ANGIOTENSIN I

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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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- Pharmacology
- Psychopharmacology
- Nitric Oxide
- Oncology / Apoptosis
- Oxidative injury
- Cell signaling
- Drug metabolism

Do not hesitate to contact our after-sales services for further information at bioreagent@bertinpharma.com

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

Angiotensin I
Enzyme Immunoassay kit
#A05882.96 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

#A11882
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 I precoated 96-well Strip Plate	Blister with zip	A08882.1 ea	1	
Angiotensin I Tracer	Green	A04882.xx dtn	1	Lyophilised
Angiotensin I Standard	Blue with red septum	A06882.1 ea	2	Lyophilised
Angiotensin I Quality Control	Green with red septum	A10882.1 ea	2	Lyophilised
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 50	Black with red septum	A09000_50.100 dtn	2	Lyophilised
Technical Booklet	-	A11882.1 ea	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 35 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.

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 I *[4-8]*

The Renin-Angiotensin system (RAS) is essential for the control of blood pressure and homeostasis. It plays an important role in cardiovascular and renal disorders.

Among the different peptides resulting from the proteolytic processing of angiotensinogen (the only substrate of Renin), the octapeptide Angiotensin II (AII) is the major hormone involved in the pathophysiology of hypertensive diseases as it mediates vasoconstrictor action.

Angiotensin I is the precursor of Angiotensin II. It is a decapeptide without apparent physiologic effect. It is converted in Angiotensin II by the Angiotensin-converting enzyme (ACE).

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

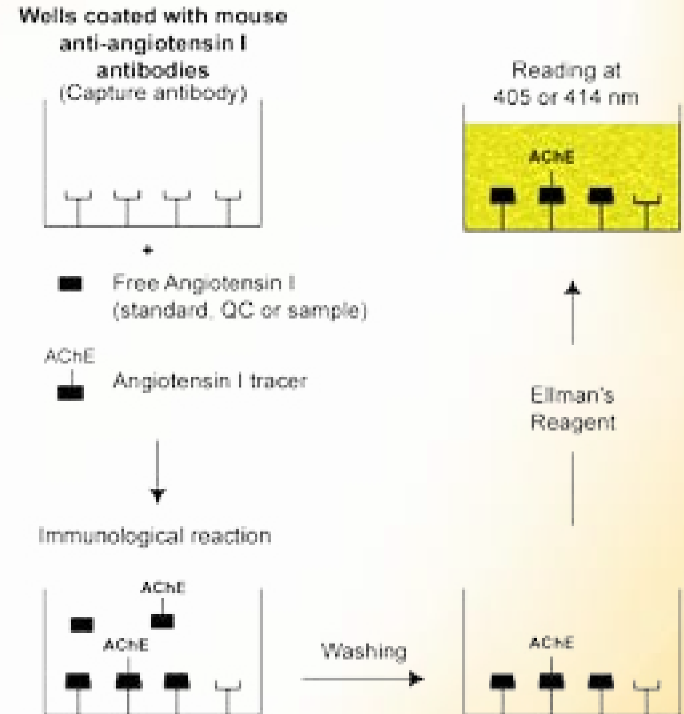
Principle of the assay

This Enzyme Immunometric Assay (EIA) is based on the competition between unlabelled (free) Angiotensin I (standard, QC, samples) and Acetylcholinesterase (AChE) linked to Angiotensin I (Tracer) for limited specific mouse anti-angiotensin I antibody sites coating the well of a plate.

Unlabelled Angiotensin I and AChE-labelled Angiotensin I bind to the antibodies coating the wells during the incubation. Then the plate is washed to remove any unbound reagent, and Ellman's Reagent (enzymatic substrate for AChE and chromogen) is added to the wells.

AChE acts on the Ellman's Reagent to form a yellow compound that strongly absorbs at 414 nm. The intensity of the colour, which is determined by spectrophotometry, is proportional to the amount of tracer bound to the well and is inversely proportional to the amount of free angiotensin I present in the well during the immunological incubation.

The principle of the assay is summarised below:

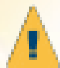


Materials and equipment required

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

For sample preparation:
- Column: Waters Sep-Pak C18 Vac 3cc cartridge (Waters Ref WAT054945)
- Methanol
- Acetic acid
- UltraPure water

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.

Blood collection

Blood samples are collected in tubes kept on ice at 4°C and usually containing EDTA-K3.

The samples are centrifuged at 3000 g for 20 minutes at 4°C.

Samples should be immediately extracted or stored at -20°C until extraction. Avoid thawing samples more than one time.

