Autoantibodies against Insulin (IAA) ELISA

Enzyme Immunoassay for the qualitative detection of circulating autoantibodies against human Insulin.

REF NM59071

Σ 12x8

For illustrative purposes only.
To perform the assay the instructions for use provided with the kit have to be used.

Distributed by:

IBL GESELLSCHAFT FÜR IMMUNCHEMIE UND IMMUNBIOLOGIE MBH
FLUGHAFENSTRASSE 52a • D-22335 HAMBURG • GERMANY
Phone: +49 (0)40 53 28 91-0 IBL@IBL-Hamburg.com
Fax: +49 (0)40 53 28 91-11 www.IBL-Hamburg.com
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1. Introduction

Insulin-dependent diabetes mellitus (IDDM) or Type I Diabetes is an autoimmune disease in which insulin deficiency is a consequence of immunological destruction of the pancreatic beta cells. In individuals who are genetically predisposed to IDDM, the immunological attack on the beta cells occurs during an asymptomatic period which is referred to as the "Prediabetic Phase". This prediabetic phase usually begins several years before the clinical onset of IDDM. During this phase, autoantibodies directed against pancreatic islet cell antigens (ICA) and/or insulin (IAA) are detected in the blood of many prediabetic subjects.

Insulin Autoantibodies (IAA) were first described in 1970 by Hirata and colleagues in a patient with spontaneous hypoglycaemia. IAA have been characterised and found to be of IgG class and possess binding constants and capacities similar to those of insulin autoantibodies from diabetic patients treated with insulin.

The role of IAA in the autoimmune mechanisms of IDDM was first suggested by Palmer and associates who found IAA in 18 % of newly diagnosed untreated IDDM patients. With an improved antibody assay, these investigators detected IAA in approximately 40 % of fresh onset, untreated IDDM patients. Other laboratories have reported a rate of 20 % to 50 % of IAA incidence among newly diagnosed IDDM patients. In addition IAA have been detected in non-diabetic patients who were genetically at higher risk for IDDM. In a study of a heterogeneous group of high risk subjects including discordant monozygotic twins and ICA-positive first degree relatives with a history of glycosuria, IAA were detected in 31 % of ICA-positive individuals. The presence of both IAA and ICA showed a greater likelihood of subsequently developing IDDM. In another study, IAA were found in 40 % of ICA-positives and 16 % of the ICA-negative first degree relatives of IDDM-patients. Furthermore, serum IAA were found in four individuals who subsequently developed IDDM. The mechanism or the physiological role of IAA in the pathogenesis of IDDM is not understood.

The most sensitive method currently being used for determination of IAA in human serum employs a radiometric competitive assay. The IAA Assay, an ELISA test for IAA determination, is simple to use and it does not require the use of radioactive materials. The IAA test is intended for research use only for the in vitro detection of circulating autoantibodies against human insulin.
2. Principle of the Test

Human insulin is immobilised onto microwells. The positive control, negative control, and one dilution of patient serum are added to the appropriate microwells. Human IgG specific antibodies to insulin in the serum sample and control bind to the insulin molecules on the microwells. After washing off unreacted serum materials, an enzyme labelled goat antibody specific to human IgG is added to the antigen-antibody complex. After thorough washing to remove the unbound enzyme, a substrate solution is added and the colour development is measured spectrophotometrically. The intensity of the colour is directly proportional to the concentration of IAA in the sample. Two quality controls (positive and negative) are provided to monitor and validate assay results.

3. Precautions

- The assay calibrators and controls are of human origin and have been tested and confirmed negative for HIV, HBsAg and HCV by FDA approved procedures. All standards, however, should be treated as potential biohazards in use and for disposal.

- The assay reagents contain sodium azide or thimerosal which may be toxic if ingested. Sodium azide may react with copper and lead piping to form highly explosive salts. On disposal, flush with large quantities of water.

- This kit is for in vitro diagnostic use only.

- Never pipette by mouth and avoid contact of reagents and specimens with skin and mucous membranes. If contact occurs, wash with a germicidical soap and copious amounts of water.

