OBESTATIN (human)

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

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

Obestatin (human)
Enzyme Immunoassay kit
#A05036.96 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

#A11036
Version: 0117

Table of contents

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

This kit contains:

Designation	Colour of cap	Item #	Quantity per kit	Form
A covered 96 well Microtiter plate, pre-coated with Obestatin MAb	Blister with zip	A08035.1ea	1	-
Streptavidin AChE Tracer	Green	A04750.100 dtn	1	Lyophilized
Obestatin (human) Biotin-Labelled Antibody	Red	A03036.100 dtn	1	Lyophilized
Obestatin (human) Standard	Blue with red septum	A06036.1 ea	2	Lyophilized
Obestatin (human) Quality Control	Green with red septum	A10036.1 ea	2	Lyophilized
Obestatin EIA Buffer	Blue	A07035.1 ea	1	Lyophilized
Concentrated Wash Buffer 400x	Silver	A17000.1 ea		Liquid
Tween 20	Transparent	A12000.1 ea	1	Liquid
Ellman's reagent 49+1	Black with red septum	A09000_49+1. 100 dtn	2	Lyophilized
Technical booklet	-	A11036	1	-
Well cover sheet	-	-		-

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.

Obestatin

Obestatin is an amidated peptide made of 23 amino-acids with a secondary conformation in alpha-helix ***[4]***. It was first isolated in 2005 from rat stomach ***[5]***. Obestatin is a preproghrelin-derived peptide and is produced by many tissues or organs like stomach ***[6]***, pancreas ***[6]***, adipose tissue ***[6]***, skeletal muscle or heart.

Obestatin was identified as an anorexigenic peptide with an action on the food intake ***[5]***. The first studies have shown that the obestatin reduced food intake and body weight. It has also been considered to be an antidiabetic peptide by positively influencing glucose and lipid metabolism ***[6]***.

NH2
Ghrelin
Obestatin
-COOH
Preproghrelin (117 AA prohormone)
1MLSSGTICSLLLLSMLWMDMAMAGSSFLSPEHQKAQQRKESKKPPAKLQPRALEGWLH
PEDRGQAEETEEELEIRFNAPFDVGIKLSGAQYQQHGRALGKFLQDILWEEVKEAPADK 117
1
28
Acyl ghrelin
1
28
Desacyl ghrelin
23
Obestatin

Obestatin reduces the apoptosis and promote the proliferation of B-cells and human pancreatic islets ***[7]***.

Due to heterogeneity of these sources, obestatin has many different functions. Indeed obestatin could have a function in the regulation of blood pressure ***[8]*** and its plasmatic concentration increases in case of hypertensive patients ***[9]***.

Finally obestatin could have a role in regulation of anxiety and improvement of the memory ***[10]***.

Principle of the assay

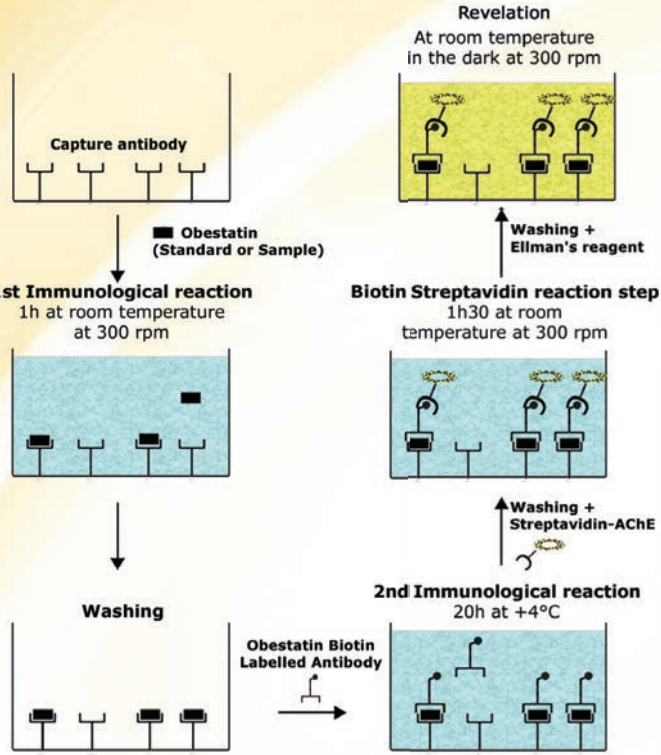
This Enzyme Immunometric Assay (EIA) is based on a sandwich technique. The plate supplied is coated with a monoclonal antibody (mAb) specific to the obestatin.

