Mercodia
Immunoassays & Services

Mercodia Glucagon ELISA

Directions for Use

10-1271-01
REAGENTS FOR 96 DETERMINATIONS

For *in vitro* diagnostic use

IVD

CE

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oder/ou/o/eller/of **Fax No +46 18-570080**

Regulatory status in China: For Research use only.
Not for use in diagnostic procedures

Manufactured by

Mercodia AB
Sylveniusgatan 8A,
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EXPLANATION OF SYMBOLS USED ON LABELS

	Reagents for 96 determinations
	Expiry date
	Store between 2–8°C
	Lot No.
	For *in vitro* diagnostic use

INTENDED USE

Mercodia Glucagon ELISA is an assay intended to measure the pancreatic hormone glucagon in plasma and serum. Glucagon measurements are used in the diagnosis and treatment of patients with various disorders of carbohydrate metabolism, including diabetes mellitus, hypoglycemia, and hyperglycemia.

SUMMARY AND EXPLANATION OF THE TEST

Glucagon is a 29 amino acid polypeptide processed from proglucagon in pancreatic alpha cells. In intestinal L-cells proglucagon is cleaved into glicentin, corresponding to proglucagon residues no. 1-69. Glicentin can further be processed into oxyntomodulin, corresponding to proglucagon residues no. 33-69. These peptides are released simultaneously upon stimulation. Moreover, a fragment of glucagon corresponding to its C-terminal part (residues no. 19-29), also designated mini-glucagon, is reported to be present in the pancreas in low amounts compared to the total glucagon content.

Glucagon is processed into several truncated forms by various endopeptidases (neprilysin, for example) and exopeptidases (DPP-4). If you are interested in measuring active glucagon (1-29), the assay should not cross-react to any of the metabolites[1], of which glucagon 3-29 is the most common. Biacore and ELISA analyses have shown that there is no cross-reactivity in the Mercodia Glucagon assays to glucagon 3-29 at, or above, physiological concentrations. This is an important finding, since glucagon 3-29 has been reported to be the major metabolite in clinical samples due, in large part, to in vitro plasma protease metabolism during sample storage[2]. The increased specificity is a primary reason why concentrations in the Mercodia glucagon assays are lower than concentrations generated by other glucagon assays.

In general, glucagon has an effect opposite that of insulin, i.e. it raises blood glucose levels. It causes the liver to convert glycogen into glucose, which is then released into the blood stream. With longer stimulation, glucagon action in the liver results in a glucose-sparing activation of free fatty acid oxidation and production of ketones. During hypoglycaemia, glucagon secretion offers a protective feedback mechanism, defending the organism against damaging effects of glucose deficiency in the brain and nerves.

PRINCIPLE OF THE PROCEDURE

Mercodia Glucagon ELISA is a solid phase two-site enzyme immunoassay. It is based on the direct sandwich technique in which two monoclonal antibodies are directed against separate antigenic determinants on the glucagon molecule. During incubation glucagon in the sample reacts with peroxidase-conjugated anti-glucagon antibodies (clone E6A11K) and anti-glucagon antibodies (clone M5F9S) bound to microplate wells. A simple washing step removes unbound enzyme labelled antibody. The bound conjugate is detected by reaction with 3,3’,5,5’-tetramethylbenzidine (TMB). The reaction is stopped by adding acid to give a colorimetric endpoint that is read spectrophotometrically.

WARNINGS AND PRECAUTIONS

- For in vitro diagnostic use.
- Regulatory Status in China: For Research Use Only. Not for use in diagnostic procedures.
- The content of this kit and their residues must not be allowed to come into contact with ruminating animals or swine.
- All samples should be handled as capable of transmitting infections.
- Each well can only be used once.
- The Stop Solution contains <5% Sulphuric acid.
  The Stop Solution is labeled:

Danger
H318 – Causes serious eye damage.
H315 – Causes skin irritation.
P280 – Wear protective gloves. Wear eye or face protection.
P264 – Wash hands thoroughly after handling.
P302 + P352 + P362 + P364 – IF ON SKIN: Wash with plenty of soap and wa ter. Take off contaminated clothing and wash it before reuse.
P332 + P313 - If skin irritation occurs: Get medical attention.
P305 + P351 + P338 + P310 – IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a POISON CENTER or physician.