- Do not smoke, eat or drink in areas where specimens or kit reagents are handled.

- Wear disposable latex gloves when handling specimens and reagents, and wash hands thoroughly afterwards. Microbial contamination of reagents or specimens may give false results.
4. Kit Components

4.1 Contents of the Kit

This IAA test kit contains reagents and materials for 96 microwells.

4.1.1 Microwell Strips (with the holder) 12 strips

4.1.2 Anti-Human IgG Enzyme Conjugate 2 vials
1.0 ml each, concentrate.

4.1.3 Sample Diluent 1 bottle
25 ml, concentrate.

4.1.4 Conjugate Diluent 1 bottle
10 ml, ready for use.

4.1.5 Positive Control 1 vial
1.5 ml, ready for use.

4.1.6 Negative Control 1 vial
1.5 ml, ready for use.

4.1.7 Substrate Solution 1 bottle
20 ml, ready for use.

4.1.8 Washing Buffer 1 bottle
20 ml, concentrate.

4.1.9 Stopping Solution (1N NaOH) 1 vial
6.0 ml, ready for use.
Material required but not provided

- Distilled or deionized water.
- Absorbent paper towels to blot dry the strips after washing steps and parafilm plastic wraps to cover strips during incubations.
- Suitable sized glass tubes for serum dilution.
- Micropipet with disposable tips to deliver 25 µl, 50 µl and 100 µl.
- A microtiter plate washer or a squeeze bottle for washing.
- 10 ml pipettes for substrate buffer and conjugate diluent delivery.
- A 500 ml graduate cylinder.
- Microtiter plate reader with 405 nm absorbance capability.

4.2 Storage and Stability of the Kit

Do not freeze test reagents, store all kit components at 4-8 °C at all times. Do not mix reagents from different kits. Do not allow reagents to stand at room temperature for extended periods of time. Store substrate tablets in the dark in an air tight container.

4.3 Preparations of Reagents

Allow all reagents and required number of strips to reach room temperature prior to use.

4.3.1 Anti-Human IgG Enzyme Conjugate

Accurately transfer 5 ml of the Conjugate Diluent into one dropper bottle containing Anti-Human IgG enzyme conjugate (concentrate). Close the bottle and mix thoroughly by inversions. Each of the two conjugate (concentrate) bottles is sufficient for 6 strips. Reconstitute as needed.
4.3.2 Sample Diluent
The concentrated Sample Diluent, when stored at 4-8 °C, may have salt crystals at the bottom of the vial. Transfer the entire contents into 200 ml of distilled/deionized water in a 250 ml container (rinse out all crystals). Mix thoroughly, label the container as diluted Sample Diluent.

4.3.3 Washing Buffer
Salt crystals may appear at the bottom of the Washing buffer concentrate vial during storage at 4-8 °C. Transfer the entire contents into 480 ml of distilled/deionized water in a 500 ml container (rinse out all crystals). Mix thoroughly and label the container as diluted Washing Buffer.

4.4 Storage and Stability of prepared Reagents

4.4.1 Anti-Human IgG Enzyme Conjugate
Store the diluted conjugate at 4-8 °C at all times. Record the date of reconstitution on the label. This diluted reagent expires 30 days after reconstitution.

4.4.2 Sample Diluent
Store the diluted Sample Diluted at 4 - 8 °C. The diluted reagent is stable until expiration shown on the vial.

4.4.3 Washing Buffer
Store the diluted Washing Buffer at 4 - 8 °C. The diluted reagent is stable until expiration shown on the vial.

4.5 Disposal of the Kit
The disposal of the kit must be made according to the national official regulations. Special information for this product are given in the Material Safety Data Sheets (see chapter 3).
4.6 Damaged Test Kits

In case of any severe damage of the test kit or components, IBL have to be informed written, latest one week after receiving the kit. Severely damaged single components should not be used for a test run. They have to be stored until a final solution has been found. After this, they should be disposed according to the official regulations.

