Version 22-04/24

User's Manual

Human Ghrelin Acylated ELISA Kit

REF **DEIA4583**

Σ **384T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

1. 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 103 to 105 more Unacylated Ghrelin in the sample.
2. Alternatively, it can be used for measuring Acylated Ghrelin in human biological samples. For this application, please contact technical support at CD.

General Description

Acetylcholinesterase (AChE) the enzymatic label for ELISA, 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.

AChE assays are revealed with Substrate Solution (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:

a. Kinetic superiority and high sensitivity: AChE shows true fi rst-order kinetics with a turnover of 64,000 sec-1. That is nearly 3 times faster than Horse Radish Peroxidase (HRP) or alkaline phosphate. AChE allows a greater sensitivity than other labeling enzymes.

b. 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.

c. 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.

d. 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 solution (Ellman' s reagent) or if it needs to be revealed again, one only needs to wash the plate, add fresh Substrate Solution (Ellman' s reagent) and proceed with a new development. Otherwise, the plate can be stored at 4°C with Wash Buffer in wals while waiting for technical advice from the Bioreagent Department.

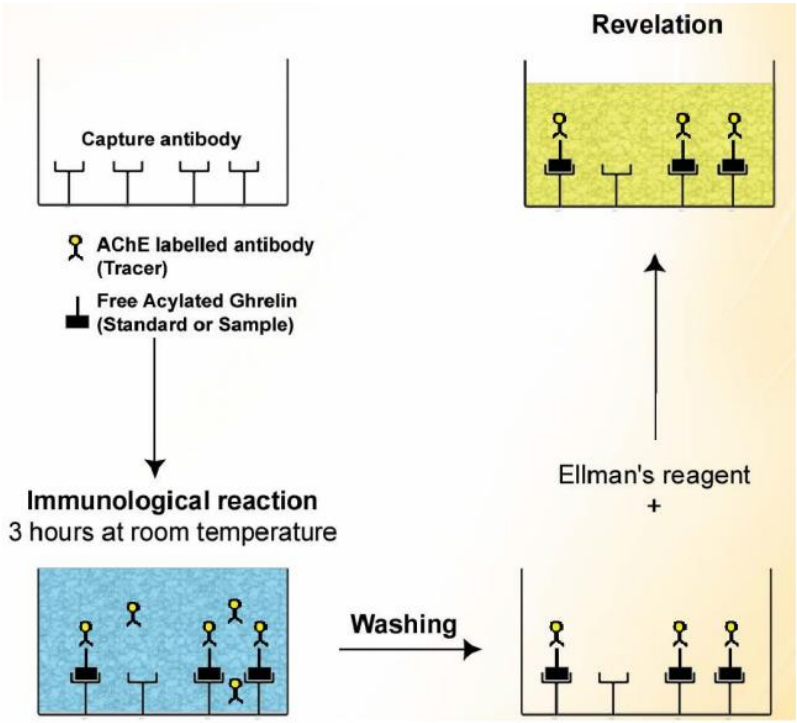
Ghrelin Ghrelin discovered in 1999, is fast becoming an endokrinology target of the millennium. Ghrelin, identifi ed in rat stomach as an endogenous ligand for the GH secretagogue receptor, is mainly produced in stomach, but has been demonstrated in many other organs. In addition to GH-releasing properties 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.

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 Essentials 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 but also in cachexia (related to cancer treatment, anorexia nervosa or ischemia) gastrin motility and may be involved in osteoporosis, somatopause, infertility and ovulation induction, neurological disorders (Alcoholism, Post Traumatic Stress disorders...) and cardiovascular diseases.

Principles of Testing

This ELISA is based on a doubleantibody sandwich technique. The wells of the plate supplied are coated with a monoclonal antibody specifi c 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 Substrate Solution (Ellman' s Reagent). AChE Tracer acts on Substrate Solution (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.



Reagents And Materials Provided

1. Acylated ghrelin precoated 384 well Microtiter plate, Blister with zip (1)
2. Conjugate Solution (Tracer for 384 well plate), Green, lyophilized (2)
3. Human Acylated Ghrelin Standard, Blue with red septum, lyophilized (1)
4. Quality Control, Green with red septum, lyophilized (1)
5. Dilution Buffer (EIA buffer), Blue, lyophilized (1)
6. Wash Solution Conc. (400x), Silver, liquid (1)

7. Substrate Solution (Ellman' s reagent), Black with red septum, lyophilized (2)
8. Tween 20, Transparent, liquid (1)
9. 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.

