

# Oxyntomodulin ELISA

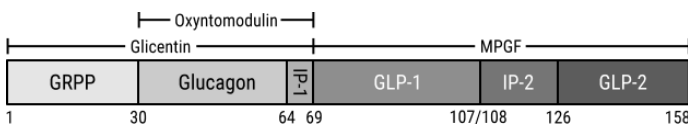
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## AL-139

### INTENDED USE

The Oxyntomodulin (OXM) enzyme linked immunosorbent assay (ELISA) kit provides materials for the quantitative measurement of Oxyntomodulin in K<sub>2</sub>EDTA and Li-Heparin plasma and other biological fluids. This kit is intended for laboratory research use only and is not for use in diagnostic or therapeutic procedures.

### SUMMARY AND EXPLANATION



Oxyntomodulin was initially identified as a moiety that exhibited glucagon like immunoreactivity and was appropriately named for its ability to modulate the gastric acid secretion in the gastric oxyntic glands.<sup>1,2</sup> Oxyntomodulin is a 37 amino acid peptide hormone secreted by the gut endocrine L-cells post-prandially<sup>3</sup>. Oxyntomodulin and Glucagon, a peptide hormone secreted by the alpha cells of the pancreas, share the identical amino acid sequence in the N-terminal 29 aa with oxyntomodulin having an additional octapeptide in the C-terminus. Upon nutrient ingestion, prohormone convertase1/3 cleaves proglucagon precursor into Oxyntomodulin, GLP-1/2 and GRPP.<sup>3</sup> Oxyntomodulin is known to bind both the GLP-1 receptor (GLP1R) and the glucagon receptor (GCGR), but with lower affinity compared to GLP-1 and glucagon.<sup>3</sup>

Oxyntomodulin causes weight loss in obese patients via suppression of food intake and increase in energy expenditure.<sup>3</sup> It is reported to have varied tissue-specific effects; and stimulates endocrine pancreas to secrete insulin, somatostatin and glucagon. Oxyntomodulin administration was also reported to increase heart-rate in mice.<sup>3</sup> Besides regulating glucose homeostasis in monogastric animals, oxyntomodulin also increases the concentration of insulin and glucose in plasma of Holstein cattle under normal physiological conditions.<sup>4</sup> Using mass-spectrometry based profiling of human plasma, it was shown that Type 2 diabetes patients have lower levels of Oxyntomodulin and levels increase more than 10-fold after gastric bypass surgery.<sup>5</sup> Oxyntomodulin is therapeutically used to lower glucose levels and suppress appetite resulting in weight loss.

### PRINCIPLE OF THE TEST

The Oxyntomodulin ELISA is a quantitative two-step sandwich type immunoassay. In first step Unknown samples are diluted on to a solid phase extraction plate. Then Calibrators, Controls and diluted samples are added to Oxyntomodulin antibody coated microtiter wells and incubated with biotinylated Oxyntomodulin antibody solution. After the first incubation and washing, the wells are incubated with streptavidin horseradish peroxidase conjugate (SHRP) solution. After the second incubation and washing step, the wells are incubated with substrate solution (TMB) followed by an acidic stopping solution. In principle, the antibody-biotin conjugate binds to the solid phase antibody-antigen complex which in turn binds to the streptavidin-enzyme

conjugate. The biotin-SHRP complex bound to the well is detected by enzyme-substrate reaction. The degree of enzymatic turnover of the substrate is determined by dual wavelength absorbance measurement at 450 nm as primary test filter and 630 nm as reference filter. The absorbance measured is directly proportional to the concentration of Oxyntomodulin in the samples and calibrators.

### MATERIALS SUPPLIED

#### CAL-139A Oxyntomodulin Calibrator A/Sample Diluent

One bottle, 11 mL, labeled Oxyntomodulin Cal. A/ Sample Diluent, containing 0 pg/mL Oxyntomodulin in protein based buffer and Pro-Clean 400. Store unopened at 2-8°C until the expiration date.

#### CAL-139B - CAL-139F Oxyntomodulin Calibrators B – F (Lyophilized)

Five vials, labeled B-F, containing concentrations of approximately 3.0 - 300 pg/mL Oxyntomodulin in protein based buffer and Pro-Clean 400. Refer to **calibration card** for exact concentrations. Store unopened at 2 to 8°C until the expiration date. Reconstitute calibrators B-F with 1 mL deionized water. Solubilize, mix well and use after reconstitution. Aliquot and freeze in plastic vials immediately for multiple use and discard after the run. Avoid repeated freeze thaws. The Oxyntomodulin concentration in the calibrators is traceable to the manufacturer's working calibrators (peptide weight/volume). Values assigned by other methodologies may be different. Such differences, if present, may be caused by inter-method bias.

