ANGIOTENSIN II

A brand name of

bertin pharma

For laboratory research use only. Not for human or veterinary diagnostic use.

Bertin Pharma also markets pre-analytical products, EIA kits, antibodies & biochemicals for:

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- Psychopharmacology
- Nitric Oxide
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- Cell signaling
- Drug metabolism

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European patent # 89 139 552
U.S. patent # 50 47 330

Angiotensin II
Enzyme Immunoassay kit
#A05880.96 wells

For research laboratory use only
Not for human diagnostic use

This assay has been developed & validated
by Bertin Pharma

Fabriqué en France
Made in France

#A11880
Version: 0117

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96 wells
Storage: -20°C
Expiry date: stated on the package

This kit contains:

Designation	Colour of cap	Item #	Quantity per kit	Form
Angiotensin II precoated 96-well Strip Plate	Blister with zip	A08880.1 ea	1	
Angiotensin II Tracer	Green	A04880.100 dtn	1	Lyophilised
Angiotensin II Standard	Blue with red septum	A06880.1 ea	2	Lyophilised
Angiotensin II Quality Control	Green with red septum	A10880.1 ea	2	Lyophilised
Glutaraldehyde	White with red septum	A13880.1 ea	1	Liquid
Borane Trimethylamine	Silver	A14880.1 ea	2	Powder
EIA Buffer	Blue	A07000.1 ea	1	Lyophilised
Wash Buffer	Silver	A17000.1 ea	1	Liquid
Tween 20	Transparent	A12000.1 ea	1	Liquid
Ellman's Reagent 49+1	Black with red septum	A09000_49+1.100 dtn	2	Lyophilised
Technical Booklet	-	A11880	1	-
Well cover Sheet	-	-	1	-

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 Ellman's Reagent.

Precaution for use

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

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.

- For research laboratory use only
- Not for human diagnostic use
- Do not pipet liquids by mouth
- Do not use kit components beyond the expiration date
- Do not eat, drink or smoke in area in which kit reagents are handled
- Avoid splashing

The total amount of reagents contains less than 100 µg of sodium azide. Flush the drains thoroughly to prevent the production of explosive metal azides.

Borane Trimethylamine is highly toxic. Handle this reagent with care.

Wearing gloves, laboratory coat and glasses is recommended when assaying kit materials and samples.

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.

Background

Acetylcholinesterase AChE® Technology

Acetylcholinesterase (AChE®), the enzymatic label for EIA, has the fastest turnover rate of any enzymatic label. This specific AChE is extracted from the electric organ of the electric eel, *Electrophorus electricus*, and is capable of massive catalytic turnover during the generation of the electrochemical discharges. The use of AChE as enzymatic label for EIA has been patented by the French academic research Institute CEA ***[1, 2, 3]***, and Bertin Pharma, formerly known as SPI-Bio, has expertise to develop and produce EIA kits using this technology.

AChE® assays are revealed with Ellman's Reagent, which contains acetylthiocholine as a substrate. The final product of the enzymatic reaction (5-thio-2-nitrobenzoic acid), is bright yellow and can be read at 405-414 nm. AChE® offers several advantages compared to enzymes conventionally used in EIAs:

- **Kinetic superiority and high sensitivity**: AChE® shows true first-order kinetics with a turnover of 64,000 sec^{-1}. That is nearly 3 times faster than Horseradish Peroxidase (HRP) or alkaline phosphatase. AChE® allows a greater sensitivity than other labeling enzymes.
- **Low background**: non-enzymatic hydrolysis of acetylthiocholine in buffer is essentially absent. So, AChE® allows a very low background and an increased signal/noise ratio compared to other substrate of enzymes which is inherently unstable.

