



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



REF EA112/96

 12 x 8

 ⁺⁸/₋₂ °C 2 – 8 °C

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Symbols

 IVD	In-Vitro-Diagnostic Device		EC Declaration of Conformity
 CONT	Contents		Expiry Date
 LOT	Lot Number		Store
	Manufactured by		Sufficient for
 REF	Catalogue Number		Consult Instructions

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'-tetramethylbenzidine (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

4.1 **Coated Wells** **STRIPS** 12 strips
8 wells per strip
coated with 21-OH

4.2 **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	1.0	10	100

4.3 **Positive Controls** **CONTROL I** + **CONTROL II** 2 vials
0.7 ml each, ready for use
values for the Controls are given on the QC certificate

4.4	Negative Control 0.7 ml, ready for use	CON -	1 vial
4.5	Reaction Enhancer 6 ml coloured red, ready for use	ENHANCER	1 vial
4.6	21-OH-Biotin 5.5 ml each, lyoph.	21-OH-BIOTIN	3 vials
4.7	Reconstitution Buffer 10 ml each, ready for use for reconstituting 21-OH-Biotin	RECONST	2 vials
4.8	Streptavidin-Peroxidase (SA-POD) 0.7 ml, 20 x concentrated	SA-POD	1 vial
4.9	Diluent 15 ml, ready for use for diluting SA-POD	DIL	1 vial
4.10	Substrate 15 ml, tetramethyl benzidine (TMB), ready for use	SUB	1 vial
4.11	Wash Buffer 125 ml, 10 x concentrated	WASH	1 vial
4.12	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

STRIPS

Before opening the packet of strip wells, 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

21-OH-BIOTIN

Immediately before use, reconstitute the contents of one vial with 5.5 ml Reconstitution Buffer. 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 (SA-POD)

SA-POD

Dilute the concentrate 1 in 20 with the Diluent 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

WASH

Dilute 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, Negative and Positive Controls and test sera into the appropriate wells. Leave one well empty for blank.
2. Pipette each 50 μl Reaction 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. 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 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. 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 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. 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) 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 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 100 µl of Stop Solution only.

7. Calculation of Results

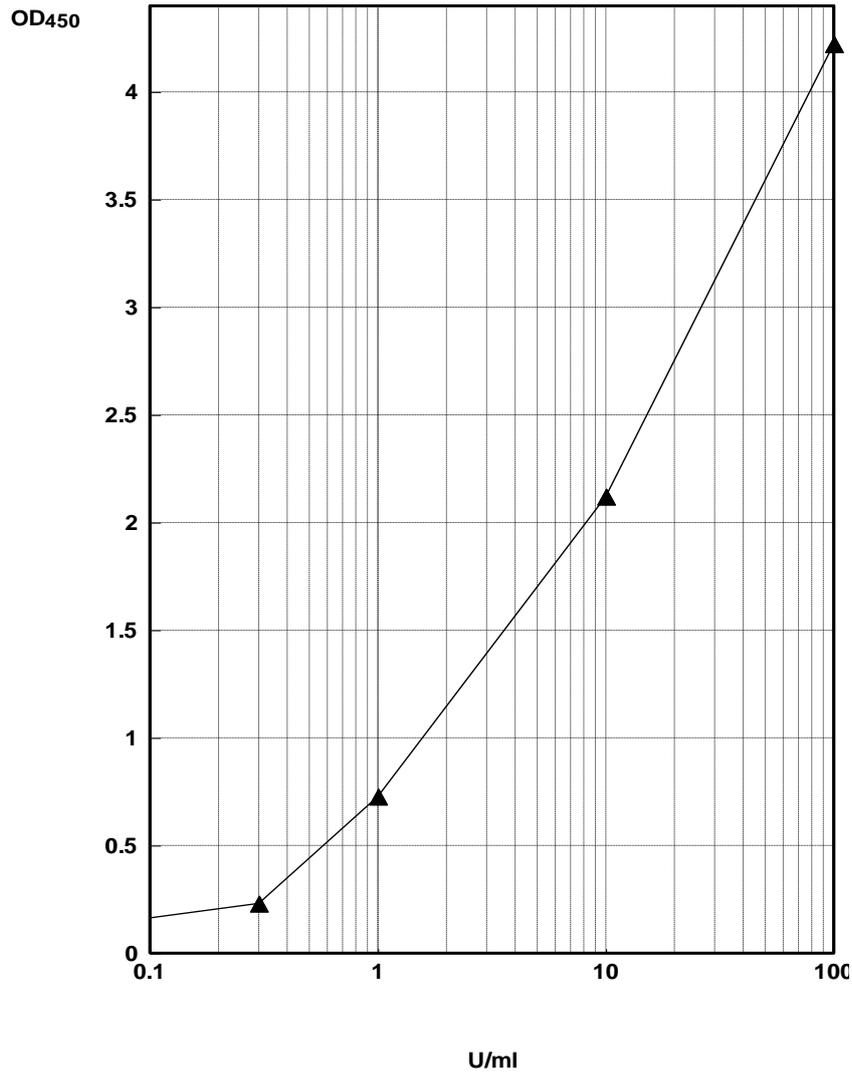
A calibration curve can be established by plotting calibrator concentration on the x-axis (log scale) against the absorbance of the calibrators on the y-axis (linear scale). The 21-OH autoantibody concentrations in patient sera can then be read off the calibration curve plotted as a spline log/lin curve (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.

Typical Example

Typical results are shown in the following table.

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



8. Clinical Evaluation

Expected Values

Negative < 0.4 U/ml
 Positive ≥ 0.4 U/ml

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.

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.

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.

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.

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, J Clin Endocrinol Metab 1995 **80**: 1502-1505 Editorial: Adrenal and gonadal autoimmune diseases

S Chen et al, J Clin Endocrinol Metab 1996 **81**: 1871-1876 Autoantibodies to steroidogenic enzymes in autoimmune polyglandular syndrome, Addison's disease, and premature ovarian failure

H Tanaka et al, J Clin Endocrinol Metab 1997 **82**: 1440-1446 Steroid 21-Hydroxylase autoantibodies: Measurements with a new immunoprecipitation assay

G Coco et al, J Clin Endocrinol Metab 2006 **91**: 1637-1645 Estimated risk for developing autoimmune Addison's disease in patients with adrenal cortex autoantibodies

E. S. Husebye et al, J. Intern. Med. 2014 **275**:104-115
Consensus Statement on the Diagnosis, Treatment and Follow-up of Patients with Primary Adrenal Insufficiency.

Pipetting Scheme

	B ₀	Calibrator A - D	Positive Control I/II	Patient Sample
Negative Control μl	50			
Calibrators μl		50		
Positive Controls μl			50	
Patient Serum μl				50

React. Enhancer μl	50	50	50	50
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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	100	100	100	100
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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	100	100	100	100
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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

TMB-Substrate μl	100	100	100	100
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Incubate for 20 minutes at RT in the dark without shaking

Stop Solution μl	50	50	50	50
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5 sec shaking on an ELISA plate shaker

Reading of absorbance at 450 nm and 405 nm within 20 minutes