RSR

ElisaRSR™ 21-OH Ab

21-Hydroxylase (21-OH) Autoantibody ELISA Kit - Instructions for use

CE

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INTENDED USE

The RSR 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.

REFERENCES

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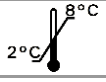
ASSAY PRINCIPLE

In RSR's 21-OH Ab ELISA kit, 21-OH Ab in patients' sera, reference preparation or calibrators (optional) 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 low absorbance values are measured at 450nm. If it is possible to read at only one wavelength 405nm may be used.

STORAGE AND PREPARATION OF TEST SERUM SAMPLES

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 (duplicate 50 µL determinations). Repeated freeze thawing or increases in storage temperature should be avoided. Do not use lipaemic or haemolysed serum samples. Do not use plasma in the assay. When required, bring test sera to room temperature and mix gently to ensure homogeneity. Centrifuge serum prior to assay (preferably for 5 min at about 10,000 rpm i.e. about 10,000 g in a microfuge) to remove particulate matter. Please do not omit this centrifugation step if sera are cloudy or contain particulates.

SYMBOLS

Symbol	Meaning
CE	EC Declaration of Conformity
IVD	In Vitro Diagnostic Device
REF	Catalogue Number
LOT	Lot Number
	Consult Instructions
	Manufactured by
	Sufficient for
	Expiry Date
	Store
CONTROL -	Negative Control
CONTROL +	Positive Control

MATERIALS REQUIRED AND NOT SUPPLIED

Pipettes capable of dispensing 50 µL and 100 µL.
Means of measuring various volumes to reconstitute or dilute reagents supplied.
Pure water.
ELISA Plate reader suitable for 96 well formats and capable of measuring at 450nm and 405nm.
ELISA Plate shaker, capable of 500 shakes/min (not an orbital shaker).
ELISA Plate cover.
ELISA Plate washing machine.

PREPARATION OF REAGENTS SUPPLIED

Store unopened kit and all kit components (A-N) at 2-8°C.

A	**21-OH Coated Wells** 12 breakapart strips of 8 wells (96 in total) in a frame and sealed in a foil bag. Allow foil bag to stand at room temperature (20-25°C) for 30 minutes before opening.
	Ensure wells are firmly fitted in the frame provided. After opening return any unused wells to the original foil bag with desiccant provided and seal with adhesive tape. Place foil bag in the self-seal plastic bag provided and store at 2-8°C for up to 6 months.
B	**Negative Control** 0.7 mL Ready for use
C1-2	**Positive Controls I & II** 2 x 0.7 mL Ready for use
D	**Reference Preparation** 0.7 mL Ready for use
E1-4	**Calibrators (optional)** 0.3, 1.0, 10, 100 u/mL (arbitrary RSR units) 4 x 0.7 mL Ready for use
F	**Reaction Enhancer** 6 mL, coloured red Ready for use
G	**21-OH–Biotin** 3 vials Lyophilised
	Reconstitute with room temperature reconstitution buffer (H) immediately before use (within 30 minutes), 5.5 mL per vial. When more than one vial is to be used, pool the vials and mix gently.
H	**Reconstitution Buffer for 21-OH-Biotin** 2 x 10 mL Ready for use
J	**Streptavidin Peroxidase (SA-POD)** 0.7 mL Concentrated
	Dilute 1 in 20 with diluent for SA-POD (K). For example, 0.5 mL (J) + 9.5 mL (K). Store for up to 16 weeks at 2-8°C after dilution.
K	**Diluent for SA-POD** 15 mL Ready for use
L	**Peroxidase Substrate (TMB)** 15 mL Ready for use
M	**Stop Solution** 12 mL Ready for use
N	**Concentrated Wash Solution** 125 mL Concentrated
	Dilute 1 in 10 with pure water before use. Store at 2-8°C up to kit expiry date.

ASSAY PROCEDURE

On day 1 allow all the reagents required for steps 1-3 to stand at room temperature (20-25°C) for at least 30 minutes prior to use.
On day 2 allow all the reagents required for steps 4-13 (except for the coated wells) to stand at room temperature (20-25°C) for at least 30 minutes prior to use. The coated wells from day 1 must remain at 2–8°C until ready to proceed with step 4 below. Do not reconstitute 21-OH-Biotin until step 5 below. A repeating Eppendorf type pipette is recommended for steps 2, 5, 8, 11, and 12.

