

Human Unacylated Ghrelin ELISA

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Use only the actual version of Product Data Sheet enclosed with the kit!

1. Intended Use

The RD194063400R Human Unacylated Ghrelin ELISA is a sandwich enzyme immunoassay for quantitative measurement of human unacylated ghrelin in EDTA plasma and buffer solution. It is intended for in vitro and research use only.

Features

- The kit measures plasma unacylated ghrelin.
- Quality controls are human-plasma based.

2. Storage, Expiration

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

3. Summary

Ghrelin, an endogenous ligand for the growth hormone secretagogue receptor, is synthesized principally in the stomach. It stimulates food intake and transduces signals to hypothalamic regulatory nuclei that controls energy homeostasis. The peptide consists of 28 amino acids, with a n-octanoylation of the serine-3 residue, which is necessary for the biological activity mentioned below. Ghrelin is present in the peripheral circulation under two forms: acylated and unacylated. The Human Unacylated Ghrelin ELISA kit specifically measures the unacylated form of ghrelin.

4. Test Principle

The BioVendor Human Unacylated Ghrelin ELISA is based on a double-antibody sandwich technique. The wells of the plate supplied with the kit 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 which recognizes the N-terminal part of unacylated ghrelin is also added to the wells. This allows the two antibodies to form a sandwich by binding on different parts of the human unacylated ghrelin.

The sandwich is immobilized on the plate so the excess reagents may be washed away. The concentration of the human unacylated ghrelin is then determined by measuring the enzymatic activity of the immobilized AChE using the Ellman's Reagent. The AChE tracer acts on the Ellman's Reagent to form a yellow compound. The intensity of the color, which is determined by spectrophotometry, is proportional to the amount of the human unacylated ghrelin present in the well during the immunological incubation.

5. Precautions

- For *in vitro* research use only.
- This kit contains components of human origin. These materials should be handled as potentially infectious, as no tests can guarantee complete absence of infectious agents.
- Avoid contact with the Substrate Solution (Ellman's Reagent). In the case of contact, wash your skin thoroughly with water.
- Wear gloves and laboratory coats when handling immunodiagnostic materials.
- The materials should not be pipetted by mouth.
- Do not drink, eat or smoke in rooms where immunodiagnostic materials are being handled.
- Do not mix different lot numbers of any kit components.

6. Reagents Supplied

<i>Kit Components</i>	<i>Quantity</i>
Antibody Coated Microtiter Strips, ready to use	96 wells
Conjugate Solution, lyophilized	1 vial
Human Unacylated Ghrelin Standard, lyophilized	2 vials
Quality Control, lyophilized	2 vials
Dilution Buffer, lyophilized	1 vial
Wash Solution Concentrate, liquid	1 vial
Tween 20, liquid	1 vial
Substrate Solution (Ellman's reagent), lyophilized	2 vials
Product Data Sheet + Certificate of Analysis	1 pc
Template Sheet + Cover Sheet	1 pc

Each kit contains sufficient reagents for 96 wells. The kit can be used in two independent runs.

7. Materials Required but Not Supplied

- EDTA-plasma collection test tubes
- Test tubes for diluting samples (polypropylene)
- Precision pipettes to deliver 20-1000 μ l and disposable tips
- Multichannel pipette 100-200 μ l (recommended)
- Microplate reader with 405 or 414 nm filter
- Orbital microplate shaker
- Software package facilitating data generation and analysis
- Microtitration plate washer (optional) [Manual washing is possible but not preferable.]
- Glassware (graduated cylinder and bottle for Wash Solution)
- Deionized (distilled) water

8. Preparation of Reagents

Dilution Buffer:

Reconstitute one vial with 50 ml of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.

Stability at 4°C is 1 month.

Human Unacylated Ghrelin Standard:

Reconstitute the vial with 1 ml of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion. The concentration of the unacylated ghrelin standard S1 is 250 pg/ml.

