AssayMax™

Human Ghrelin ELISA Kit

Assaypro LLC
3400 Harry S Truman Blvd
St. Charles, MO 63301
T (636) 447-9175
F (636) 395-7419
www.assaypro.com

For any questions regarding troubleshooting or performing the assay, please contact our support team at support@assaypro.com.

Thank you for choosing Assaypro.

Assay Summary

Step 1. Add 50 µl of Standard or Sample per well.
Incubate 2 hours.

Step 2. Wash, then add 50 µl of Biotinylated Antibody per well.
Incubate 1 hour.

Step 3. Wash, then add 50 µl of SP Conjugate per well.
Incubate 30 minutes.

Step 4. Wash, then add 50 µl of Chromogen Substrate per well.
Incubate 15 minutes.

Step 5. Add 50 µl of Stop Solution per well.
Read at 450 nm immediately.

Symbol Key

Consult instructions for use.

Assay Template

	A	B	C	D	E	F	G	H
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								

AssayMax™ Human Ghrelin ELISA Kit

Catalog No. EG3780-1

Sample insert for reference use only

Introduction

Ghrelin, colloquially known as the "hunger hormone," is a peptide produced in the gastrointestinal tract (1-2). It functions as a neuropeptide by regulating hunger and participating in the regulation of energy use and distribution. Higher levels of ghrelin contribute to the increase in appetite and metabolic function. Ghrelin suppression is related to weight loss and is a potential treatment of obesity via gastric bypass (3). Like other metabolically related hormones, ghrelin is released in a circadian fashion, suggesting that ghrelin levels can indicate interruptions in circadian rhythm (4). Elevated ghrelin levels have been observed in eating disorders and cachexia associated with chronic heart failure, liver cirrhosis, and cancer (1, 5). The administration of synthetic ghrelin is being investigated as a potential treatment for cachexia and hemodialysis patients (6-7). In animal models, ghrelin has been shown to suppress seizures, and it may also be useful in treating gastroparesis (8-9).

Principle of the Assay

The AssayMax™ Human Ghrelin ELISA (Enzyme-Linked Immunosorbent Assay) Kit is designed for detection of ghrelin in human **plasma and serum samples**. This assay employs a quantitative **sandwich enzyme immunoassay** technique that measures human ghrelin in approximately 4 hours. A polyclonal antibody specific for human ghrelin has been pre-coated onto a 96-well microplate with removable strips. Ghrelin in standards and samples is sandwiched by the immobilized antibody and a biotinylated polyclonal antibody specific for human ghrelin, which is recognized by a streptavidin-peroxidase (SP) conjugate. All unbound material is washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Caution and Warning

- This product is for **Research Use Only** and is not intended for use in diagnostic procedures.
- Prepare all reagents (diluent buffer, wash buffer, standard, biotinylated antibody, and SP conjugate), as instructed, prior to running the assay.

- Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this insert. However, the user should determine the optimal dilution factor.
- Spin down the SP conjugate vial and the biotinylated antibody vial before opening and using contents.
- The Stop Solution is an acidic solution.
- The kit should not be used beyond the expiration date.

Reagents

- **Human Ghrelin Microplate:** A 96-well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against human ghrelin.
- **Sealing Tapes:** Each kit contains 3 precut, pressure sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **Human Ghrelin Standard:** Human ghrelin in a buffered protein base (19.2 ng, lyophilized, 2 vials).
- **Biotinylated Human Ghrelin Antibody (100x):** A 100-fold concentrated biotinylated polyclonal antibody against human ghrelin (60 µl).
- **EIA Diluent Concentrate (10x):** A 10-fold concentrated buffered protein base (20 ml).
- **Wash Buffer Concentrate (20x):** A 20-fold concentrated buffered surfactant (30 ml, 2 bottles).
- **SP Conjugate (100x):** A 100-fold concentrate (80 µl).
- **Chromogen Substrate (1x):** A stabilized peroxidase chromogen substrate tetramethylbenzidine (7 ml).
- **Stop Solution (1x):** A 0.5 N hydrochloric acid solution to stop the chromogen substrate reaction (11 ml).