Day 1	**1.**	Pipette **50 µL** (in duplicate) of patient sera, negative control (B), positive controls (C1–2), reference preparation (D) and (if used) calibrators (E1-4) into respective wells (A). Leave one well empty for blank (see step 13).
	2.	Pipette **50 µL** reaction enhancer (F) into each well (except blank).
	3.	Cover the frame and shake the wells on an ELISA plate shaker (500 shakes per min) for 1 minute. Incubate overnight (16-20 hours) at 2-8°C without shaking.
Day 2	**4.**	Aspirate and wash/aspirate the wells three times with diluted wash solution (N) by use of an ELISA plate washing machine.
	5.	Reconstitute 21-OH-Biotin (G) using reconstitution buffer (H) which has reached room temperature. Pipette **100 µL** into each well (except blank).
	6.	Cover the frame and shake the wells for 1 hour at room temperature on an ELISA plate shaker (500 shakes per min).
	7.	Repeat wash step 4.
	8.	Pipette **100 µL** of diluted SA-POD (J) into each well (except blank).
	9.	Cover the frame and shake the wells for 20 minutes at room temperature on an ELISA plate shaker (500 shakes per min).
	10.	Repeat wash step 4.
	11.	Pipette **100 µL** of TMB (L) into each well (including blank) and incubate for 20 minutes in the dark at room temperature without shaking.
	12.	Pipette **50 µL** of stop solution (M) into each well (including blank), cover the frame and shake for approximately 5 seconds on an ELISA plate shaker. Ensure substrate incubations are the same for each well.

	13.	Within 20 minutes, read the absorbance of each well at 450nm and 405nm using an ELISA plate reader, blanked against the well containing **100 µL** of TMB (L) and **50 µL** stop solution (M) **only**.

RESULT ANALYSIS

Calculation of results without calibrators

Index Calculation

The index values are calculated as follows:

$$\text{Index} = \frac{\text{test sample absorbance at 450nm}}{\text{reference preparation absorbance at 450nm}} \times 100$$

The index value can also be calculated using absorbance data at 405nm.

TYPICAL RESULTS (Example only; not to be used for calculation of actual results)

	A450 nm	Index value	A405 nm	Index value
Reference Preparation (D)	0.728	100	0.232	100
Negative Control (B)	0.090	12	0.028	12
Positive Control (C1)	0.464	64	0.151	65
Positive Control (C2)	1.684	231	0.541	233

ASSAY CUT OFF

Negative	< 45
Positive	≥ 45

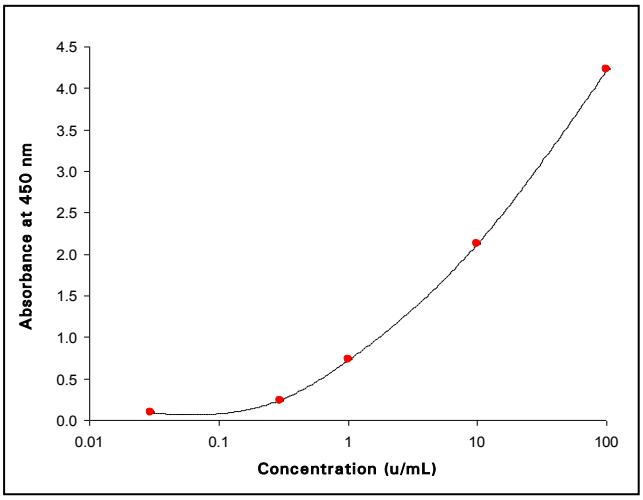
Calculation of results with calibrators (optional)

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 Ab concentrations in patients' sera can then be read off the calibration curve [plotted at RSR as a spline log/lin curve (smoothing factor = 0)]. Other data reduction systems can be used. The negative control (B) can be assigned a value of 0.03 u/mL to assist in computer processing of assay results.

TYPICAL RESULTS (Example only; not for calculation of actual results)

	A450 nm	Conc. u/mL	A405 nm	Conc. u/mL
Negative Control (B)	0.090		0.028	
E1	0.231	0.3	0.073	0.3
E2	0.728	1	0.232	1
E3	2.121	10	0.679	10
E4	4.223	100	1.242	100
Positive Control (C1)	0.464	0.57	0.151	0.59
Positive Control (C2)	1.684	5.37	0.541	5.32

For absorbance readings at 450nm above 3.0, the absorbance reading at 405nm can be converted to 450nm absorbance values by multiplying by the appropriate factor (3.4 in the case of equipment used at RSR).



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

ASSAY CUT OFF

Negative	< 0.4 u/mL
Positive	≥ 0.4 u/mL

This cut off and the cut off based on index value (see above) has been validated at RSR. However each laboratory should establish its own normal and pathological reference ranges for 21-OH Ab levels. Also it is recommended that each laboratory include its own panel of control samples in the assay.

CLINICAL EVALUATION

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.

