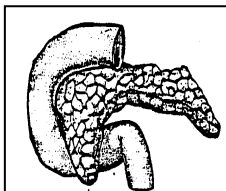


Isletest™-IAA

REF 7011



Qualitative ELISA Test for the Detection of Circulating Autoantibodies Against Human Insulin

March 2019

I. INTRODUCTION AND INTENDED USE

The Isletest™ - IAA test detects the presence of specific human IgG to human insulin. It is a screening test which, together with other clinical information, may be useful as an aid to the diagnosis of Type I Diabetes.

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 ⁽¹⁾ 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 hypoglycemia ⁽²⁾. IAA have been characterized, and found to be of IgG class similar to those insulin autoantibodies from diabetic patients treated with insulin ^(3,4). The role of IAA in the autoimmune mechanisms of IDDM was first suggested by Palmer and associates ⁽⁵⁾ who found IAA in 18% of newly diagnosed untreated IDDM patients. With an improved radiometric antibody assay, these investigators detected IAA in approximately 40% of fresh onset, untreated IDDM patients ⁽⁶⁾. Other laboratories have reported a rate of 20% to 50% of IAA incidence among newly diagnosed IDDM patients ⁽⁷⁻¹²⁾. In a study of a heterogeneous group of high risk subjects (non-diabetic patients genetically at higher risk for IDDM including discordant monozygotic twins and ICA-positive first degree relatives) IAA were detected in 31% of ICA-positive individuals. It was reported that the presence of both IAA and ICA showed an increased likelihood of those

individuals subsequently developing IDDM ⁽¹³⁾. In another study, IAA were found in 40% of ICA-positives and 16% of the ICA-negative first degree relatives of IDDM patients ⁽¹⁴⁾. Furthermore, serum IAA were found in four individuals who subsequently developed IDDM ⁽⁹⁾. The mechanism or the physiological role of IAA in the pathogenesis of IDDM is not yet understood.

The most sensitive method currently being used for determination of IAA in human serum employs a radiometric competitive assay ⁽¹⁵⁾. The Isletest™-IAA is an enzyme-linked immunosorbent assay (ELISA) for IAA determination. It is simple to use and does not require the use of radioactive materials. The Isletest™-IAA is intended for the *in vitro* detection of circulating autoantibodies against human insulin.

II. PRINCIPLE OF THE TEST

Human insulin is immobilized onto microwells. The reference, positive, and negative controls, and diluted patient serum samples are added to the appropriate microwells. Human IgG specific antibodies to insulin in the serum sample and controls bind to the insulin molecules on the microwells. After washing off unreacted serum materials, an enzyme (alkaline phosphatase) labeled goat antibody specific to human IgG is added to the antigen-antibody complex. After thorough washing to remove the unbound enzyme, a substrate (PNPP) solution is added and the color development is measured spectrophotometrically. The intensity of the color 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.

III. WARNING AND PRECAUTIONS

All reagents provided with the kit are for *in vitro* diagnostic use only.

1. Potential Biohazardous Material

The matrix of the Calibrators and Controls is human serum. The human serum used has been found non-reactive to HbsAg, anti-HIV 1/2 and anti-HCV when tested with FDA licensed reagents. Because there is no test method that can offer complete assurance that HIV, Hepatitis B virus or other infectious agents are absent, these reagents should be handled as if potentially infectious.

2. Sodium Azide

Some reagents contain sodium azide as a preservative. Sodium azide may react with lead, copper or brass to form explosive metal azides. When disposing of these materials, always flush with large volumes of water to prevent azide buildup.

3. Stopping Solution

Stopping Solution consists of 1N NaOH. This is a strong base and should be handled with caution. It can cause burns and should be handled with gloves. Wear eye protection and appropriate protective clothing. Avoid inhalation. Dilute a spill with water before absorbing the spill with paper towels.

4. Substrate Solution

Substrate Solution consists of para-Nitrophenylphosphate (PNPP), a non-proteinaceous chromogenic substrate used in this ELISA test. On occasion substrate may display a yellowish color. This color will not interfere with test results.

Precautions

1. Do not freeze test reagents, store all kit components at 2-8°C at all times.
2. Positive and Negative Controls must be run each time the test is performed.
3. Use only clear serum as test specimens. The test sample should not have gross turbidity, hemolysis, or microbial contamination.
4. All samples should be analyzed in duplicate.
5. Do not mix reagents from different lots.
6. Do not use expired reagents.
7. Do not allow reagents to stand at room temperature for extended periods of time.
8. Do not expose substrate solution to light.
9. Careful pipetting technique is necessary for reproducible and accurate results.