Use seven polypropylene tubes to prepare the set of standards S2 to S8 from the reconstituted lyophilized standard S1 by serial dilution with Dilution Buffer as follows:

<i>Standard to be prepared</i>	<i>Dilution buffer volume</i>	<i>Standard to be added</i>
S1 (250 pg/ml)	-----	-----
S2 (125 pg/ml)	500 µl	S1: 500 µl
S3 (62.5 pg/ml)	500 µl	S2: 500 µl
S4 (31.3 pg/ml)	500 µl	S3: 500 µl
S5 (15.6 pg/ml)	500 µl	S4: 500 µl
S6 (7.81 pg/ml)	500 µl	S5: 500 µl
S7 (3.91 pg/ml)	500 µl	S6: 500 µl
S8 (1.96 pg/ml)	500 µl	S7: 500 µl

Stability at -20°C is 1 week.

Quality Control:

Reconstitute the vial with 1 ml of distilled or deionized water. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.

Stability at -20°C is 1 week.

Conjugate Solution (Anti-unacylated ghrelin-AChE tracer):

Reconstitute one vial with 10 ml of Dilution Buffer. Allow it to stand 5 minutes until completely dissolved and then mix thoroughly by gentle inversion.

Stability at 4°C is 1 week.

Wash Solution:

Dilute 1 ml of Wash Solution Concentrate to 400 ml with distilled or deionized water. Add 200 µl of Tween 20 (use a magnetic stirrer to mix the contents).

Stability at 4°C is 1 week.

Substrate Solution (Ellman's Reagent):

Reconstitute one vial with 49 ml of distilled or deionized water and 1 ml of Wash Solution Concentrate five minutes before use. The tube contents should be thoroughly mixed. Stability at 4°C and in the dark is 1 day.

9. Preparation of Samples

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. 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.

For assaying the acylated ghrelin, please refer to the section "Blood collection" of the protocol of the RD194062400 Human Acylated Ghrelin ELISA.

Sample preparation:

Plasma samples may be directly assayed (without any extraction procedure) after being diluted at least to 1:10 in the Dilution Buffer in order to avoid matrix effect.

10. Assay Procedure

All reagents and samples need to reach room temperature prior to the assay

- 1) Select strips sufficient for your assay and wash the wells 5-times with the prepared Wash Solution (0.3 ml per well) just before further using the strips.
Place the unused strips back in the packet and store at 4°C.
- 2) Left four wells empty for blanking the Substrate Solution (BI).
- 3) Pipette 100 µl of prepared Dilution buffer into four Non Specific Binding wells (NSB).
- 4) Pipette 100 µl of each of the eight prepared standards (S1 to S8) and Quality Control, preferably in duplicates into the respective wells.
- 5) Pipette 100 µl of diluted samples into the appropriate wells, preferably in duplicates (see example of work sheet).
- 6) Pipette 100 µl of Conjugate Solution to each well, except the Blank (BI) wells.
- 7) Cover the plate with adhesive sheet.
- 8) Incubate the plate 3 hours at room temperature (ca. 25°C) or 20 hours at 4°C.
The long incubation period allows the increase of the assay sensitivity: 0.2 pg/ml versus 0.6 pg/ml for short incubation.
- 9) Reconstitute the Substrate Solution.
- 10) Wash the wells 5-times with the Wash Solution (0.3 ml per well). Do not empty the wells after the fifth washing step, but shake the plate slightly for 5 minutes using an orbital shaker.
- 11) Repeat washing the wells 5-times with the wash buffer (0.3 ml per well).
- 12) Add 200 µl of Substrate Solution into each well including the blank (BI) wells.
- 13) Incubate the plate in darkness at room temperature (using an orbital shaker is optimal for the color development).
- 14) Determine the absorbance by reading the plate at 405 to 414 nm: 30 minutes after adding the Substrate Solution for long first incubation period (20 hours at +4°C) or 30 to 60 minutes after adding the Substrate Solution for short first incubation period (3 hours at room temperature).

Example of work sheet:

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

11. Calculations

Most microtiter plate readers perform automatic calculations of analyte concentration. The calibration curve is constructed by plotting the absorbance (Y) of standards versus *log* of the known concentration (X) of standards, using the four-parameter function. The results are reported as the unacylated ghrelin concentration in samples (pg/ml). Make sure that your plate reader has subtracted the absorbance readings of the blank wells from absorbance readings of the rest of the plate. If not, do it manually.

Alternatively, the *logit log* function can be used to linearize the calibration curve (i.e. *logit* of absorbance (Y) is plotted versus *log* of the known concentration (X) of standards).