Lower Detection Limit

The negative control was assayed 20 times and the mean and standard deviation calculated. The lower detection limit at +2 standard deviations was 0.13 u/mL, the index value was 12.

Intra Assay Precision

Sample	Mean u/mL (n = 25)	CV (%)	Mean index (n = 25)	CV (%)
1	0.30	2.7	39	2.2
2	0.89	6.1	92	4.8
3	2.0	6.3	154	3.5
4	5.4	18.1	249	7.3
5	55	9.9	512	2.3

Inter Assay Precision

Sample	Mean u/mL (n = 20)	CV (%)	Mean index (n = 20)	CV (%)
A	0.39	4.1	43	4.9
B	1.0	7.4	102	4.6
C	2.7	17.9	164	8.9
D	10.7	11.5	284	6.2
E	58.7	14.0	500	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 (GADAb positive) gave an index value of 409 and a concentration of 44 u/mL. This sample was assayed in RSR's 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 an index value of 60 and 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 an index value of 68 and 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.

SAFETY CONSIDERATIONS

Streptavidin Peroxidase (SA-POD) and Reaction Enhancer

Signal word: Warning

Hazard statement(s)

H317: May cause an allergic skin reaction

Precautionary statement(s)

P261: Avoid breathing mist, vapours

P272: Contaminated work clothing should not be allowed out of the workplace

P280: Wear protective gloves/protective clothing/ eye protection/face protection

P302 + P352: IF ON SKIN: Wash with plenty of soap and water

P333 + P313: If skin irritation or rash occurs: Get medical advice/attention

P362 + P364: Take off contaminated clothing and wash it before reuse

P501: Dispose of contents/container to hazardous or special waste collection point, in accordance with local, regional, national and/or international regulation

Peroxidase Substrate (TMB)

Signal word: Danger

Hazard statement(s)

H360D: May damage the unborn child

Precautionary statement(s)

P202: Do not handle until all safety precautions have been read and understood

P280: Wear protective gloves/protective clothing/ eye protection/face protection

P308 + P313: IF exposed or concerned: Get medical advice/attention

P501: Dispose of contents/container to hazardous or special waste collection point, in accordance with local, regional, national and/or international regulation

This kit is intended for use by professional persons only. Follow the instructions carefully. Observe expiry dates stated on the labels and the specified shelf life for coated wells, diluted and reconstituted reagents. Refer to the Safety Data Sheet for more detailed safety information. Avoid all actions likely to lead to ingestion. Avoid contact with skin and clothing. Wear protective clothing. Material of human origin used in the preparation of the kit has been tested and found non-reactive for HIV1 and 2 and HCV antibodies and HBsAg but should, none-the-less, be handled as potentially infectious. Wash hands thoroughly if contamination has occurred and before leaving the laboratory. Sterilise all potentially contaminated waste, including test specimens before disposal. Material of animal origin used in the preparation of the kit has been obtained from animals certified as healthy but these materials should be handled as potentially infectious. Some components contain small quantities of sodium azide as preservative. As with all kit components, avoid ingestion, inhalation, injection and contact with skin, eyes and clothing. Avoid formation of heavy metal azides in the drainage system by flushing any kit component away with copious amounts of water.

ASSAY PLAN

	Allow all reagents and samples to reach room temperature (20 – 25°C) before use	
Day 1	Pipette:	**50 µL** negative and positive controls (B and C1-2), reference preparation (D) or calibrators (if used E1-4) and patient sera (except blank)
	Pipette:	**50 µL** reaction enhancer (F) (except blank)
	Mix:	Shake on an ELISA plate shaker at 500 shakes/min for 1 minute
	Incubate:	Overnight (16 – 20 hours) at 2 – 8°C without shaking
Day 2	Aspirate/Wash:	ELISA plate (A) three times
	Pipette:	**100 µL** 21-OH-Biotin (G) reconstituted with room temperature reconstitution buffer (H) into each well (except blank)
	Incubate:	1 hour at room temperature on an ELISA plate shaker at 500 shakes/min
	Aspirate/Wash:	ELISA plate (A) three times
	Pipette:	**100 µL** SA-POD (J) (diluted 1:20) into each well (except blank)
	Incubate:	20 minutes at room temperature on an ELISA plate shaker at 500 shakes/min
	Aspirate/Wash:	ELISA plate (A) three times
	Pipette:	**100 µL** TMB (L) into each well (including blank)
	Incubate:	20 minutes at room temperature **in the dark (without shaking)**
	Pipette:	**50 µL** stop solution (M) into each well (including blank) and shake for 5 seconds
	Read absorbance at 450nm and 405nm within 20 minutes of adding stop solution	