Medizym® anti-GAD

Enzyme immunoassay for the determination of autoantibodies to Glutamic Acid Decarboxylase (GAD65) in human serum

INTENDED USE

Type 1 diabetes, also known as insulin-dependent diabetes mellitus (IDDM), results from a chronic autoimmune destruction of the insulin-secreting pancreatic beta cells, probably initiated by exposure of genetically susceptible host to environmental agents. Autoimmune destruction of beta cells is thought to be completely asymptomatic until 80-90% of the cells are lost. This process may take years to complete and may occur at any time in all ages.

During the preclinical phase, this autoimmune process is marked by circulating autoantibodies to beta cell antigens. These autoantibodies such as anti-insulin (IAA), anti-glutamic acid decarboxylase (GAD) and anti-tyrosine phosphatase ICA 512 (IA2), are present years before the onset of type 1 diabetes and prior to clinical symptoms.

GAD, the enzyme that catalyzes the conversion of glutamate to GABA, has been identified in two isoforms, molecular weight 65.000 (GAD65) and 67.000 (GAD67). Although GAD autoantibodies are found in type 1 diabetes and in the rare neurological disorder Stiff-man syndrome (SMS), the GAD autoantibodies profile in the two diseases differs. Autoantibodies of SMS patients recognize a combination of linear and conformational epitopes of GAD while GAD65 autoantibodies in patients with type 1 diabetes are predominantly directed to the conformational epitopes. GAD65 autoantibodies (GAD65 Abs) are present in 70-80% of newly diagnosed patients with type 1 diabetes.

The combination of the autoantibodies to GAD65 and IA2 is highly relevant for risk assessment of type 1 diabetes in children and adolescence. These tests in combination are more sensitive and predictive than ICA in risk groups, e.g. relatives of patients with type 1 diabetes.

GAD65 Abs also occur in a subset of adults with type 2 diabetes. These patients can have pronounced hyperglycemia, and after therapy with oral hypoglycemic agents for several months to years they may become insulin dependent. Therefore, these patients are thought to have a slowly progressive form of type 1 diabetes, often called latent diabetes or latent autoimmune diabetes in adults (LADA).

The presence of GAD65 Abs in sera of such patients is a sensitive and specific marker for future insulin dependency.

LITERATURE

- Winter WE, Harris N; Schatz D: Immunological markers in the diagnosis and prediction of autoimmune Type 1a diabetes. Clinical Diabetes 2002; 20: 183-191

PRINCIPLE of the TEST

Medizym® anti-GAD is an enzyme immunoassay for the quantitative determination of autoantibodies to glutamic acid decarboxylase (GAD65 Abs) in human serum.

The assay system uses the ability of GAD65 Abs acting divaletly and forming a bridge between immobilized GAD65 and liquid-phase GAD65-Biotin. In the first step GAD65 Ab from the sample bind to GAD65 coated on the microtiter plate. In a second step GAD65-Biotin binds to this complex. The bound GAD65-Biotin correlates with the amount of GAD65 Abs in patient’s serum. Unbound GAD65-Biotin is removed by washing.

The bound GAD65-Biotin could be quantified by addition of Streptavidin-peroxidase and a colorogenic substrate (TMB) and reading the optical density (OD) at 450 nm.
The expiry date of each component is reported on its respective label that and control sera assayed in duplicates.

Sufficient for the analysis of 40 unknown samples as well as for calibrators at 2 - 8 °C, preferably in the original kit box.

Upon receipt, all components of the Medizym® anti-GAD have to be kept initially aliquot samples and keep at - 20 °C.

The samples may be kept at 2 - 8 °C up to three days. Long-term storage requires - 20 °C.