Storage Condition

- Upon arrival, immediately store components of the kit at recommended temperatures up to the expiration date.
- Store SP Conjugate and Biotinylated Antibody at -20°C.
- Store Standard, Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8°C.
- Unused microplate wells may be returned to the foil pouch with the desiccant packs and resealed. May be stored for up to 30 days in a vacuum desiccator.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm
- Pipettes (1-20 µl, 20-200 µl, 200-1000 µl, and multiple channel)
- Deionized or distilled reagent grade water

Sample Collection, Preparation, and Storage

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Sample collection and processing should be performed as quickly as possible. Keep on ice when not in use. It is recommended that a protease inhibitor cocktail be added to the sample. For example: O-phenanthroline 0.44 mM, EDTA 25 mM, p-hydroxymercuribenzoic acid 1mM, and pepstatin A 0.12 mM. The user may need to optimize the concentration of the above reagents. Centrifuge samples at 3000 x *g* for 10 minutes and collect plasma. The sample is suggested for use at 1x; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles (EDTA or Heparin can also be used as an anticoagulant).
- **Serum:** Samples should be collected into a serum separator tube. Sample collection and processing should be performed as quickly as possible. Keep on ice when not in use. It is recommended that a protease inhibitor cocktail be added to the sample. For example: O-phenanthroline 0.44 mM, EDTA 25 mM, p-hydroxymercuribenzoic acid 1mM, and pepstatin A 0.12 mM. The user may need to optimize the concentration of the above reagents. After clot formation, centrifuge samples at 3000 x *g* for 10 minutes and remove serum. The sample is suggested for use at 1x; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

Applicable samples may also include biofluids, cell culture, and tissue homogenates. If necessary, user should determine optimal dilution factor depending on application needs.

Refer to Dilution Guidelines for further instruction.

Guidelines for Dilutions of 100-fold or Greater (*for reference only; please follow the insert for specific dilution suggested)*	
100x	**10000x**
A) 4 µl sample : 396 µl buffer (100x) = 100-fold dilution *Assuming the needed volume is less than or equal to 400 µl.*	A) 4 µl sample : 396 µl buffer (100x) B) 4 µl of A : 396 µl buffer (100x) = 10000-fold dilution *Assuming the needed volume is less than or equal to 400 µl.*
1000x	**100000x**
A) 4 µl sample : 396 µl buffer (100x) B) 24 µl of A : 216 µl buffer (10x) = 1000-fold dilution *Assuming the needed volume is less than or equal to 240 µl.*	A) 4 µl sample : 396 µl buffer (100x) B) 4 µl of A : 396 µl buffer (100x) C) 24 µl of B : 216 µl buffer (10x) = 100000-fold dilution *Assuming the needed volume is less than or equal to 240 µl.*

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- **EIA Diluent Concentrate (10x):** Dilute the EIA Diluent Concentrate 10-fold with reagent grade water to produce a 1x solution. When diluting the concentrate, make sure to rinse the bottle thoroughly to extract any precipitates left in the bottle. Mix the 1x solution gently until the crystals have completely dissolved. Store for up to 30 days at 2-8°C.
- **Human Ghrelin Standard:** Reconstitute the Human Ghrelin Standard (19.2 ng) with 0.6 ml of EIA Diluent to generate a 32 ng/ml standard stock solution. Allow the vial to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare duplicate or triplicate standard points by serially diluting from the standard stock solution (32 ng/ml) 4-fold with EIA Diluent to produce 8, 2, 0.5, and 0.125 ng/ml solutions. EIA Diluent serves as the zero standard (0 ng/ml). Reconstitute a new vial for each assay.

Standard Point	Dilution	[Ghrelin] (ng/ml)
P1	1 part Standard (32 ng/ml)	32
P2	1 part P1 + 3 parts EIA Diluent	8.0
P3	1 part P2 + 3 parts EIA Diluent	2.0
P4	1 part P3 + 3 parts EIA Diluent	0.5
P5	1 part P4 + 3 parts EIA Diluent	0.125
P6	EIA Diluent	0.0

- **Biotinylated Human Ghrelin Antibody (100x):** Spin down the antibody briefly and dilute the desired amount of the antibody 100-fold with EIA Diluent to produce a 1x solution. The undiluted antibody should be stored at -20°C.
- **Wash Buffer Concentrate (20x):** Dilute the Wash Buffer Concentrate 20-fold with reagent grade water to produce a 1x solution. When diluting the concentrate, make sure to rinse the bottle thoroughly to extract any precipitates left in the bottle. Mix the 1x solution gently until the crystals have completely dissolved.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 100-fold with EIA Diluent to produce a 1x solution. The undiluted conjugate should be stored at -20°C.

