ACYLATED GHRELIN (human)
EASY SAMPLING

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

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- 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

Acylated Ghrelin (human)
Easy Sampling Enzyme Immunoassay kit
#A05306.96 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

Version: 0115
Ref. #A11306

Table of contents

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

This kit contains

	Colour of cap	Item #	Quantity per kit	Form
Strip 96 well Microtiter plate, pre-coated with anti-Ghrelin mouse monoclonal antibody Easy Sampling	blister with zip	A08306.1 ea	1	-
Acylated Ghrelin (human, rat) Tracer Easy Sampling	green	A04306.100 dtn	1	lyophilised
Acylated Ghrelin (human) Standard	blue with red septum	A06106.1 ea	2	lyophilised
Acylated Ghrelin (human) Quality Control	green with red septum	A10106.1 ea	2	lyophilised
Acylated Ghrelin EIA Buffer	blue	A07106.1 ea	1	lyophilised
Wash Buffer concentrated 400x	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
Instruction booklet	-	A11306	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.

Precaution for use

Users are recommended to 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

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 it's 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 Horse Radish 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.

Ghrelin

Ghrelin discovered in 1999, is fast becoming an endocrinology target of the millennium. Ghrelin, identified in rat stomach as an endogenous ligand for the GH secretagogue receptor, is mainly produced in stomach, but has been demonstrated in many other organs ***[4, 5]***.

In addition to GH-releasing properties and its orexant action, Ghrelin could act as an hormone having effects on gastric motility (similarity with the peptide hormone motilin), acidic secretion, cardiovascular action, antiproliferative effects, pancreatic and glucose metabolism function, sleep ***[6, 7, 8]***...

Ghrelin gene raises to mRNA prepro-ghrelin of 117 amino acids. This precursor is processed into Ghrelin, 28 amino acids (human).

Before being secreted, this peptide is octanoylated at Ser 3 by GOAT (Ghrelin Octanoyl Acyl Transferase). This step is essential for biological activity making GOAT a perfect target for drugs in feeding behaviour. Interestingly, the potential therapeutic importance of this hormone is not restricted to regulation of food intake ***[9]*** but also in cachexia (related to cancer treatment, anorexia nervosa or ischemia) ***[10]*** gastric motility and may be involved in osteoporosis, somatopause, infertility and ovulation induction, neurological disorders (Alcoholism, Post Traumatic Stress disorders...) ***[11]*** and cardiovascular diseases.

Principle of the assay

This Enzyme Immunometric Assay (EIA) is based on a double-antibody sandwich technique. The wells of the plate supplied are coated with a monoclonal antibody specific to the C-terminal part of Ghrelin.
This antibody will bind to any Ghrelin introduced into the wells (standard or sample). After a washing step, the acetylcholinesterase (AChE) - Fab' conjugate (Tracer) which recognises the N-terminal part of Acylated Ghrelin is then added to the wells.

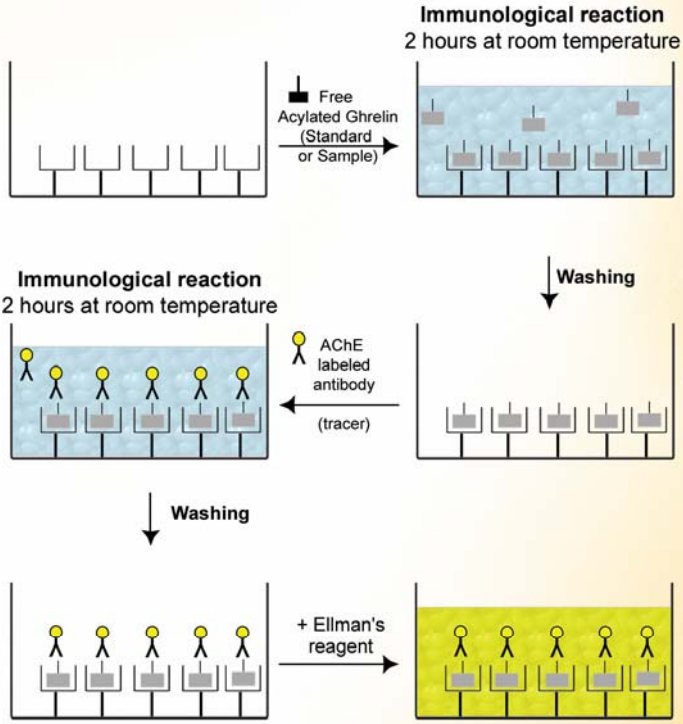
The two antibodies then form a sandwich by binding on different parts of the Acylated Ghrelin.
The sandwich is immobilised on the plate so reagents in excess may be washed away.