- **Wide dynamic range**: AChE® is a stable enzyme and its activity remains constant for many hours as, unlike other enzymes, its substrate is not suicidal. This permits simultaneous assays of high diluted and very concentrated samples.
- **Versatility:** AChE® is a completely stable enzyme, unlike peroxidase which is suicidal. Thus, if a plate is accidentally dropped after dispatch of the AChE® substrate (Ellman's Reagent) or if it needs to be revealed again, one only needs to wash the plate, add fresh Ellman's Reagent and proceed with a new development. Otherwise, the plate can be stored at +4°C with wash buffer in wells while waiting for technical advice from the Bioreagent Department.

Angiotensin

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 ***[4]***.

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

Principle of the assay

The principle of this Enzyme ImmunoAssay (EIA) is summarised on the following page.

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

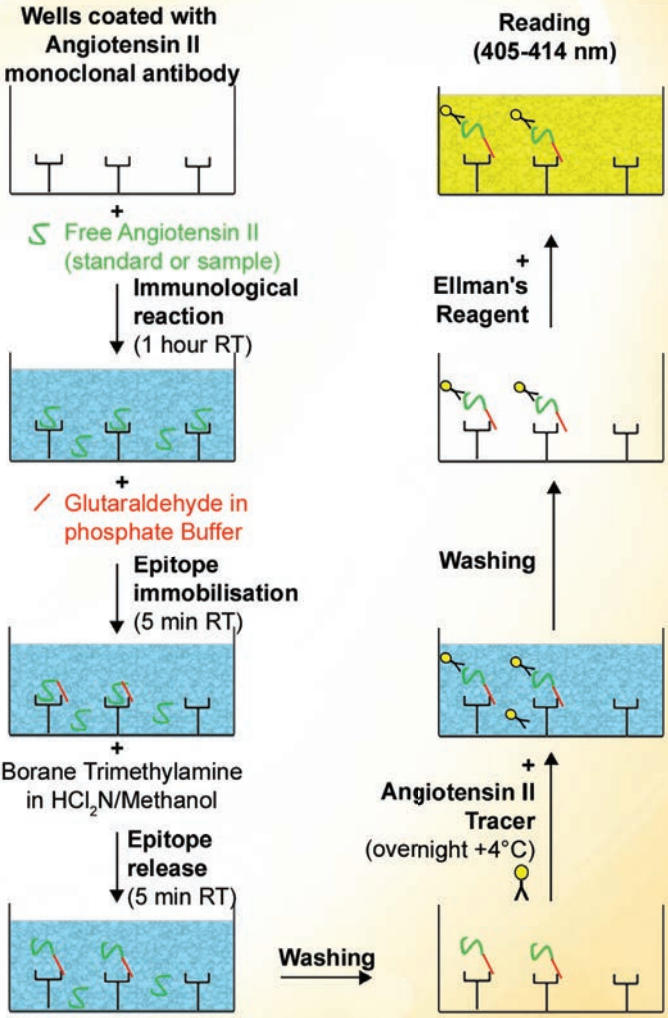
After immunological reaction with Angiotensin II and washing, the trapped molecule is covalently linked to the plate by the glutaraldehyde via amino groups ***[5, 6, 8]***.

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

The plate is then washed and Ellman's Reagent (enzymatic substrate for AChE and chromogen) is added to the wells.

The AChE tracer acts on the Ellman's Reagent to form a yellow compound.

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


Wells coated with Angiotensin II monoclonal antibody
+
Free Angiotensin II (standard or sample)
Immunological reaction
(1 hour RT)
+
Glutaraldehyde in phosphate Buffer
Epitope immobilisation
(5 min RT)
+
Borane Trimethylamine in HCl2N/Methanol
Epitope release
(5 min RT)
Washing
+
Angiotensin II Tracer
(overnight +4°C)
Washing
+
Ellman's Reagent
Reading
(405-414 nm)

Materials and equipment required

In addition to standard laboratory equipment, the following material is required:

For sample preparation:

- Phenyl-cartridges 3 mL with 500 mg resin #D30004
- Methanol
- HCl
- UltraPure water
- Inhibitor cocktail #D05006

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 30-300µL
- UltraPure water #A07001.1L
- Polypropylene tubes

Water used to prepare all EIA reagents and buffers must be UltraPure (deionized & free from organic contaminant traces).
Otherwise, organic contamination can significantly affect the enzymatic activity of the tracer Acetylcholinesterase (AChE).
Do not use distilled water, HPLC-grade water or sterile water.