Assay Procedure

- Prepare all reagents, standard solutions, and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-25°C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccants inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 50 µl of Human Ghrelin Standard or sample to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for 2 hours. Start the timer after the last addition.
- Wash the microplate manually or automatically using a microplate washer. Invert the plate and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If washing manually, wash five times with 200 µl of Wash Buffer per well. Invert the plate each time and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If using a microplate washer, wash six times with 300 µl of Wash Buffer per well; invert the plate and hit 4-5 times on absorbent material to completely remove the liquid.
- Add 50 µl of Biotinylated Human Ghrelin Antibody to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for 1 hour.
- Wash the microplate as described above.
- Add 50 µl of SP Conjugate to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash the microplate as described above.
- Add 50 µl of Chromogen Substrate to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed.

Incubate in ambient light for 15 minutes or until the optimal blue color density develops.

- Add 50 µl of Stop Solution to each well. The color will change from blue to yellow. Gently tap plate to ensure thorough mixing. Break any bubbles that may have formed.
- Read the absorbance on a microplate reader at a wavelength of 450 nm **immediately**. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample.
- To generate a standard curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance (OD) on the y-axis. The best fit line can be determined by regression analysis using log-log or four-parameter logistic curve fit.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

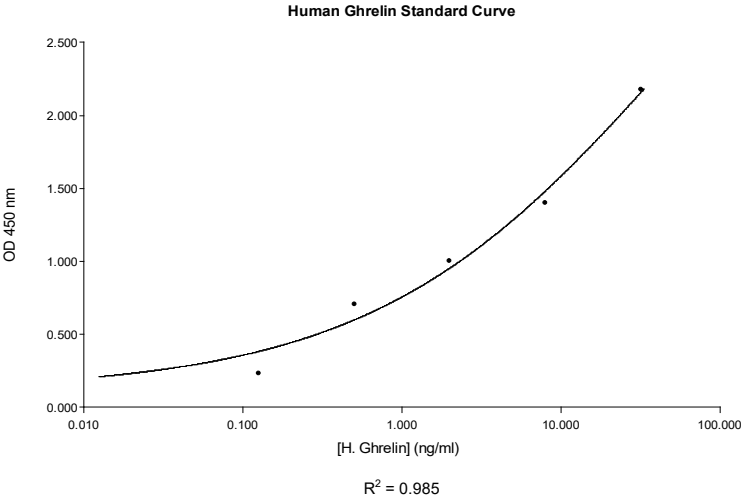
Typical Data

- The typical data is provided for reference only. Individual laboratory means may vary from the values listed. Variations between laboratories may be caused by technique differences.

Standard Point	ng/ml	OD	Average OD
P1	32	2.202 2.156	2.179
P2	8.0	1.437 1.377	1.407
P3	2.0	1.023 0.991	1.007
P4	0.5	0.718 0.698	0.708
P5	0.125	0.242 0.236	0.239
P6	0.0	0.152 0.146	0.149

Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.



Performance Characteristics

- The minimum detectable dose of human ghrelin as calculated by 2SD from the mean of a zero standard was established to be 78 pg/ml.
- Intra-assay precision was determined by testing three plasma samples twenty times in one assay.
- Inter-assay precision was determined by testing three plasma samples in twenty assays.