5. Specimen

5.1 Collection

Serum
Collect 5-10 ml of blood by venipuncture into a clot (red top) tube. Separate serum by centrifugation. Serum samples may be stored at 2-8 °C. Excessive hemolysis and presence of large clots or microbial growth in the test specimen may interfere with the performance of the test.

5.2 Storage

Serum
The serum samples obtained should be applied in the assay after storing for up to 24 hours at 2 - 8°C. If stored for longer periods the samples should be stored in aliquots at –20°C. Repeated freeze-thaw cycles have to be avoided.

5.3 Pretreatment

After removing assay reagents from the refrigerator, allow them to reach room temperature before pipetting. Sample preparation should be performed in glass tubes.
Dilution of samples
- Accurately pipet 25 µl of sample into 2.5 ml of the diluted Sample Diluent into an already labelled glass tube.
- Mix thoroughly.

6. Assay Procedure

GENERAL REMARKS:
All reagents and specimens must be allowed to come to room temperature before use. All reagents must be mixed without foaming. Once the test has been started, all steps should be completed without interruption. Use new disposable plastic pipette tips for each reagent, standard or specimen in order to avoid cross contamination. Before starting the assay, it is recommended that all reagents be ready, caps removed, all needed wells secured in holder, etc. This will ensure equal elapsed time for each pipetting step without interruption.

6.1 Assay Procedure

6.1.1 Assemble the number of strips needed for a test run in the holder provided. The microwell strip must be snapped in place firmly with its square side sitting on the numerical side of the holder or it will fall out and break.

6.1.2 Familiarise yourself with the indexing system of wells, e.g. A1, B1, C1, D1, etc.

6.1.3 Dispense 100 µl of Negative Control into microwells C1 and D1.

6.1.4 Dispense 100 µl of Positive Control into microwells E1 and F1.

6.1.5 Add 100 µl of diluted patient serum (see 5.3) to microwells G1 and H1. For more patient samples, use additional strips and add diluted samples to microwells in duplicate. There should be 100 µl of solution in each microwell to be assayed except A1 and B1 which are empty at this point and will be used later.
6.1.6 Any well strips not used should be stored in the ziplock bag provided for the next run.

6.1.7 Cover the plate with a parafilm/plastic wrap (to prevent contamination) and leave at 2-8 °C overnight (12-16 h).

6.1.8 The next morning, decant the solution from all wells. Blot the plate dry by tapping gently on a paper towel. If an automatic plate washer is being used, wash each well with 300 µl (0.3 ml) of the diluted Washing Buffer. When a squeeze bottle is used, fill the wells with diluted Washing Buffer carefully and then drain the buffer from the microwells. Repeat the procedure two more times and blot dry the plate with a paper towel.

6.1.9 Add 100 µl of Anti-Human IgG Enzyme Conjugate (see 4.3.1) to all microwells except wells A1 and B1.

6.1.10 Cover the plate with a parafilm/plastic wrap and let it stand at 25 °C ± 1 °C for one hour.

6.1.11 After incubation, repeat the washing step (see 6.8) and blot dry the microwells.

6.1.12 Add 100 µl of Substrate Solution to all microwells including wells A1 and B1. Be sure to dispense the substrate solution at a rapid steady pace without any interruption.

6.1.13 Leave the plate in the dark for 30 minutes at 25 °C ± 1 °C.

6.1.14 After 30 minutes promptly add 50 µl of the Stopping Solution into each well.

6.1.15 Set up the microtiter plate reader to read at 405 nm absorbance according to manufacturer's instructions. Blank the plate reader with well A1 or B1 and read the absorbance at 405 nm.

6.1.16 Calculate the data according to the following section.
6.2 Calculation of Results

Record the spectrophotometric readings [optical density (O.D.) in absorbance units] as shown in the example IAA test report form (see page 13). The actual O.D. reading from your IAA assay may be different.

- Calculate the average reading of a sample of control done in duplicate.

- The average reading of the negative control data is N, positive control data is P, and sample data is S.

