ElisaRSR™ GADAb
Glutamic Acid Decarboxylase (GAD) Autoantibody ELISA kit from RSR – Instructions for use
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INTENDED USE
The RSR GAD65 autoantibody (GADAb) ELISA kit is intended for use by professional persons only, for the quantitative determination of GADAb in human serum. Autoantibodies to pancreatic beta cell antigens are important serological markers of type 1 diabetes mellitus (type 1 DM). The antigens recognised by these antibodies include insulin, glutamic acid decarboxylase (GAD65 kDa isoform), the islet cell antigen IA-2 or ICA-512 and zinc transporter 8 (ZnT8).

REFERENCES
H. Brookin et al
A Sensitive non-isotopic assay for GAD65 autoantibodies
Clinica Chimica Acta 2003 331:55-59
S. Chen et al
Sensitive non-isotopic assays for autoantibodies to IA2 and to a combination of both IA2 and GAD65.
Clinica Chimica Acta 2005 357:74-83
E. Nilson et al
Calcium addition to EDTA plasma eliminates falsely positive results in the RSR GADAb ELISA.
Clinica Chimica Acta 388 (2008) 130-134
K. Rahmati et al
A Comparison of Serum and EDTA Plasma in the Measurement of Glutamic Acid Decarboxylase Autoantibodies (GADA) and Autoantibodies to Islet Antigen-2 (IA-2A) Using the RSR Radioimmunoassay (RIA) and Enzyme Linked Immunosorbent Assay (ELISA) Kits.
Clin. Lab. 2008 54:227-235
C. Törn et al
Diabetes Antibody Standardization Program: evaluation of assays for autoantibodies to glutamic acid decarboxylase and islet antigen-2.
Diabetologia 2008 51:846-852

PATENTS
The following patents apply:

ASSAY PRINCIPLE
In RSR’s GADAb ELISA, GADAb in patients’ sera, calibrators and controls are allowed to interact with GAD65 coated onto ELISA plate wells. After a 1 hour incubation, the samples are discarded leaving GADAb bound to the immobilised GAD65 on the plate. GAD65-Biotin is added in a 2nd incubation step where, through the ability of GADAb in the samples to act divally, a bridge is formed between GAD65 immobilised on the plate and GAD65-Biotin. The amount of GAD65-Biotin bound is then determined in a 3rd incubation step by addition of Streptavidin Peroxidase, which binds specifically to Biotin. Excess, unbound Streptavidin Peroxidase is then washed away and addition of 3,3’,5,5’–tetramethylbenzidine (TMB) results in formation of a blue colour. This reaction is stopped by addition of stop solution causing the well contents to turn yellow. The absorbance of the yellow reaction mixture at 450 nm and 405 nm is then read using an ELISA plate reader. A higher absorbance indicates the presence of GADAb in the test sample. Reading at 405 nm allows quantitation of high absorbances (and should be used for concentrations of 200 u/mL or more). Low values (less than 10 u/mL) should be read off the 450 nm calibration curve. If it is possible to read at only one wavelength 405nm may be used. The measuring interval is 5 – 2000 u/mL (units are NIBSC 97/550).

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. 50 µL is sufficient for one assay (duplicate 25 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 10-15,000 rpm in a microfuge) to remove particulate matter. Please do not omit this centrifugation step if sera are cloudy or contain particulates.

SYMBOLS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC</td>
<td>EC Declaration of Conformity</td>
</tr>
<tr>
<td>IVD</td>
<td>In Vitro Diagnostic Device</td>
</tr>
<tr>
<td>REF</td>
<td>Catalogue Number</td>
</tr>
<tr>
<td>LOT</td>
<td>Lot Number</td>
</tr>
<tr>
<td>ⓦ</td>
<td>Consult Instructions</td>
</tr>
<tr>
<td>Manufactured By</td>
<td></td>
</tr>
</tbody>
</table>

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Sufficient for

Expiry Date

Store

CONTROL - Negative Control

CONTROL + Positive Control

MATERIALS REQUIRED AND NOT SUPPLIED
Pipettes capable of dispensing 25 μL and 100 μL.
Means of measuring out various volumes to reconstitute or dilute reagents.
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 capable of measuring at 450nm and 405nm.