Repeated freezing and thawing should be avoided. For multiple use,

Materials required
- Precision pipettes 10 - 100 µl
- Multi-channel pipette
- Disposable pipette tips
- 8 channel wash comb or microplate washer
- Micro plate reader with optical filters for 450 nm and 620 or 690 nm
- Graduated cylinders
- Distilled or de-ionized water
- Absorbent paper or paper towel
- Foil

Size and storage
Medizym® anti-GAD has been designed for 96 determinations. This is sufficient for the analysis of 40 unknown samples as well as for calibrators and control sera assayed in duplicates.

The expiry date of each component is reported on its respective label that of the complete kit on the box label.

Upon receipt, all components of the Medizym® anti-GAD have to be kept at 2 - 8 °C, preferably in the original kit box.

Preparation before use
Allow samples to reach room temperature prior to assay. Take care to agitate serum samples gently in order to ensure homogeneity.

Please, handle carefully with the following components:

A Allow the sealed microplate to reach room temperature before opening. Unused wells should be stored refrigerated and protected from moisture in the original bag carefully resealed for 16 weeks.

B Prepare a sufficient amount of washing solution by diluting the concentrated wash buffer (B) 1 + 9 with distilled or de-ionized water. For example, dilute 50 ml of the concentrate with 450 ml of distilled water. B should be free of crystals before dilution, otherwise dissolve by warming up to max. 37 °C. The diluted washing solution can be stored at 2 - 8 °C up to 30 days.

D Prepare a sufficient amount of Streptavidin-peroxidase solution by diluting SA-POD concentrate (D) 1 + 99 (0.05 ml SA-POD concentrate with 4.95 ml diluent for SA-POD (G). The SA-POD solution prepared is stable up to 16 weeks at 2 - 8 °C.

E Avoid exposure of substrate solution (E) to light.

H Prepare the GAD-Biotin solution by diluting x ml of GAD-Biotin concentrate (H) with y ml of diluent for GAD-Biotin (J).

Use prepared solution within the day of diluting.

Materials required
- Microtiter plate
- Vacuum sealed with desiccant
- Concentrated wash buffer sufficient for 1000 ml
- Streptavidin-peroxidase (SA-POD) sufficient for 20.0 ml
- Substrate (3,3’,5,5’-Tetramethylbenzidin)
- Stop solution (0.25 M sulfuric acid)
- Diluent for SA-POD (D)
- GAD65-Biotin
- Diluent for GAD65-Biotin (H)
- positive control concentration: see leaflet
- calibrators concentrations see leaflet

ASSAYS PROCEDURE

1. Pipette into the corresponding wells according to assay scheme - 25 µl patient's samples and control serum (C II).

2. Cover the plate and incubate for 60 min at room temperature (18 - 25 °C) while shaking > 500 rpm.

3. Aspirate or “flick out” by striking the wells sharply onto absorbent paper to remove any residual droplets. Wash 3 times with 300 µl washing solution (diluted from B) with 5 seconds soaking time each.

4. Add 100 µl of diluted GAD65-Biotin solution (prepared from H and J) to each well.

5. Cover the plate and incubate for 60 min at room temperature (18 - 25 °C) while shaking > 500 rpm.

6. Aspirate or “flick out” by striking the wells sharply onto absorbent paper to remove any residual droplets. Wash 3 times with 300 µl washing solution (diluted from B) with 5 seconds soaking time each.

7. Add 100 µl diluted SA-POD (prepared from D and G) to each well.

8. Cover the plate and incubate for 20 min at room temperature (18 - 25 °C) while shaking > 500 rpm.

9. Aspirate or “flick out” by striking the wells sharply onto absorbent paper to remove any residual droplets. Wash 3 times with 300 µl washing solution (diluted from B) with 5 seconds soaking time each.