**Note: The calibrators are not corrected for the dilution factor.**

#### CTR-139-I & CTR-139-II Oxyntomodulin Controls I & II

Two vials, labeled Levels I and II containing low and high Oxyntomodulin concentrations in protein based buffer and Pro-Clean 400. Refer to **calibration card** for exact concentrations. Store unopened at 2 to 8°C until the expiration date. Reconstitute control Levels I and II with 1 mL deionized water. Solubilize, mix well and use after reconstitution. Aliquot and freeze immediately in plastic vials for multiple use and discard after a run. Avoid repeated freeze thaws.

#### PLT-139 Oxyntomodulin Antibody Coated Microtitration strips

One strip-holder, containing 12 strips and 96 microtitration wells with anti-Oxyntomodulin antibody immobilized to the inside wall of each well. Store at 2-8°C until expiration date in the resealable pouch with a desiccant to protect from moisture.

#### PLT-SPE-02 Solid Phase Extraction Plate

One strip-holder, containing 12 strips and 96 microtitration wells with secondary mouse monoclonal antibody immobilized to the inside wall of each well. Store at 2-8°C until expiration date in the resealable pouch with a desiccant to protect from moisture.

**Note: Samples should be diluted on the PLT-SPE-02 and the results should be corrected for the dilution factor.**

#### BCR-139 Oxyntomodulin Biotin Conjugate Ready-To-Use (RTU)

One bottle, 12mL containing biotinylated anti-Oxyntomodulin antibody in a protein-based buffer with a non-mercury preservative. Store at 2-8°C until expiration date.

**SAR-139 Oxyntomodulin Streptavidin-Enzyme Conjugate-Ready-To-Use (RTU)**

One bottle, 12 mL, containing streptavidin-HRP (horseradish peroxidase) in a protein-based buffer and a non-mercury preservative. Store at 2-8°C until expiration date.

**TMB-100 TMB Chromogen Solution**

One bottle, 12 mL, containing a solution of tetramethylbenzidine (TMB) in buffer with hydrogen peroxide. Store at 2-8°C until expiration date.

**STP-100 Stopping Solution**

One bottle, 12 mL, containing 0.2 M sulfuric acid. Store at 2-30°C until expiration date.

**WSH-100 Wash Concentrate A**

One bottle, 60 mL, containing buffered saline with a nonionic detergent. Store at 2-30°C until expiration date. Dilute 25-fold with deionized water prior to use.

**MATERIALS REQUIRED BUT NOT PROVIDED**

1. Microplate absorbance reader capable of absorbance measurement at 450 nm, 405 nm and 630 nm.
2. Microplate orbital shaker.
3. Microplate washer.
4. Semi-automated/manual precision pipette to deliver 10–250 µL.
5. Vortex mixer.
6. Deionized water.
7. Repeater Pipette

**WARNINGS AND PRECAUTIONS**

*For Research Use Only. Not for use in diagnostic procedures.*

The following precautions should be observed:

- a) Follow good laboratory practice.
- b) Use personal protective equipment. Wear lab coats and disposable gloves when handling immunoassay materials.
- c) Handle and dispose of all reagents and material in compliance with applicable regulations

**WARNING: Potential Biohazardous Material**

This reagent may contain some human source material (e.g. serum) or materials used in conjunction with human source materials. Handle all reagents and patient samples at a Biosafety Level 2, as recommended for any potentially infectious human material in the Centers for Disease Control/National Institutes of Health manual "Biosafety in Microbiological and Biomedical Laboratories," 5<sup>th</sup> Edition, 2007.<sup>6</sup>

**WARNING: Potential Chemical Hazard**

Some reagents in this kit contain Pro-Clean 400 and Sodium azide<sup>7</sup> as a preservative. Pro-Clean 400 and Sodium azide in concentrated amounts are irritants to skin and mucous membranes.

For further information regarding hazardous substances in the kit, please refer to the MSDS, either at AnshLabs.com or by request.

**SAMPLE COLLECTION**

- a) K<sub>2</sub>EDTA plasma is the recommended sample type.