PREPARATION OF REAGENTS SUPPLIED
Store unopened kit and components at 2-8°C

<p>| | |</p>
<table>
<thead>
<tr>
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</thead>
</table>
| **A** | **B1-6** Calibrators  
5, 18, 35, 120, 250, 2000 u/mL  
(units are NIBSC 97/550)  
6 x 0.7 mL  
Ready to use |
| **A** | **C** Positive Control  
(see label for concentration range)  
0.7 mL  
Ready to use |
| **A** | **D** Negative Control  
0.7 mL  
Ready to use |
| **A** | **E** GADαs-Biotin  
3 vials  
Lyophilised  
Reconstitute each vial with 5.5 mL GAD Biotin reconstitution buffer (F). When more than one vial is used, pool the vials and mix gently before use. Store at 2 – 8°C for up to 3 days after reconstitution. |
| **A** | **F** Reconstitution Buffer for GADαs-Biotin  
2 x 15 mL, coloured red  
Ready to use |
| **G** | Streptavidin Peroxidase (SA-POD)  
1 x 0.7 mL  
Concentrated  
Dilute 1 in 20 with diluent for diluting SA-POD (H). For example, 0.5 mL (G) + 9.5 mL (H). Store at 2 – 8°C for up to 16 weeks after dilution. |
| **H** | Diluent for SA-POD  
15 mL  
Ready to use |
| **I** | Peroxidase Substrate (TMB)  
15 mL  
Ready to use |
| **J** | Concentrated Wash Solution  
125 mL  
Concentrated  
Dilute 10 X with pure water before use. Store at 2 – 8°C up to kit expiry date. |
| **K** | Stop Solution  
12 mL  
Ready to use |

ASSAY PROCEDURE
Allow all reagents to stand at room temperature (20-25°C) for at least 30 minutes before use. A repeating Eppendorf type pipette is recommended for steps 4, 7, 10 and 11.

1. Pipette 25 μL of patient sera, calibrators (B1-6) and controls (C and D) into respective wells, in duplicate, leaving one well empty for blank (see step 12).
2. Cover the frame and shake the wells for 1 hour at room temperature on an ELISA plate shaker (500 shakes per min.).
3. Use an ELISA plate washer to aspirate and wash the wells three times with diluted wash solution (J). If a plate washer is not available, discard the well contents by briskly inverting the frame of wells over a suitable receptacle, wash three times manually and finally tap the inverted wells gently on a clean dry absorbent surface.
4. Pipette 100 μL of reconstituted GADαs-Biotin (E) into each well (except blank). Avoid splashing the material out of the wells during addition.
5. Cover the frame, and incubate at room temperature for 1 hour on an ELISA plate shaker (500 shakes per min).
6. Repeat wash step 3.
7. Pipette 100 μL of diluted SA-POD (G) into each well (except blank).
8. Cover the frame and incubate at room temperature for 20 minutes on an ELISA plate shaker (500 shakes per min).
9. Repeat wash step 3. If manual washing is being carried out use one additional wash step with pure water (to remove any foam) before finally tapping the inverted wells dry.
10. Pipette 100 µL of TMB (I) into each well (including blank) and incubate in the dark at room temperature for 20 minutes without shaking.

11. Pipette 100 µL stop solution (K) to each well (including blank) cover the frame and shake for approximately 5 seconds on a plate shaker. Ensure substrate incubations are the same for each well.

12. Within 15 minutes, read the absorbance of each well at 450nm and 405 nm using an ELISA plate reader, blanked against the well containing 100 µL of TMB (I) and 100 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 GADAb 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 0.5 u/mL to assist in computer processing of assay results. Most test sera will have values below 250 u/mL and the 2000 u/mL calibrator need not always be included. Samples with high Ab concentrations can be diluted in GADAb negative serum or the kit negative control (D). For example, 20 µL of sample plus 180 µL of diluent to give a 10x dilution. Other dilutions (e.g. 100x) can be prepared from a 10x dilution or otherwise as appropriate. Some sera will not dilute in a linear way according to the kit calibrators (standardised against NIBSC 97/550).

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

<table>
<thead>
<tr>
<th>Calibrator</th>
<th>A450 nm</th>
<th>Conc. u/mL</th>
<th>A405 nm</th>
<th>Conc. u/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>0.199</td>
<td>5</td>
<td>0.061</td>
<td>5</td>
</tr>
<tr>
<td>B2</td>
<td>0.527</td>
<td>18</td>
<td>0.164</td>
<td>18</td>
</tr>
<tr>
<td>B3</td>
<td>0.975</td>
<td>35</td>
<td>0.301</td>
<td>35</td>
</tr>
<tr>
<td>B4</td>
<td>2.794</td>
<td>120</td>
<td>0.843</td>
<td>120</td>
</tr>
<tr>
<td>B5</td>
<td>4.264</td>
<td>250</td>
<td>1.254</td>
<td>250</td>
</tr>
<tr>
<td>B6</td>
<td>5.671</td>
<td>2000</td>
<td>1.668</td>
<td>2000</td>
</tr>
<tr>
<td>Negative Control (D)</td>
<td>0.035</td>
<td>0</td>
<td>0.012</td>
<td>0</td>
</tr>
<tr>
<td>Positive Control (C)</td>
<td>1.374</td>
<td>49.2</td>
<td>0.418</td>
<td>49.6</td>
</tr>
</tbody>
</table>

Absorbance readings 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).