10. Add 100 µl substrate solution (E) to each well and shake shortly.

11. Incubate for 20 min in the dark at room temperature.

12. Add 100 µl stop solution (F) after exact 20 min to each well. Shake the plates for 5 seconds > 200 rpm.

13. Read the optical density at 450 nm versus 620 or 690 nm within 15 min after adding the stop solution.

Please note that the washing procedure is crucial. Insufficient washing will result to poor precision and falsely elevated OD readings. Without shaking the ODS will be measured about 20 % lower with a loss of sensitivity.
DATA PROCESSING

The standard curve is established by plotting the mean OD-values of the calibrators 0 - 5 on the ordinate, y-axis, versus their respective GAD65 Ab-concentrations on the abscissa, x-axis. In addition negative control (CI) should be used (see below).

The GAD65 Abs concentrations of the controls and the unknown samples are directly read off in IU/ml from the measured OD450 values.

Medizym® anti-GAD may be used also with Computer Assisted Analysis using software able to curves with spline smoothing fit.

TYPICAL EXAMPLE

Do not use for evaluation!

<table>
<thead>
<tr>
<th>Sample</th>
<th>OD (a) 450 nm</th>
<th>OD (b) 450 nm</th>
<th>OD (mean)</th>
<th>IU / ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrator 0</td>
<td>0.077</td>
<td>0.073</td>
<td>0.075</td>
<td>1</td>
</tr>
<tr>
<td>Calibrator 1</td>
<td>0.199</td>
<td>0.195</td>
<td>0.197</td>
<td>5</td>
</tr>
<tr>
<td>Calibrator 2</td>
<td>0.386</td>
<td>0.393</td>
<td>0.390</td>
<td>15</td>
</tr>
<tr>
<td>Calibrator 3</td>
<td>0.685</td>
<td>0.667</td>
<td>0.676</td>
<td>35</td>
</tr>
<tr>
<td>Calibrator 4</td>
<td>1.206</td>
<td>1.296</td>
<td>1.251</td>
<td>100</td>
</tr>
<tr>
<td>Standard 5</td>
<td>2.399</td>
<td>2.471</td>
<td>2.435</td>
<td>250</td>
</tr>
<tr>
<td>Control CI</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Patient 1</td>
<td>1.056</td>
<td>0.868</td>
<td>0.962</td>
<td>79</td>
</tr>
</tbody>
</table>

STANDARD CURVE

Typical example

REFERENCE VALUES

<table>
<thead>
<tr>
<th>Medizym® anti-GAD</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>negative</td>
<td>&lt; 5.0 IU/ml</td>
</tr>
<tr>
<td>positive</td>
<td>≥ 5.0 IU/ml</td>
</tr>
</tbody>
</table>

It is recommended that each laboratory establishes its own normal and pathological reference ranges for serum anti-GAD65 Abs antibodies levels as usually done for other diagnostic parameters, too. Therefore, the abovementioned reference values provide only a guide.

CHARACTERISTIC ASSAY DATA

Calibration

The Medizym® anti-GAD is calibrated against the WHO reference preparation NIBSC 97/550 and concentrations of GAD65 Abs are therefore expressed in IU/ml.

Linearity

Anti-GAD positive human sera, diluted in GAD Ab free serum samples, show theoretically expected values when tested in Medizym® anti-GAD.

On the basis of the heterogeneous nature of the autoantibody population and in view of epitope specificity and affinity of the autoantibodies the theoretical values expected by dilution with GAD65 Abs free human serum do not correspond with the measured concentrations in some cases.

Specificity and sensitivity

Using the cut-off 5 IU/ml the Medizym® anti-GAD a sensitivity of 90 % (69 patients with newly onset type 1 diabetes) and a specificity of 93 % (56 healthy blood donors) has been determined.

In comparison to a reference assay, relative sensitivity of 96% and relative specificity of 93% was found.

Detection limits

The analytical sensitivity (lower detection limit, 0 ± 3 SD) was established to be < 1 IU/ml.

