ElisaRSR™ 21-OH Ab

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

FOR RESEARCH USE ONLY

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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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Editorial: Adrenal and Gonadal Autoimmune Diseases
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S. Chen et al
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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

ASSAY PRINCIPLE

In RSR's 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 RSR units).

STORAGE AND PREPARATION OF SERUM SAMPLES

Sera to be analysed should be assayed soon after separation or stored, preferably in aliquots, at or below –20ºC. 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, thaw test sera at room temperature and mix gently to ensure homogeneity. Centrifuge serum prior to assay (preferably for 5 min at 10-15,000 g in a microfuge) to remove particulate matter. Please do not omit this centrifugation step if sera are cloudy or contain particulates.

IFU SYMBOLS

Symbol	Meaning
RUO	For Research Use Only
REF	Catalogue Number
LOT	Lot Number
	Consult Instructions
	Manufactured by
	Sufficient for
	Expiry Date
	Store

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 at 2-8°C.

A	**21-OH Coated Wells** 12 breakapart strips of 8 wells (96 in total) in a frame and sealed in 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 14 days.
B1-4	**Calibrators** 0.3, 1.0, 10, 100 u/mL (arbitrary RSR units) 4 x 0.7 mL Ready for use
C1-2	**Positive Controls I & II** **(see label for concentration range)** 2 x 0.7 mL Ready for use
D	**Negative Control** 0.7 mL Ready for use
E	**Reaction Enhancer** 6 mL, coloured red Ready for use
F	**21-OH–Biotin** 3 vials Lyophilised
	Immediately before use, reconstitute with reconstitution buffer for 21-OH-Biotin (G), 5.5 mL per vial. When more than one vial is to be used, pool the vials and mix gently.
G	**Reconstitution Buffer for 21-OH-Biotin** 2 x 15 mL Ready for use
H	**Streptavidin Peroxidase (SA-POD)** 0.7 mL Concentrated
	Dilute 1 in 20 with diluent for diluting SA-POD (I). For example, 0.5 mL (H) + 9.5 mL (I). Store for up to 16 weeks at 2-8°C after dilution.
I	**Diluent for SA-POD** 15 mL Ready for use
J	**Peroxidase Substrate (TMB)** 15 mL Ready for use
K	**Stop Solution** 12 mL Ready for use
L	**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

Allow all reagents to stand at room temperature (20-25°C) for at least 30 minutes prior to use. Do not reconstitute 21-OH-Biotin until step 5 below. An Eppendorf type repeating pipette is recommended for steps 2, 5, 8, 11, and 12.

Day 1	**1.**	Pipette **50 μL** (in duplicate) of patient sera, calibrators (B1-4) and controls (C1-2 and D) into respective wells. Leave one well empty for blank (see step 13).
	2.	Pipette **50 μL** reaction enhancer (E) 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 (L) by use of a plate washing machine.
	5.	Reconstitute 21-OH-Biotin (F) and 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.
Day 2 continued	**8.**	Pipette **100 μL** of diluted SA-POD (H) 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 (J) into each well (including blank) and incubate for 20 minutes in the dark at room temperature without shaking.
	12.	Pipette **50 μL** stop solution (K) to 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.	Read the absorbance of each well at 450nm and 405nm (within 5 minutes of completing step 12) using an ELISA plate reader, blanked against the well containing **100 μL** of TMB (J) and **50 μL** stop solution (K) **only**.

RESULT ANALYSIS

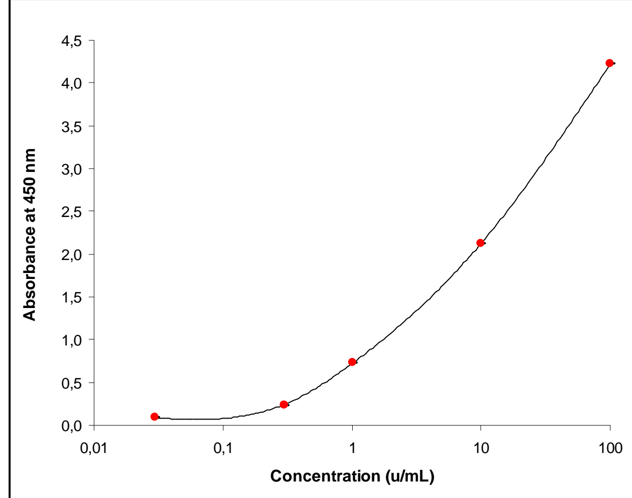
A calibration curve can be established by plotting calibrator concentration on the x-axis (log scale) against the absorbance of the calibrators on the y-axis (linear scale). The 21-OH Ab concentrations in patients' sera can then be read off the calibration curve [plotted at RSR as a spline log/lin curve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

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.

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

	A450 nm	Conc. u/mL	A405 nm	Conc. u/mL
Negative Control (D)	0.090		0.028	
B1	0.231	0.3	0.073	0.3
B2	0.728	1	0.232	1
B3	2.121	10	0.679	10
B4	4.223	100	1.242	100
Positive Control (CI)	0.464	0.57	0.151	0.59
Positive Control (CII)	1.684	5.37	0.541	5.32

Absorbance readings at 405nm can be converted to 450nm absorbances by multiplying by the appropriate factor (3.4 in the case of equipment used at RSR).



ASSAY CUT OFF

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

CLINICAL EVALUATION

Clinical Specificity

Sera from 211 individual healthy blood donors were tested in the RSR 21-OH Ab ELISA kit. 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 the RSR 21-OH Ab RIA when anti-human IgM-agarose was used instead of Protein A.

Clinical Sensitivity

Sera from 63 patients with autoimmune Addison's disease were tested in the 21-OH Ab ELISA.

51 (81%) were identified as being positive for 21-OH Ab.

Clinical Accuracy

Analysis of 162 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, acetylcholine receptor or from rheumatoid factor. A serum sample from a further patient with Type 1 DM (GAD Ab positive) gave a value of 44 u/mL. This sample was assayed in the RSR's 21-OH Ab RIA kit and was positive with a 21-OH Ab concentration of 100 u/mL

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.

SAFETY CONSIDERATIONS

This kit is intended for use by professional persons only. Follow the instructions carefully. Observe expiry dates stated on the labels and the specified stability for coated wells, diluted and reconstituted reagents. Refer to 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. 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

Day 1	Allow all reagents and samples to reach room temperature (20 – 25°C) before use	
	Pipette:	**50 µL** Calibrators, controls and patient sera
	Pipette:	**50 µL** Reaction enhancer (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:	Plate three times
	Pipette:	**100 µL** 21-OH-Biotin (reconstituted) into each well (except blank)
	Incubate:	1 Hour at room temperature on an ELISA plate shaker at 500 shakes/min
	Aspirate/Wash:	Plate three times
	Pipette:	**100 µL** SA-POD (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:	Plate three times
	Pipette:	**100 µL** TMB into each well (including blank)
	Incubate:	20 Minutes at room temperature **in the dark** without shaking
	Pipette:	**50 µL** Stop solution into each well (including blank) and shake for 5 seconds
	Read absorbance at 450nm and 405nm promptly (within 5 minutes)	