The concentration of Acylated Ghrelin (human) 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 414 nm.

The intensity of the colour, which is determined by spectrophotometry, is proportional to the amount of Acylated Ghrelin (human) present in the well during the immunological incubation.

This EIA so called Easy Sampling EIA kit works with any sample collected on any kind of protease inhibitors, without extraction but a simple dilution.

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 sample preparation

- EDTA tubes for blood collection
- Protease inhibitor (AEBSF, PMSF, Aprotinin, Pefabloc®, P800, PHMB ...)
- UltraPure water #A07001

For the assay

- Precision micropipettes (20 to 1000 µL)
- Spectrophotometer plate reader (405 or 414 nm filter)
- Microplate washer (or washbottles)
- Orbital microplate shaker able to perform at 600 rpm
- Multichannel pipette and disposable tips 30-300 µL
- UltraPure water (Ref. #A07001)
- 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 Acylated Ghrelin in buffer and in human plasma samples (see validation data at the end of this booklet).

General precautions

- All samples must be free of 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 containing EDTA and a protease inhibitor to prevent the degradation of Acylated Ghrelin.

- **Choice of protease inhibitor**
  We suggest adding AEBSF at 0.2 mg/mL blood during blood collection.
  We suggest preparing a 100 times concentrated solution of protease inhibitor and then adding 10 µL of this solution per mL of blood. For example, for the AEBSF, prepare a mother solution at 20 mg/mL in UltraPure water and add 10 µL of this solution per mL of blood.
  The mother solution may be stored one month at -20°C.
  We suggest using aliquots for AEBSF solution in order to avoid freezing/thawing cycles.

Other protease inhibitors could be used with the assay like Aprotinin (up to 0,6 TIU/mL blood), PMSF (around 0.1 mg/mL blood according to literature), PHMB, Pefabloc® or Pefabloc SC® (up to 0.2 mg/mL blood) as indicated in the section "Protease inhibitor compatibility table" at the end of this booklet. For the use of these different products, please refer to the vendor's instructions.

> Collection tubes are mixed by inversion 5 folds.

Samples should be kept on ice between collection and centrifugation (15 minutes max).

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

> The best way is to assay the samples within 3 weeks after the collection date. Moreover, we suggest using aliquots for plasma samples (we suggest 250 µl per aliquot) in order to avoid freezing/thawing cycles.

Plasma samples prepared as above-mentioned can be assayed for Acylated Ghrelin with Acylated Ghrelin Easy Sampling EIA kit or for Unacylated Ghrelin with Unacylated Ghrelin Easy Sampling EIA kit.

Sample preparation

Plasma samples may be assayed directly without any extraction procedure after being diluted at **least to 1:2 in EIA Buffer** in order to avoid matrix effect.

Reagent preparation

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

EIA Buffer

Reconstitute the vial #A07106 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

Acylated Ghrelin (human) Standard

Reconstitute the Standard vial #A06106 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 250 pg/mL.