Materials Required But Not Supplied

1. Precision micropipettes (20 to 1000 µL) or a robotic platform
2. Spectrophotometer plate reader (405 or 414 nm filter)
3. Microplate washer (or wash-bottles)
4. Orbital Microplate shaker
5. Multichanel pipette and disposable tips 30-300 µL
6. Ultra pure water
7. Polypropylene tubes

Note: Water used to prepare all ELISA reagents and buffers must be Ultra Pure, deionized & free from organic contaminants traces. Otherwise, organic contamination can signifi cantly affect the enzymatic activity of the tracer AcetylCholinesterase. Do not use distilled water, HPLC-grade water or sterile water.

Storage

Store the kit at -20°C. Under these conditions, the kit is stable until expiration date (see label on the box).

Specimen Collection And Preparation

1. **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.

2. **Sample preparation**

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

Plate Preparation

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

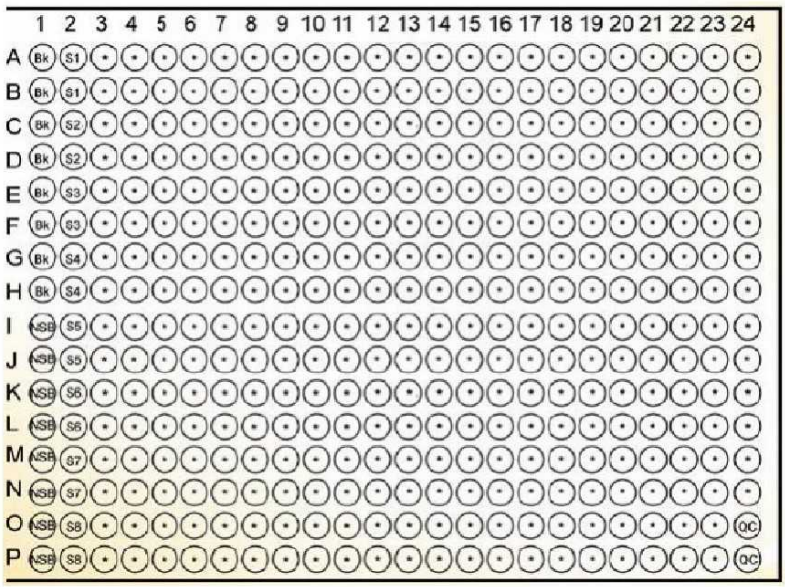
Rinse each well 5 times with the Wash Buffer 65 µL/well.

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.

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.



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

Reagent Preparation

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

1. Dilution Buffer

Reconstitute the vial Dilution buffer 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**

2. Acylated Ghrelin (human) Standard

Reconstitute the Standard vial 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 Dilution Buffer into each tube. Then prepare the standards by serial dilutions as follows:

Standard	Volume of Standard	Volume of DILUTION 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

3. Acylated Ghrelin (human) Quality Control

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

Reconstitute the Quality Control vial 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**

4. Acylated Ghrelin Conjugate Solution

Reconstitute the vial Conjugate Solution with 6 mL of Dilution Buffer. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. **Stability at 4°C, 1 week**

5. Wash Buffer

Dilute 2 mL of concentrated Wash Buffer with 800 mL of UltraPure water. Add 400 µL of Tween 20. Use a magnetic stirring bar to mix the content. **Stability at 4°C, 1 week**

6. Substrate Solution (Ellman' s Reagent)

5 minutes before use (development of the plate), reconstitute one vial of Substrate Solution (Ellman' s Reagent_49+1) with 49 of UltraPure water and 1 mL of concentrated Wash Buffer. The tube kontent should be thoroughly mixed. **Stability at 4°C and in the dark: 24 hours**

Assay Procedure

1. **Plate preparation**

(refer to the section **Plate preparation**)

2. **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).

3. **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, conjugate, 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.

a. Dilution Buffer

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

b. 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.

c. Quality Control and samples

Dispense 25 µL in duplicate or in simplicate to appropriate wells. Highly concentrated samples may be diluted in Dilution Buffer.

d. Acylated Ghrelin Conjugate Solution

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

4. **Developing and reading the plate**

a. Reconstitute Substrate Solution (Ellman' s reagent) as mentioned in the Reagent preparation section.

b. 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.

c. Add 50 µL of Substrate Solution (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.

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

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

After addition of Substrate Solution (Ellman' s reagent), the absorbance has to be checked periodically (every 30 minutes) until the maximum absorbance 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
DILUTION 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				

Calculation

Make sure that your plate reader has subtracted the absorbance readings of the blank well (absorbance of Substrate Solution (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.