- b) Sample handling, processing, and storage requirements depend on the brand of blood collection tube that you use. Please reference the manufacturer's instructions for guidance. Each laboratory should determine the acceptability of its own blood collection tubes and serum separation products.
- c) Samples must be stored at -20°C or -80°C to avoid loss of bioactivity and contamination.
- d) Avoid assaying lipemic, hemolyzed or icteric samples.
- e) Avoid repeated freezing and thawing of samples. Thaw samples no more than 2 times.
- f) For shipping, place specimens in leak proof containers in biohazard specimen bags with appropriate specimen identification and test requisition information in the outside pocket of the biohazard specimen bag. Follow DOT and IATA requirements when shipping specimens.

**PROCEDURAL NOTES**

1. A thorough understanding of this package insert is necessary for successful use of the Oxyntomodulin ELISA assay. It is the user's responsibility to validate the assay for their purpose. Accurate results will only be obtained by using precise laboratory techniques and following the package insert.
2. A calibration curve must be included with each assay.
3. Bring all kit reagents to room temperature before use. Thoroughly mix the reagents before use by gentle inversion. Do not mix various lots of any kit component and do not use any component beyond the expiration date.
4. Use a clean disposable pipette tip for each reagent, calibrator, control or sample. Avoid microbial contamination of reagents, contamination of the substrate solutions with the HRP conjugates. The enzyme used as the label is inactivated by oxygen, and is highly sensitive to microbial contamination, sodium azide, hypochlorous acid and aromatic chlorohydrocarbons often found in laboratory water supplies. Use deionized water.
5. Incomplete washing will adversely affect the outcome and assay precision. Care should be taken to add TMB into the wells to minimize potential assay drift due to variation in the TMB incubation time. Avoid exposure of the reagents to excessive heat or direct sunlight.

**PREPARATION OF REAGENTS**

1. **Oxyntomodulin Calibrators B-F and Oxyntomodulin Controls I & II:** Tap and reconstitute Oxyntomodulin Calibrators B-F and Oxyntomodulin Controls I & II each with **1 mL** deionized water and solubilize for 15 minutes, vortex and use.
2. **Wash Solution:** Dilute wash concentrate 25-fold with deionized water. The wash solution is stable for one month at room temperature when stored in a tightly sealed bottle.
3. **Microtitration Wells:** Select the number of coated wells required for the assay. The remaining unused wells should be placed in the resealable pouch with a desiccant. The pouch must be resealed to protect from moisture.

**ASSAY PROCEDURE**

Allow all specimens and reagents to reach room temperature (23 ± 2°C) and mix thoroughly by gentle inversion before use. Calibrators, controls, and unknowns should be assayed in duplicate.

**Sample treatment and dilution protocol:**

Label the PLT-SPE-02 microtitration strips to be used in the order in which the diluted samples will be transferred to the Oxyntomodulin (PLT-139) plate.

**Note: Do not add Oxyntomodulin calibrator A-F and controls on Solid phase Extraction plate PLT-SPE-02.**

- Pipette **20 µL** of samples on **PLT-SPE-02** in the designated wells.
- Add **80 µL** of Oxyntomodulin Calibrator A/ Sample Diluent (CAL-139A) to the **PLT-SPE-02** plate.
- Incubate the PLT-SPE-02 plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **60 minutes** at room temperature ( $23 \pm 2^\circ\text{C}$ ). **DO NOT WASH THE PLATE.**
- Gently mix the reconstituted Oxyntomodulin Calibrators B-F and Oxyntomodulin Controls I & II. See Preparation of Reagents section of this package insert for reconstitution.
- Pipette **50 µL** of the reconstituted Calibrator and Control to the **designated wells of Oxyntomodulin plate (PLT-139).**
- Pipette **50 µL diluted samples** from PLT-SPE-02 to the **designated wells of Oxyntomodulin plate (PLT-139).** *Dilution factor after samples transfer is 5X.*
- Add **100 µL** of the **Oxyntomodulin Biotin Conjugate Ready-To-Use (RTU)** to each well using a repeater pipette.
- Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **90 minutes** at room temperature ( $23 \pm 2^\circ\text{C}$ ).
- Aspirate and wash each strip **5 times (350 µL/per well)** with **Wash Solution** using an automatic microplate washer.
- Add **100 µL** of the **Streptavidin-Enzyme Conjugate-RTU** to each well using a repeater pipette.
- Incubate the plate, shaking at a fast speed (**600-800 rpm**) on an orbital microplate shaker, for **30 minutes** at room temperature ( $23 \pm 2^\circ\text{C}$ ).
- Aspirate and wash each strip **5 times (350 µL/per well)** with the **Wash Solution** using an automatic microplate washer.
- Add **100 µL** of the **TMB chromogen** solution to each well using a repeater pipette. Avoid exposure to direct sunlight
- Incubate the wells, shaking at **600–800 rpm** on an orbital microplate shaker, for **8-12 min** at room temperature ( $23 \pm 2^\circ\text{C}$ ).
- NOTE: Visually monitor the color development to optimize the incubation time
- Add **100 µL** of the **Stopping Solution** to each well using a repeater pipette. Read the absorbance of the solution in the wells within **20 minutes**, using a microplate reader set to **450 nm**.