	Intra-Assay Precision			**Inter-Assay Precision**		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
CV (%)	5.0%	5.7%	6.7%	10.1%	11.0%	11.6%
Average CV (%)	5.8%			10.9%		

Recovery

Standard Added Value	0.2 – 8 ng/ml
Recovery %	87 – 112%
Average Recovery %	96%

Cross-Reactivity

Species	Cross-Reactivity (%)
Canine	50%
Bovine	None
Equine	100%
Monkey	90%
Mouse	20%
Rat	50%
Swine	80%
Rabbit	None

Troubleshooting

Issue	Causes	Course of Action
Low Precision	Use of improper components	• Check the expiration date listed before use. • Do not interchange components from different lots.
	Improper wash step	• Check that the correct wash buffer is being used. • Check that all wells are empty after aspiration. • Check that the microplate washer is dispensing properly. • If washing by pipette, check for proper pipetting technique.
	Splashing of reagents while loading wells	• Pipette properly in a controlled and careful manner.
	Inconsistent volumes loaded into wells	• Pipette properly in a controlled and careful manner. • Check pipette calibration. • Check pipette for proper performance.
	Insufficient mixing of reagent dilutions	• Thoroughly agitate the lyophilized components after reconstitution. • Thoroughly mix dilutions.
	Improperly sealed microplate	• Check the microplate pouch for proper sealing. • Check that the microplate pouch has no punctures. • Check that three desiccants are inside the microplate pouch prior to sealing.
Unexpectedly Low or High Signal Intensity	Microplate was left unattended between steps	• Each step of the procedure should be performed uninterrupted.
	Omission of step	• Consult the provided procedure for complete list of steps.
	Steps performed in incorrect order	• Consult the provided procedure for the correct order.
	Insufficient amount of reagents added to wells	• Check pipette calibration. • Check pipette for proper performance.
	Wash step was skipped	• Consult the provided procedure for all wash steps.
	Improper wash buffer	• Check that the correct wash buffer is being used.
	Improper reagent preparation	• Consult reagent preparation section for the correct dilutions of all reagents.
	Insufficient or prolonged incubation periods	• Consult the provided procedure for correct incubation time.

Deficient Standard Curve Fit	Non-optimal sample dilution	• Sandwich ELISA: If samples generate OD values higher than the highest standard point (P1), dilute samples further and repeat the assay. • Competitive ELISA: If samples generate OD values lower than the highest standard point (P1), dilute samples further and repeat the assay. • User should determine the optimal dilution factor for samples.
	Contamination of reagents	• A new tip must be used for each addition of different samples or reagents during the assay procedure.
	Contents of wells evaporate	• Verify that the sealing film is firmly in place before placing the assay in the incubator or at room temperature.
	Improper pipetting	• Pipette properly in a controlled and careful manner. • Check pipette calibration. • Check pipette for proper performance.
	Insufficient mixing of reagent dilutions	• Thoroughly agitate the lyophilized components after reconstitution. • Thoroughly mix dilutions.

References

(1) Inui A *et al*. (2004) *FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology*. 18(3):439-56. doi:10.1096/fj.03-0641 rev. PMID 15003990.

(2) Sakata I, Sakai T. (2010) *International Journal of Peptides*. 2010:1. doi:10.1155/2010/945056. PMC 2925405. PMID 20798855.

(3) Cummings DE *et al*. (2002) *The New England Journal of Medicine*. 346(21):1623-30. doi:10.1056/NEJMoa012908. PMID 12023994.

(4) Fonken LK, Nelson RJ. (2014) *Endocrine Reviews*. 35(4):648-70. doi:10.1210/er.2013-1051. PMID 24673196.

(5) Garcia JM *et al*. (2005) *The Journal of Clinical Endocrinology and Metabolism*. 90(5):2920-6. doi:10.1210/jc.2004-1788. PMID 15713718.

(6) Ruperto M *et al*. (2014) *Nutrición Hospitalaria*. 29(4):735-50. doi:10.3305/nh.2014.29.4.7222 (inactive 2015-02-01). PMID 24679014.

(7) Berardi E *et al*. (2014) *Frontiers in Physiology*. 5:119. doi:10.3389/fphys.2014.00119. PMC 3986550. PMID 24782779.

(8) Clynen E *et al*. (2014) *Molecular Neurobiology*. 50(2):626-46. doi:10.1007/s12035-014-8669-x. PMC 4182642. PMID 24705860.

(9) Hasler WL. (2014) *Expert Opinion on Emerging Drugs*. 19(2):261-79. doi:10.1517/14728214.2014.899353. PMID 24669936.

Version 1.7