- UltraPure Water #A07001.1L may be purchased from Bertin Pharma.

Sample collection and preparation

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.

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 EIA Buffer. The assay can be performed without any modifications.

When assaying less concentrated samples (when samples cannot be diluted with EIA 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.

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 ***[7]***.

Bertin Pharma offers the inhibitor cocktail ready to use #D05006, 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.

Extraction protocol

- Pre-wash phenyl cartridges with 1 mL of methanol, followed by 1 mL of water.
- Pass 2 ml of plasma through the cartridge and then wash it with 1 mL of water.
- Elute absorbed Angiotensin peptides with 0.5 mL of methanol.
- Evaporate the methanol to dryness either by vacuum centrifugation or by evaporation under a stream of dry nitrogen.
- Add 0.5 mL of EIA buffer, vortex and centrifuge at 3,000 g for 10 minutes at 4°C.

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 known volume (a)
(X+Y)/b = concentration of the 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 EIA are respectively equal to (X/a)z and [(X+Y)/b]z.

> **Example**

- Volume of the unspiked sample: a = 1mL
- Final volume of the spiked sample: b = 2 mL
- Concentration determined by EIA for the unspiked sample: (X/a)z = 8 pg/mL
- Concentration determined by EIA for the spiked sample: [(X+Y)/b]z = 16 pg/mL
- Quantity of spike: Y = 30 pg in 1mL

$Xz = 8 \Leftrightarrow z = 8/X$
$[(X+30)/2]z = 16 \Leftrightarrow [(X+30)]z = 32$
thus,
$[(X+30)]8/X = 32 \Leftrightarrow X+30 = 4X \Leftrightarrow 3X = 30 \Leftrightarrow X = 10$
and
$Xz = 8 \Leftrightarrow z = 0.8$

> **Note**

To minimise the 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).

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 Ellman's Reagent.

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

EIA Buffer

Reconstitute the vial #A07000 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.

Angiotensin II Standard (calibrated with the standard WHO 86/538)

Reconstitute one Angiotensin II Standard vial #A06880 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 EIA buffer into each tube. Then prepare the seven standards by serial dilutions as follows:

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

Stability at 4°C: 24 hours.

▷ Angiotensin II Quality Control

Reconstitute one vial #A10880 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.

▷ Angiotensin II Tracer

Reconstitute the vial #A04880 with 10 mL of EIA Buffer. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.
Stability at +4°C: 1 month.

Glutaraldehyde

Dilute 0,125 mL of concentrated Wash Buffer #A17000 with 4,875 mL of UltraPure Water. Just before use add 100 µL of glutaraldehyde #A13880 then mix thoroughly by gentle inversion.
Stability at 4°C: 24 hours.

Borane Trimethylamine

Just before use, reconstitute one vial #A14880 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.

Wash Buffer

Dilute 2 mL of concentrated Wash Buffer #A17000 with 800 mL of UltraPure water. Add 400 µL of Tween 20 #A12000. Use a magnetic stirring bar to mix the content.
Stability at +4°C: 1 month.

Ellman's Reagent

5 minutes before use (development of the plate), reconstitute one vial of Ellman's Reagent #A09000_49+1 with 49 mL of UltraPure water and 1 mL of concentrated Wash Buffer #A17000. The tube content should be thoroughly mixed.
Stability at +4°C and in the dark: 24 hours.

Assay procedure

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

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.

