ACYLATED GHRELIN (human)
384 wells

A brand name of bertin pharma

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

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- **Psychopharmacology**
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- **Oncology / Apoptosis**
- **Oxidative injury**
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- **Drug metabolism**

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

Acylated Ghrelin (human)
384-well Enzyme Immunoassay kit
#A05106.384 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. #A11106.384 dtn

Table of contents

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

This kit contains

	Colour of cap	Item #	Quantity per kit	Form
Acylated Ghrelin precoated 384 well Microtiter plate	blister with zip	A08106.384 dtn	1	-
Acylated Ghrelin Tracer for 384 well plate	green	A04106.384 dtn	2	lyophilised
Acylated Ghrelin (human) Standard	blue with red septum	A06106.384 dtn	1	lyophilised
Acylated Ghrelin (human) Quality Control	green with red septum	A10106.384 dtn	1	lyophilised
Acylated Ghrelin EIA Buffer	blue	A07106.384 dtn	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	-	A11106.384 dtn	1	-
Well cover sheet	-	-	1	-

Each kit contains sufficient reagents for 384 wells. This allows for the construction of one standard curve in duplicate and the assay of 176 samples in duplicate or screening of 352 samples in simplicate.

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

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.

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

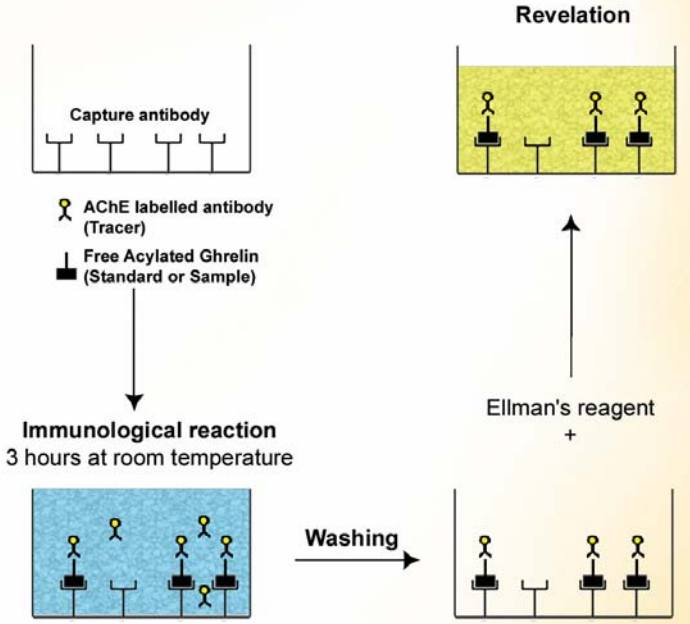
This Enzyme Immunometric Assay (EIA) is based on a double-antibody sandwich technique. The wells of the plate supplied are coated with a monoclonal antibody specific to the C-terminal part of Ghrelin.
This antibody will bind to any Ghrelin introduced into the wells (standard or sample). The acetylcholinesterase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

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) or a robotic platform
- Spectrophotometer plate reader (405 or 414 nm filter)
- Microplate washer (or washbottles)
- Orbital microplate shaker
- 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 developed to screen GOAT activity by monitoring Acylated Ghrelin production in cell culture media.

Cells over-expressing GOAT are cultivated in large excess of Unacylated Ghrelin, GOAT transforming Unacylated Ghrelin into Acylated Ghrelin.

The kit detects Acylated Ghrelin with 10^3 to 10^5 more Unacylated Ghrelin in the sample.

> Alternatively, it can be used for measuring Acylated Ghrelin in human biological samples.

For this application, please contact technical support at bioreagent@bertinpharma.com.

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.

Sample preparation

No extraction or dilution are necessary providing that the sample concentration is within the standard curve range. Otherwise, dilute with Acylated Ghrelin EIA Buffer.

Reagent preparation

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

Acylated Ghrelin 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 #A06106.384 dtn 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 #A10106.384 dtn 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

Reconstitute each vial #A04106.384 dtn with 6 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 follow the instructions hereafter.

Plate preparation

Prepare the Wash Buffer as indicated in the reagent preparation section.
Open the plate packet.

Rinse each well 5 times with the Wash Buffer 65 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.
Note that the first row is dedicated to 8 Blank wells and 8 Non Specific Binding wells (NSB).

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 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 25 µL to Non Specific Binding (NSB) wells and 50 µL to the Blank (Bk) wells.

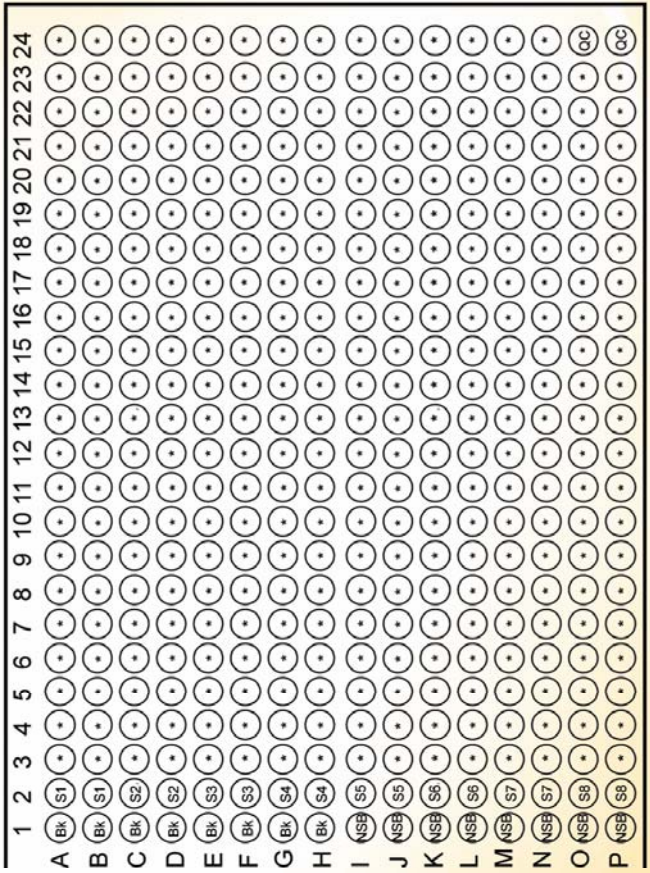
> **Acylated Ghrelin (human) Standards**
Dispense 25 µL of each of the eight standards S1 to S8 in duplicate to appropriate wells.
Start with the lowest concentration standard S8 and equilibrate the tip in the next higher standard before pipetting.