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

Standard	Volume of Standard	Volume of EIA Buffer	Standard concentration pg/mL
S1	-	-	250
S2	500 µL of S1	500 µL	125
S3	500 µL of S2	500 µL	62.5
S4	500 µL of S3	500 µL	31.3
S5	500 µL of S4	500 µL	15.6
S6	500 µL of S5	500 µL	7.8
S7	500 µL of S6	500 µL	3.9
S8	500 µL of S7	500 µL	2.0

Stability at 4°C: 1 week

▷ Acylated Ghrelin (human) Quality Control

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

Reconstitute the Quality Control vial #A10106 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

▷ Acylated Ghrelin Tracer Easy Sampling

Reconstitute the vial #A04306 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 week

Wash Buffer

Dilute 2 mL of concentrated Wash Buffer #A17000 with 800 mL of UltraPure water. Add 400 µL of Tween20 #A12000. Use a magnetic stirring bar to mix the content.
Stability at +4°C: 1 week

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. 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 and place the unused strips back in the packet, store at +4°C for 1 month maximum.

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

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

Distribution of reagents and samples

A plate set-up is suggested on the following page.
The contents of each well may be recorded on the template sheet provided at the end of this technical booklet.

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, antiserum 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.

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

> **Acylated Ghrelin (human) Standards**
Dispense 100 µL of each of the eight standards S1 to S8 in duplicate to appropriate wells.
Start with the lowest concentration standard S8 and equilibrate the tip in the next higher standard before pipetting.

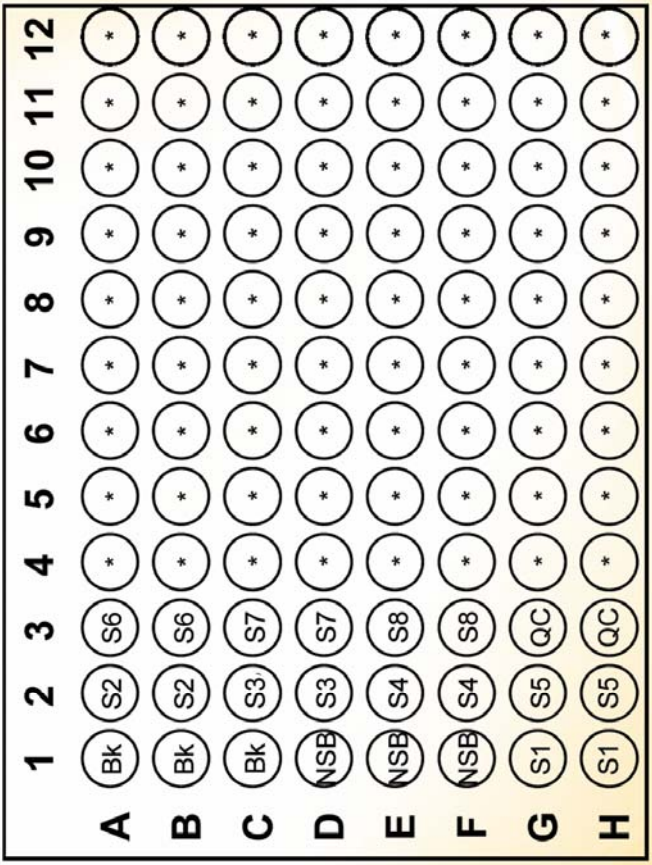
> **Quality Control and samples**
Dispense 100 µL in duplicate to appropriate wells. Highly concentrated samples may be diluted in EIA Buffer.

▷ Incubating the plate

Cover the plate with the cover sheet and incubate for 2 hours at room temperature on an orbital shaker (at 600 rpm).

▷ Washing the plate

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



Bk : Blank
NSB : Non Specific Binding
* : Samples

S1-S8 : Standards 1-8
QC : Quality Control

Pipetting the reagents

- **Acylated Ghrelin Tracer**
  Dispense 100 µL to each well, **except** blank (Bk) wells.

