DIAGNOSTIKA GMBH

Instructions for Use

21-Hydroxylase Antibody ELISA

Addison's Disease, Autoimmune Polyglandular Syndrome

Enzyme Immuno Assay
for the Quantitative Determination of
Autoantibodies to Steroid 21-Hydroxylase (21-OH)
in Serum

CE IVD

REF	EA112/96
Σ	12 x 8
	2 – 8 °C

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Symbols

IVD	In Vitro Diagnostic Medical Device	CE	EC Declaration of conformity
CONT	Content		Expiry Date
LOT	Lot Number		Store at
	Manufactured by	Σ	Sufficient for ... determinations
REF	Catalogue Number		Consult Instructions for Use

Hazard Pictograms

Warning

1 Introduction and Principle of the Test

The 21-hydroxylase autoantibody (21-OH Ab) ELISA kit is intended for use by professional persons only, for the quantitative determination of 21-OH Ab in human serum. Autoimmune destruction of the adrenal cortex is the most common cause of Addison's disease and autoantibodies to the adrenal specific enzyme steroid 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 syndromes (APS) type I or type II.

In the 21-OH Ab ELISA kit, 21-OH Ab in patients' sera, calibrators and controls are allowed to interact with 21-OH coated onto ELISA plate wells. After a 16 - 20 hour incubation, the samples are discarded leaving 21-OH Ab bound to the 21-OH coated on the wells. 21-OH-Biotin is added in a 2nd incubation step where, through the ability of 21-OH Ab to act divalently, a bridge is formed between the 21-OH immobilised on the plate and 21-OH-Biotin. The amount of 21-OH-Biotin bound is then determined in a 3rd incubation step involving addition of streptavidin peroxidase (SA-POD), which binds specifically to Biotin. Excess, unbound SA-POD is then washed away and addition of the peroxidase substrate 3,3',5,5'-tetramethlybenzidine (TMB) results in formation of a blue colour. This reaction is stopped by the addition of a stop solution, causing the well contents to turn yellow. The absorbance of the yellow reaction mixture at 450nm and 405nm is then read using an ELISA plate reader. A higher absorbance indicates the presence of 21-OH Ab in the test sample. Reading at 405nm allows quantitation of high absorbances. It is recommended that values below 1 U/ml should be measured at 450nm. If it is possible to read at only one wavelength 405nm may be used. The measuring interval is 0.3 – 100 U/ml (arbitrary units).

2 Precautions

- For in vitro use only.
- Some reagents contain sodium azide as preservative (<0.1%). Avoid skin contact.
- All reagents of human origin used in this kit are tested for HIV I/II antibodies, HCV and HBsAg and found to be negative. However, because no test method can offer complete assurance that infectious agents are absent, these reagents should be handled as potentially biohazardous materials.
- Material of animal origin used in the preparation of the kit have been obtained from certified healthy animals but these materials should be handled as potentially infectious.

3 Storage and Stability

On arrival, store the kit at 2-8 °C. Once opened the kit is stable until its expiry date. For stability of prepared reagents refer to Preparation of Reagents.

4 Contents of the Kit

Coated Wells **STRIPS** 12 Strips

8 wells per strip
coated with 21-Hydroxylase

Calibrators A - D **CAL A – CAL D** 4 vials

0.7 ml each, ready for use
Concentrations (arbitrary Units):

Calibrator	A	B	C	D
U/ml	0.3	1	10	100

Positive Controls **CON I & CON II** 2 vials

0.7 ml each, ready for use,
values for the controls are given on the QC certificate

Negative Control **CON -** 1 vial

0.7 ml, ready for use

Reaction Enhancer 6 ml, coloured red, ready for use	**ENHANCER**		1 vial
21-OH Biotin 5.5 ml each, lyoph.	**21-OH-BIOTIN**		3 vials
Reconstitution Buffer 10 ml each, ready for use, for reconstituting 21-OH-Biotin	**RECONST**		2 vials
Streptavidin-Peroxidase 0.7 ml, 20 x concentrated	**SA-POD**		1 vial Warning
Diluent 15 ml, ready for use, for diluting SA-POD	**DIL**		1 vial
Substrate 15 ml, tetramethyl benzidine (TMB), ready for use	**SUB**		1 vial
Wash Buffer 125 ml, 10 x concentrated	**WASH**		1 vial
Stop Solution 12 ml, ready for use	**STOP**		1 vial