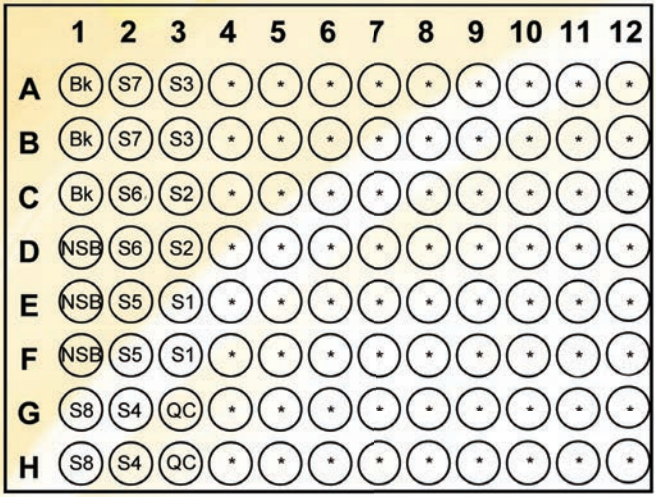
Rinse each well 5 times with Wash Buffer (300 µL/well).

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.

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.



Bk : Blank
NSB : Non Specific Binding
S1-S8 : Standards 1-8
*/QC : Samples or Quality Controls

▷ 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, tracer, 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.

> **EIA Buffer**
Dispense 100 µL to Non Specific Binding (NSB) wells.

> **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.

> **Angiotensin II Quality Control and Samples**
Dispense 100 µL in duplicate to appropriate wells. Highly concentrated samples may be diluted in EIA Buffer.

▷ Incubating the plate

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

▷ Pipetting the reagent

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

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

Washing the plate

> Empty the plate by turning it over.

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

> At the end of the last washing step, remove the buffer from the wells by inverting the plate and shaking out the last drops on a paper towel.

Pipetting the reagent

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

Incubating the plate

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

Developing and reading the plate

> Reconstitute Ellman's Reagent as mentioned in the Reagent preparation section.

> Empty the plate by turning over. Rinse each well five times with 300 µL Wash Buffer. The 5th time, slightly shake the plate for 10 minutes on an orbital shaker. 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.

> Add 200µL of 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.

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

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

> **After addition of 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**.

Enzyme Immunometric Assay Protocol			
Steps	Blank	NSB	Standard, QC and Samples
Wash the plate 5 times			
Incubation (1 h. RT)	_	100 µL of EIA Buffer	100 µL of standard, QC or sample
Epitope immobilisation (5 min. RT)	–	50 µL of Glutaraldehyde in phosphate buffer	
Glutaraldehyde neutralisation (5 min. RT)	–	50 µL of Borane Trimethylamine in HCl 2N/Methanol (50/50, v/v)	
Wash the plate 5 times			
Incubation (overnight 4°C)	_	100 µL of Tracer	
Wash the plate 5 times, shake 10 min, wash 5 times & discard liquid from the wells			
Development	200 µL of Ellman's Reagent		
Incubate with an orbital shaker in the dark at room temperature			
Read the plate between 405 and 414 nm			

Data analysis

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

- Calculate the average absorbance for each NSB, standard and sample.
- For each standard, plot the absorbance on *y* axis versus the concentration on *x* axis. Draw a best-fit line through the points.
- To determine the concentration of your samples, find the absorbance value of each sample on the *y* axis.
- Read the corresponding value on the *x* axis which is the concentration of your unknown sample.
- Samples with a concentration greater than 125 pg/mL should be re-assayed after dilution in EIA Buffer.
- 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).

Acceptable range

- Non-specific Binding <150 mAU
- Limit of detection <5 pg/mL
- Quality Control ±25% of the expected concentration (see the label on QC vial)

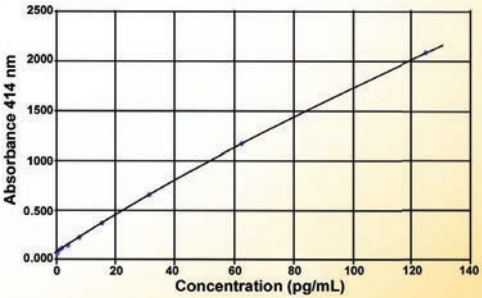
Typical results

The following data are for demonstration purpose only. Your data may be different and still correct.