**NOTE:** Zero calibrator should be programmed as "Blank" while reading the optical density. If instrument has a wavelength correction, set the instrument to dual wavelength measurement at 450 nm with background wavelength correction at 630 nm.

## RESULTS

**NOTE:** The results in this package insert were calculated by plotting the **log optical density (OD) data on the y-axis and log OXM concentration on X-axis** using a cubic regression curve-fit. Alternatively, log vs. log quadratic regression curve-fit can be used. Other data reduction methods may give slightly different results.

- Calculate the mean optical density (OD) for each calibrator, Control, or Unknown.
- Optimum results can be obtained at incubation temperature of ( $23 \pm 2^\circ\text{C}$ ).
- Plot the log of the mean OD readings for each of the Calibrators along the y-axis versus log of the Oxyntomodulin concentrations in pg/mL along the x-axis, using a cubic regression curve-fit.
- Determine the Oxyntomodulin concentrations of the Controls and **diluted unknowns (1:5)** from the calibration curve by matching their mean OD readings with the corresponding Oxyntomodulin concentrations.
- Any sample reading higher than the highest Calibrator should be appropriately further diluted with the 0 pg/mL (Cal. A / Sample Diluent) and re-assayed.
- Any sample reading lower than the analytical sensitivity should be reported as such.

- The measured concentrations of the unknown samples should be multiplied by the dilution factor (5 folds).**

## LIMITATIONS

The reagents supplied in this kit are optimized to measure Oxyntomodulin levels in human EDTA plasma. If there is evidence of microbial contamination or excessive turbidity in a reagent, discard the vial. For assays employing antibodies, the possibility exists for interference by heterophile antibodies in the samples.<sup>8</sup>

## QUALITY CONTROL

- Each laboratory should establish mean values and acceptable ranges to assure proper performance.
- Oxyntomodulin ELISA controls or other commercial controls should fall within established confidence limits.
- The confidence limits for Oxyntomodulin controls are printed on the **Calibration Card**.
- A full calibration curve, low- and high-level controls, should be included in each assay.
- TMB should be colorless. Development of any color may indicate reagent contamination or instability.

## REPRESENTATIVE CALIBRATION CURVE DATA

Well Number	Well Contents Calibrators	Mean Absorbance	Conc. (pg/mL)
A1, A2	A	0.018 (Blank)	0
B1, B2	B	0.043	3.0
C1, C2	C	0.130	10.0
D1, D2	D	0.467	36.0
E1, E2	E	1.401	111.0
F1, F2	F	3.218	290.0

**CAUTION:** The above data must not be employed in lieu of data obtained by the user in the laboratory.

## ANALYTICAL CHARACTERISTICS

All analytical characteristics are stated in pg/mL.

1 pg/mL Oxyntomodulin = 226 pMol/L or 0.226 nMol/L

### Analytical Sensitivity:

The analytical sensitivity in the Oxyntomodulin ELISA assay, as calculated by the interpolation of mean plus two standard deviations of 16 replicates of calibrator A (0 pg/mL) and calibrator B (3.0 pg/mL), is **0.243 pg/mL**.

### Imprecision:

Reproducibility of the Oxyntomodulin ELISA assay was determined in a study using four K<sub>2</sub>EDTA sample pools. The study included a total of 8 assays, four replicates of each per assay (n=32). Representative data were calculated and are presented in the following table.