Additional materials and equipment required but not provided:

- Pipettes for 50 µl and 100 µl
- ELISA plate cover
- ELISA plate shaker capable of 500 shakes per min (not an orbital shaker)
- Pure water
- Microtiter plate reader (450 nm and 405 nm)

5 Specimen Collection and Storage

Sera to be analysed should be assayed soon after separation or stored (preferably in aliquots) at or below -20 °C.

100 µl is sufficient for one assay. Subsequent freezing and thawing or increase in storage temperature should be avoided. Incorrect storage of serum samples can lead to loss of 21-OH autoantibodies. Do not use grossly haemolysed or lipaemic serum samples. Do not use plasma in the assay.

When required, thaw test sera at room temperature and mix gently to ensure homogeneity. Centrifuge the serum prior to assay (preferably for 5 min at 10-15,000 g in a microfuge) to remove any particulate matter. Please do not omit this centrifugation step if sera are cloudy or contain particulates.

6 Test Procedure

6.1 Preparation of Reagents

MT strips

Before opening the packet of strip wells [STRIPS], allow it to stand at room temperature for 30 minutes. After opening, return any unused wells in the original foil packet (reseal with adhesive tape) and in the self-seal plastic bag with the desiccant provided. Store at 2-8 °C for up to 6 months.

21-OH-Biotin

Immediately before use, reconstitute the contents of one vial [21-OH-BIOTIN] with 5.5 ml Reconstitution Buffer [RECONST]. If more than 1 vial of 21-OH-Biotin is going to be used, pool the contents of each vial after reconstitution and mix gently before use. Discard any unused reagent, storage and subsequent use of the prepared 21-OH-Biotin is not possible.

Streptavidin-Peroxidase

Dilute the concentrate [SA-POD] 1 in 20 with the Diluent [DIL] provided (e.g. 0.5 ml SA-POD + 9.5 ml Diluent). Store at 2-8 °C for up to 16 weeks after dilution.

Wash Buffer

Dilute Wash Buffer [WASH] 1 in 10 with pure water before use. Store at 2-8 °C after dilution up to kit expiry date.

6.2 Assay Procedure

Calculate the number of individual ELISA plate wells needed for the assay. Allow all the reagents supplied, including the appropriate number of packets of strips, to reach room temperature (at least 30 min), remove the number of strip wells required and fit them firmly into the frame provided. Negative and Positive Controls in duplicate must always be included in each assay run.

1. Pipette each 50 µl (in duplicate) of Calibrators A – D [CAL A] – [CAL D], Negative [CON -] and Positive Controls [CON I] & [CON II] and test sera into the appropriate wells [STRIPS]. Leave one well empty for blank.
2. Pipette each 50 µl Reaction Enhancer [ENHANCER] into the appropriate wells. Leave one well empty for blank. Cover the frame and shake on an ELISA plate shaker plate (500 shakes per min) for 1 minute. Incubate over night (16-20 hours) at 2 – 8 °C without shaking.
3. After the overnight incubation aspirate or discard the reagent from the wells, and wash the wells three times with diluted Wash Buffer [WASH]. Tap the inverted wells gently on a clean dry absorbent surface to remove any droplets of Wash Buffer.
4. Pipette 100 µl of reconstituted 21-OH-Biotin [21-OH-BIOTIN] into each well (except blank), cover the plate and incubate for 1 hours at room temperature (20 – 25 °C) with shaking on an ELISA plate shaker (500 shakes per min).
5. After the 1 hour incubation with 21-OH-Biotin, aspirate or discard the reagent from the wells, and wash the wells three times with diluted Wash Buffer [WASH]. Tap the inverted wells gently on a clean dry absorbent surface to remove any droplets of Wash Buffer.
6. Pipette 100 µl of ready-for-use diluted SA-POD [SA-POD] into each well (except blank). Cover the plate and incubate for 20 minutes at room temperature (20–25 °C) with shaking on an ELISA plate shaker (500 shakes per min).
7. Aspirate or discard the reagent from the wells, and wash the wells three times with diluted Wash Buffer [WASH]. Tap the inverted wells gently on a clean dry absorbent surface to remove any droplets of Wash Buffer.