Obestatin from the standard or the samples is going to bind to the mAb coated on the plate and then is detected by a second mAb labelled with biotin also specific for the obestatin. The immunological complex (mAb-obestatin-mAb_biotin) is revealed by the interaction between biotin and streptavidin labelled with AChE (Tracer).

The concentration of obestatin is determined by measuring the enzymatic activity of immobilized tracer using Ellman's Reagent. AChE tracer acts on Ellman's Reagent to form a yellow compound that strongly absorbs at 405 or at 414 nm.

The intensity of colour, which is determined by spectrophotometry, is proportional to the amount of obestatin present in the well during the immunological reaction.

The principle of the assay is summarised below:



Materials and equipment required

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

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 (item number #A07001.1L)
- Polypropylene tubes

Water used to prepare all EIA reagents and buffers must be ultra pure, deionized & free from organic contaminants traces.
Otherwise, organic contamination can significantly affect the enzymatic activity of the tracer AcetylCholinesterase.
Do not use distilled water, HPLC-grade water or sterile water.

- UltraPure water may be purchased from Bertin Pharma (item #A07001.1L)

Sample collection and preparation

This assay has been validated to measure obestatin in plasma (K_3-EDTA) or in Obestatin EIA Buffer.

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.

Sample collection

Blood samples are collected in tubes containing K_3-EDTA. then, they are centrifuged at 3,500 rpm for 10 minutes at +4°C and supernatants are transfered in separate tubes. Samples should be quickly assayed or stored at -20°C for later use.

Sample preparation

Plasma samples may be assayed directly without any extraction procedure after being diluted at least to **1:8 in Obestatin EIA Buffer** in order to avoid matrix effect.

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.

Obestatin EIA Buffer

Reconstitute the vial #A07035 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

Obestatin (human) Standard

Reconstitute the vial #A06036 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 4000 pg/mL.

Prepare seven propylene tubes for the other standards and add 500 µL of Obestatin EIA Buffer into each tube. Then prepare the standards by serial dilutions as follows:

Standard	Volume of Standard	Volume of Obestatin EIA Buffer	Standard concentration pg/mL
S1	-	-	4000 pg/mL
S2	500 µL of S1	500 µL	2000 pg/mL
S3	500 µL of S2	500 µL	1000 pg/mL
S4	500 µL of S3	500 µL	500 pg/mL
S5	500 µL of S4	500 µL	250 pg/mL
S6	500 µL of S5	500 µL	125 pg/mL
S7	500 µL of S6	500 µL	62.5 pg/mL
S8	500 µL of S7	500 µL	31.3 pg/mL

Stability at 4°C: 8 days

▷ Obestatin (human) Quality Control

The Quality Control provided in this kit has been prepared by spiking Obestatin (human) peptide in Obestatin EIA Buffer.

Reconstitute the vial #A10036 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: 8 days

▷ Obestatin (human) Biotin-Labelled Antibody

Reconstitute the vial #A03036 with 10 mL of Obestatin EIA Buffer. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.
Stability at +4°C: 1 month

Steptavidin AChE Tracer

Reconstitute the vial #A04750 with 10 mL of Obestatin 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 3.5 mL of concentrated Wash Buffer #A17000 with 1400 mL of UltraPure water. Add 700 µL of Tween20 #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 Elman'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 and to follow 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.

Put unused strips back in the zip lock bag with the absorbant pocket and properly close zip lock bag.
Store at -20°C for 1 month.

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

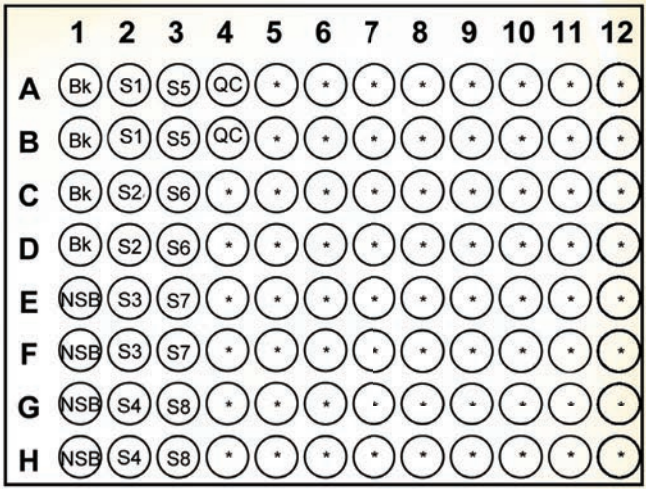
Just before distributing samples, remove the buffer from the wells by inverting the plate and shaking out the last drops on a paper towel.

Plate set-up

A plate set-up is suggested hereafter.

