



G A D

EXPORT ONLY

Qualitative ELISA Test for the Detection of Circulating Autoantibodies Against GAD Antigens

I. INTENDED USE

The GAD test is an *in vitro* qualitative ELISA assay for the detection of circulating autoantibodies to glutamic acid decarboxylase (GAD) antigen in prediabetic high risk individuals as well as IDDM diabetic patients.

II. BACKGROUND

Insulin - dependent diabetes mellitus (IDDM), Type 1, is caused by the autoimmune destruction of the beta cells of the pancreas (1, 2). This selective autoimmune pathogenesis causes complete elimination of insulin secretion. The immunological evidence was demonstrated by the presence of specific islet cell autoantibodies in IDDM patient's sera (3). At least three autoantibodies have been identified against antigenic components of the islet cells in Type 1 diabetic patients. These autoantibodies are directed specifically to islet cell antigenic component(s) (4), glutamic acid decarboxylase (5) and insulin (6).

Glutamic acid decarboxylase (GAD) is the biosynthetic enzyme for the neurotransmitter inhibitor gamma-amino butyric acid, GABA (7). Two forms of GAD, 65 Kda and 67 Kda, are produced by a single gene and are highly homogenous (8-10). 65-Kda GAD and 67-Kda GAD are identified in brain and Islet cells and are differentially expressed in human, rat and mouse pancreas (11,12).

Since diabetes is a chronic autoimmune disease involving beta-cell destruction, early and accurate prediction of the onset of the disease at the preclinical (asymptomatic) stage will help to intervene in the islet cells destruction and to preserve the maximum possible beta-cell mass. The screening of high-risk populations, for all the three autoantibodies (ICA, IAA and GAD) will help to either prevent or to slow down the onset of the disease. A high-risk (asymptomatic) population, positive to two or more autoantibodies, is vulnerable for developing IDDM, usually in the next 5-7 years (13,14).

III. PRINCIPLE OF THE TEST

A purified GAD antigen is immobilized onto microwells. GAD specific IgG antibodies present in the patient's serum sample are allowed to react with the antigen. The excess /unbound serum proteins are washed-off from the microwells. An enzyme (alkaline phosphatase) labeled goat-antibody, specific to human IgG is added to the GAD-antibody complex. After washing off excess unreacted enzyme conjugate from the microwells, a substrate (PNPP) is added and the color generated is measured spectrophotometrically. The intensity of the color developed gives directly the concentration of GAD autoantibodies in the test serum sample. GAD positive and negative controls serve as an internal quality control to ensure valid results.

IV. 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.

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.**

V. REAGENTS AND MATERIALS

Materials Supplied:

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

VI. 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 ml, 50 ml and 100 ml.
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.

VII. 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.

VIII. REAGENTS PREPARATION AND STORAGE

1. GAD-Enzyme Conjugate Reconstitution:

Accurately transfer 5 ml of the conjugate diluent into the bottle containing 1.0 ml of the enzyme conjugate (concentrate). Close the bottle and mix thoroughly by inversions. Store the diluted conjugate at 2-8°C when not in use. Record the date of reconstitution on the label. This diluted reagent expires 30 days after reconstitution. Each bottle contains enough conjugate for 6 strips. Reconstitute as needed.

2. Sample Diluent Buffer:

Transfer the entire contents (25ml) into 100 ml of distilled/deionized water in a suitable container. Mix thoroughly; label the container as Sample Diluent, and store at 2-8°C. The diluted reagent is stable until the expiration shown on the vial.

3. Wash Solution:

Transfer the entire contents into 480 ml of distilled/deionized water in a 500 ml container. Mix thoroughly; label the container as 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 into an already labeled glass tube. Mix thoroughly.

IX. ASSAY PROCEDURE

The test kit contains 12 microwell strips coated with purified GAD antigen. 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 of 42 serum samples 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 number A1, B1, C1, D1, etc. and label the strips used with a marking pen.
3. Dispense 100 µl (0.1 ml) of calibrators, positive and negative controls, and the diluted serum samples into the appropriate microwells. Wells A1 and B1 are reserved for blank and contain no sample.
4. Cover the plate with a parafilm/plastic wrap (to prevent contamination) and incubate the plate for 1 hour at room temperature (25°C ± 1°C).
5. After a 1 hr incubation, dump the contents in the microwells and blot the plate dry by tapping gently onto a paper towel a few times. If an automatic plate washer is being used, wash each well 3 times with 300 µl (0.3 ml) of the wash buffer solution. If a squeeze bottle is used, fill the wells with the wash buffer carefully and then dump the buffer from the microwells. Avoid air bubbles in the well during washing. Repeat the washing procedure two more times (i.e. total 3 times). Blot the plate onto paper towel a few times at the end of each wash.
6. Add 100 µl (0.1 ml) of reconstituted Enzyme Conjugate reagent (see #1; Section VIII, Reagent Preparation) to all microwells except wells A1 and B1.
7. Cover the plate with a parafilm/plastic wrap and incubate it in the dark at room temperature (25°C ± 1°C) for one hour.
8. At the end of the incubation, wash the microwells three times as described earlier (see step # 4).
9. 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.
10. Cover the plate and leave it in the dark for 30 minutes at room temperature (25° ± 1°C).
11. At the end of 30 minutes after substrate addition, add 50 µl (0.05 ml) of the stopping solution into each well at a rapid steady pace without interruption.
12. Blank the plate reader and read the absorbance of the plate at 405 nm. A1 or B1 wells can be used to blank the plate reader. They have no sample, no conjugate, only substrate reagent and stopping solution.
13. Calculate the data according to Section X.