- The Enzyme Conjugate Buffer, Cal 0, 1, 2, 3, 4, 5 and Wash Buffer contain <0.06% 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-2H-isothiazol-3-one (3:1). The Enzyme Conjugate Buffer, the Calibrators and Wash Buffer are labeled:

Warning
H317 – May cause an allergic skin reaction.
P280 – Wear protective gloves.
P261 – Avoid breathing vapour.
P272 – Contaminated work clothing should not be allowed out of the workplace.
P302 + P352 – IF ON SKIN: Wash with plenty of soap and water.
P333 + P313 – If skin irritation or rash occurs: Get medical attention.
P501 – Dispose of contents and container in accordance with all local, regional, national and international regulations.

MATERIAL REQUIRED BUT NOT PROVIDED

- Pipettes with appropriate volumes (repeating pipettes preferred for addition of enzyme conjugate 1X solution, Substrate TMB and Stop Solution)
- Tubes, beakers and cylinders for reagent preparation
- Redistilled water
- Magnetic stirrer
- Vortex mixer
- Microplate reader with 450 nm filter
- Microplate shaker (700–900 cycles per minute, orbital movement)
- Refrigerator (2–8°C) with room for microplate shaker
- Microplate washing device with overflow function (recommended but not required)

REAGENTS FOR 1 X 96 KIT

Each Mercodia Glucagon ELISA kit (10-1271-01) contains a plate sealer and reagents for 96 wells, sufficient for 42 samples and one calibrator curve in duplicate. For larger series of assays, use pooled reagents from packages bearing identical lot numbers. The expiry date for the complete kit is stated on the outer label. The recommended storage temperature is 2–8°C.

Coated Plate Mouse monoclonal anti-glucagon	1 plate	96 wells 8-well strips	Ready for Use
For unused microplate strips, reseal the bag using adhesive tape, store at 2–8°C and use within 2 months.			
Calibrators 1, 2, 3, 4, 5 Synthetic glucagon Color coded yellow Concentration stated on vial label.	5 vials	1000 µL	Lyophilized Add 1000 µL redistilled water per vial.
Reconstituted Calibrators are stable for 1 month at 2-8°C. If reconstituted Calibrators are to be used for longer than 1 month, aliquote and store at -20°C. Aliquoted Calibrators are stable for at least 2 months at -20°C. Avoid repeated freeze/thaw cycles.			
Calibrator 0 Color coded yellow	1 vial	5 mL	Ready for Use
Enzyme Conjugate 11X Mouse monoclonal anti-glucagon	1 bottle	2.2 mL	Preparation, see below
Enzyme Conjugate Buffer Color coded blue.	1 bottle	22 mL	Ready for use
Wash Buffer 21X Storage after dilution: 2-8°C for 2 months.	1 bottle	50 mL	Dilute with 1000 mL redistilled water to make wash buffer 1X solution.
Substrate TMB Colorless solution *Note! Light sensitive!*	1 bottle	22 mL	Ready for Use
Stop Solution 0.5 M H_2SO_4	1 vial	7 mL	Ready for Use

Preparation of enzyme conjugate 1X solution

Prepare the needed volume of enzyme conjugate 1X solution by dilution of Enzyme Conjugate 11X (1+10) in Enzyme Conjugate Buffer according to the table below. When preparing enzyme conjugate 1X solution for the whole plate, pour all of the Enzyme Conjugate Buffer into the Enzyme Conjugate 11X bottle. Mix gently.
Use within 1 week.

Number of strips	Enzyme Conjugate 11X	Enzyme Conjugate Buffer
12 strips	1 bottle	1 bottle
8 strips	1300 µL	13 mL
4 strips	700 µL	7 mL

SPECIMEN COLLECTION AND HANDLING

Serum or plasma can be used. However, glucagon in serum or EDTA plasma samples will be sensitive to storage conditions and freeze-thaw cycles. It is recommended to keep samples on ice when thawing them and preparing the assay. Return to freezer as soon as possible. Addition of aprotinin to EDTA plasma samples will not improve stability.