We suggest to assay each Blank and each Non-Specific Binding in four different wells.

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: Quality Control *: Samples

▷ Pipetting the reagents

All samples and reagents must reach room temperature prior to performing the assay. Use different tips to pipette the buffer, standard, sample, tracer, biotin-labelled antibody and other reagents.

Before pipetting, equilibrate the pipette tips in each reagent.

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

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

> **Obestatin (human) Standards**
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.

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

▷ Incubating the plate

Incubate the plate 1 hour at room temperature under agitation on an orbital plate shaker at 300 rpm.

▷ Washing the plate

Rinse each well 5 times with the Wash Buffer (300 µL/well).
Just before distributing reagents, remove the buffer from the wells by inverting the plate and shaking out the last drops on a paper towel.

▷ Pipetting the reagents

> **Obestatin Biotin-Labelled Antibody**
Dispense 100 µL into each well, except Blank (Bk) wells.

Incubating the plate

Incubate the plate overnight (20 hours) at +4°C.

Bring the plate back to room temperature 1 hour before the end of the incubation time

Washing the plate

Rinse each well 5 times with the 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.

Pipetting the reagents

> **Streptavidin-AChE Tracer**
> Dispense 100 µL into each well, except Blank (Bk) wells.

Incubating the plate

Incubate the plate 1 hour 30 minutes at room temperature under agitation on an orbital plate shaker at 300 rpm.

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 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 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 of the STD1 is between 2.500-3.000 A.U (blank subtracted).

Enzyme Immunoassay Protocole (volumes are in µL)					
Volume / Wells	Blank	NSB	Standard	QC	Sample
Obestatin EIA Buffer	-	100	-	-	-
Standard	-	-	100	-	-
QC	-	-	-	100	-
Sample	-	-	-	-	100
Cover plate, incubate 1 hour at RT under agitation at 300 rpm					
Wash strips 5 times, with 300 µL and discard the liquid from the wells					
Biotin-Labelled Ab	-	100			
Cover plate, incubate overnight (20 hours) at +4°C					
Wash strips 5 times, with 300 µL and discard the liquid from the well					
Streptavidin AChE Tracer	-	100			
Cover plate, incubate 1 hour 30 under agitation at 300 rpm					
Wash strips 5 times, with 300 µL and discard the liquid from the well					
Ellman's reagent	200				
Incubate with an orbital shaker in the dark at RT					
Read the plate between 405 and 414 nm					

Data analysis

Make sure that your plate reader has subtracted the absorbance readings of the blank well (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 4000 pg/mL should be re-assayed after dilution in Obestatin EIA Buffer.
- Most plate readers are supplied with curve-fitting software capable of graphing these data (logit/log or 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).**
- **If the NSB is lower than 0.200 A.U.**

Acceptable range

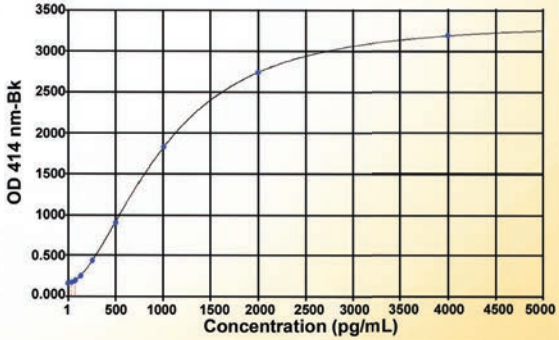
- NSB absorbance < 0.200 A.U.
- Limit of detection < 500.0 pg/mL
- QC sample: ±25% of the expected concentration (see the label of QC vial)

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: 60 minutes developing at RT, reading at 414 nm. A 5-parameter logistic fitting with a 1/Y ponderation was used to determine the concentrations.

Standard	Obestatin (human) pg/mL	Absorbance A.U.
S1	4,000.0	3.192
S2	2,000.0	2.737
S3	1,000.0	1.833
S4	500.0	0.906
S5	250.0	0.437
S6	125.0	0.242
S7	62.5	0.180
S8	31.3	0.164
NSB	0	0.146

Typical obestatin (human) standard curve



Assay validation and characteristics

The Enzyme Immunometric assay of Obestatin (human) has been validated in plasma collected on K_3-EDTA.

For additional information regarding the validation of imunoassay for protein biomarkers in biological samples, please refer to bibliography ***[11, 12]***.

> The **Limit of Detection (LOD)**, calculated as the concentration of Obestatin (human) corresponding to the NSB average plus three standard deviations is 62.5 pg/mL.

Due to the minimal plasma dilution (1:8), the limit of detection in plasma is less than 500.0 pg/mL.

