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Use only the current version of Product Data Sheet enclosed with the kit!
1  **INTENDED USE**

The IA-2 autoantibody (IA-2 Ab) ELISA kit is intended for use by professional persons only, for the quantitative determination of IA-2 autoantibodies 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 (GAD$_{65}$ kDa isoform) and an islet cell antigen IA-2 or ICA-512.

2  **ASSAY PRINCIPLE**

In 's IA-2 Ab ELISA, IA-2 autoantibodies in patients’ sera, calibrators and controls are allowed to interact with IA-2 coated onto ELISA plate wells. After a 16 - 20 hour incubation, the samples are discarded leaving IA-2 autoantibodies bound to the IA-2 coated wells. IA-2 Biotin is added in a 2$^{nd}$ incubation step where, through the ability of IA-2 autoantibodies to act divalenty, a bridge is formed between the IA-2 immobilised on the plate and IA-2 Biotin. The amount of IA-2 Biotin is then determined in a third incubation step by the 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 from blue to yellow. The absorbance of the yellow reaction mixture at 405nm and 450nm is then read using an ELISA plate reader. A higher absorbance indicates the presence of IA-2 autoantibody in the test sample. Reading at 405nm allows quantitation of high absorbances. Low values (less than 30 units per mL) should be read off the 450nm calibrator curve.

3  **STORAGE AND PREPARATION OF SERUM SAMPLES**

Sera to be analysed should be assayed soon after separation or stored, preferably in aliquots, at or below –20°C. 100μL is sufficient for one assay (duplicate 50μL determinations). Repeated freeze thawing or increases in storage temperature must be avoided. Do not use lipaemic or haemolysed serum samples. EDTA plasma may be used 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.
4 MATERIALS REQUIRED AND NOT SUPPLIED

- Pipettes capable of dispensing 25 µL, 50µL and 100µL.
- 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 an orbital shaker).
- ELISA Plate cover

5 PREPARATION OF REAGENTS SUPPLIED

A IA-2 Coated Wells
12 breakapart strips of 8 wells (96 in total) in a frame and sealed in foil bag.
Fit stripwells firmly into frame provided
After opening return any unused wells to the original foil packet and seal with adhesive tape. Place foil bag in the self-seal plastic bag with desiccant provided, and store at 2-8°C for up to 16 weeks.

B1-6 Calibrators
7.5, 35, 120, 350, 4000 u/mL
(units are NIBSC 97/550)
5 x 0.7 mL
Ready for use

C Positive Control
(see certificate of analysis for concentration range)
0.7 mL
Ready for use

D Negative Control
0.7 mL
Ready for use
E Reaction Enhancer
4.0 mL, coloured red
Ready for use

F IA-2 Biotin
3 vials
Lyophilised
Reconstitute to volume indicated on certificate of analysis using buffer for reconstituting IA-2 Biotin (G). When more than one vial is used, pool the vials and mix gently before use. Store at 2–8°C and use on day of reconstitution.

G Buffer for IA-2 Biotin
2x15 mL, coloured blue
Ready for use

H Streptavidin Peroxidase (SA-POD)
1 x 0.7 mL
Concentrated
Dilute 1 in 20 with diluent for diluting SAPOD (I). For example, 0.5mL (H) + 9.5mL (I). Store at 2–8°C for up to 20 weeks after dilution.

I Diluent for SAPOD
15 mL
Ready for use

J Peroxidase Substrate (TMB)
15 mL
Ready for use

K Concentrated wash solution
125 mL
Concentrated
Dilute 10 X with pure water before use. Store at 2 – 8°C up to kit expiry date.

L Stop solution
12 mL
Ready for use
ASSAY PROCEDURE

Allow all reagents to stand at room temperature for at least 30 minutes before use. A repeating Eppendorf type pipette is recommended for steps 2, 5, 8, 10 & 11.

1. Pipette 50 μL of patient sera, calibrators (B1-5) and controls (C and D) into respective wells, in duplicate.

2. Pipette 25 μL of reaction enhancer into each well.

3. Cover the frame and shake the wells for 5 seconds at 500 shakes per min then incubate overnight, without shaking, for 16-20 hours at 2–8°C.

4. After incubation, aspirate samples by use of a plate washing machine or discard the samples by briskly inverting the frame of stripwells over a suitable receptacle. Wash the wells three times with diluted wash solution (K), and aspirate the wash by use of a plate washing machine or discard the wash by briskly inverting the frame of stripwells over a suitable receptacle. Tap the inverted wells gently on a clean dry absorbent surface to remove excess wash solution (not necessary when an automatic plate washer is used).

5. Pipette 100 μL of reconstituted IA-2 Biotin into each well. Avoid splashing the material out of the wells during addition.

6. Cover the plate, and incubate at 2-8°C for 1 hour without shaking.

7. Repeat wash step 4.

8. Pipette 100 μL of diluted Streptavidin Peroxidase (H) into each well and incubate at room temperature for 20 minutes on an ELISA plate shaker (500 shakes per min).

9. After incubation, discard the samples by briskly inverting the frame of stripwells over a suitable receptacle. Wash the wells three times with diluted wash solution (K) followed by once with pure water (to remove any foam) and tap the inverted wells gently on a clean dry absorbent surface to remove excess wash solution (if a plate washing machine is used, the plate can be washed 3 times with diluted wash solution (K) only).

10. Pipette 100 μL of TMB (J) into each well and incubate in the dark at room temperature for 20 minutes without shaking.

11. Pipette 100 μL stop solution (L) into each well and shake the plate for approximately 5 seconds on a plate shaker. Ensure substrate incubations are the same for each well.