These data were obtained using all reagents as supplied in this kit under the following conditions: 1 hour developing at +20°C, reading at 414 nm. A linear-curve fitting was used to determine the concentrations.

	Angiotensin II (pg/mL)	Absorbance (mAU)
Standard S1	125	2,069
Standard S2	62.5	1,156
Standard S3	31.25	636
Standard S4	15.63	359
Standard S5	7.81	211
Standard S6	3.9	140
Standard S7	1.95	101
Standard S8	0.98	82
NSB	-	57

Typical Angiotensin II standard curve



Assay validation and characteristics

The Enzyme Immunometric Assay of Angiotensin II has been validated for its use in plasma after extraction.

For additional information regarding the validation of immunoassay for protein biomarkers in biological samples, please refer to bibliography ***[9, 10]***.

- The **Minimum Detectable Concentration** (MDC) or Sensitivity of Angiotensin II corresponding to the NSB average plus three standard deviations (n = 8) is: 1 pg/mL.

- **Limit of quantification**: 2 pg/mL

- Quality Control (QC) samples **intra & inter-assay variation**: established by measuring each QC five times per assay and in six different assays (i.e. 30 assays per QC)

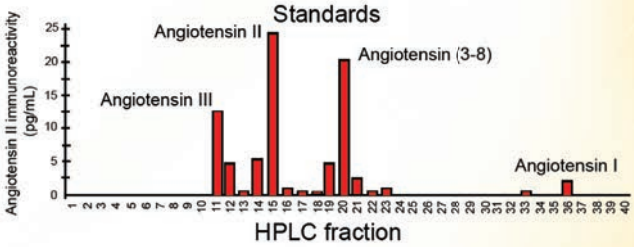
pg/mL	Intra-assay coefficient of variation	Inter-assay coefficient of variation
100	7 %	7 %
20	2 %	5 %
5	6 %	10 %
2	10 %	14.5 %

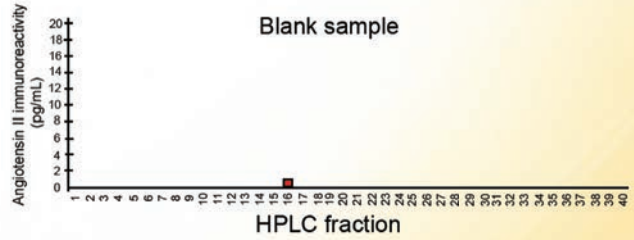
- **Cross-reactivity *[5]***
  The peptidic sequence for Angiotensin II is highly conserved across mammalian species, this is why our kit cross-reacts with all mammalian samples.

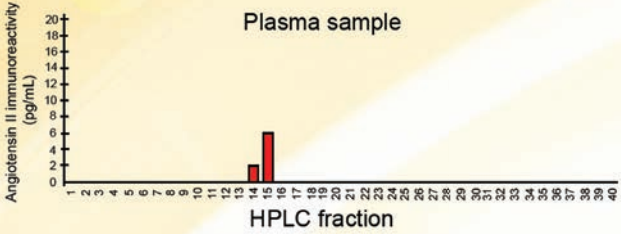
Angiotensin II	100 %
Angiotensin I	4 %
Angiotensin III	36 %
Angiotensin 3-8	33 %
Angiotensin 1-7	<0.01 %

> Specificity

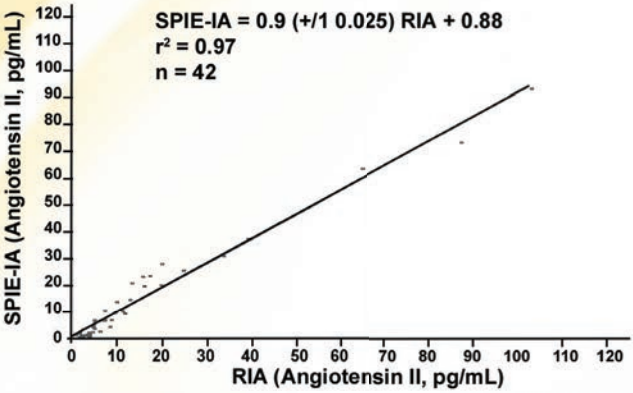
Comparison of HPLC profiles of Angiotensin standards, a blank sample and a plasma sample.