Incubating the plate

Cover the plate with the cover sheet and incubate for 2 hours at room temperature on an orbital shaker (at 600 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 Wash Buffer. The 5th time, slightly shake the plate for 5 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 96 well. Cover the plate with 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.

Easy Sampling Enzyme Immunoassay Protocole (volumes are in µL)				
	Blank	NSB	Standard	Sample or QC
EIA Buffer	-	100	-	-
Standard	-	-	100	-
Sample or QC	-	-	-	100
Cover plate, incubate 2 hours at 600 rpm				
Wash plate 5 times, shake 5 min, wash 5 times & discard liquid from the wells				
Tracer	-	100	100	100
Cover plate, incubate 2 hours at 600 rpm				
Wash plate 5 times, shake 5 min, wash 5 times & discard liquid from the wells				
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 not, do it now.

- 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. Do not forget to integrate the dilution factor of your own samples (due notably to the minimal dilution for the assay 1:2).
- Samples with a concentration greater than 250 pg/mL should be re-assayed after dilution in 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 (written on the label of the QC vial)

Acceptable range

- Non Specific Binding < 50 mA.U.
- Limit of detection in the sample before dilution <2 pg/mL
- QC sample: ±25% of the expected concentration (see the label of QC vial)

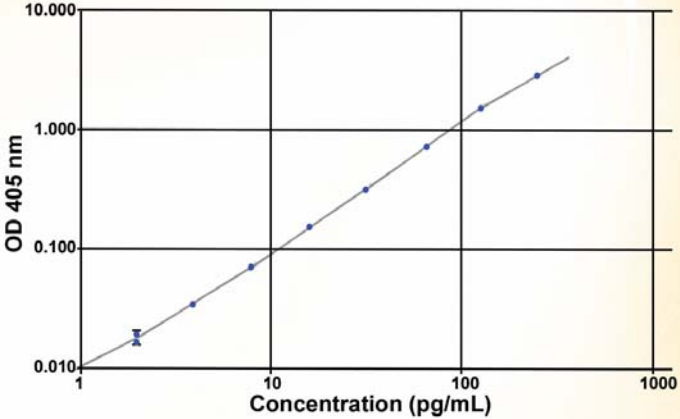
Typical results

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 developping, reading at 414 nm. A 5-parameter logistic fitting with ponderation
1/Y2 was used to determine the concentrations.

	Acylated Ghrelin (human) pg/mL	Absorbance (mAU)
Standard S1	250	2875
Standard S2	125	1518
Standard S3	62.5	741
Standard S4	31.3	324
Standard S5	15.6	163
Standard S6	7.8	81
Standard S7	3.9	43
Standard S8	2.0	28
Blank	0	10

Typical Acylated Ghrelin (human) Easy Sampling standard curve



Assay validation and characteristics

The Enzyme Immunometric assay of Acylated Ghrelin (human) has been validated for its use in buffer and in plasma (without extraction but diluted at least 1:2). A 5-parameter logistic fitting with ponderation 1/Y2 was used to determine the concentrations.

For additional information regarding the validation of imunoassay for protein biomarkers in biological samples, please refer to bibliography ***[12, 13]***.

- The **limit of detection**, calculated as the concentration of Acylated Ghrelin corresponding to the NSB average (n = 8) plus three standard deviations is 2 pg/mL. Due to the minimal plasma dilution (1:2), the limit of detection in the samples is 4 pg/mL.