The actual concentration of unacylated ghrelin in the original plasma sample has been assessed by multiplying the assay result by the dilution factor 10 (e.g. 13.5 pg/ml x 10 gives 135 pg/ml).

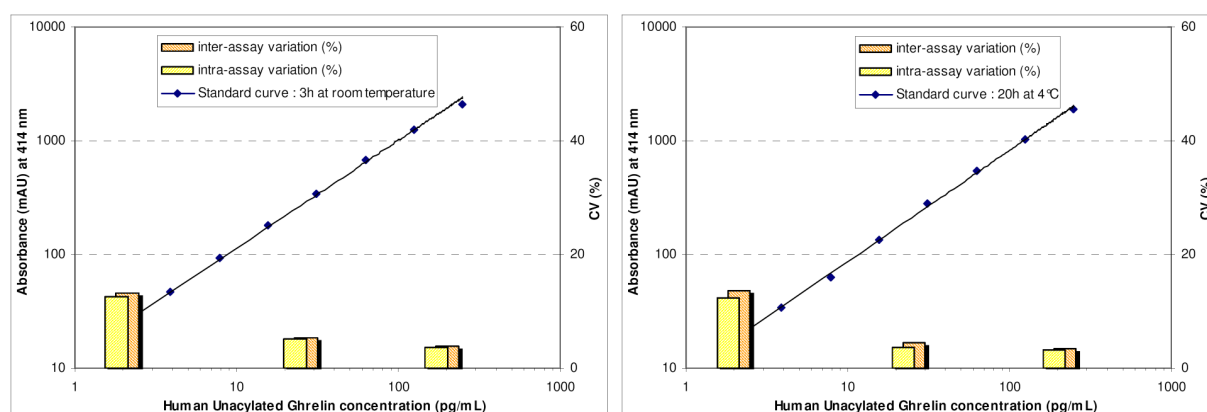
12. Limits of Assay

Results exceeding concentration 250 pg/ml should be repeated with more diluted samples (e.g. 1:20). In this case, dilution factor need to be taken into consideration in calculating of the concentrations.

Non Specific Binding Absorbance <0.050 is acceptable.

13. Performance Characteristics

Typical analytical data of BioVendor Human Unacylated Ghrelin ELISA are presented in this chapter. 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: 30 minutes developing at room temperature for long first incubation period (20h at +4°C) and 60 minutes developing for short first incubation period (3h at RT), reading at 414 nm. A 4-parameter logistic fitting was used to determine the concentrations.



Example data:

Ghrelin standard (pg/ml)	Absorbances	
	Short first incubation period (3h RT)	Long first incubation period (20h +4°C)
250	2.083	1.880
125	1.242	1.020
62.5	0.679	0.539
31.3	0.343	0.277
15.6	0.180	0.135
7.81	0.093	0.063
3.91	0.047	0.034
1.95	0.021	0.018

- **Sensitivity**

The Human Unacylated Ghrelin ELISA has been validated for its use in buffer and in plasma (without extraction but diluted at least 1:5). A sigmoidal 4-parameter logistic fitting was used to determine the concentrations.

The Limit of Determination, calculated as the concentration of unacylated ghrelin corresponding to the NSB average (n = 8) plus three standard deviations is 0.2 pg/ml and 0.6 pg/ml for long and short first incubation period, respectively.

The Limit of Quantification. Due to the minimal plasma dilution needed (1:10), the limits of quantification in the samples are 2 pg/ml (20h at +4°C) and 6 pg/ml (3h at RT), respectively.

- **Precision**

Intra-assay (Within-Run), Inter-assay (Run-to-Run) and Spiking Recovery

The intra-assay and inter-assay variations were studied on 30 human plasma (free of ghrelin) spiked samples for each level of QC. QC's were prepared as 10x concentrated from a pool of human plasma and then diluted to 1:10 in Dilution Buffer before assay. Replicate samples (n=6) at each of the three validation levels were analyzed along with the calibration curve for a total of 5 independent runs.