IV. REAGENTS AND MATERIALS

Materials Supplied:

1. **PLA IAA** = IAA-Microwell Strips (with the holder)12 strips
2. **CONJ ENZ 6X** = IAA- IgG Enzyme Conjugate (conc.).. 2 x 1.0 ml
3. **DIL SPE 5X** = IAA-Sample Diluent (concentrate) 1 x 25.0 ml
4. **CONJ ENZ DIL** = Isletest Conjugate Diluent..... 1 x 10.0 ml
5. **CTRL REF IAA** = IAA-Ref. Control (human serum) 1 x 1.5 ml
6. **CTRL + IAA** = IAA-Positive Control (human serum) 1 x 1.5 ml
7. **CTRL - IAA** = IAA-Negative Control (human serum) 1 x 1.5 ml
8. **SUBS PNPP** = Isletest Substrate Solution (PNPP) 1 x 15.0 ml
9. **BUF WASH 25X** = Isletest Washing Buffer (conc.)..... 1 x 20.0 ml
10. **SOLN STP** = Isletest Stopping Solution (1N NaOH) 1 x 6.0 ml

V. ADDITIONAL MATERIALS REQUIRED BUT NOT SUPPLIED

1. Distilled or deionized water.
2. Absorbent paper towels to blot dry the strips after washing and parafilm/plastic wraps to cover strips during incubations.
3. Suitable sized glass tubes for serum dilution.
4. Micropipet with disposable tips to deliver 10 µl, 50 µl and 100 µl.
5. A microtiter plate washer or a squeeze bottle for washing.
6. 5 ml pipets for conjugate diluent delivery.
7. A 500 ml graduate cylinder.
8. Microtiter plate reader with 405 nm absorbance capability.
9. Plastic label tape, to tape unused wells before assay.

VI. SPECIMEN COLLECTION

Collect 5-10 ml of blood by venipuncture into a clot (red top) tube. Serum separators may be used. Separate serum by centrifugation. Serum samples may be stored at 2-8°C. Excessive hemolysis and the presence of large clots or microbial growth in the test specimen may interfere with the performance of the test. Freeze the serum sample at -20°C if it cannot be analyzed within 24 hours.

VII. REAGENT PREPARATION AND STORAGE

1. IAA-IgG Enzyme Conjugate Reconstitution:

Accurately transfer 5 ml of the Isletest Conjugate Diluent into one bottle containing IAA-IgG Enzyme Conjugate (concentrate). Close the bottle and mix thoroughly by inversions. Store the diluted conjugate at 2-8°C at all times. Record the date of reconstitution on the label. **This diluted reagent expires 30 days after reconstitution.** Each of the two conjugate (concentrate) bottles is sufficient for 6 strips. Reconstitute as needed.

2. IAA-Sample Diluent Buffer:

If precipitate is present in the sample diluent buffer concentrate due to storage at lower temperature such as 2-8°C, dissolve by placing the vial in a 37°C water bath for 30 minutes. Transfer the entire contents (25ml) into 100 ml of distilled/deionized water in a suitable container. Mix thoroughly; label the container as IAA-Sample Diluent, and store at 2-8°C. The diluted reagent is stable until the expiration shown on the vial.

3. Isletest Wash Solution:

If crystals are present in the Wash Buffer concentrate due to storage at a lower temperature such as 2-8°C, dissolve by placing the vial in a 37°C water bath or incubator for 30 minutes. Transfer the entire contents into 480 ml of distilled/deionized water in a 500 ml container. Mix thoroughly; label the container as Isletest wash, and store at 2-8°C. The diluted reagent is stable until the expiration shown on the vial.

4. Serum Sample Preparation:

Accurately pipet 10 µl (0.010 ml) of serum sample into 1.0 ml of the Working Sample Diluent in an already labeled glass tube. Mix thoroughly.

VIII. ASSAY PROCEDURE

The test kit contains 12 microwell strips coated with human insulin. The number of microwell strips used in each assay depends upon the number of serum samples to be tested. If 12 microwell strips are used, a total 45 patient sera can be tested in duplicate with this kit.

IMPORTANT NOTE: Bring all the reagents, including serum samples, to room temperature (25°C) before starting the assay. Incubation temperatures varying by greater than ± 1°C can definitely affect results.

1. Assemble the number of strips needed for a test run in the holder provided. The microwell strip must be snapped firmly in place or it may fall out and break.
2. Familiarize yourself with the indexing system of wells, e.g. well #A1, B1, C1, D1, etc. and label the strips used with a marking pen.
3. Dispense 100 µl of IAA-Reference Control into microwell C1 and D1
4. Dispense 100 µl of IAA-Negative Control into microwells E1 and F1.
5. Dispense 100 µl of IAA-Positive Control into microwells G1 and H1.