Serum

Collect blood by venipuncture, allow to clot and separate the serum by centrifugation. Store samples at -80°C and avoid freeze-thaw cycles. Avoid storing samples at room temperature or 2-8°C.

Plasma

EDTA plasma

Collect blood by venipuncture into tubes containing EDTA as anticoagulant, and separate the plasma fraction by centrifugation. Store samples at -80°C and avoid freeze-thaw cycles. Avoid storing samples at room temperature or 2-8°C.

Stabilized EDTA plasma

For studies in which low levels of glucagon need to be detected, it may be beneficial to use sample collection tubes specifically optimized for stabilization, since this will prevent the degradation of glucagon. Store samples at -80°C and avoid freeze-thaw cycles. Avoid storing samples at room temperature or 2-8°C.

Cell culture medium

Note that different chemicals used in cell culture media can interfere with the assay (such as sodium azide (NaN_3) and beta-mercaptoethanol).

Preparation of samples

No dilution is normally required, however, samples above the obtained value of Calibrator 5 should be diluted with Calibrator 0. *Note!* Buffers containing sodium azide (NaN_3) cannot be used for sample dilution.

TEST PROCEDURE

Prepare a calibrator curve for each assay run.

1. Prepare enzyme conjugate 1X solution and wash buffer 1X solution.
2. Prepare sufficient microplate wells to accommodate Calibrators, controls and samples in duplicate.
3. Pipette 25 µL each of Calibrators, controls and samples into appropriate wells.
4. Add 200 µL enzyme conjugate 1X solution to each well and attach the plate sealer.
5. Incubate on a plate shaker (700-900 rpm) over night (18-22h) at 2–8°C.
6. Wash 6 times with 700 µL wash buffer 1X solution per well using an automatic plate washer with overflow-wash function. After final wash, invert and tap the plate firmly against absorbent paper. Do not include soak step in washing procedure.
   Or manually,
   Discard the reaction volume by inverting the microplate over a sink. Add 350 µL wash solution to each well. Discard the wash buffer 1X solution, tap firmly several times against absorbent paper to remove excess liquid. Repeat 5 times. Avoid prolonged soaking during washing procedure.
7. Add 200 µL Substrate TMB.
8. Incubate on the bench for 15 minutes at room temperature (18–25°C).
9. Add 50 µL Stop Solution to each well.
   Place plate on a shaker for approximately 5 seconds to ensure mixing.
10. Read optical density at 450 nm and calculate results.
    Read within 30 minutes.

Note! Be extra careful not to contaminate the Substrate TMB with enzyme conjugate solution.

INTERNAL QUALITY CONTROL

Commercial controls and/or internal serum pools with low, intermediate and high glucagon concentrations should routinely be assayed as samples and results charted from day to day. It is good laboratory practice to record the following data for each assay: kit lot number, dilution and/or reconstitution dates of kit components, OD values for the blank, Calibrators and controls.

Laboratories should follow government regulations or accreditation requirements for quality control frequency.

CALCULATION OF RESULTS

The concentration of glucagon is obtained by plotting the absorbance of the Calibrators, except for Cal 0, versus their concentration. It is important to use an appropriate curve fitting model that represent the true dose-response relationship to get accurate results.

The Mercodia Glucagon ELISA is validated with five parameter logistic with automatic weighting using $1/y^2$.

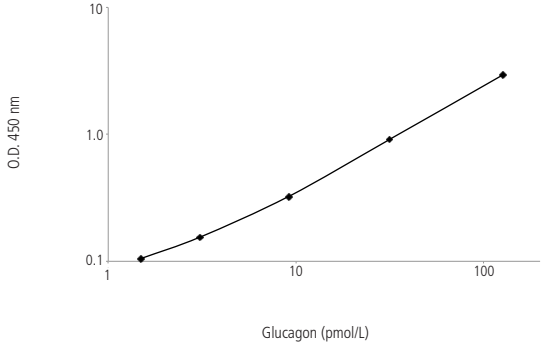
Example of results

Wells	Identity	A_{450}	Mean conc. pmol/L
1A–B	Calibrator 0	0.067/0.069	
1C–D	Calibrator 1*	0.106/0.104	
1E–F	Calibrator 2*	0.152/0.157	
1G–H	Calibrator 3*	0.331/0.316	
2A–B	Calibrator 4*	0.902/0.923	
2C–D	Calibrator 5*	2.949/2.945	
2E–F	Sample 1	0.154/0.155	3.14
2G–H	Sample 2	0.191/0.193	4.43
3A–B	Sample 3	0.569/0.562	18.1

* Concentration stated on vial label.