<i>Samples</i>	<i>Expected concentrations in diluted QC (pg/ml)</i>	<i>Observed concentrations (pg/ml)</i>	<i>Intra-assay CV (%)</i>	<i>Inter-assay CV (%)</i>	<i>Recovery O/E (%)</i>	<i>Confidence Interval ($\alpha = 0.05$)</i>
Incubation 20 hours at +4°C						
QC1	2	1.74	11.8	13.2	86.8	86.8 ± 5.8
QC2	25	21.6	3.20	3.80	86.5	86.5 ± 1.8
QC3	200	186	3.90	4.00	89.7	89.7 ± 1.4
Incubation 3 hours at room temperature						
QC1	2	1.78	12.10	12.60	88.9	88.9 ± 4.9
QC2	25	20.6	4.40	4.50	82.2	82.2 ± 1.5
QC3	200	176	4.30	4.60	87.8	87.8 ± 1.8

- **Linearity**

Human plasma samples were diluted 1:10. Afterwards, four independent dilutions (n=3) were performed and the samples were assayed.

<i>Samples</i>	<i>Dilution factor</i>	<i>Unacylated ghrelin measured (pg/ml)</i>	<i>Corrected Concentrations (pg/ml)</i>	<i>Recovery (%)</i>	<i>Mean Recovery (%)</i>
1	1:10	66.2	662	-	98.9
	1:20	34.2	684	103	
	1:50	13.8	690	104	
	1:100	6.22	622	94.0	
	1:200	3.12	624	94.3	
2	1:10	53.2	532	-	97.5
	1:20	26.6	532	100	
	1:50	10.7	535	101	
	1:100	5.09	509	96	
	1:200	2.49	498	94	
3	1:10	64.8	648	-	96.8
	1:20	33.6	672	104	
	1:50	13.4	670	103	
	1:100	5.92	592	91.4	
	1:200	2.88	576	88.9	

- **Effect of Sample Matrix**

Five individual lots of human plasma samples were tested. Validation samples (n=3) were prepared five times, concentrated in each matrix (free of ghrelin) and then diluted to 1:10 in order to obtain a final concentration of 25 pg/ml. Sample concentrations were read from a calibration curve derived from a pool of human plasmas.

<i>Sample</i>	<i>Expected (pg/ml)</i>	<i>Observed (pg/ml)</i>	<i>Recovery (%)</i>	<i>Mean Recovery (%)</i>
1	25	25.3	101	99.8
2		25.7	103	
3		25.5	102	
4		23.9	95.6	
5		24.3	97.2	

- **Effect of Freezing/Thawing (sample stability)**

Five human plasma samples (n=3) were analyzed just after collection and dilution to 1:10 before the assay (expected value) and after 1, 2 and 3 freeze/thaw cycles.

Samples	Expected value (pg/ml)	Observed 1 cycle (pg/ml)	Observed 2 cycles (pg/ml)	Observed 3 cycles (pg/ml)	Mean recovery O/E (%)
1	288	308	301	310	106
2	264	272	269	278	103
3	389	417	407	446	109
4	556	577	561	614	105
5	210	226	225	221	107

- **Specificity**

The Human Unacylated Ghrelin ELISA is highly specific. The cross-reactivity values for related peptides have been as follows:

Cross-reactivity:

- Ghrelin (Des-Octanoyl-Ser3) (Rat): 100 %
- Ghrelin (Octanoyl-Ser3) (Human): <0.06 %
- Ghrelin (Octanoyl-Ser3) (Rat, Mouse): <0.03 %
- 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 %

14. Troubleshooting and FAQs

1/ Weak signal in all wells

Possible explanations:

- Omission of a reagent or a step
- Improper preparation or storage of a reagent
- Assay performed before reagents were allowed to come to room temperature
- Incubation in wrong conditions (time or temperature) or reading time too short

2/ High signal and background (NSB) in the wells

Possible explanations:

- Improper or inadequate washing
- Overdeveloping; substrate incubation time should be decreased
- Contamination of NSB wells with standard or sample

3/ High coefficient of variation (CV)

Possible explanation:

