INSTRUCTION MANUAL

(April 29, 2016)

21-Hydroxylase Autoantibodies ELISA Assay

CE REF 3789

Enzyme immunoassay for the determination of **autoantibodies** to 21-Hydroxylase (21-OH) in human serum

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IFU symbols non-radioactive assays MEDIPAN GMBH

		CE	EC Declaration of Conformity
REF	Catalogue number	LOT	Batch code
	Expiry date		Manufactured by
	Consult accompanying documents		Consult operating instruction
	Store at		Biological risk
MP	Microtiter plate	WASHB	Wash buffer
CONJ	Conjugate	SUB	Substrate
STOP	Stop solution	BUF D	Diluent for start buffer
START	Start buffer	BUF H	Diluent for conjugate
CONTROL	Control serum	CAL	Calibrators

INTENDED USE

The Medizym® anti-21-OH ELISA kit is intended for the quantitative determination of autoantibodies to 21-hydroxylase (21-OH Abs) in human serum. For research use only.

Autoimmune destruction of the adrenal cortex is the most common cause of Addison's disease and autoantibodies to corticosteroid producing cells in adrenal cortex. The antibodies recognizing the adrenal specific enzyme 21-hydroxylase are important markers of adrenal autoimmunity. This can be the case if the disease presents as Addison's disease or as part of the autoimmune polyglandular syndrome together with diabetes mellitus type I and thyroid disorders.

LITERATURE

- J. Furmaniak and B. Rees Smith: Editorial: Adrenal and Gonadal Autoimmune Diseases. J. Clin. Endocrinol. Metab. 1995 80: 1502 – 1505
- S. Chen et al: Autoantibodies to Steroidogenic Enzymes in Autoimmune Polyglandular Syndrome, Addison's Disease, and Premature Ovarian Failure. J. Clin. Endocrinol. Metab. 1996 81: 1871-1876
- H. Tanaka et al: Steroid 21-Hydroxylase Autoantibodies: Measurements with a New Immunoprecipitation Assay. J. Clin. Endocrinol. Metab. 1997 82: 1440-1446
- G. Coco et al: Estimated Risk for Developing Autoimmune Addison's Disease in Patients with Adrenal Cortex Autoantibodies. J. Clin. Endocrinol. Metab. 2006 91: 1637-1645
- E. S. Husebye et al: Consensus Statement on the Diagnosis, Treatment and Follow-up of Patient with Primary Adrenal Insufficiency. J. Intern. Med. 2014 275(2):104-15

PRINCIPLE of the TEST

Medizym® anti-21-OH is an enzyme immunoassay for the quantitative determination of autoantibodies to 21-hydroxylase (21-OH) in human serum.

The assay utilizes the ability of 21-OH Abs to act divalently, and to form a bridge between immobilized 21-OH and 21-OH-Biotin in the fluid phase.

In the first step, 21-OH Ab present in samples binds with 21-OH immobilized onto the microtiter plate. In the second step, 21-OH-Biotin binds to this complex. The amount of 21-OH-Biotin bound correlates with the level of antibodies present in patient samples. Unbound 21-OH-Biotin is then removed by washing.

Bound 21-OH-Biotin can then be quantified by addition of streptavidin peroxidase (SA-POD) and a colorigenic substrate (TMB), and reading the optical density at 450nm.

PATIENT SAMPLES

Specimen collection and storage

Blood is taken by venipuncture. After clotting, the serum is separated by centrifugation. Do not use lipaemic or hemolytic serum samples. Do not use plasma samples in the test.

The samples may be kept at 2 - 8 °C up to three days. Long-term storage requires storage at- 20 °C.

Repeated freezing and thawing should be avoided. For multiple use, initially aliquot samples and store at - 20 °C.