8. Pipette 100 µl of Substrate (TMB) [SUB] into each well (including blank) and incubate for 20 minutes at room temperature in the dark without shaking.
9. Stop the substrate reaction by addition of 50 µl of Stop Solution [STOP] to each well (including blank) and shake the plate for about 5 seconds on a plate shaker to ensure uniformity of the solution in each well.
10. Read the absorbance at 450 nm and at 405 nm within 5 to 10 minutes after addition of Stop Solution using an ELISA plate reader blanked against a well containing 100 µl Substrate plus 50 µl of Stop Solution only.

7 Calculation of Results

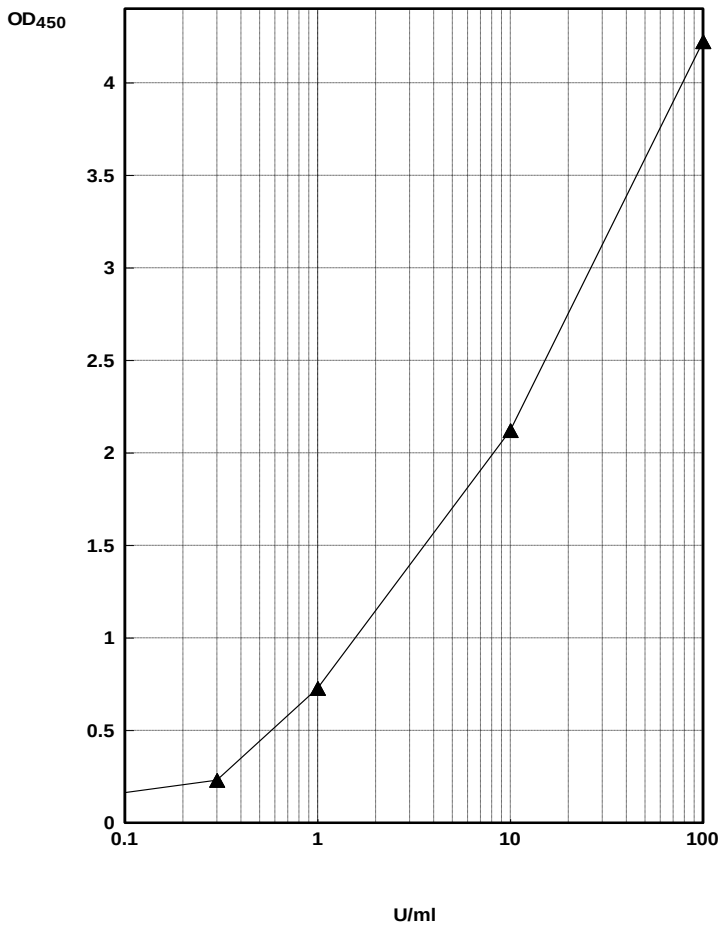
A calibration curve can be established by plotting calibrator concentration on the x-axis (log scale) against the absorbance of the calibrators on the y-axis (linear scale). The 21-OH autoantibody concentrations in patient sera can then be read off the calibration curve plotted as a spline log/lin curve (smoothing factor = 0). Other data reduction systems can be used. The negative control can be assigned a value of 0.03 U/ml to assist in computer processing of assay results.

Samples with 21-OH autoantibody concentrations above 100 U/ml can be diluted (e.g 10 x and/or 100 x) in 21-OH antibody negative serum. Some sera will not dilute in a linear way.