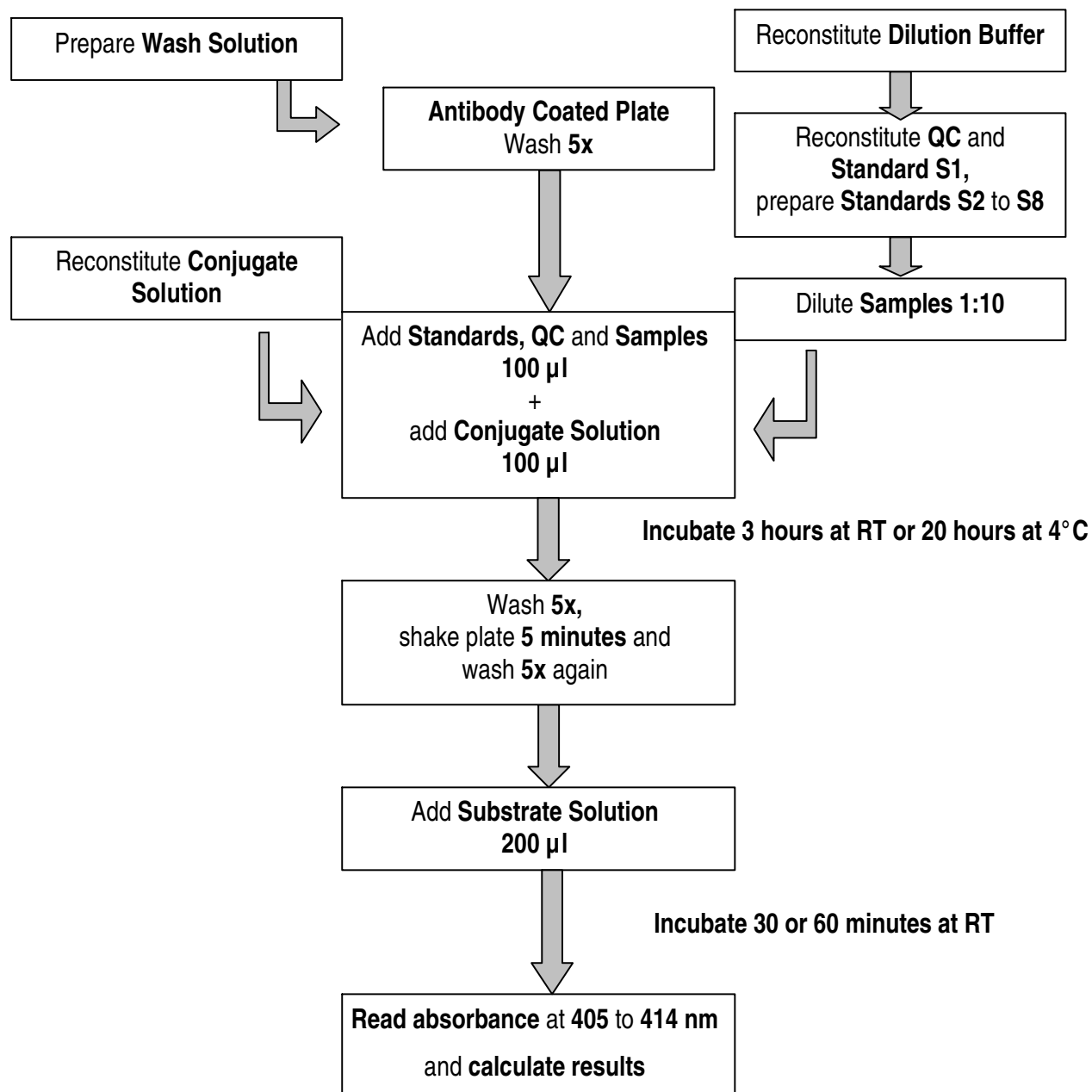
- Improper or inadequate washing
- Poor pipetting technique

15. References

Grassi J. & Pradelles Ph. Compounds labelled by the acetylcholinesterase of *Electrophorus Electricus*. Its preparation process and its use as a tracer or marker in enzyme-immunological determinations. *United States patent, N° 1,047,330. September 10, 1991*

Notes:

Assay Procedure Summary



The BioVendor RD194063400 Human Unacylated Ghrelin ELISA is manufactured and distributed on agreement of the Société de Pharmacologie et d'Immunologie - BIO, Parc d'activités du Pas du Lac 10 bis, Avenue Ampère, F-78180 Montigny le Bretonneux, FRANCE