Extraction

Buffer preparation

Wash Buffer: acetic acid 1% in water (dilute 1 mL of acetic acid 100% in 99 mL of UltraPpure water).

Elution buffer: Acetic acid 1% in methanol (dilute 1 mL of acetic acid 100% in 99 mL of methanol).

Protocol

Dilute at ½ the sample in the Washing Buffer: 500 µL of the sample + 500 µL of Wash Buffer.

Equilibrate the column with 2 mL of methanol.

Wash the column 3 times with 2 mL of Wash Buffer.

Load the column with the diluted sample at 1/2, the maximum volume which is could be loaded is 3 mL.

Wash the column twice with 2 mL of Wash Buffer.

Dry the column.

Elute the sample with 1 mL of Elution Buffer.

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

Resuspend the sample with 500 µL of EIA buffer.

Vortex and centrifuge at 3000 g for 5 minutes at +4°C.

Collect the supernatant.

> 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 I (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 used for spiking 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 35 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 EIA Buffer 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 I Standard

Reconstitute one Angiotensin I Standard vial #A06882 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 2000 pg/mL.

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

Standard	Volume of Standard	Volume of Assay Buffer	Standard concentration (pg/mL)
S1	-	-	2000
S2	500 µL of S1	500 µL	1000
S3	500 µL of S2	500 µL	500
S4	500 µL of S3	500 µL	250
S5	500 µL of S4	500 µL	125
S6	500 µL of S5	500 µL	62.5
S7	500 µL of S6	500 µL	31.2
S8	500 µL of S7	500 µL	15.6

Stability at 4°C: 1 week.

▷ Angiotensin I Quality Control

Reconstitute one Angiotensin I Quality Control vial #A10882 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: 1 week.

▷ Angiotensin I AChE Tracer

Reconstitute the Angiotensin I AChE Tracer vial #A04882 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.

▷ 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_50 with 50 mL of UltraPure water. 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 4 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.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Bk	S7	S3	·	·	·	·	·	·	·	·	·
B	Bk	S6	S2	·	·	·	·	·	·	·	·	·
C	B0	S6	S2	·	·	·	·	·	·	·	·	·
D	B0	S5	S1	·	·	·	·	·	·	·	·	·
E	B0	S5	S1	·	·	·	·	·	·	·	·	·
F	S8	S4	·	·	·	·	·	·	·	·	·	·
G	S8	S4	·	·	·	·	·	·	·	·	·	QC
H	S7	S3	·	·	·	·	·	·	·	·	·	QC

Bk: Blank B0: Maximum Binding
S1-S8: Standards 1-8 *: Samples QC: 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 Maximum Binding (B0) wells.

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

> **Angiotensin I AChE Tracer**
Dispense 100 µL to each well, **except** Blank (Bk) wells.

Incubating the plate

Cover the plate with the 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 it over. Rinse each well 4 times with 300 µL of 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 aluminum sheet and incubate in the dark at room temperature, on 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 (B0 wells) has reached a minimum of 0.2 A.U. (blank subtracted).

Enzyme Immunoassay Protocol (volumes are in µL)					
Volume \ Wells	Blank	B0	Standard	QC	Sample
Wash the plate 4 times					
EIA Buffer	-	100	-	-	-
Standard	-	-	100	-	-
QC	-	-	-	100	-
Sample	-	-	-	-	100
Tracer	-	100			
Cover plate, incubate overnight at +4°C					
Wash strips 4 times & discard liquid from the wells					
Ellman's Reagent	200				
Incubate on an orbital shaker in the dark at RT					
Read the plate between 405 and 414 nm					

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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 not, do it now.

- Calculate the average absorbance for each B0, standard, quality control and sample.
- Calculate the B/B0 (%) for each standard, QC and sample: (average absorbance of standards, QC or sample divided by average absorbance of B0) & multiplied by 100.
- Using a semi-log graph paper, plot the B/B0 (%) for each standard point (y axis) versus the concentration (x axis). Draw a best-fit line through the points.
- To determine the concentration of your samples, find the B/0 (%) value 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 2000 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 +/- 35% of the expected concentration (see the label of QC vial).