6. Add 100 µl of diluted patient serum (see #4, Section VII, Reagent Preparation) to microwells A2 and B2. 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.
7. Any wells not used on the strip should be properly covered and saved for the next run. Any well strips not used should be stored with the desiccant in the ziplock bag provided at 2-8°C for the next run.
8. Cover the plate with a parafilm/plastic wrap (to prevent contamination) and leave at 2-8°C overnight (12-16 hrs.).
9. The next morning, discard the solution into sink by quick decantation. Blot the plate dry by tapping gently on a paper towel. If an automatic plate washer is being used, wash each well 3 times with 300 µl of the Wash Solution (prepared under Section VII, #3). If a squeeze bottle is used, fill the wells with Wash Solution carefully and decant the buffer from the microwells. Repeat the procedure two more times and blot the plate dry with a paper towel.
10. Add 100 µl of IAA-IgG Enzyme Conjugate reagent (see #1, section VII, Reagent Preparation) to all microwells except wells A1 and B1.
11. Cover the plate with a parafilm/plastic wrap and let it stand at $25^{\circ} \pm 1$ C for one hour.
12. After incubation, repeat the washing step (step #9) and blot dry the microwells.
13. Add 0.1 ml (100 µl) of Substrate Solution to all microwells including wells A1 and B1. Be sure to dispense the substrate reagent at a rapid steady pace without any interruption. On occasion substrate will display a yellowish color. This color may not interfere with test results.
14. Cover the plate and leave it in the dark for 30 minutes at room temperature ($25^{\circ} \pm 1^{\circ}$ C).
15. After 30 minutes promptly add 50 µl of the Stopping Solution into each well at a rapid steady pace without any interruption.
16. Set up microplate reader to read the absorbance at 405 nm according to manufacturing instructions, and blank the plate reader with well A1 or B1.
17. Calculate the data according to Section IX.

IX. CALCULATION OF DATA

Record the spectrophotometric readings [optical density (OD) in absorbance units] as shown in the example Isletest IAA Sample Data. The actual OD reading from your Isletest-IAA may be different. This is only an example.

1. Calculate the average O.D. reading of the Reference, Negative and Positive Controls and Patient samples done in duplicate.

Average OD: Reference (\bar{R}), Negative (\bar{N}), Positive (\bar{P}),
Samples (\bar{S})

2. Divide the average O.D. of Samples and Controls by the \bar{R} value. This gives a Ratio Value for each sample.

Interpretations:

<u>IAA Ratio Value (U/mL)</u>	<u>Result</u>
< 0.95	Negative
> 1.05	Positive
0.95 – 1.05	Indeterminate (Borderline)

Samples with Ratio values < 0.95 show an insignificant level of IAA antibodies, value > 1.05 show a high level of IAA antibodies. Samples with values between 0.95 and 1.05 are considered as indeterminate. The suggestion is to repeat indeterminate samples or to run in parallel with a new sample taken at a later date.

ISLETEST™ - IAA SAMPLE DATA

Section A: Control Results

Data (Net O.D.)			Ratio Value	Result
Controls	O.D.	Ave. O.D.		
Reference Ctrl	1.224	$\bar{R} = 1.247$	1.00	
	1.271			
Negative Ctrl	0.498	$\bar{N} = 0.481$	0.39	Negative
	0.464			
Positive Ctrl	1.855	$\bar{P} = 1.809$	1.45	Positive
	1.764			

Note: For a valid test, Ratio Value, $\bar{N} < 0.95$ and $\bar{P} > 1.05$
Repeat the test if results are not valid.

Section B: Patient Sample Results

Data (Net O.D.)			Ratio Value	Result
Sample	O.D.	Ave. O.D.		
Reference Ctrl	1.224	$\bar{R} = 1.247$	1.00	
	1.271			
1	1.994	$\bar{S}_1 = 2.002$	1.61	Positive
	2.010			
2	0.540	$\bar{S}_2 = 0.541$	0.43	Negative
	0.542			
3	1.280	$\bar{S}_3 = 1.270$	1.02	Indeterminate
	1.261			

X. QUALITY CONTROL

Negative and Positive Controls must be run along with unknown samples each time in order for the results to be valid. The Negative Control should show a ratio value < 0.95 Units/ml and the Positive Control should show a value > 1.05 Units/ml.

XI. PERFORMANCE CHARACTERISTICS

The Isletest™-IAA is a qualitative test designed to detect the presence of 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 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 (see references 6-12).

XII. CLINICAL SIGNIFICANCE

This *in vitro* test procedure detects the presence of human insulin autoantibodies in 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% of the Reference Control OD) 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.

XIII. LIMITATIONS AND SOURCES OF ERROR

1. This a qualitative screening test only.
2. Old substrate solution may give high background color.
3. Poor reproducibility may result from:
 - a. Inconsistent delivery.
 - b. Improper storage of reagents.
 - c. Improper reconstitution of reagents.
 - d. Faulty washing of microwells.
 - e. Inconsistent/defective instrument.
 - f. 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.

XIV. LITERATURE

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XV. SYMBOLS

	Storage Temperature
	Lot Code
	Expiration
	Manufacturer
	Authorized Representative
	Caution, see instructions
	For in vitro diagnostic use
	Catalog No.

XVI. ORDERING INFORMATION

Contact:

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2°C  8°C



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