> **Quality Control and samples**
Dispense 25 µL in duplicate or in simplicate to appropriate wells. Highly concentrated samples may be diluted in EIA Buffer.

> **Acylated Ghrelin Tracer**
Dispense 25 µL to each well, **except** the 8 Blank (Bk) wells.

▷ Incubating the plate

Cover the plate with the cover sheet and incubate for 3 hours at room temperature.
A longer immunological reaction (20 hours at +4°C) is also possible, increasing the sensitivity of the assay.



Bk: Blank

NSB: Non Specific Binding

*: Samples

S1-S8: Standards 1-8

QC: Quality Control

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 65 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 50 µL of Ellman's reagent to each 384 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 (Standard 1 or Control) has reached a minimum of 0.3 A.U. blank subtracted.

384-well Enzyme Immunoassay Protocole (volumes are in µL)				
	Blank	NSB	Standard	Sample or QC
EIA Buffer	50	25	-	-
Standard	-	-	25	-
Sample or QC	-	-	-	25
Tracer	-	25	25	25
Cover plate, incubate 3 hours at RT				
Wash plate 5 times, shake 5 min, wash 5 times & discard liquid from the wells				
Ellman's reagent	50			
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.
- 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.

One vial of Quality Control is 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 < 60 mA.U.
- Sensitivity: <5 pg/mL
- Limit of detection in the sample before dilution <8 pg/mL
- QC sample: ±25% of the expected concentration (see the label of QC vial)
- False positive: <1%
  Determined as samples giving a signal 35% lower than expected for a QC at 200 pg/mL, corresponding to 35% inhibition of GOAT.

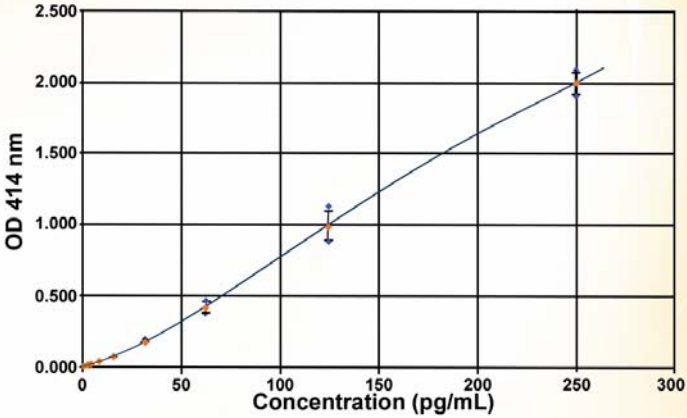
Typical results

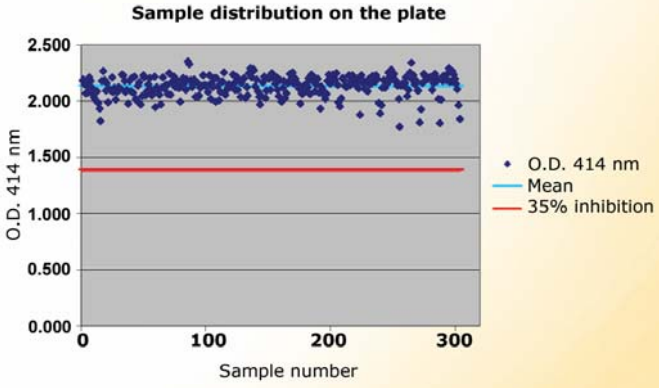
The following data are for demonstration purpose only. Your data may be different and still correct.

These data were obtained using all reagents as supplied in this kit under the following conditions: 3 hours at room temperature immunological incubation, then 90 minutes developping at room temperature, reading at 414 nm. A 4-parameter logistic fitting was used to determine the concentrations.

	Acylated Ghrelin (human) pg/mL	Absorbance (mAU)
Standard S1	250	1596
Standard S2	125	924
Standard S3	62.5	506
Standard S4	31.3	239
Standard S5	15.6	97
Standard S6	7.8	31
Standard S7	3.9	18
Standard S8	2.0	12

Typical Acylated Ghrelin (human) 384 wells standard curve





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.
- **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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Acylated and unAcylated Ghrelin impair skeletal muscle atrophy in mice.
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> Additional readings

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- Wasse L et al
The influence of vigorous running and cycling exercise on hunger perceptions and plasma acylated ghrelin concentrations in lean young men
Appl. Physiol. Nutr. Metab.(2013) dx.doi.org/10.1139/apnm-2012-0154

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Clin Biochem. (2013) DOI: 10.1016/j.clinbiochem.2013.01.015

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Influence of resistance and aerobic exercise on hunger, circulating levels of acylated ghrelin, and peptide YY in healthy males
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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 bio-chemicals 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.

For further information, please send your request to
bioreagent@bertinpharma.com

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