TEST COMPONENTS for 96 DETERMINATIONS

A MP	**Microtiter plate** 12 breakable strips of 8 wells, coated with 21-hydroxylase		Vacuum sealed with desiccant
B WASHB	**Concentrated wash buffer** Sufficient for 1250 ml		**125 ml** concentrate
D CONJ	**Streptavidin-peroxidase (SA-POD)** Sufficient for 14 ml		**0.7 ml** concentrate
E SUB	**Substrate (TMB)** (3,3´,5,5´-Tetramethylbenzidine)		**15 ml** ready for use
F STOP	**Stop solution** (0.25 M sulfuric acid)		**12 ml** ready for use
G BUF D	**Diluent for SA-POD (D)**		**15 ml** ready for use
H START	**21-OH-Biotin**		**3 vials** lyophilized
J BUF H	**Diluent for 21-OH-Biotin (H)**		**2x 15 ml** ready for use colored blue
K ENH	**Enhancer**		**6 ml** ready for use colored red
C I CONTROL	**Negative control** For concentration: see leaflet	L	**0,7 ml** ready for use
C II, C III CONTROL	**Positive controls** For concentrations: see leaflet	L	**2 vials** **0,7 ml** each ready for use
1 - 4 CAL	**Calibrators** For concentrations: see leaflet	L	**4 vials** **0,7 ml** each ready for use

Materials required in addition

- Precision pipettes 10 - 100 µl
- Multi-channel pipette
- Disposable pipette tips
- 8 channel wash comb or microplate washer
- Micro plate reader with optical filters for 450 nm and 620 or 690 nm
- Graduated cylinders
- Distilled or de-ionized water
- Absorbent paper or paper towel
- Adhesive foil

Size and storage

Medizym® anti-21-OH has been designed for 96 determinations. This is sufficient for the analysis of 41 unknown samples as well as for calibrators and control sera assayed in duplicate.

The expiry date of each component is reported on its respective label, that of the complete kit on the box label. The maximum shelf life is still limited to 6 months in the moment.

Upon receipt, all components of the Medizym® anti-21-OH have to be kept at 2 - 8 °C, preferably in the original kit box.

Preparation before use

Allow samples to reach room temperature prior to assay. Allow all reagents to reach room temperature prior to assay, except 21-OH-Biotin (D) and 21-OH-Biotin reconstitution buffer (F). Take care to agitate serum samples gently in order to ensure homogeneity.

Please perform the following steps with care:

A Allow the sealed microplate to reach room temperature before opening. Unused wells should be stored refrigerated and protected from moisture in the original bag carefully resealed for max. 6 months.

B Prepare a sufficient amount of washing solution by diluting the concentrated wash buffer (B) 1 + 9 with distilled or de-ionized water. For example, dilute 50 ml of the concentrate with 450 ml of distilled water. B should be free of crystals before dilution, otherwise dissolve by warming up to max. 37°C. The diluted washing solution can be stored at 2 - 8 °C up to 30 days.

D Prepare a sufficient amount of streptavidin-peroxidase solution by diluting SA-POD concentrate (D) 1 + 19 (eg. 0.5 ml SA-POD concentrate with 9.5 ml diluent for SA-POD (G). The SA-POD solution prepared is stable up to 16 weeks at 2 - 8 °C.

E Avoid exposure of the substrate solution (E) to light.

H Prepare a sufficient amount of 21-OH-Biotin solution by reconstitution of one vial lyophilized 21-OH-Biotin (H) with 5.5 ml diluent for 21-OH-Biotin (J) immediately before use. Do not store the 21-OH-Biotin solution.

ASSAY PROCEDURE

- Duplicates are recommended.

1. Pipette into the corresponding wells according to assay scheme
   - **50 µl** negative control (C I) and calibrators (1 - 4)
   - **50 µl** control sera (C II, C III)
   - **50 µl** of patient samples.
2. Pipette **50 µl** Enhancer (K) into each well.
3. Cover the plate, shake for **1 min** at **500 rpm** on a plate shaker and incubate overnight, for **16 – 20 hours**, at **2 - 8°C**.
4. Prepare sufficient amount of reagents (B, D/G, H/J)
5. Aspirate or “flick out” by striking the wells sharply onto absorbent paper to remove any residual droplets. Wash **3 times** with **300 µl** washing solution (diluted from B) with 5 seconds soaking time each.
6. Pipette **100 µl** of reconstituted 21-OH-Biotin solution (prepared from H and J) to each well.
7. Cover the plate and incubate for **60 min** at room temperature (18 - 25 °C) while shaking at **500 rpm**.
8. Repeat washing step 5.
9. Add **100 µl** reconstituted SA-POD (prepared from D and G) to each well.
10. Cover the plate and incubate for **20 min** at room temperature (18 - 25 °C) while shaking at **500 rpm**.
11. Repeat washing step 5.
12. Add **100 µl** substrate solution (E) to each well.
13. Incubate for **20 min** in the **dark** at room temperature, without shaking.
14. Add **50 µl** stop solution (F) to each well, shortly shake the plate.
15. Read the optical density **at 450 nm** against **620 or 690 nm** with a micro plate reader, **within 20 minutes** after adding the stop solution.