- Calculate the cut-off point of each run:

  \[
  \text{Cut-off Point} = N \times 2.5
  \]

Enter either (+) or (-) by comparing the average sample O.D. value (S) with the calculated cut-off point value as shown in the example below. The borderline cases (within 5 % above the cut-off point) should be tested again every 6 months with the previous serum sample as reference. These samples should be stored at -20 °C, or below, until the next test run.

6.3 Automation

There is no protocol available for the Insulin Autoantibody (IAA) ELISA at the moment.
IAA Test Report Form (Sample)

Kit-/Lot-No. __________ Expiration Date: __________
Assay Date: __________ Operator: __________

Section A: Control Results

<table>
<thead>
<tr>
<th>Data</th>
<th>Cut-off Value</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls O.D.</td>
<td>Average O.D. X = (2.5 x N)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>0.245</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.233</td>
<td>N = 0.239 X = 0.597</td>
</tr>
<tr>
<td>Positive Control</td>
<td>1.231</td>
<td></td>
</tr>
<tr>
<td>Positive Control</td>
<td>1.123</td>
<td>P = 1.177 Positive</td>
</tr>
</tbody>
</table>

Interpretation:
1. Negative Control (N) should read O.D. not more than 0.600.
2. The Average O.D. of the Positive Control (P) should read O.D. more than the cut-off point (X).

Section B: Patient Sample Results

<table>
<thead>
<tr>
<th>Patient</th>
<th>Data</th>
<th>Average O.D.</th>
<th>Results (Cut-off Point: X = 0.597)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O.D.</td>
<td>(Average O.D. &gt; X)</td>
<td>Positive (+)</td>
</tr>
<tr>
<td>1</td>
<td>0.225</td>
<td>S₁ = 0.227</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.229</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.435</td>
<td>S₂ = 0.415</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>0.395</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.788</td>
<td>S₃ = 0.810</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.832</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.662</td>
<td>S₄ = 0.665</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0.668</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Date: __________ Reported by: __________
7. Assay characteristics

7.1 Expected values

The IAA assay is designed to detect circulating autoantibodies to human insulin. The antigen coated on the wells does not react with other autoantibodies such as islet cell autoantibodies, anti-thyroglobulin and anti-rheumatoid factor.

In a study of 100 serum samples randomly selected out of patient sera submitted to our clinical laboratory, two were found to contain measurable IAA titers. In addition, of the forty newly diagnosed IDDM patients, 40 % were found to be IAA-positive by this ELISA method. These values are in close agreement with the published estimates.

7.2 Accuracy

7.2.1 Quality Control

The controls (negative and positive) have to be run with the unknown sample each time for valid test results. For test results to be valid, the optical density for the negative control should show not more than 0.600. Positive control O.D. should not read less than the cut-off point (X) value and is run to indicate that all reagents are working properly. Invalid results should not be reported and the test should be repeated.

It is also recommended to make use of national or international Quality Assessment programs in order to ensure the accuracy of the results.

Employ appropriate statistical methods for analysing control values and trends. If the results of the assay do not fit to the established acceptable ranges of control materials patient results should be considered invalid. In this case, please check the following technical areas: Pipetting and timing devices; photometer, expiration dates of reagents, storage and incubation conditions, aspiration and washing methods.

After checking the above mentioned items without finding any error contact your distributor or IBL-Hamburg directly.
8. Limitations of Use

8.1 Interfering Substances

Any improper handling of samples or modification of this test might influence the results. Interferences caused by improper sample handling are explained in the chapters 'Specimen - Collection'.

Azide and thimerosal at concentrations higher than 0.1 % interfere in this assay. Therefore control sera or samples containing higher concentrations of the above mentioned components may give false results.

8.2 High-Dose-Hook Effect

There exists no High-Dose-Hook effect in a competitive assay. In case of sandwich assays the probability of a High-Dose-Hook effect is reduced if the antibody and antigen containing solutions are added in a sequential order.

8.3 Limitations and sources of error

The test results are valid only if all controls are within the specified ranges and if all other test parameters are also within the given assay specifications. In case of any doubt or concern please contact IBL-Hamburg.