Calibrator curve

A typical calibrator curve is shown here. Do not use this curve to determine actual assay results.



Conversion factor

1 pmol/L = 3.5 pg/mL

LIMITATIONS OF THE PROCEDURE

Grossly lipemic or icteric samples do not interfere in the assay. High levels of hemoglobin (>500 mg/dL) can interfere in the assay. As with all diagnostic tests, a definitive clinical diagnosis should not be based on the results of a single test, but should be made by the physician after all clinical findings have been evaluated.

When measuring samples where the levels of oxyntomodulin, glicentin or proglucagon 1-61 are above the normal physiological range, or if either of these peptides have been injected into the subjects prior to sampling, an alternative protocol (see below) can be used to decrease the crossreactivity from these peptides. Increased levels of glicentin and oxyntomodulin are commonly found in post-bariatric surgery patients. Increased levels of proglucagon 1-61 is commonly found in patients with kidney failure.

The alternative protocol is for research use only and is described in TechNote TN34-0158. The protocol requires one extra vial of Conjugate Buffer (art nr 20-7055). Please contact info_europe@mercodia.com, or your local sales representative, for more information.

EXPECTED VALUES

Good practice dictates that each laboratory establishes its own expected range of values. The following results may serve as a guide until the laboratory has gathered sufficient data of its own. Fasting levels for 121 tested, apparently healthy individuals, yielded a median of 6.5 pmol/L and central 95% reference range of ≤1.5-18 pmol/L analyzed in plasma. [7]

PERFORMANCE CHARACTERISTICS

The assay has been validated according to FDA[8], EMA[9], and CLSI[10-14] guidelines. Selected studies are presented here. Additional data can be obtained from Mercodia.

Validation of Curve Fit

The curve fitting was validated with Five Parameter Logistics with $1/y^2$ weighting. Four Parameter Logistics with $1/s^2$ weighting, and Cubic spline will also give acceptable results.

Analytical sensitivity and Range of Quantification

The detection limit is 1 pmol/L as determined by the methodology described in ISO11843-Part 4.[15]

Lower Limit of Quantification, LLOQ, is 1.7 pmol/L as determined according to FDA/EMA guidelines.

The Upper Limit of Quantification, ULOQ, is 130 pmol/L as determined according to FDA/EMA guidelines.

Precision and Accuracy

QC samples were analyzed in 4 replicates over 12 different occasions on one kitlot and one instrument system by one laboratory technician.

			Coefficient of variation	
Sample	Mean value pmol/L	Accuracy %	Repeatability %*	Within laboratory precision %**
QC_{LLOQ}	1.7	99	11	16
QC_{Low}	4.6	102	8.7	11
QC_{Medium}	49	99	3.1	5.8
QC_{High}	99	99	3.1	5.0
QC_{ULOQ}	130	97	5.3	6.2

*Within-run variation

**Between-run variation

Analytical Specificity

- **Cross-reactivity**

  The following cross-reactions were found:

Substance	Cross-reaction %	Concentrations tested pmol/L
Glicentin	0.8	300
Oxyntomodulin	4.4	135
Mini-Glucagon	< 0.1	1600
GLP-1	< 0.3	500
GLP-2	< 0.3	500
GRPP	< 0.0005	300000
GlucaGen (recombinant glucagon for injection)	100	100

- **Interference**
  Recovery values at LLOQ and ULOQ concentration of glucagon is presented below. The substance is concluded to interfere if the recovery value is not within 100 ± 25 % of the nominal concentration. [8]

	Concentration (pM)	Recovery (%) LLOQ	ULOQ
Mini-Glucagon	3	90	106
	12	107	102
	30	83	104
	120	81	99
	300	84	100
Glicentin	3	92	107
	12	93	107
	30	106	105
	120	154	111
	300	220	119
Oxyntomodulin	3	105	111
	12	120	110
	30	172	111
	120	463	116
	300	1577	128
GLP-1	5	88	104
	25	97	104
	125	86	102
	250	90	103
	1000	85	100

Selectivity
Lipemic or haemolyzed samples do not interfere in the assay.
High levels of haemoglobin (> 500 mg/dl) can interfere in the assay.