- **Intra-assay & inter-assay variations** and recovery

	Inter-assay			Intra-assay		
QC levels after 1:5 dilution (pg/mL)	**Mean of observed concentrations (pg/mL)**	**CV (%)**	**Recovery (%)**	**Mean of observed concentrations (pg/mL)**	**CV (%)**	**Recovery (%)**
200 (ULOQ)	217.0	10.0	8.6	228.0	2.6	13.8
150 (HQC)	163.0	7.0	8.5	167.0	2.8	11.6
50 (MQC)	45.9	13.5	-8.3	45.8	4.8	-8.3
10 (LQC)	9.6	20.1	-4.2	9.0	15.1	-10.1
5 (LLOQ)	5.1	27.6	-2.2	4.2	21.6	-16.6

ULOQ: Upper Limit of Quantification HQC: High QCMQC: Mid QC
LQC: Low QC LLOQ: Lower Limit of Quantification

The intra-assay and inter-assay variations were studied on a pool of human plasma containing AEBSF 0.4 mg/mL (free of Ghrelin) spiked samples for each level of QC. QC were prepared five times concentrated from a pool of human plasma and then diluted to 1:5 in EIA Buffer before assay.
For within-run precision and accuracy, the number of replicates (***n***) is equal to 6 for each levels of QC, the five QC samples were analysed along with the calibration curve for a unique experiment.
For between-run precision and accuracy, the number of replicates (n) is equal to 6 for each levels of QC, the five QC samples were analysed along with the calibration curve for a total of 9 independent experiments.

> Selectivity

Matrix	Mean of measured concentration (pg/mL)	CV (%)	Recovery (%)
1	6.49	3.18	29.7
2	6.05	3.53	20.9
3	6.14	4.95	22.7
4	6.35	2.18	26.9
5 (haemolysed)	3.96	10.70	-20.7
6	5.44	3.83	8.8
7	5.54	6.69	8.8
8	5.71	9.73	14.2
9	5.13	10.70	2.5
10 (haemolysed)	3.66	4.26	-26.7

Selectivity was tested by spiking 10 sources of samples matrix containing AEBSF at 0.4 mg/mL at the LLOQ (n=3).

These sources included 2 haemolysed samples (matrix 5 and 10). QC samples (n=3) were prepared five times concentrated in each matrix (free of Ghrelin) and then diluted to 1:5 in EIA buffer in order to obtain a final concentration of 5 pg/mL and analysed against a calibration curve.

> Specificity

Specificity was tested by adding AEBSF at 0.4 mg/mL (recommended use concentration = reference) and 2 mg/mL (high concentration) or aprotinin at 1.2 TIU/mL with or without HCl 0.1 N final into sample matrix (a pool of human plasma samples) and measuring the accuracy of the Acylated Ghrelin (human) at both LLOQ and ULOQ (n=3).

Matrix	QC level after 1:5 dilution (pg/mL)	Mean of measured concentration (pg/mL)	CV (%)	Recovery (%)
AEBSF 0.4 mg/mL	5	5.25	9.23	4.91
	200	224	1.36	11.80
AEBSF 0.4 mg/mL + HCl 0.1N	5	5.17	7.62	3.32
	200	228	0.97	14.00
AEBSF 2 mg/mL	5	4.35	13.30	-13.00
	200	200	1.09	-0.13
AEBSF 2 mg/mL + HCl 0.1N	5	4.91	11.20	-1.72
	200	209	4.81	4.48
Aprotinin 1.2 TIU/mL	5	4.38	5.05	-12.50
	200	211	1.32	5.66
Aprotinin 1.2 TIU/mL + HCl 0.1N	5	4.88	3.99	-2.31
	200	215	3.54	7.31

> Dilution tests

Dilution linearity was tested by spiking a pool of human plasma samples (free of Ghrelin) containing AEBSF at 0.4 mg/mL at 2000 pg/mL (n=3) and measuring precision and accuracy after serial dilution in EIA buffer to bring the Acylated Ghrelin concentrations into the validated range for analysis (between ULOQ and LLOQ).