Please note that the washing procedure is crucial. Insufficient washing will result to poor precision and falsely elevated OD readings. Without shaking the ODs will be measured about 20% lower with a loss of sensitivity.

DATA PROCESSING

The standard curve is established by plotting the mean OD-values of the calibrators 1 - 4 on the y-axis, against their respective 21-OH-Ab concentrations on the x-axis. In addition, the negative control (C I) should be included (see below).

The 21-OH Ab concentrations of the controls and the unknown samples are read directly in U/ml from the measured OD values.

High concentrations of 21-OH Abs could be measured by reading absorbencies at 405 nm instead of 450 nm.

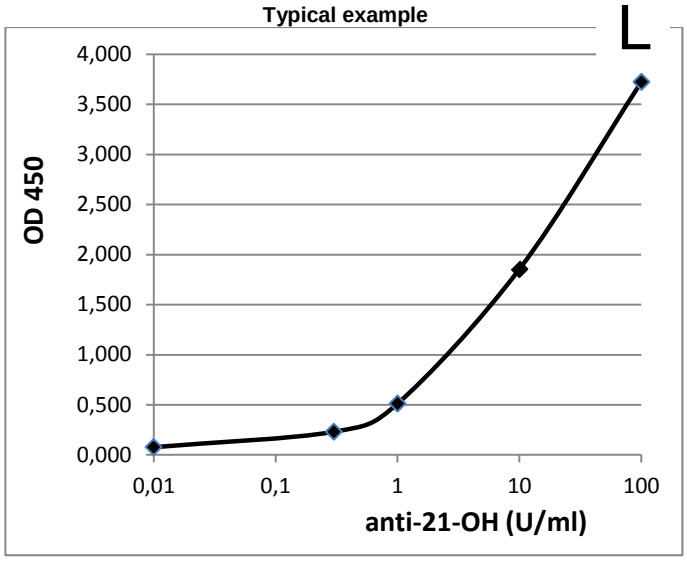
Medizym® anti-21-OH may also be used with computer assisted analysis software, able to produce curves with a spline smoothing fit.

TYPICAL EXAMPLE

Do not use for evaluation!

Sample	OD (a) 450 nm	OD (b) 450 nm	OD (MW)	U / ml
Control CI	0.076	0.078	0.077	**0.01**
Standard 1	0.226	0.237	0.231	**0.3**
Standard 2	0.523	0.501	0.512	**1**
Standard 3	1.872	1.838	1.855	**10**
Standard 4	3.698	3.748	3.723	**100**
Patient 1	0.517	0.547	0.532	1.1

STANDARD CURVE



REFERENCE VALUES

Medizym® anti-21-OH	
Negative	< 0.4 U/ml
Positive	≥ 0.4 U/ml

Healthy individuals should be tested negative with the Medizym® anti-21-OH assay. However, 21-OH antibodies may also be present in apparently healthy persons.

It is recommended that each laboratory establishes its own normal and pathological ranges for anti-21-OH antibody levels, as is usually done for other diagnostic parameters. Therefore, the abovementioned reference values provide only a guide.

CHARACTERISTIC ASSAY DATA

Calibration

Due to the lack of an international reference standard for 21-OH antibodies, Medizym® anti-21-OH is calibrated in arbitrary units (U/ml).

Linearity

Anti-21-OH positive human serum samples diluted with 21-OH Ab-free human serum, measured with the Medizym® anti-21-OH assay, show the theoretically expected values.
On the basic of the heterogeneous nature of the autoantibody population and in view of epitope specificity and affinity of the autoantibodies exceptions are possible in some cases.