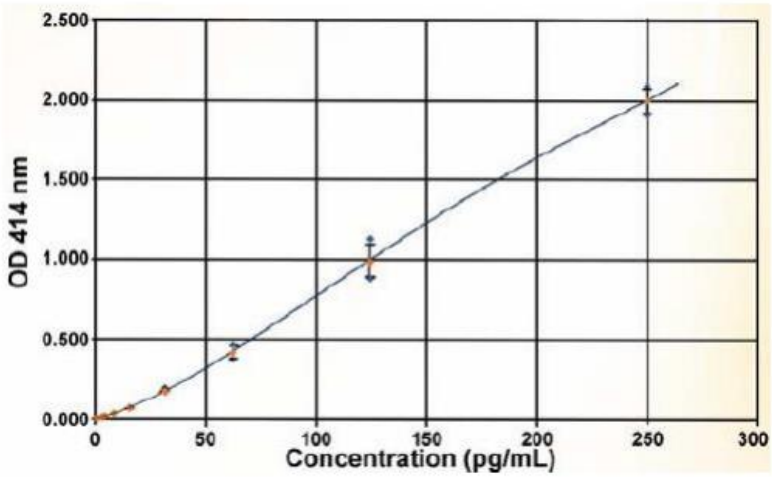
To determine the concentration of your samples, find the absorbance value of each sample on the y axis.

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 Dilution 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.

Note: One 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 Quality Control Sheet)

Typical Standard Curve



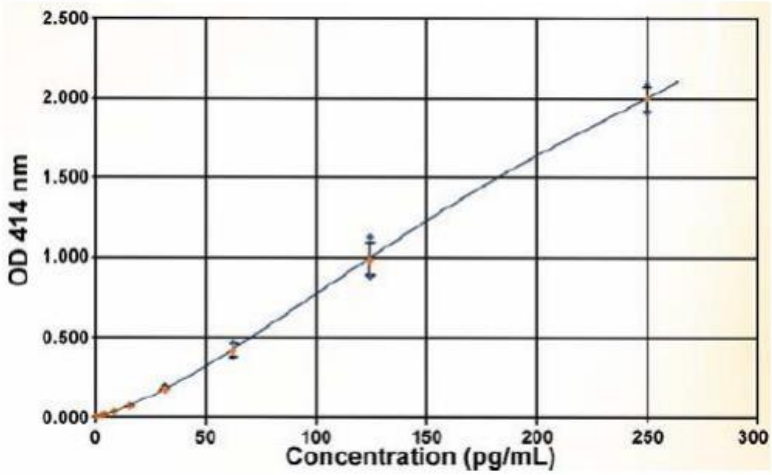
Reference Values

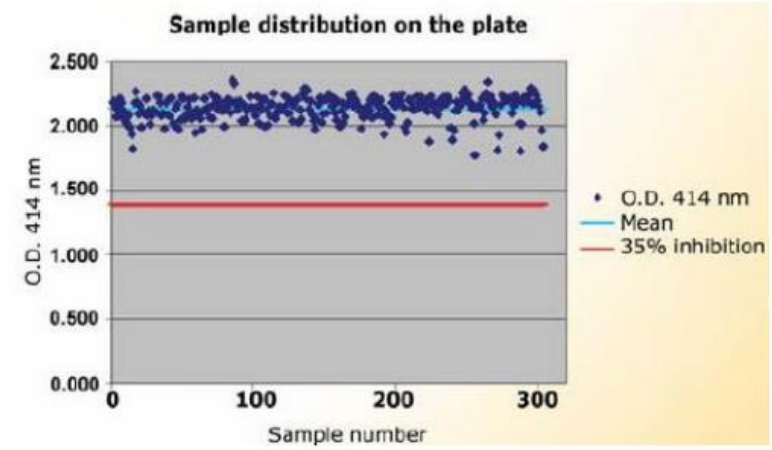
The following data are for demonstration purpose only. Your data may be different and still correct.

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 Human Acylated ghrelin 384 wells standard curve





Detection Range

2-250 pg/ml

Detection Limit

Limit of detection in the sample before dilution <8 pg/mL

Sensitivity

Sensitivity: <5 pg/mL

Specificity

Non Specific Binding < 60 mA.U.

Precautions

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 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 material and samples.

Possible Problems and Interference Elimination

1. **Absorbance values 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 enought.
2. **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.
3. **If a plate is accidentally dropped after dispatch of the AChE substrate solution (Ellman' s reagent) or if it needs to be revealed again:** one only needs to wash the plate, add fresh Substrate Solution 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.