Old substrate solution may give high background colour. Poor reproducibility may result from:
- Improper reconstitution of reagents;
- Improper storage of reagents;
- Faulty washing of microwells;
- Inconsistent/defective instrument;
- Using outdated reagents.

Therefore, it is very essential that the instructions are followed carefully and consistently. For better reproducibility, test conditions and test equipment should not vary extremely.
9. Legal Aspects

9.1 Reliability of Results

The test must be performed exactly as per the manufacturer’s instructions for use. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable national standards and/or laws. This is especially relevant for the use of control reagents. It is important to always include, within the test procedure, a sufficient number of controls for validating the accuracy and precision of the test.

9.2 Complaints

Complaints will only be accepted in written format (preferably on the manufacturer's complaint form) and only if all details of the test kit, as well as the test results, are included. A copy of the complaint form is available from IBL-Hamburg upon request.

9.3 Therapeutical Consequences

Therapeutical consequences should never be based on laboratory results alone even if all test results are in agreement with the items as stated under point 9.1. Any laboratory result is only a part of the total clinical picture of a patient.

Only in cases where the laboratory results are in acceptable agreement with the overall clinical picture of the patient should therapeutical consequences be derived.

The test result itself should never be the sole determinant for deriving any therapeutical consequences.

This in vitro test procedure detects the presence of human insulin autoantibodies in the patient sera. Results obtained by using this procedure alone must not be used for the diagnosis of IDDM.

Save the weak positive and borderline samples (within 5 % above the cut-off point) and store at -20 °C. Fresh samples from these patients should be tested again every six months together with the previous serum samples.

This is a screening test only. The diagnosis of IDDM should be made with data from the patient's medical history, clinical symptoms, and results of other tests.
9.4 Liability

Any modification of the test kit and/or exchange or mixture of any components of different lots from one test kit to another could negatively affect the intended results and validity of the overall test. Such modification and/or exchanges invalidate any claim for replacement. Claims submitted due to customer misinterpretation of laboratory results subject to point 9.3. are also invalid. Regardless, in the event of any claim, the manufacturer’s liability is not to exceed the value of the test kit. Any damage caused to the test kit during transportation is not subject to the liability of the manufacturer.
10. References


1. Preparation of Reagents (Volumens stated for 48 determinations)

- **Anti–Human IgG Enzyme Conjugate**: Accurately transfer 5 ml of the Conjugate Diluent into one dropper bottle containing Anti-Human IgG enzyme conjugate (concentrate). Close the bottle and mix thoroughly by inversions. Store the diluted conjugate at 4-8 °C at all times. Record the date of reconstitution on the label. This diluted reagent expires 30 days after reconstitution.

- **Sample Diluent**: The concentrated Sample Diluent, when stored at 4-8 °C, may have salt crystals at the bottom of the vial. Transfer the entire contents into 200 ml of distilled/deionized water in a 250 ml container (rinse out all crystals). Mix thoroughly, label the container as diluted Sample Diluent. Store the diluted Sample Diluted at 4 - 8 °C.

- **Washing Buffer**: Salt crystals may appear at the bottom of the Washing buffer concentrate vial during storage at 4-8 °C. Transfer the entire contents into 480 ml of distilled/deionized water in a 500 ml container (rinse out all crystals). Mix thoroughly and label the container as diluted Washing Buffer. Store the diluted Washing Buffer at 4 - 8 °C.

2. Specimen Collection and Storage

Samples: Serum
Storage: for up to 24 h at 2 - 8 °C, longer storage at –20°C. Avoid repeated freezing and thawing.

3. Assay Procedure

Allow reagents to reach room temperature. Unused reagents should be stored at 2-8°C.