Sample	Mean conc.	Within run		Between run		Total	
	(pg/mL)	SD	%CV	SD	%CV	SD	%CV
Pool-1	13.072	0.408	3.12%	0.650	4.97%	0.767	5.87%
Pool-2	33.079	0.697	2.11%	1.260	3.81%	1.440	4.35%
Pool-3	111.957	2.571	2.30%	3.609	3.22%	4.431	3.96%
Pool-4	9.039	0.285	3.15%	0.358	3.96%	0.457	5.06%

### Linearity:

Samples containing various Oxyntomodulin levels were serially diluted 1:2, 1:4 and 1:8 in Calibrator A/Sample Diluent. Neat and Diluted samples were then added to PLT-SPE-02 and assayed as per the procedure outlined in steps 1-6 in assay procedure. Oxyntomodulin Ag was serially diluted in calibrator A/Sample

diluent and added to PLT-139 as per assay procedure. The percent (%) recovery on individual samples is represented in the following table.

Sample	Dilution Factor	Expected Conc. (pg/mL)	Observed Conc. (pg/mL)	% Recovery
Antigen	Neat	296.0	NA	NA
	1:2	148.0	145.70	98%
	1:4	74.0	68.79	93%
	1:8	37.0	38.17	103%
Sample-1	1:5	177.87	NA	NA
	1:10	88.93	97.33	109%
	1:20	44.47	48.66	109%
Sample-2	1:5	191.45	NA	NA
	1:10	95.73	81.44	85%
	1:20	47.86	42.61	89%
	1:40	23.93	21.55	90%

#### Recovery:

Known amounts of Oxyntomodulin were added to five K<sub>2</sub>EDTA samples containing different levels of endogenous Oxyntomodulin. The concentration of Oxyntomodulin was determined before and after the addition of exogenous Oxyntomodulin and the percent recovery was calculated.

Sample	Endogenous Conc. (pg/mL)	Expected Conc. (pg/mL)	Observed Conc. (pg/mL)	% Recovery
1	14.60	28.37	27.97	99%
		42.14	40.33	96%
		55.91	51.95	93%
2	0.001	14.50	15.55	107%
		29.00	29.02	100%
		43.50	43.01	99%
3	0.001	14.50	13.26	91%
		29.00	26.43	91%
		43.50	38.01	87%
4	12.76	26.62	26.54	100%
		40.48	39.23	97%
		54.35	50.39	93%
5	23.96	37.26	37.78	101%
		50.56	49.07	97%
		63.86	58.82	92%

#### Analytical Specificity:

Monoclonal antibody pair used in the assay detects human, bovine, equine, canine, rabbit, goat, sheep, mouse, squirrel and monkey Oxyntomodulin. Cross-reactivity to other closely related analyte is represented in the table below.

Cross-Reactant	Concentration	% Cross-reactivity
Glucagon (1-29)	1000 ng/mL	Non-Detectable
GLP-1 (7-36)	1000 ng/mL	Non-Detectable
GLP-1 (9-36)	1000 ng/mL	Non-Detectable
GLP-2 (1-34)	1000 ng/mL	Non-Detectable
GRPP	1000 ng/mL	Non-Detectable
MPGF	1000 ng/mL	Non-Detectable
Glicentin	300 pg/mL	< 7.4%
Insulin	1000 pg/mL	Non-Detectable
C-peptide	1000 pg/mL	Non-Detectable
Thyroglobulin	1000 pg/mL	Non-Detectable
Pro-glucagon KO serum (n=3)	NA	Non-Detectable
Wild Type Mice Serum (n=3)	103-246 pg/mL	100%
Oxyntomodulin (1-37)	100 pg/mL	100%

#### Interference:

When potential interferents (hemoglobin, triglycerides, bilirubin and biotin) were added at least at two times their physiological concentration to control

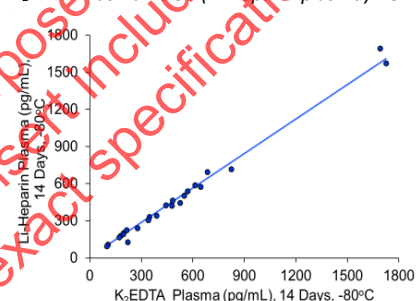
sample, Oxyntomodulin concentration were within  $\pm 10\%$  of the control as represented in the following table.

