DRG® Anti-GAD ELISA (EIA-1910)

Revised 14 June 2011 rm (Vers. 6.1) **RUO in the USA**

Please use only the valid version of the package insert provided with the kit.

This kit is intended for Research Use Only.

This kit is not intended for diagnostic purposes.

1 INTENDED USE

The GAD ELISA is ELISA test for the detection of circulating autoantibodies to glutamic acid decarboxylase (GAD-65) antigen.

2 PRINCIPLE OF THE TEST

A purified GAD antigen is immobilized onto microwells. GAD specific IgG antibodies present in the 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 result.

3 WARNING AND PRECAUTIONS

1. Potential Biohazardous Materials

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.

4 REAGENT AND MATERIALS

Materials Supplied:

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

5 ADDITIONAL MATERIALS REQUIRED BUT NOT SUPPLIED

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

6 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 should 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.

7 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**

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 (25 ml) 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 until use. The diluted reagent is stable until the expiration shown on the vial. Please note that the precipitate seen in the concentrate has no affect on the performance of the test and will not be present in the 1X working solution.

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 solution, and store at 2-8°C until use. 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.

8 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 microwell strips needed for the test run in the holder provided. The microwell strips 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 an 1 hour 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 washing 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 8, 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 100 µl (0.1 ml) 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 incubate in the dark for 30 minutes at room temperature (25°C ± 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 the Section 10.

9 CALCULATION OF RESULTS

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 serum sample using its absorbance value and extrapolating from the DRC on the X-axis.

For automatic calculations, absorbance of each 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 DRG Units/ml.

GAD ELISA SAMPLE DATA

Section A: Calibrator Values and Control Results

Controls	Ave. OD	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)

10 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.

11 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

LITERATURE

1. Cooke, A. (1990). An overview on the possible mechanism of destruction of the insulin-producing beta cell. Curr. Top. Microbiol. Immunol. 164:125-142.
2. Harrison, L.C., I.L. Campbell, P.G. Colman, et al (1990). Type 1 diabetes: Immunology and Immunotherapy. Adv. Endocrinol. Metab. 1:35-94.

3. Bottazzo, G.F., A. Florin-Christensen, and D. Doniach (1974). Islet cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. Lancet, ii:1279-1283.
4. Colman, P.G., R.C. Nayak, I.L. Campbell, and g.s. Eisenbarth (1988). Binding of cytoplasmic islet cell antibodies is blocked by human pancreatic glycolipin extract. Diabetes, 37:645-652.
5. Baekkeskou, S., J.H. Nielson, and B. Marner, et al. (1982). Autoantibodies in newly diagnosed diabetic children immunoprecipitate human pancreatic islet cell proteins. Nature, 298:167-169.
6. Palmer, J.P., C.M. Aspilin, P. Clemons, et al. (1983). Insulin antibodies in insulin-dependent diabetes before insulin treatment. Science, 222:1337—1339.
7. Erlander, M.G. and A. J. Tobin (1991). The structure and functional heterogenicity of glutamic acid decarboxylase: a review. Neurochem. Res., 16:215-226.
8. Bu, D.F., M.G. Erlander, B.C. Hitz, N.J. Tillakaratne, et al. (1992). Two human glutamic decarboxylases, 65-kda GAD and 67-kda GAD, are encoded by a single gene. Proc. Natl. Acad. Sci. USA, 89:2115-2119.
9. Christgau, S., H. Schierbeck, H.J. Aunstoot, L. Aaggard, et al. (1991). Pancreatic B-Cells express two autoantigenic forms of glutamic acid decarboxylase, a 65-kda hydrophilic form and a 64-kda amphiphilic form which can be both membrane-bound and soluble. J. Biol. Chem., 266:21257-21269.
10. Clare-Salzler, M.J., A.J. Tobin, and D.LO. Kaufman (1992). Glutamate decarboxylase: an autoantigen in IDDM. Diabetes Care, 15:132-135.
11. Giorda, R., M. Peakman, K.C. Tan, D. Vergani, and M. Trucco (1991). Clutamic acid decarboxylase expression in islets and brain. Lancet, 338:1469-1470.
12. Kim, J., W. Richter, H.J. Aanstoot, Y. Shi, Q. Fu, et al. (1993). Differential expression of GAD 65 and GAD 67 in humans, rat and mouse pancreatic islets. Diabetes, 42:1799-1808.
13. Dean, B.M., F. Becker, J.M. McNally, A.C. Tarn, et al. (1986). Insulin autoantibodies in the pre-diabetic period: correlation with islet cell antibodies and development of diabetes. Diabetologia, 29:339-342.
14. Komalesh, M., A. Karasik, and S. Srikanta (1986). Islet cell antibodies as predictors of insulin-dependent diabetes mellitus. Pract. Cardiol., 12:79-91.

Version_May 2011~rm