Clinical specificity

Sera from 211 individual healthy blood donors were tested in the Medizym® anti-21-OH. 210 (99.5%) sera were identified as being negative for 21-OH Ab. The healthy blood donor serum that was 21-OH Ab positive was also positive in another assay.

Clinical sensitivity

Sera from 63 patients with autoimmune Addison's disease were tested in the Medizym® anti-21-OH. 51 (81%) were identified as being positive for 21-OH antibodies.

Lower Detection Limit

The analytical sensitivity (lower detection limit, negative control +2 standard deviations) has been found at 0.13 U/ml.

Clinical accuracy

Analysis of 185 sera from patients with autoimmune diseases other than Addison's disease indicated no interference from autoantibodies to thyroglobulin, thyroid peroxidase, TSH receptor, glutamic acid decarboxylase, zinc transporter 8, aquaporin-4, voltage gated potassium channel, double stranded DNA, acetylcholine receptor or from rheumatoid factor.

No interference was observed when samples were spiked with the following materials; haemoglobin up to 500 mg/dl, bilirubin up to 20 mg/dl or intralipid up to 3000 mg/dl.

Intra and inter assay variation

Intra-assay			Inter-assay		
sample No.	Mean (U/ml)	CV (%)	sample No.	Mean (U/ml)	CV (%)
1	0.30	2.7	A	0.39	4.1
2	0.89	6.1	B	1.0	7.4
3	2.0	6.3	C	2.7	17.9
4	5.4	18.1	D	10.7	11.5
5	55	9.9	E	58.7	14.4

LIMITATIONS of the METHOD

For research use only. Not for use in diagnostic or clinical procedures.

Medizym® anti-21-OH

Bring all reagents to room temperature. Gently mix all reagents to ensure homogeneity.

ASSAY SCHEME

Step	Activity	Material	CI / CAL	C II, CIII	Patients 1, 2 etc.
1	Pipette	CAL, controls, samples	50 µl	50 µl	50 µl
2	Pipette	Enhancer (K)	50 µl	50 µl	50 µl
3	Cover and incubate	Plate	Shake for 1 min at 500 rpm, incubate at 2 - 8 °C **overnight (16 - 20 h)**		
4	Prepare	Reagents B, D/G, H/J			
5	Wash		Aspirate or decant plate		
	Pipette	Washing solution	3 x 300 µl	3 x 300 µl	3 x 300 µl
6	Pipette	21-OH-Biotin solution (H + J)	100 µl	100 µl	100 µl
7	Incubate	Plate	**1 hour** at room temperature **with shaking (500 rpm)**		
8	Wash		Aspirate or decant plate		
	Pipette	Washing solution	3 x 300 µl	3 x 300 µl	3 x 300 µl
9	Pipette	SA-POD solution (D + G)	100 µl	100 µl	100 µl
10	Cover and incubate	Plate	**20 min** at room temperature **with shaking (500 rpm)**		
11	Wash		Aspirate or decant plate		
	Pipette	Washing solution	3 x 300 µl	3 x 300 µl	3 x 300 µl
12	Pipette	Substrate (E)	100 µl	100 µl	100 µl
13	Incubate	Plate	**20 min** at room temperature **in the dark** without shaking		
14	Pipette and mix	Stop solution (F)	50 µl	50 µl	50 µl
15	Measure OD		at 450 nm against 620 nm (690 nm) within 20 min		

SAFETY PRECAUTIONS

- **This kit is for research use only**. Follow the working instructions carefully. This instruction manual is valid only for the present kit with the given composition. An exchange of single components is not in agreement with CE regulations.
- The expiration dates stated on the respective labels are to be observed. The same relates to the stability stated for reconstituted reagents.
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid time shift during pipetting of reagents.
- All reagents should be kept at 2 - 8 °C before use in the original shipping container.
- Some of the reagents contain small amounts (< 0.1 % w/v) of sodium azide as a preservative. They must not be swallowed or allowed to come into contact with skin or mucosa.
- Source materials derived from human body fluids or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
- Since the kit contains potentially hazardous materials, the following precautions should be observed:
  - Do not smoke, eat or drink while handling kit material,
  - Always use protective gloves,
  - Never pipette material by mouth,
  - Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.
- In any case GLP should be applied with all general and individual regulations to the use of this kit.