Dilution factor	Theoretical concentration (pg/mL)	Measured concentration (pg/mL)	Corrected concentration (pg/mL)	Recovery (%)	Mean recovery (%)	CV%
1:10	200	188.6	1886	-5.69	-1.33	5.27
		206.6	2066	3.32		
		196.8	1968	-1.62		
1:20	100	90.9	1 817	-9.14	-5.98	
		99.7	1 995	-0.25		
		91.5	1 829	-8.55		
1:40	50	46.3	1 852	-7.42	-8.64	
		46.7	1 868	-6.61		
		44.1	1 763	-11.90		
1:80	25	22.2	1 776	-11.20	-9.60	
		22.2	1 776	-11.20		
		23.4	1 872	-6.41		
1:160	12.5	11.1	1 768	-11.60	-4.32	
		12.7	2 028	1.39		
		12.2	1 945	-2.74		

> Parallelism

Parallelism between the calibration standard curve and serial diluted samples was tested by diluting 3 samples containing AEBSF at 0.4 mg/mL in EIA buffer (n=3) to bring the Acylated Ghrelin concentrations into the validated range for analysis (between ULOQ and LLOQ).

Sample	Dilution series	Dilution factor	Measured concentration (pg/mL)	Corrected concentrations (pg/mL)	CV (%)
1	1	1:5	13.30	66.3	29.3
		1:10	6.60	66.0	
		1:20	5.32	106.0	
	2	1:5	13.00	65.0	12.7
		1:10	6.01	60.1	
		1:20	2.52	50.4	
	3	1:5	10.80	54.0	10.0
		1:10	4.70	47.0	
		1:20	2.87	57.3	
2	1	1:2	13.80	27.6	18.8
		1:5	4.06	20.3	
		1:10	2.02	20.2	
	2	1:2	13.50	27.0	11.8
		1:5	4.32	21.6	
		1:10	2.29	22.9	
	3	1:2	15.20	30.4	16.7
		1:5	4.52	22.6	
		1:10	2.34	23.4	

Sample	Dilution series	Dilution factor	Measured concentration (pg/mL)	Corrected concentrations (pg/mL)	CV (%)
3	1	1:2	14.80	29.7	15.5
		1:5	4.72	23.6	
		1:10	2.24	22.4	
	2	1:2	14.20	28.4	12.0
		1:5	4.72	34.4	
		1:10	2.29	22.9	
	3	1:2	13.90	27.7	9.2
		1:5	4.82	24.1	
		1:10	2.34	23.4	

> Stability test (freezing/thawing, 24h at +5°C and 24h at +20/+25°C)

Stability of Acylated Ghrelin was evaluated by using Low and High QC samples. These QC samples (n=3) were prepared from a pool of human plasma (free of Ghrelin) containing AEBSF at 0.4 mg/mL or Aprotinin at 1.2 TIU/mL and then frozen at -20°C for freeze/thaw stability or stored 24h at +5°C or at 20/25°C for short-term stability.

Conditions	QC level after 1:5 dilution (pg/mL)	Mean of measured concentration (pg/mL)	CV (pg/mL)	Recovery (%)
Freeze/thaw 1 cycle AEBSF 0.4 mg/mL	10	9.5	3.48	-4.53
	150	165	1.93	10.30
Freeze/thaw 3 cycles AEBSF 0.4 mg/mL	10	11.0	10.90	10.10
	150	157	8.31	4.89
Freeze/thaw 1 cycle Aprotinin 1.2 TIU/mL	10	10.3	9.12	3.18
	150	156	8.81	4.18
Freeze/thaw 3 cycles Aprotinin 1.2TIU/mL	10	9.5	12.90	-4.49
	150	150	3.79	-0.15
24h at +5°C AEBSF 0.4 mg/mL	10	8.0	3.52	-20.20
	150	138	3.91	8.27
24h at 20/25°C AEBSF 0.4 mg/mL	10	3.3	7.82	-66.80
	150	65.4	3.90	-56.40

> Long term stability (3 and 6 months at -20°C & -80°C)

Conditions	QC level after 1:10 dilution (pg/mL)	Mean of measured concentration (pg/mL)	CV (pg/mL)	Recovery (%)
3 months at -20°C	10	8.33	4.17	-16.70
	150	159	4.23	6.28
3 months at -80°C	10	9.64	5.66	-3.60
	150	171	2.19	14.20
6 months at -20°C	10	In progress		
	150	In progress		
6 months at -80°C	10	In progress		
	150	In progress		