> **Inter-assay variation**

	QC n°1	QC n°2	QC n°3
Means of measured concentrations (pg/mL)	142.0	225.7	744.1
Means of measured concentrations (pg/mL) X dil	1135.6	1805.4	5952.8
Cv %	16.2%	13.9%	5.6%

Unspiked plasma (K_3-EDTA) have been diluted at 1:8 in Obestatin EIA Buffer. The number of replicates (n) is equal to 5, for the three quality controls. The three validation levels were analysed along with the calibration curve for a total of 5 independent runs.

> Intra-assay variation

	QC n°1	QC n°2	QC n°3
Means of measured concentrations (pg/mL)	172.1	265.7	731.5
Means of measured concentrations (pg/mL) X dil	1377.1	2125.8	5851.9
Cv %	6.9%	2.3%	1.8%

Unspiked plasma (K_3-EDTA) has been diluted at 1:8 in Obestatin EIA Buffer. The number of replicates (n) is equal to 20, for the three quality controls. The three validation levels were analysed along with the calibration curve for a unique experiment.

> Cross-reactivity

Obestatin (mouse, rat)	19.1 %
Obestatin (dog)	10.1 %
Obestatin (pig)	0.0 %

> Linearity

Single donor plasma are spiked with 6,000 pg/mL of Obestatin (human) and then diluted by serial dilutions in Obestatin EIA Buffer. Each dilution is run in duplicate.

Matrix	Dilution (1:x)	Endogenous obestatin (human) measured conc (pg/mL)	Spiked obestatin (human) (pg/mL)	Endogenous obestatin (human) + Spiked measured conc (pg/mL)	Endogenous obestatin (human) + Spiked measured conc X Dilution (pg/mL)	Accuracy (%)	CV %
1		1297.3	6000.0	-	-	-	-
	8	-	-	740.3	5922.6	77.1%	13.2%
	16	-	-	420.4	6725.6	90.5%	
	32	-	-	229.1	7331.5	100.6%	
	64	-	-	106.6	6823.9	92.1%	
	128	-	-	66.0	8454.3	119.3%	
2		1334.7	6000.0	-	-	-	-
	8	-	-	886.8	7094.4	96.0%	6.1%
	16	-	-	421.3	6740.8	90.1%	
	32	-	-	211.7	6775.7	90.7%	
	64	-	-	121.8	7796.2	107.7%	
	128	-	-	57.4	7343.0	100.2%	
3		5122.5	6000.0	-	-	-	-
	8	-	-	1388.2	11105.6	99.7%	16.9%
	16	-	-	738.1	11810.1	111.5%	
	32	-	-	364.1	11651.0	108.8%	
	64	-	-	245.9	15736.4	176.9%	
	128	-	-	ND	ND	N/A	

ND: range outside of the standard curve

> Stability test

			1	2	3
Number of freezing / thawing cycles	0	pg/mL	596.3	4590.2	9051.0
		Accuracy	N/A	N/A	N/A
	1	pg/mL	444.6	4719.1	8875.8
		Accuracy	74.7%	102.8%	98.1%
	2	pg/mL	445.7	4384.3	8822.1
		Accuracy	74.7%	95.5%	97.5%
	3	pg/mL	631.0	4369.7	9255.1
		Accuracy	105.8%	95.2%	101.8%
	4	pg/mL	525.5	4455.9	9217.3
		Accuracy	88.1%	97.1%	101.8%
24 hous at RT		pg/mL	647.7	4420.2	8779.7
		Accuracy	108.6%	96.3%	97.0%
Means		pg/mL	548.5	4489.9	9000.2
CV %			15.0%	2.8%	2.1%

Each sample is diluted at 1:8 in Obestatin EIA Buffer.

The concentration indicated in the table above is the measured concentration multiplied by the dilution factor 1:8.

Troubleshooting

> **Absorbance values are too low**:
- organic contamination of water,
- one reagent has not been dispensed,
- incorrect preparation/dilution,
- assay performed before reagents reached room temperature,
- reading time not long enough.

> **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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1 2 3 4 5 6 7 8 9 10 11 12
A
B
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Bertin Pharma, over the last decades, has been developing and marketing over 100 biomarker assays, pre-analytical products, kits, antibodies and biochemicals thanks to its innovative work in research and development.
Our core areas are orientated to inflammation, oxidative injury, endocrinology, diabetes, obesity, hypertension, neurodegenerative diseases, HIV, prion diseases, pharmacokinetics and metabolism.

Bertin Pharma is active worldwide either with direct sales or through our qualified and trained international distribution network from the United States to Japan.

We are able to provide you with local technical support to use at ease our products.

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