Acceptable range

- B0 absorbance >200 mAU blank subtracted in the conditions indicated above.
- IC50: 112.5 to 187.5 pg/mL (mean: 150 pg/mL)
- QC sample: ±35% of the expected concentration (see the label of QC vial)

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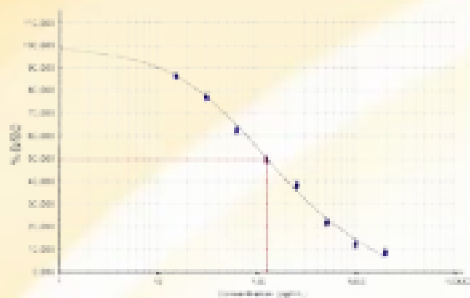
Typical results

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

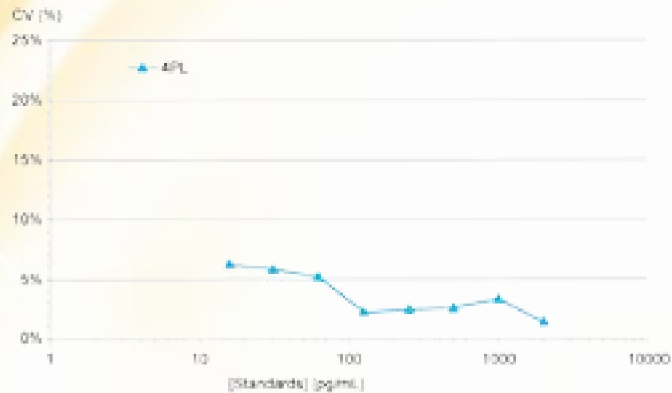
These data were obtained using all reagents as supplied in this kit under the following conditions: 45 minutes developing at +20°C, reading at 414 nm. A 4-parameter curve fitting was used to determine the concentrations.

	Angiotensin I pg/mL	Absorbance (mAU)	B/B0 (%)
Standard S1	2000	60	8.6
Standard S2	1000	88	12.8
Standard S3	500	156	22.5
Standard S4	250	269	38.9
Standard S5	125	347	50.2
Standard S6	62.5	438	63.3
Standard S7	31.25	533	77.1
Standard S8	15.625	600	86.7
B0	0	692	100
QC	150 ±35%	331	-

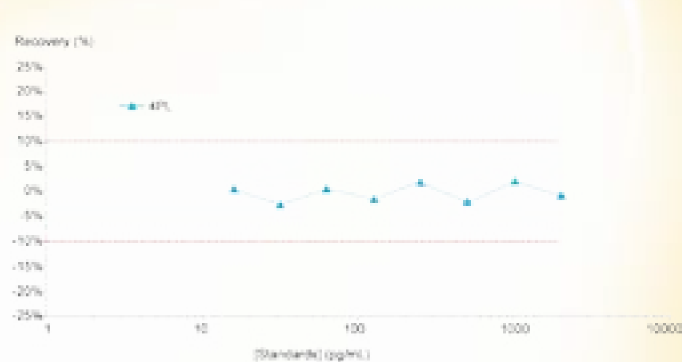
Typical Angiotensin I standard curve



> Precision profile



> Recovery profile



Assay validation and characteristics

The Enzyme Immunometric Assay of Angiotensin I 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 ***[5, 6]***.

> The **Limit of detection (LOD)** of Angiotensin I corresponding to the B0 average minus three standard deviations is around 10 pg/mL..

> The **IC50** is the concentration in Angiotensin I corresponding to 50 % of the maximum Binding is around : 150 pg/mL.

> **Inter-assay variation** (n = 4) in EIA Buffer

Sample level (pg/mL)	Mean of observed concentrations (pg/mL)	Coefficient of Variation (%)
1300	1402	8.99
200	187	6.54
35	39.5	3.2

> **Intra-assay variation** (n = 10) in EIA Buffer

Sample level (pg/mL)	Mean of observed concentrations (pg/mL)	Coefficient of Variation (%)
1300	1326	4.58
200	180	5.47
35	28.2	15.0

The intra-assay and inter-assay variations were studied on human plasma spiked with Angiotensin I at each level of QC.

For the intra-assay validation, the number of replicates (n) is equal to 10 for each level of QC, the three validation levels were analysed along with the calibration curve for a unique experiments.

For the inter-assay validation, the number of replicates (n) is equal to 4 for each levels of QC, the three validation levels were analysed along with the calibration curve for a total of 6 independent runs.