X. CALCULATION OF DATA

For manual calculations, prepare a dose response curve (DRC) on linear graph paper, plotting each calibrator value (as indicated on the calibrator vial label) on the X-axis and its corresponding absorbance value on the Y-axis. Draw a line to represent the best-fit straight line between the three points. Determine the GAD value of each patient's serum using its absorbance value and extrapolating from the DRC on the X-axis.

For automatic calculations, absorbance of each patient's serum sample must be converted into GAD values using a best-fit linear regression computer program. The GAD values indicated on each label of the calibrators should be entered as standards. The values are expressed as Units/ml.

The GAD value of each sample is interpreted as follows:

GAD Value	Result
<1.00	Negative
>1.05	Positive
1.00-1.05	Indeterminate (borderline)

A positive result (>1.05) indicates the presence of GAD autoantibodies in the patient's serum sample. A negative result (<1.00) indicates the absence of GAD autoantibodies or below the limit of resolution of the test. If an indeterminate (borderline) value is obtained (1.00-1.05), the sample should be retested. If a repetition of the test shows a negative value, the sample should be considered as negative. If the repetition shows a positive result, treat the serum sample as positive. If a borderline value is obtained after repeating the test, another sample should be taken at a later time for testing according to physician's instructions.

GAD SAMPLE DATA

Section A: Calibrator Values and Control Results

Controls	Ave. O.D.	GAD Value	Result
Calibrator #1	0.346	0.613	
Calibrator #2	0.634	1.124	
Calibrator #3	1.687	2.991	
Negative Control	0.188	0.32	-
Positive Control	1.24	2.2	+

NOTE: Do not use this data for actual experimental values. This is only a sample.

GAD Value: Negative	< 1.00 U/ ml
Positive	>1.050 U/ml
Indeterminate	1.00-1.050 U/ml (borderline)

Section B: Patient Sample Results

Sample	Ave.O.D.	GAD Value	Result
1	0.375	0.664	-
2	0.273	0.484	-
3	0.662	1.173	+

XI. 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 value < 1.0 Units/ml and the Positive Control should be > 1.0 Units/ml.

XII. PERFORMANCE CHARACTERISTICS**Cross-reactivity**

Interference from ANA, DNA, and Rheumatoid factors was not significantly observed. Serum samples with anti-Tg and Anti-TPO autoantibodies showed little or no cross-reactivity.

Precision

The reliability of the GAD ELISA test was assessed by examining its reproducibility using confirmed clinical samples for GAD autoantibody.

Intra-Assay (within run):				
Sample	N	Mean GAD Value	S.D.	%C.V.
1	20	0.560	0.038	5.4
2	20	1.771	0.035	4.8

Inter-Assay (between runs):				
Sample	N	Mean GAD Value	S.D.	%C.V.
1	10	0.424	0.074	4.6
2	10	1.542	0.040	4.5

Specificity and Sensitivity

A total of 99 confirmed patient serum samples were tested for GAD autoantibodies. Out of these samples, 39 were confirmed negatives and the remaining 60 samples were confirmed positives. The GAD ELISA test results of the samples are presented in the following table:

Total No. of Samples Tested	Total Negative ¹	Total Positive ²	False Positive ³	False Negative ⁴
99	34	51	5	9

- (1) Negative by both GAD ELISA and reference test
- (2) Positive by both GAD ELISA and reference test
- (3) Positive by GAD ELISA and Negative by reference test
- (4) Negative by GAD ELISA and Positive by reference test

Percent Accuracy: 85.8% Percent Specificity: 87.1%
Percent Sensitivity: 85.0%

Recovery

Recovery studies were performed with the GAD ELISA using previously confirmed serum samples of known values of GAD autoantibody.

Autoantibody added (GAD Value)	Autoantibody recovered (GAD Value)	% Recovery
3.620	3.432	94.5%
1.491	1.594	106.9%
0.915	0.825	90.2%
1.180	1.080	91.5%

XIII. LIMITATIONS AND SOURCES OF ERROR

1. Although a higher GAD titer will produce a higher OD reading, the test is designed for the semi-quantitative determination of the GAD autoantibodies in test serum samples.
2. Poor test reproducibility may result from:
 - a. Inconsistent delivery of reagents
 - b. Improper storage of reagents
 - c. Improper reconstitution of reagents
 - d. Incomplete washing of microwells
 - e. Substrate reagent old or exposed to light
 - f. Unstable /defective Spectrophotometer
 - g. Error in following the assay procedure

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: **MP Biomedicals, INC.**
Diagnostics Division
13 Mountain View Avenue
Orangeburg, NY 10962-1294

Customer Service: (800) 888-7008
FAX: (949) 260-1079
www.mpbio.com



“Authorized Representative”
according to IVDD 98/79/ EC

MP Biomedicals
n.v.-s.a., Doornveld, 10
B-1731 Asse-Relegem, Belgium