Interferents	Analyte Conc.	Unspiked Sample Value (pg/mL)	Spiked Sample Value (pg/mL)	% Difference
Hemoglobin	1.35 mg/mL	26.38	26.60	0.8
		48.77	47.25	-3.1
		83.62	83.62	0.0
Triglycerides	10.0 mg/mL	25.44	23.07	-9.3
		46.67	45.78	-1.9
		81.65	78.17	-4.2
Bilirubin	0.60 mg/mL	22.05	22.22	0.8
		42.49	40.21	-5.3
		69.27	68.13	-1.6
Biotin	1200 ng/mL	5.23	5.30	1.4
	600 ng/mL	5.74	5.23	-8.9
	200 ng/mL	5.59	5.30	-5.2

#### Sample Type:

Twenty-four matched K<sub>2</sub>EDTA and Lithium-heparin plasma specimens in the range of 100-1600 pg/mL were compared in AnshLabs Oxyntomodulin ELISA assay (AL-139). Passing Bablok analysis of the results yielded the following Regression:

$$K_2EDTA\ Plasma = 0.93 (Li-Heparin\ plasma) + 9.13, (r=0.99; P<0.0001)$$

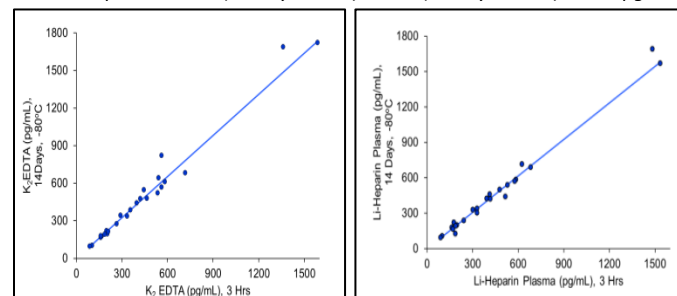


#### Plasma Stability:

Twenty-five K<sub>2</sub>EDTA and Twenty-four Lithium-heparin plasma specimens in the range of 100-1600 pg/mL were aliquoted stored at -80°C for 14 days and compared to freshly drawn plasma samples in Oxyntomodulin ELISA assay (AL-139). Passing Bablok analysis of the results yielded the following Regression:

$$K_2EDTA\ Plasma\ (Freshly\ Drawn) = 1.09\ (14\ Days,\ -80^\circ C) - 2.99\ pg/mL$$

$$Lithium-Heparin\ Plasma\ (Freshly\ Drawn) = 1.03\ (14\ Days,\ -80^\circ C) + 0.66\ pg/mL$$

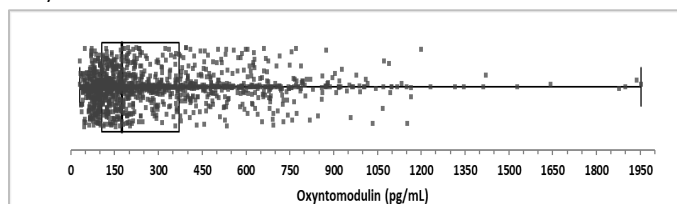


#### Expected Ranges:

Seven matched Fasting and non-fasting (30-45 mins after meal) K<sub>2</sub>EDTA and Lithium-heparin plasma specimens from participants were analyzed in Ansh Labs Oxyntomodulin assay (AL-139) and their mean and 95% confidence interval were calculated and are represented below:

Sample Type	n	Mean (pg/mL)	95% CI
Li-Heparin (Fasting)	7	268.50	123.58-413.41
Li-Heparin (Non-Fasting)	7	682.23	238.81-1125.66
K <sub>2</sub> EDTA (Fasting)	7	283.29	125.41-441.16
K <sub>2</sub> EDTA (Non-Fasting)	7	721.50	285.88-1157.12

Expected Oxyntomodulin concentration in undifferentiated population (diabetic and non-diabetic) was calculated by evaluating 1534 samples in Ansh Labs Oxyntomodulin ELISA. The frequency distribution was calculated using Analyse-It® for Microsoft Excel and is shown below.



n	Oxyntomodulin (pg/mL)			
	Mean	Median	Range	95% CI
1534	277.7	175.7	29.9 - 1953.8	49.5 - 948.1
Quantile		Oxyntomodulin (pg/mL)		
0.100		77.4		
0.200		97.0		
0.300		118.1		
0.400		141.7		
0.500		175.7		
0.600		222.6		
0.700		309.9		
0.800		441.4		
0.900		635.9		

NOTE: It is recommended that each laboratory should determine the reference range(s) for its own patient population. The results of this assay should be used in conjunction with other relevant and applicable clinical information.

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