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

12 x 8

2 – 8 °C

DLD Gesellschaft für Diagnostika und medizinische Geräte mbH
Adlerhorst 15 • 22459 Hamburg • Germany • Tel.: +49 40 5558710 • Fax: +49 40 55587111
Internet: http://www.dld-diagnostika.de • e-mail: contact@dld-diagnostika.de
Contents

1. Introduction and Principle of the Test  Page  3
2. Precautions  Page  4
3. Storage and Stability  Page  4
4. Contents of the Kit  Page  4
5. Specimen Collection and Storage  Page  6
6. Test Procedure  Page  6
7. Calculation of Results, Typical Example  Page  8
8. Clinical Evaluation  Page  9
9. Literature  Page  11
   Pipetting Scheme  Page  12

Symbols

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tr>
<td>IVD</td>
<td>In-Vitro-Diagnostic Device</td>
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<td>CONT</td>
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<td>Lot Number</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>Store</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td>Consult Instructions</td>
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</table>
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**

   8 wells per strip coated with 21-OH

   **STRIPS**

   12 strips

4.2 **Calibrators A – D**

   0.7 ml each, ready for use

   **CAL A – CAL D**

   4 vials

   Concentrations (arbitrary Units):

<table>
<thead>
<tr>
<th>Calibrator</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>U/ml</td>
<td>0.3</td>
<td>1.0</td>
<td>10</td>
<td>100</td>
</tr>
</tbody>
</table>

4.3 **Positive Controls**

   0.7 ml each, ready for use

   **CONTROL I + CONTROL II**

   2 vials

   values for the Controls are given on the QC certificate
4.4 **Negative Control**  
**CON**-  
0.7 ml, ready for use

4.5 **Reaction Enhancer**  
**ENHANCER**  
6 ml coloured red, ready for use

4.6 **21-OH-Biotin**  
**21-OH-BIOTIN**  
5.5 ml each, lyoph.

4.7 **Reconstitution Buffer**  
**RECONST**  
10 ml each, ready for use  
for reconstituting 21-OH-Biotin

4.8 **Streptavidin-Peroxidase (SA-POD)**  
**SA-POD**  
0.7 ml, 20 x concentrated

4.9 **Diluent**  
**DIL**  
15 ml, ready for use  
for diluting SA-POD

4.10 **Substrate**  
**SUB**  
15 ml, tetramethyl benzidine (TMB), ready for use

4.11 **Wash Buffer**  
**WASH**  
125 ml, 10 x concentrated

4.12 **Stop Solution**  
**STOP**  
12 ml, ready for use

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, 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 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)

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

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.

<table>
<thead>
<tr>
<th></th>
<th>OD 450 nm</th>
<th>Conc. U/ml</th>
<th>OD 450 nm</th>
<th>Conc. U/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neg. Control</td>
<td>0.090</td>
<td>0.028</td>
<td>0.073</td>
<td>0.3</td>
</tr>
<tr>
<td>Calibrator A</td>
<td>0.231</td>
<td>0.3</td>
<td>0.232</td>
<td>1</td>
</tr>
<tr>
<td>Calibrator B</td>
<td>0.728</td>
<td>1</td>
<td>0.679</td>
<td>10</td>
</tr>
<tr>
<td>Calibrator C</td>
<td>2.121</td>
<td>10</td>
<td>1.242</td>
<td>100</td>
</tr>
<tr>
<td>Calibrator D</td>
<td>4.223</td>
<td>100</td>
<td>0.151</td>
<td>0.59</td>
</tr>
<tr>
<td>Control I</td>
<td>0.464</td>
<td>0.57</td>
<td>0.541</td>
<td>5.32</td>
</tr>
<tr>
<td>Control II</td>
<td>1.684</td>
<td>5.37</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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)

<table>
<thead>
<tr>
<th>sample</th>
<th>mean U/ml</th>
<th>cv (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.30</td>
<td>2.7</td>
</tr>
<tr>
<td>2</td>
<td>0.89</td>
<td>6.1</td>
</tr>
<tr>
<td>3</td>
<td>2.0</td>
<td>6.3</td>
</tr>
<tr>
<td>4</td>
<td>5.4</td>
<td>18.1</td>
</tr>
<tr>
<td>5</td>
<td>55.0</td>
<td>9.9</td>
</tr>
</tbody>
</table>

Inter-Assay Precision (n=20)

<table>
<thead>
<tr>
<th>sample</th>
<th>mean U/ml</th>
<th>cv (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.39</td>
<td>4.1</td>
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<tr>
<td>2</td>
<td>1.0</td>
<td>7.4</td>
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<td>3</td>
<td>2.7</td>
<td>17.9</td>
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<tr>
<td>4</td>
<td>10.7</td>
<td>11.5</td>
</tr>
<tr>
<td>5</td>
<td>58.7</td>
<td>14.0</td>
</tr>
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</table>
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


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


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

# Pipetting Scheme

<table>
<thead>
<tr>
<th></th>
<th>B₀</th>
<th>Calibrator A - D</th>
<th>Positive Control I/II</th>
<th>Patient Sample</th>
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<tbody>
<tr>
<td>Negative Control</td>
<td>µl</td>
<td>50</td>
<td></td>
<td></td>
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<tr>
<td>Calibrators</td>
<td>µl</td>
<td>50</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Positive Controls</td>
<td>µl</td>
<td></td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Patient Serum</td>
<td>µl</td>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>React. Enhancer</td>
<td>µl</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

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 |

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 |

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 |

Incubate for 20 minutes at RT in the dark without shaking

| Stop Solution | µl | 50 | 50 | 50 | 50 |

5 sec shaking on an ELISA plate shaker

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