- **A: Dilution of Samples** (in Glass Tubes)

  - Pipet **2.5 ml of Sample Diluent** into each labelled tube.
  - Add **25 µl of Serum Sample** into the respective tubes. Mix thoroughly.
B: ELISA

1. Pipet 100 µl of Negative Control, Positive Control and diluted Samples. Let two wells empty for substrate blank.

2. Seal plate and incubate for 12 - 16 hours (over night) at 2 – 8 °C.

3. Empty plate, wash 3 x with 300 µl of diluted Washing Buffer, eliminate residual fluids. *

4. Pipet 100 µl of Anti-Human IgG Enzyme Conjugate (except wells for blank).

5. Seal plate and incubate for 1 h at room temperature (25 + 1 °C).

6. Empty plate, wash 3 x with diluted Washing Buffer, eliminate residual fluids.*

7. Pipet 100 µl of Substrate Solution into all wells (including wells for blank).

8. Seal plate and incubate for 30 min. at room temperature (25 + 1 °C) in the dark.

9. Pipet 50 µl of Stopping Solution.**

10. Briefly mix contents, read the optical density at 405 nm (reference wave length 600-650 nm) within 60 min. after stopping.

* Wash procedure is essential for the assay results.

** Stopping solution should be pipetted after incubation in the same time intervals as the substrate reagent.

Calculate the average reading of a sample of control done in duplicate. The average reading of the negative control data is N, positive control data is P, and sample data is S.

Calculate the cut-off point of each run:

Cut-off Point = N x 2,5

Enter either (+) or (-) by comparing the average sample O.D. value (S) with the calculated cut-off point value as shown in the example below. The borderline cases (within 5 % above the cut-off point) should be tested again every 6 months with the previous serum sample as reference. These samples should be stored at -20 °C, or below, until the next test run.

4. Expected values

In a study of 100 serum samples randomly selected out of patient sera submitted to our clinical laboratory, two were found to contain measurable IAA titres. In addition, of the forty newly diagnosed IDDM patients, 40 % were found to be IAA-positive by this ELISA method. These values are in close agreement with the published estimates.

Attention: For comprehensive information about the test procedure please refer to the detailed instructions for use available on the Internet (http://www.IBL-Hamburg.com) or upon request from IBL in Hamburg.

June, 11th 2003/K1/L1
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<tr>
<td></td>
<td>Read instructions before use. / Arbeitsanleitung lesen. / Lire la fiche technique avant emploi. / Lea las instrucciones antes de usar. / Leggere le istruzioni prima dell’uso. / Διαβάστε τις οδηγίες πριν την χρήση.</td>
</tr>
<tr>
<td></td>
<td>Keep away from heat or direct sun light. / Vor Hitze und direkter Sonneneinstrahlung schützen. / Garder à l’abri de la chaleur et de toute exposition lumineuse. / Manténgase alejado del calor o la luz solar directa. / Manter longe do calor ou luz solar directa. / Να φυλάσσεται μακριά από θερμότητα και άμεση επαφή με το φως του ηλίου.</td>
</tr>
<tr>
<td></td>
<td>Store at: / Lagern bei: / Stocker à: / Almacene a: / Armazenar a: / Conservare a: / Αποθήκευση στους:</td>
</tr>
<tr>
<td></td>
<td>Manufacturer: / Hersteller: / Fabricant: / Productor: / Fabricante: / Fabbricante: / Παραγωγός:</td>
</tr>
<tr>
<td></td>
<td>Caution! / Vorsicht! / Attention! / ¡Precaución! / Cuidado! / Attenzione! / Προσοχή!</td>
</tr>
</tbody>
</table>

Symbols of the kit components see MATERIALS SUPPLIED.

Die Symbole der Komponenten sind im Kapitel KOMPONENTEN DES KITS beschrieben.

Voir MATERIEL FOURNIS pour les symbôles des composants du kit.

Símbolos de los componentes del juego de reactivos, vea MATERIALES SUMINISTRADOS.

Para símbolos dos componentes do kit ver MATERIAIS FORNECIDOS.

Per i simboli dei componenti del kit si veda COMPONENTI DEL KIT.

Για τα σύμβολα των συστατικών του κιτ συμβουλευτείτε το ΠΑΡΕΧΟΜΕΝΑ ΥΛΙΚΑ.

Symbols Version 2.01 / 2005-10-19