> **Comparison with RIA** on 29 samples



Troubleshooting

> **Absorbance values are too low**:
- incubation in wrong conditions (time or temperature),
- reading time too short,
- Tracer, Glutaraldehyde, Wash Buffer or Borane Trimethylamine have not been dispensed,
- organic contamination of water.

> **NSB value too high**:
- contamination of NSB wells with Angiotensin II standard,
- inefficient washing,
- Borane Trimethylamine has been dispensed not on time or not at all.

> **High dispersion of duplicates**:
- poor pipetting technique
- irregular plate washing.

> **If a plate is accidentally dropped after dispatch of the AChE® substrate (Ellman's Reagent) or if it needs to be revealed again:**
- one only needs to wash the plate, add fresh Ellman's Reagent and proceed with a new development.
- Otherwise, the plate can be stored at +4°C with wash buffer in wells while waiting for technical advice from the Bioreagent Department.

These are a few examples of troubleshooting that may occur. If you need further explanation, Bertin Pharma will be happy to assist you. Feel free to contact our technical support staff by

phone (+33 (0)139 306 036), fax (+33 (0)139 306 299) or E-mail (bioreagent@bertinpharma.com), and be sure to indicate the batch number of the kit (see outside the box).

Bertin Pharma proposes EIA Training kit #B05005 and EIA workshop upon request. For further information, please contact our Marketing Department by phone (+33 (0)139 306 260) or E-mail (marketing@bertinpharma.com).

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Additional readings

List of publications quoting the use of this kit.

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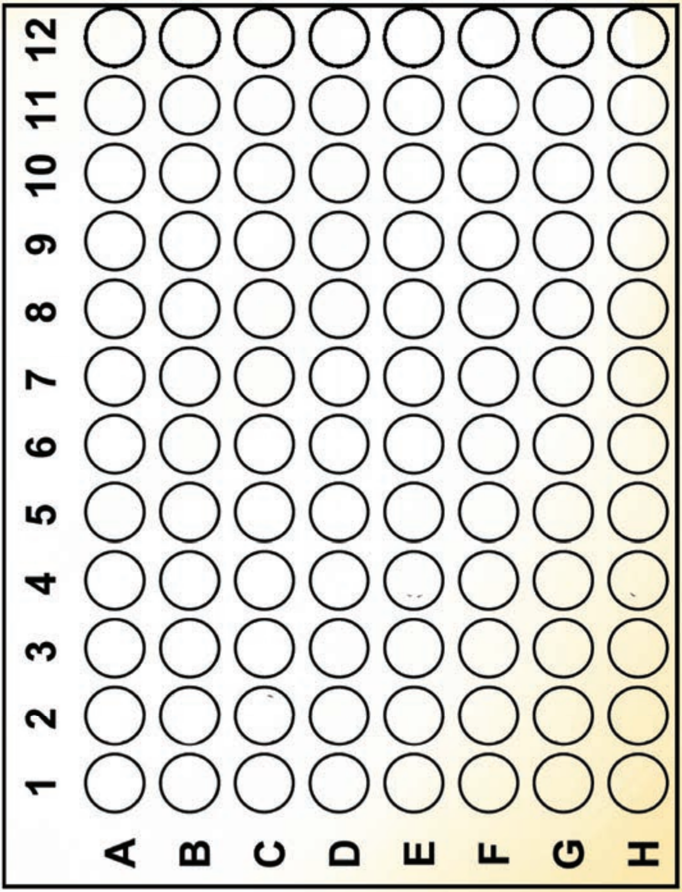
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