Mercodia Glucagon ELISA

Directions for Use

Important changes made in this version

Page 7 **Test procedure**

The changes made to the protocol has affected several parts throughout this document

10-1271-01
Reagents for 96 determinations

For *in vitro* diagnostic use in EU/EEA, UK, US and Canada

IVD

CE

Gebrauchsanweisung auf Deutsch finden Sie unter folgendem Link:
Veuillez trouver le mode d'emploi en français à:
Podrá encontrar las instrucciones de uso en español en:
Le istruzioni per l'uso sono reperibili in italiano all'indirizzo:
For danske brugsanvisning gå til:
För svensk bruksanvisning gå till:
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Ga voor de Nederlandse vertaling naar:
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https://www.mercodia.com/product/glucagon-elisa/
oder/ou/o/eller/of **email: info-global@mercodia.com**

Regulatory status in the rest of the world: For research use only.
Not for use in diagnostic procedures.

Manufactured by

Mercodia AB
Sylveniusgatan 8A
SE-754 50 Uppsala
Sweden

Explanation of symbols used on labels

	Reagents for 96 determinations
	Expiry date
	Store between 2-8ºC
	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 (residues 33-61) 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. 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.

Mercodia Glucagon ELISA was launched in 2013 and has since contributed to advancing research by, for the first time, allowing specific and sensitive measurements of glucagon. A few years later, a glicentin ELISA was launched that made it possible to accurately measure circulating glicentin, and with help of this assay it was shown that levels of glicentin are increasing during an oGTT in gastrectomy patients to levels not seen in any patient group before[1,2]. Levels of oxyntomodulin are also increasing, but not to the same extent. When such high levels of glicentin appear in the circulation, also a low cross-reactivity will disturb the measurement of glucagon and give rise to falsely high levels.

After discussions with different research groups a sequential protocol was developed that removes the small amount of cross-reactivity to glicentin. As of October 1st 2020, the assay protocol of Mercodia Glucagon ELISA 10-1271-01 is updated to make the sequential protocol default, abolishing cross reactivity to glicentin.

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 the first incubation glucagon in the sample reacts with anti-glucagon antibodies bound to microplate wells. After washing, peroxidase conjugated anti-glucagon antibodies are added. After a second incubation and a simple washing step, 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 in EU/EEA, UK, US and Canada.
- Regulatory Status in the rest of the world: For Research Use Only. Not for use in diagnostic procedures.
- 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 water. 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, Wash Buffer and Assay 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, Wash Buffer and Assay 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, Assay Buffer, 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

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
Assay Buffer Color coded red	1 bottle	22 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 vial	1 vial
8 strips	1300 µL	13 mL
4 strips	700 µL	7 mL

Specimen collection and handling

Serum, plasma or cellculture medium can be used. However, glucagon in serum, EDTA plasma and cell culture medium 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). Avoid freeze/thaw, do not store samples in room temperature, samples should be kept on ice during use. Samples should be diluted at least 2X with Calibrator 0.

Preparation of samples

No dilution is normally required for serum and plasma, but samples above Calibrator 5 should be diluted with Calibrator 0. Dilution in Calibrator 0 is recommended for all cell culture medium samples.

Test procedure

Prepare a calibrator curve for each assay run. All reagents and samples must be brought to room temperature before use.

1. Reconstitute Calibrators with 1000 µL redistilled water.
2. Pipette 25 µL each of Calibrators, controls and samples in duplicate into appropriate wells.
3. Add 200 µL Assay Buffer to each well and attach the plate sealer.
4. Incubate on plate shaker (700-900 rpm) over night (18-22 h) at 2-8°C.
5. Prepare wash buffer 1X solution by diluting Wash Buffer 21X with 1000 mL redistilled water. Prepare the needed volume of enzyme conjugate 1X solution by dilution of Enzyme Conjugate 11X (1+10) in Enzyme Conjugate Buffer.
6. Wash 6 x 700 µL using an automatic plate washer with overflow wash function. The chosen program should fill all the wells with wash buffer in each cycle and ensure that the wells are never left without wash buffer (e.g. Plate Mode). Do not use additional soak! Invert and tap the plate firmly against absorbent paper after the final wash.
   Or manually,
   Discard the reaction volume by inverting the microplate over a sink.
   Hold the plate vertically over a sink and fill the wells by spraying wash buffer into the wells with a wash bottle. 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.
   For more information se Technical Note No: 34-0106 Instruction for manual washing procedure for microplates (available online).
7. Add 200 µL enzyme conjugate 1X solution to each well.
8. Incubate on plate shaker (700-900 rpm) 1h at room temperature (18-25°C).
9. Wash as described in step 6.
10. Add 200 µL Substrate TMB per well.
11. Incubate on the bench for 30 min at room temperature (18-25°C).
12. Add 50 µL Stop Solution to each well. Place plate on a shaker for approximately 5 seconds to ensure mixing.
13. Read optical density at 450 nm.
    Read within 30 minutes.
14. Apply curve fitting directly on raw data ($A_{450\ nm}$).
    Preferably use 5-parametric logistic regression with automatic weighing on relative weights ($1/y^2$).