Intra - and inter-assay variation

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Mean Concentration (IU/ml)</th>
<th>CV (%)</th>
<th>Sample no.</th>
<th>Mean Concentration (IU/ml)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17</td>
<td>9.0</td>
<td>5</td>
<td>15</td>
<td>9.1</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>6.1</td>
<td>6</td>
<td>76</td>
<td>5.5</td>
</tr>
<tr>
<td>3</td>
<td>183</td>
<td>5.7</td>
<td>7</td>
<td>186</td>
<td>5.3</td>
</tr>
</tbody>
</table>

LIMITATIONS of the METHOD

Healthy individuals should be tested negative by using the Medizym® anti-GAD. However, GAD65 Abs may also be present in apparently healthy persons.

Patients suffering from the rare neurological disorder Stiff-man Syndrome (SMS) may also show antibodies to GAD65 in serum and cerebrospinal fluid. These patients demonstrate much higher titers compared with patients with type 1 diabetes. Samples from patients with suspicion of SMS should be prediluted 1:50 or 1:100 with GAD65 Abs negative sera before testing.

Any clinical diagnosis should not be based on the results of in vitro diagnostic method alone. Physicians are supposed to consider all clinical and laboratory findings possible to state a diagnosis.
**ASSAY SCHEME**

Bring all reagents to room temperature. Gently mix all reagents to ensure homogeneity.

<table>
<thead>
<tr>
<th>Step</th>
<th>Activity</th>
<th>Material</th>
<th>CAL 0-5</th>
<th>C II</th>
<th>Patients 1, 2 etc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pipette</td>
<td>Samples</td>
<td>25 µl</td>
<td>25 µl</td>
<td>25 µl</td>
</tr>
<tr>
<td>2</td>
<td>Incubate</td>
<td>Plate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>at room temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>with shaking (&gt; 500 rpm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Aspirate or decant</td>
<td>Washing solution</td>
<td>3 x 300 µl</td>
<td>3 x 300 µl</td>
<td>3 x 300 µl</td>
</tr>
<tr>
<td>4</td>
<td>Pipette</td>
<td>GAD$_{ae}$-Biotin solution</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(prepared from H and J)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Incubate</td>
<td>Plate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 hour</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>at room temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>with shaking (&gt; 500 rpm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Aspirate or decant</td>
<td>Washing solution</td>
<td>3 x 300 µl</td>
<td>3 x 300 µl</td>
<td>3 x 300 µl</td>
</tr>
<tr>
<td>7</td>
<td>Pipette</td>
<td>SA-POD solution</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(prepared from D and G)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Incubate</td>
<td>Plate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>at room temperature</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>with shaking (&gt; 500 rpm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Aspirate or decant</td>
<td>Washing solution</td>
<td>3 x 300 µl</td>
<td>3 x 300 µl</td>
<td>3 x 300 µl</td>
</tr>
<tr>
<td>10</td>
<td>Pipette</td>
<td>Substrate</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>11</td>
<td>Incubate</td>
<td>Plate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>20 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>at room temperature</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>in the dark</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Pipette and mix</td>
<td>Stop solution</td>
<td>100 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>13</td>
<td>Measure OD</td>
<td></td>
<td>at 450 nm versus 620 nm (or 690 nm) within 15 min</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**SAFETY PRECAUTIONS**

- **This kit is for Research Use Only.** Follow the working instructions carefully. This instruction manual is valid only for the present kit with the given composition. An exchange of single components is not in agreement with CE regulations.
- The expiration dates stated on the respective labels are to be observed. The same relates to the stability stated for reconstituted reagents.
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid time shift during pipetting of reagents.
- All reagents should be kept at 2 - 8 °C before use in the original shipping container.
- Some of the reagents contain small amounts (< 0.1 % w/v) of and Neolone 10M as a preservative. They must not be swallowed or allowed to come into contact with skin or mucosa.
- Source materials derived from human body fluids or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
- Since the kit contains potentially hazardous materials, the following precautions should be observed:
  - Do not smoke, eat or drink while handling kit material,
  - Always use protective gloves,
  - Never pipette material by mouth,
  - Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.
- In any case GLP should be applied with all general and individual regulations to the use of this kit.