Parallelism
P800 samples spiked with Glucagon to high concentrations within the measuring range were diluted 1/2, 1/4 and 1/8.

Mean recovery for parallelism is 91 % (78-112 %) with precision between samples in the dilution series ≤ 15 %.

Dilutional linearity
P800 samples were spiked above the highest calibrator concentration and subsequently diluted for analysis in the assay. Nominal values were used for calculation.

Mean recovery for dilutional linearity is 83 % (80-87%) with a precision of the final concentration across all dilutions of 6 %.

High Dose Hook Effect
Samples with a concentration up to 8 µM can be measured without giving falsely low results.

CALIBRATION
Mercodia Glucagon ELISA is calibrated against WHO 1st International reference preparation 69/194.

WARRANTY
The performance data presented here was obtained using the procedure indicated. Any change or modification in the procedure not recommended by Mercodia AB may affect the results, in which event Mercodia AB disclaims all warranties expressed, implied or statutory, including the implied warranty of merchantability and fitness for use.

Mercodia AB and its authorized distributors, in such event, shall not be liable for damages indirect or consequential.

REFERENCES

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11. EP15-A3 User Verification of Precision and Estimation of Bias; Approved Guideline —Third Edition
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14. EP17-A2 Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures; Approved Guideline—Second Edition
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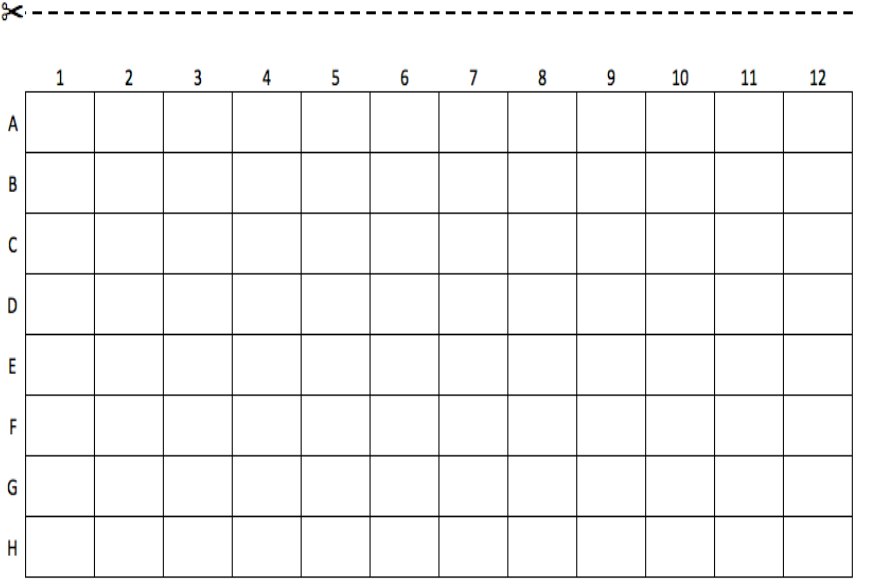
Further references can be found on our website: www.mercodia.com

NOTES



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

Experiment: Date: KIT LOT#:

SUMMARY PROTOCOL SHEET

Mercodia Glucagon ELISA

Add Calibrators, controls* and samples	25 µL
Add enzyme conjugate 1X solution and attach plate sealer	200 µL
Incubate	Overnight (18-22 h) at 2-8°C on a plate shaker, 700-900 rpm
Wash plate with wash buffer 1X solution	700 µL, 6 times
Add Substrate TMB	200 µL
Incubate	15 minutes at 18-25°C
Add Stop Solution	50 µL Shake for 5 seconds to ensure mixing
Measure A_{450}	Evaluate results

* Not included

For full details, see page 7.

For technical support please contact: support@mercodia.com

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