12. Read the absorbance of each well at 405nm and then 450nm using an ELISA plate reader, blanked against a well containing 100 μL of TMB (J) and 100 μL Stop solution (L) only.
7 RESULTS 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 IA-2 Autoantibody concentrations in patient sera can then be read off the calibration curve. Other data reduction systems can be used.

Samples with high IA-2 Ab concentrations can be diluted in kit negative control (D). For example, 15 μL of sample plus 135 μL of negative control 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 divt in a linear way.

8 TYPICAL RESULTS

Example only, not for calculation of actual results.

<table>
<thead>
<tr>
<th>Calibrator</th>
<th>450nm Absorbance</th>
<th>405nm Absorbance</th>
<th>U/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>0.15</td>
<td>0.044</td>
<td>7.5</td>
</tr>
<tr>
<td>B2</td>
<td>0.59</td>
<td>0.176</td>
<td>35</td>
</tr>
<tr>
<td>B3</td>
<td>2.24</td>
<td>0.666</td>
<td>120</td>
</tr>
<tr>
<td>B4</td>
<td>6.32</td>
<td>1.859</td>
<td>350</td>
</tr>
<tr>
<td>B5</td>
<td>9.12</td>
<td>2.682</td>
<td>4000</td>
</tr>
<tr>
<td>Negative Control D</td>
<td>0.02</td>
<td>0.003</td>
<td>0</td>
</tr>
<tr>
<td>Positive Control C</td>
<td>3.05</td>
<td>0.904</td>
<td>158</td>
</tr>
</tbody>
</table>
9 ASSAY CUT OFF

<table>
<thead>
<tr>
<th>U/mL</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>&lt; 7.5 U/mL</td>
</tr>
<tr>
<td>Positive</td>
<td>≥ 7.5 U/mL</td>
</tr>
</tbody>
</table>
10.1 Clinical Specificity and Sensitivity

In the DASP 2005 study the IA-2 Ab ELISA kit showed 99% (n=100) specificity and 66% (n=50) sensitivity.

10.2 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 0.15 u/mL.

10.3 Inter Assay Precision

<table>
<thead>
<tr>
<th>Sample</th>
<th>U/mL</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19.9</td>
<td>9.8</td>
</tr>
<tr>
<td>2</td>
<td>42.7</td>
<td>4.9</td>
</tr>
</tbody>
</table>

10.4 Intra Assay Precision

<table>
<thead>
<tr>
<th>Sample</th>
<th>U/mL</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57.6</td>
<td>1.9</td>
</tr>
<tr>
<td>2</td>
<td>72.1</td>
<td>3.5</td>
</tr>
</tbody>
</table>

10.5 Clinical Accuracy

Analysis of sera from patients with autoimmune diseases other than type 1 DM indicated no interference from autoantibodies to thyroglobulin; thyroid peroxidase; dsDNA; TSH receptor or from rheumatoid factor.

10.6 Interference

No interference was observed when samples were spiked with the following materials; haemoglobin up to 5 mg/mL or bilirubin up to 20 mg/dL. Interference was observed with intralipid at 1000 and 3000 mg/dL.

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 IA-2 Ab levels.
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 stability for reconstituted reagents. Refer to Materials Safety Data Sheet for more detailed safety information. Materials of human origin used in the preparation of the kit have 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. 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.
Allow all reagents and samples to reach room temperature before use

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
<th>Volume</th>
<th>Temperature</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipette</td>
<td>Calibrators, Controls and Patient Sera</td>
<td>50 μL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pipette</td>
<td>Reaction Enhancer</td>
<td>25 μL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mix</td>
<td>Shake on an ELISA plate shaker at 500 shakes/min for 5 seconds</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incubate</td>
<td>Overnight (16 – 20 hours) at 2 – 8°C, without shaking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirate/Decant</td>
<td>Plate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wash</td>
<td>Plate three times and tap dry on absorbent material</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pipette</td>
<td>IA-2 Biotin (reconstituted) into each well</td>
<td>100 μL</td>
<td>2 - 8°C, without shaking</td>
<td></td>
</tr>
<tr>
<td>Incubate</td>
<td>1 hour at</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirate/Decant</td>
<td>Plate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wash</td>
<td>Plate three times and tap dry on absorbent material</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pipette</td>
<td>SAPOD (diluted 1:20) into each well</td>
<td>100 μL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incubate</td>
<td>20 minutes at room temperature with shaking at 500 shakes/min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirate/Decant</td>
<td>Plate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wash</td>
<td>Plate three times and rinse with pure water and tap dry on absorbent material</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pipette</td>
<td>TMB into each well</td>
<td>100 μL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incubate</td>
<td>20 minutes at room temperature in the dark</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pipette</td>
<td>stop solution into each well and shake for 5 seconds</td>
<td>100 μL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Read absorbance at 405 nm and then 450 nm

It is not necessary to tap dry the plates after washing when an automatic plate washer is used. Also the pure water wash can be omitted from the final wash step when using an automatic washer.
REFERENCES

S. Chen et al. Sensitive non isotopic assays for autoantibodies to IA-2 and to a combination of both IA-2 and GAD
E. Nilson et al. Calcium addition to EDTA plasma eliminates falsely positive results in the GADA Elisa
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 Radioimmunoassay (RIA) and Enzyme Linked Immunosorbent Assay (ELISA) Kits.
C. Torn et al. Diabetes Antibody Standardization Program: evaluation of assays for autoantibodies to glutamic acid decarboxylase and islet antigen-2.