$$Interference\ (\%) = \frac{[Ang\ I(Spiked\ sample)] - [AngI(Native\ sample)]}{[AngI(Native\ sample)]} \times 100$$

Interferent	Added Interferent Concentration	% Interference
Haemoglobin	1 g/l	-3.0
	2 g/l	-3.8
Bilirubin Unconjugated	20 µM (12 mg/l)	0
	500 µM (300 mg/l)	0
Bilirubin Conjugated*	20 µM (16 mg/l)	+3.0
	500 µM (400 mg/l)	+13.5
Haemoglobin + Bilirubin	1 g/l + 20 µM	-0.4
	1 g/l + 500 µM	-0.1
	2 g/l + 20 µM	-3.9
	2 g/l + 500 µM	-12.4
Triglycerides (2C-10 C)	3.7 mM	+4.8
	37 mM	+16.9
Triglycerides (8C-16 C)	3.7 mM	-0.6
	37 mM	+2.2
HSA	40 g/l	-2.2
	60 g/l	-9.6

*Taurobilirubin

INTRA-ASSAY PRECISION

Four samples were assayed 14 times each on the same standard curve. The results (in ng/ml) are tabulated below:

Sample	Mean	SD	CV%
1	2.3	0.2	8.7
2	3.6	0.2	6.8
3	7.0	0.4	6.3
4	13.3	0.9	7.0

INTER-ASSAY PRECISION

Four samples were assayed in ten different tests. The results (in ng/ml) are tabulated below:

Sample	Mean	SD	CV%
1	0.48	0.03	7.12
2	0.82	0.04	5.32
3	9.46	0.45	4.81
4	11.70	0.64	5.44

COMPARATIVE STUDIES

The PRA ELISA kit (y) was compared with a competitor's PRA RIA kit (x). The comparison of 73 plasma samples yielded the following linear regression results:

$y = 0.93x - 0.08$, $r = 0.97$

EXPECTED NORMAL VALUES

Each laboratory should collect data and establish their own range of expected normal values. Data presented here were from samples incubated at pH 6.0 during the generation step (Brossaud and Corcuff, 2009).

N	PRA Mean (ng/ml.h)	PRA Range (10th-90th percentile) (ng/ml.h)
533	0.75	0.06-4.69

REFERENCES

1. Brossaud J, Corcuff JB. Pre-Analytical and Analytical Considerations for the Determination of Plasma Renin Activity. *Clin Chim Acta*. 2009; 410(1–2):90–2.
2. Bystrom CE, et al. Plasma Renin Activity by LC-MS/MS: Development of a Prototypical Clinical Assay Reveals a Subpopulation of Human Plasma Samples With Substantial Peptidase Activity. *Clin Chem*. 2010; 56(10):1561–9.
3. Campbell DJ, et al. Activity Assays and Immunoassays for Plasma Renin and Prorenin: Information Provided and Precautions Necessary for Accurate Measurement. *Clin Chem*. 2009; 55(5):867–77.
4. Cartledge S, Lawson N. Aldosterone and Renin Measurements. *Ann Clin Biochem*. 2000; 37(Pt 3):262–78.
5. Derkx FH, et al. Two-step Prorenin-Renin Conversion. Isolation of an Intermediary Form of Activated Prorenin. *J Biol Chem*. 1987; 262(6):2472–7.
6. Hartman D, et al. Direct Renin Assay and Plasma Renin Activity Assay Compared. *Clin Chem*. 2004; 50(11):2159–61.
7. Pimenta E, Calhoum D. Response to "Effective" Plasma Renin Activity: A Derived Measure for Assessing Residual Plasma Renin Activity in Patients Taking Angiotensin- Converting Enzyme Inhibitors or Angiotensin Receptor Blockers. *Hypertension*. 2010; 55:e17.
8. Reudelhuber TL. Prorenin, Renin, and Their Receptor: Moving Targets. *Hypertension*. 2010; 55(5):1071–4.
9. Sealey JE, et al. Plasma Renin Methodology: Inadequate Sensitivity and Accuracy of Direct Renin Assay for Clinical Applications Compared With the Traditional Enzymatic Plasma Renin Activity Assay. *J Hypertens*. 1995; 13(1):27–30.
10. Sealey JE. Plasma Renin Activity and Plasma Prorenin Assays. *Clin Chem*. 1991; 37(10 Pt 2):1811–9.
11. Sealey JE, et al. Plasma Renin and Aldosterone Measurements in Low Renin Hypertensive States. *Trends Endocrinol Metab*. 2005; 16(3):86–91.
12. Ulmer PS, Meikle AW. Sample Requirements for Plasma Renin Activity and Immunoreactive Renin. *Clin Chem*. 2000; 46(9):1442–4.

Symbols:

(thermometer +2/+8 °C)	Storage temperature	(factory)	Manufacturer	(Σ triangle)	Contains sufficient for \<n\> tests
(hourglass)	Expiry date	LOT	Batch code		
(book with i)	Consult instructions for use	CONT	Content		
(caution triangle)	Caution	REF	Catalogue number	RUO	For research use only!