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

Internal quality control

Commercial controls and/or internal plasma 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. It is every laboratory's responsibility to try out the functionality of the chosen curve fitting model and used software. Note that weighting of the curve fit is important to get a proper fit at the low range of the standard curve, especially when the measuring range is wide.

The Mercodia Glucagon ELISA is validated with Five parameter logistic with weighting $1/y^2$, using Magellan (Tecan) software.

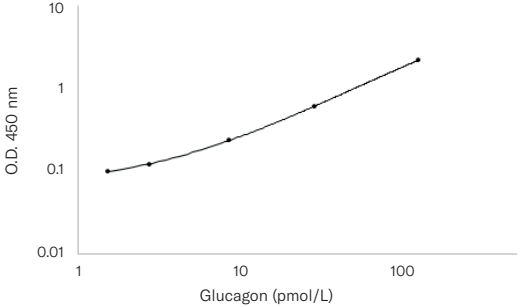
Example of results

Wells	Identity	$A_{450\ nm}$	Mean conc. pmol/L
1A–B	Calibrator 0	0.066/0.067	
1C–D	Calibrator 1*	0.099/0.100	
1E–F	Calibrator 2*	0.121/0.117	
1G–H	Calibrator 3*	0.238/0.239	
2A–B	Calibrator 4*	0.597/0.624	
2C–D	Calibrator 5*	2.235/2.179	
2E–F	Sample 1	0.141/0.138	3.68
2G–H	Sample 2	0.283/0.276	11.1
3A–B	Sample 3	1.929/1.780	99.3

*Concentration stated on vial label.

Example of 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 samples do not interfere in the assay. Certain levels of hemoglobin (>50 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.

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[10-13] guidelines with a fit for purpose approach. 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.

Sensitivity and Range of Quantification

The detection limit is 0.75 pmol/L as determined by the methodology described in ISO11843-Part 4.[14]

Lower Limit of Quantification, LLOQ, is 1.5 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 (P800) were analyzed in 4 replicates over 6 different occasions on one kitlot and one instrument system by three laboratory technicians.

			Coefficient of variation	
Sample	Mean value pmol/L	Accuracy %	Repeatability %*	Within laboratory precision %**
QC_{LLOQ}	1.5	96	5.6	8.3
QC_{Low}	4.3	90	6.3	13
QC_{Medium}	11	105	14	16
QC_{High}	101	101	2.1	10
QC_{ULOQ}	130	96	5.1	7.0

*Within-run variation
**Between-run variation

Analytical Specificity

- **Cross-reactivity**
  The following cross-reactions were found:

Substance	Cross-reaction %	Concentrations tested pmol/L
Glicentin	n.d.	300
Oxyntomodulin	4.0	300
Proglucagon 1-61	n.d.	25
Glucagon 3-29	n.d.	250

n.d. = not detected

- **Interference**

  Interference data at low (1.5 pmol/L) and high (102 pmol/L) concentrations of glucagon are presented below. The substance is concluded to interfere if the recovery value is not within 100 ± 25 % of the nominal concentration.

	Concentration (pmol/L)	Recovery %	
		Low conc.	High conc.
Glicentin	3	77	107
	12	<Cal 1	104
	30	89	114
	120	<Cal 1	107
	300	117	114
Oxyntomodulin	3	106	96
	12	118	97
	30	171	114
	120	455	112
	300	1293	136
Proglucagon 1-61	5	132	110
	23	126	111
Glucagon 3-29	5	102	110
	25	104	109
	125	113	102

Selectivity

Lipemic samples do not interfere in the assay.
Certain levels of hemoglobin (>50 mg/dL) can interfere in the assay.

Parallelism

Ten P800 samples spiked with Glucagon to high concentrations within the measuring range were diluted 1/2, 1/4, 1/8 and 1/16. Mean recovery for parallelism is 98 % (85-108 %) with precision between samples in the dilution series ≤ 7 %.

Five P800 samples with high endogen Glucagon concentrations were diluted 1/2, 1/4 and 1/8. Mean recovery for parallelism is 100 % (84-119 %) with precision between samples in the dilution series ≤ 10 %.

Dilutional linearity

Cell culture medium 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 105 % (102-110%) with a precision of the final concentration across all dilutions of 4 %.

High Dose Hook Effect

Samples with a concentration up to 50 nmol/L 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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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 of protocol sheet
Mercodia Glucagon ELISA

Add Calibrators, controls* and samples	25 µL
Add Assay Buffer and attach plate sealer	200 µL
Incubate	Overnight (18-22h) at 2-8°C on a plate shaker, 700-900 rpm
Wash plate with wash buffer 1X solution	700 µL, 6 times, using Plate Mode
Add enzyme conjugate 1X solution	200 µL
Incubate	1h (700-900 rpm) at 18-25°C
Wash plate with wash buffer 1X solution	700 µL, 6 times, using Plate Mode
Add Substrate TMB	200 µL
Incubate	30 min on the bench at 18-25°C
Add Stop Solution	50 µL Shake for 5 seconds to ensure mixing
Measure $A_{450\ nm}$	Evaluate results using 5-parametric logistic regression with automatic weighing on relative weights ($1/y^2$)

*not included

For full details see page 7

For technical support please contact: support@mercodia.com

31-3185
Version 12.0
2021-02-01