Example

Typical results are shown in the following table.

	OD 450 nm	Conc. U/ml	OD 405 nm	Conc. U/ml
CON -	0.090		0.028	
CAL A	0.231	0.3	0.073	0.3
CAL B	0.728	1	0.232	1
CAL C	2.121	10	0.679	10
CAL D	4.223	100	1.242	100
CON I	0.464	0.57	0.151	0.59
CON II	1.684	5.37	0.541	5.32



8 Clinical Evaluation

8.1 Expected Values

Negative < 0.4 U/ml

Positive ≥ 0.4 U/ml

8.2 Clinical Specificity

Sera from 928 healthy blood donors were tested in the 21-OH Ab ELISA kit. 922 (99.4%) sera were identified as being negative for 21-OH Ab. The remaining 6 (0.6%) of healthy blood donor sera (0.59, 0.93, 1.2, 2.4, >100 and >100 U/ml) were all found to contain IgM antibodies to 21-OH.

8.3 Clinical Sensitivity

Sera from 100 patients diagnosed with autoimmune Addison's disease were tested in the 21-OH Ab ELISA kit. 86(86%) were identified as being positive for 21-OH Ab.

8.4 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. A serum sample from a further patient with Type 1 DM (GAD Ab positive) gave a concentration of 44 U/ml. This sample was assayed in the 21-OH Ab RIA kit and was positive with a 21-OH Ab concentration of 100 U/ml. A serum sample from a further patient with Type 1 DM (ZnT8 Ab positive) gave a concentration of 0.53 U/ml. This sample was assayed in the 21 OH Ab RIA and was negative. A further sample that was ACHRAB positive gave a concentration of 0.61 U/ml.

8.5 Interference

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

8.6 Reproducibility

Intra-Assay Precision (n=25)

sample	mean U/ml	cv (%)
1	0.30	2.7
2	0.89	6.1
3	2.0	6.3
4	5.4	18.1
5	55.0	9.9

Inter-Assay Precision (n=20)

sample	mean U/ml	cv (%)
1	0.39	4.1
2	1.0	7.4
3	2.7	17.9
4	10.7	11.5
5	58.7	14.0

The data quoted in these instructions should be used for guidance only. It is recommended that each laboratory include its own panel of control samples in the assay. Each laboratory should establish its own normal and pathological reference ranges for 21-OH Ab levels.

9 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 polygladualar 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 Patients with Primary Adrenal Insufficiency.
  J. Intern. Med. 2014 275:104-115

10 Changes to declare

IFU has been re-formatted.

In sections 6, 7 and 8 and in the pipetting scheme the names of components as indicated on labels have been included to provide more clarity. Further, the blank was included in the pipetting scheme.

No changes have been made to kit components or execution of protocols.

Pipetting Scheme

		B_0	Negative Control	Calibrators	Positive Controls	Samples
Blank	µl	0				
CON -	µl		50			
CAL A – CAL D	µl			50		
CON I & II	µl				50	
Patient sample	µl					50

ENHANCER	µl	0	50	50	50	50

Shake on an ELISA plate shaker at 500 shakes for 1 minute
Incubate for overnight (16 - 20 hours) at 2 - 8°C without shaking
Aspirate / discard and wash three times with each 300 µl Wash Buffer

21-OH-Biotin	µl	0	100	100	100	100

Incubate for 1 hour at RT on a shaker 500 shakes/min
Aspirate / discard and wash three times with each 300 µl Wash Buffer

SA-POD dil. 1:20.	µl	0	100	100	100	100

Incubate for 20 min at room temperature on a shaker 500 shakes/min
Aspirate / discard and wash three times with each 300 µl Wash Buffer

SUB	µl	100	100	100	100	100

Incubate for 20 minutes at RT in the dark without shaking

STOP	µl	50	50	50	50	50

5 seconds shaking on an ELISA plate shaker
Reading of absorbance at 450 nm and 405 nm within 20 minutes