ASSAY CUT OFF

<table>
<thead>
<tr>
<th>Cut off</th>
<th>u/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>&lt; 5 u/mL</td>
</tr>
<tr>
<td>Positive</td>
<td>≥ 5 u/mL</td>
</tr>
</tbody>
</table>

This cut off has been validated at RSR. However each laboratory should establish its own normal and pathological reference ranges for GADAb levels. Also it is recommended that each laboratory include its own panel of control samples in the assay.

CLINICAL EVALUATION
Clinical Specificity and Sensitivity
In the DASP 2005 study the RSR GADAb ELISA kit achieved 98% (n=100) specificity and 92% (n=50) sensitivity.

Lower Detection Limit
The kit negative control was assayed 20 times and the mean and standard deviation calculated. The lower detection limit at +2 standard deviations was 0.57 u/mL.

Inter Assay Precision

<table>
<thead>
<tr>
<th>Sample</th>
<th>u/mL (n=20)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>97</td>
<td>5.7</td>
</tr>
<tr>
<td>B</td>
<td>21</td>
<td>5.2</td>
</tr>
<tr>
<td>C</td>
<td>5.7</td>
<td>6.4</td>
</tr>
</tbody>
</table>

Intra Assay Precision

<table>
<thead>
<tr>
<th>Sample</th>
<th>u/mL (n=25)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>97</td>
<td>7.3</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>8.5</td>
</tr>
<tr>
<td>3</td>
<td>7.0</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Clinical Accuracy
Analysis of sera from patients with autoimmune diseases other than type 1 DM disease indicated no interference from autoantibodies to thyroglobulin or thyroid peroxidase (n=10) or TSH receptor (n=20). One sample positive for dsDNA (n=10) and one sample positive for rheumatoid factor (n=30) were positive for GADAb.

Interference
No interference was observed when samples were spiked with the following materials; haemoglobin at 5 mg/mL, bilirubin at 20 mg/dL or Intralipid up to 3000 mg/dL.
SAFETY CONSIDERATIONS

Streptavidin Peroxidase (SA-POD)
Signal word: Warning
Hazard statement(s)
H317: May cause an allergic skin reaction
Precautionary statement(s)
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

 Peroxidase Substrate (TMB)
Signal word: Danger
Hazard statement(s)
H360D: May damage the unborn child
Precautionary statement(s)
P280: Wear protective gloves/protective clothing/eye protection/face protection
P308 + P313: IF exposed or concerned: Get medical advice/attention

This kit is intended for in vitro use by professional persons only. Follow the instructions carefully. Observe expiry dates stated on the labels and the specified shelf life for coated wells, reconstituted and diluted reagents. Refer to Safety Data Sheet for more detailed safety information. 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 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 or contact with skin, eyes and clothing. Avoid formation of heavy metal azides in the drainage system by flushing any kit components away with copious amounts of water.

ASSAY PLAN

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipette</td>
<td>25 μL calibrators (B1-6), controls (C and D) and patient sera (except blank)</td>
</tr>
<tr>
<td>Incubate</td>
<td>1 hour at room temperature on an ELISA plate shaker at 500 shakes/min</td>
</tr>
<tr>
<td>Aspirate/Decant</td>
<td>ELISA plate (A)</td>
</tr>
<tr>
<td>Wash</td>
<td>ELISA plate (A) three times and tap dry on absorbent material(^1)</td>
</tr>
<tr>
<td>Pipette</td>
<td>100 μL GAD65-Biotin (E) (reconstituted) into each well (except blank)</td>
</tr>
<tr>
<td>Incubate</td>
<td>1 hour at room temperature on an ELISA plate shaker at 500 shakes/min</td>
</tr>
<tr>
<td>Aspirate/Decant</td>
<td>ELISA plate (A)</td>
</tr>
<tr>
<td>Wash</td>
<td>ELISA plate (A) three times and tap dry on absorbent material(^1)</td>
</tr>
<tr>
<td>Pipette</td>
<td>100 μL SA-POD (G) (diluted 1:20) into each well (except blank)</td>
</tr>
<tr>
<td>Incubate</td>
<td>20 minutes at room temperature on an ELISA plate shaker at 500 shakes/min</td>
</tr>
<tr>
<td>Aspirate/Decant</td>
<td>ELISA plate (A)</td>
</tr>
<tr>
<td>Wash</td>
<td>ELISA plate (A) three times, rinse with pure water and tap dry on absorbent material(^1)</td>
</tr>
<tr>
<td>Pipette</td>
<td>100 μL TMB (I) into each well (including blank)</td>
</tr>
<tr>
<td>Incubate</td>
<td>20 minutes at room temperature in the dark (without shaking)</td>
</tr>
<tr>
<td>Pipette</td>
<td>100 μL stop solution (K) into each well (including blank) and shake for 5 seconds</td>
</tr>
<tr>
<td>Read absorbance</td>
<td>at 450 nm and 405 nm, within 15 minutes of adding stop solution</td>
</tr>
</tbody>
</table>

\(^1\) It is not necessary to tap dry the plates after washing when an automatic plate washer is used. The pure water wash can be omitted when using an automatic washer.