> **Cross-reactivity**

Angiotensin I	100 %
Angiotensinogen	< 0.001%
Angiotensin II	< 0.001%

> Dilution test

Samples	Dilution factor	Unspiked values (pg/mL)	Picogramme of Angio I spiked per mL of plasma	Theorical values (pg/mL)	Measured concentration (pg/mL)	Corrected concentrations (pg/mL)	Recovery %	Total CV (%)
1	-	1280	2000	3280	-	-	-	4.0
	1	-	-	-	-	-	-	
	1:2	-	-	-	1562	3124	-4,8	
	1:4	-	-	-	762	3048	-7,1	
	1:8	-	-	-	381	3048	-7,1	
	1:16	-	-	-	204	3264	-0,5	
	1:32	-	-	-	104	3328	1,5	
2	-	1483	2000	3483	-	-	-	5.8
	1	-	-	-	-	-	-	
	1:2	-	-	-	1663	3326	-4,8	
	1:4	-	-	-	878	3512	0,9	
	1:8	-	-	-	400	3200	-8,6	
	1:16	-	-	-	210	3360	-3,8	
	1:32	-	-	-	93,7	2998,4	-14,8	

Samples	Dilution factor	Unspiked values (pg/mL)	Picogramme of Angio I spiked per mL of plasma	Theorical values (pg/mL)	Measured concentration (pg/mL)	Corrected concentrations (pg/mL)	Recovery %	Total CV (%)
3	-	1280	2000	3280	-	-	-	9.0
	1	-	-	-	-	-	-	
	1:2	-	-	-	1623	3246	-1,0	
	1:4	-	-	-	893	3572	8,9	
	1:8	-	-	-	394	3152	-3,9	
	1:16	-	-	-	237	3792	15,6	
	1:32	-	-	-	121	3872	18,0	

1 mL of three human plasma were spiked with 2000 pg of Angiotensin I, extracted and diluted at ½ in serial dilution. Each dilution were tested in duplicate and analysed against a calibration curve.

Parallelism

Samples	Dilution factor	Angio I measured concentration (pg/mL)	Angio I concentration corrected by the dilution factor (pg/mL)	CV %
1	1	1215	1215	9.8
	1:2	597	1195	
	1:4	298	1192	
	1:8	135	1082	
	1:16	58.2	931	
	1:32	33.5	1071	
2	1	1035	1035	7.0
	1:2	503	1006	
	1:4	267	1068	
	1:8	131	1051	
	1:16	66.0	1055	
	1:32	38.1	1220	
3	1	1525	1510	16.5
	1:2	755	1302	
	1:4	326	1394	
	1:8	174	1364	
	1:16	85	1467	
	1:32	46	1510	

Two human plasma samples were extracted and diluted at 1:2 by serial dilution, each dilution were tested in triplicate and analysed against a calibration curve.

Troubleshooting

Absorbance values are too low:

- incubation in wrong conditions (time or temperature),
- reading time too short,
- one reagent has not been dispensed,
- assay performed before reagents reached room temperature,
- incorrect preparation / dilution,
- organic contaminatin of water.

High signal and background in all wells:

- inefficient washing,
- overdeveloping (incubation time should be reduced),
- high ambient temperature.

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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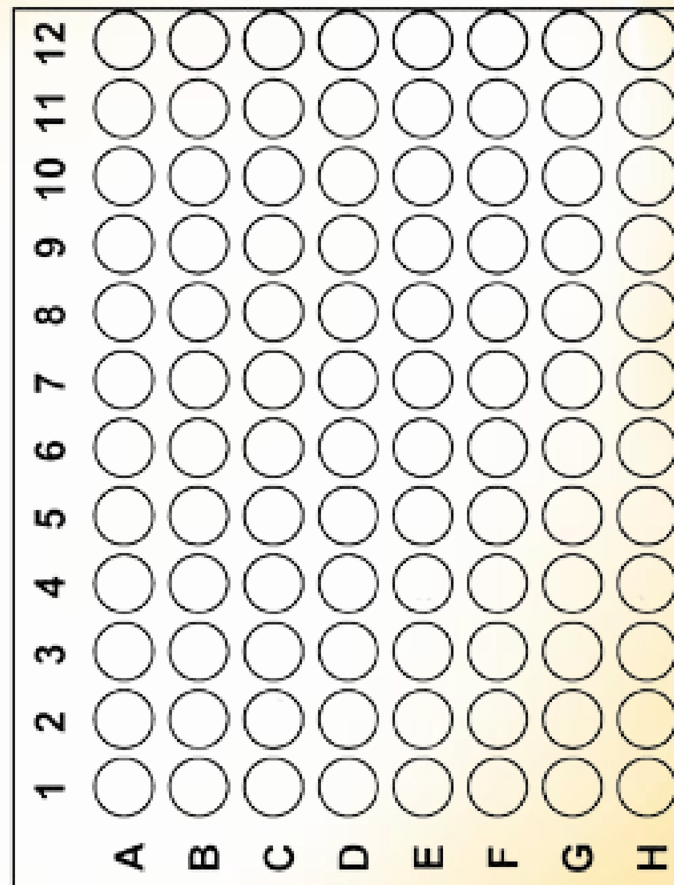
A05882 - Angiotensin I



A05882 - Angiotensin I

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