> Cross-reactivity

Acylated Ghrelin (rat)	100%
Acylated Ghrelin (dog)	85%
Unacylated Ghrelin (human)	<1%
Unacylated Ghrelin (rat)	<1 %
Unacylated Ghrelin (dog)	<1%
Ghrelin (1-14) (human)	<0.001 %
Ghrelin (1-11) (rat)	<0.001 %
Ghrelin (17-28) (human, rat)	<0.001 %
GHRF (human)	<0.001 %
Insulin (human)	<0.001 %
Motiline	<0.001 %
Leptin (human)	<0.001 %
Somatostatine	<0.001 %
CRF (human, rat)	<0.001 %
Glucagon (human, rat)	<0.001 %

> Protease Inhibitor compatibility table

	AEBSF	PMSF	Pefabloc	P800	Aprotinin	PHMB
A05306.96 wells	YES	YES	YES	YES	YES	YES
A05106.96 wells	NO	YES	NO	NO	YES	YES

Plasma samples were collected on different protease inhibitors according to vendors instruction and measured with the appropriate kit. Recovery is different from one inhibitor to the other and it belongs to the end user to define according to its needs which inhibitor to be used. Acidification has also been tested with most inhibitors and may also change recovery, but will not affect the assay performances providing that 1:5 dilution with EIA Buffer or neutralisation is performed.

> Related products

Item Reference	Designation	Application
A05106.96 wells	Acylated Ghrelin (human) Express EIA kit	PHMB, PMSF, Aprotinin samples
A05106.384 wells	Acylated Ghrelin (human) 384w EIA kit	GOAT inhibitor screening
A05117.96 wells	Acylated Ghrelin (mouse, rat) Express EIA kit	PHMB, PMSF, Aprotinin samples
A05118.96 wells	UnAcylated Ghrelin (mouse, rat) Express EIA kit	PHMB, PMSF, Aprotinin samples
A05119.96 wells	UnAcylated Ghrelin (human) Express EIA kit	PHMB, PMSF, Aprotinin samples
A05306.96 wells	Acylated Ghrelin (human) Easy Sampling EIA kit	Any kind of sample
A05317.96 wells	Acylated Ghrelin (mouse, rat) Easy Sampling EIA kit	
A05318.96 wells	UnAcylated Ghrelin (mouse, rat) Easy sampling EIA kit	
A05319.96 wells	UnAcylated Ghrelin (human) Easy Sampling EIA kit	
A05320.96 wells	UnAcylated Ghrelin (dog) Easy Sampling EIA kit	
A05321.96 wells	Acylated Ghrelin (dog) Easy Sampling EIA Kit	
A05401.96 wells	Acylated Ghrelin (pig) EIA kit	PHMB, PMSF, Aprotinin samples
A05402.96 wells	UnAcylated Ghrelin (pig) EIA kit	PHMB, PMSF, Aprotinin samples
D31009	Sampling Tubes with PHMB	Sample preparation

Assay trouble shooting

- **Absorbance values too low**: organic contamination of water, incubation in wrong conditions (time or temperature), reading time not long enough.
  Standard or Tracer or Ellman's reagent have not been dispensed.
- **High signal and background in all wells**: Inefficient washing or overdeveloping (incubation time should be reduced) or high ambient temperature. It is necessary to check for ambient temperature especially in case of high NSB.
- **High dispersion of duplicates**: Poor pipetting technique or 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 trouble shooting 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

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Parc d'activités du Pas du Lac - 10 bis avenue Ampère
F-78180 Montigny-le-Bretonneux - France
Tel: +33 (0)139 306 036 - Fax: +33 (0)139 306 299
bioreagent@bertinpharma.com - www.bertinpharma.com