ImmunoDiagnostics Limited

Glutamic Acid Decarboxylase (GAD) Autoantibody ELISA kit from ImmunoDiagnostics

For the quantitative determination of human anti-GAD65 ELISA (IgG class antibodies) in serum (plasma samples are not recommended)

This package insert must be read in its entirety before using this product

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Instructions for use

Introduction

Glutamic acid decarboxylase (GAD) autoantibodies are found in 70% to 80% of individuals with new-onset type 1 diabetes, making it the most frequent autoantibody in autoimmune diabetes. GAD autoantibodies can be detected in serum for many years post diagnosis, and high concentrations of GAD autoantibodies have been considered as a marker of faster β-cell exhaustion in these patients. Furthermore, GAD autoantibodies in non-diabetic individuals predicts the later development of type 1 diabetes. Besides autoimmune diabetes, GAD autoantibodies also exist in Stiff Man Syndrome, autoimmune poly-endocrinopathies, and some of Grave's Disease patients.

[Intended use]

Immunodiagnostics (IMD) GAD autoantibody ELISA kit is a highly sensitive and specific ELISA kit for precision detection and quantitative measurement of GAD autoantibody (GADA) titres in human serum (plasma samples are not recommended). This product is intended for use by professional persons only.

[Assay Principle]

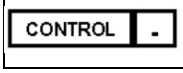
In IMD's GADA ELISA, recombinant GAD protein coated onto plate wells can specifically recognize the GAD autoantibodies in human sera and calibrators. After a 1-hour incubation, GAD autoantibodies are captured by immobilized GAD protein while the unbound components were discarded and washed away. Afterwards, biotinylated GAD protein (GAD-Biotin) is added for another round of incubation for 1 hour, wherein the GAD-Biotin detects GAD autoantibodies previously bound to GAD protein on the plate. After removal of nonspecific bindings, bound GAD-Biotin is revealed by addition of streptavidin horseradish peroxidase (STV-HRP), which specifically binds with biotin, followed by the substrate 3,3′,5,5′-Tetramethylbenzidine (TMB), which results in formation of a blue color. Color reaction will be further stopped by 2M H_2SO_4, transforming the blue color to yellow signals. The absorbance of yellow reaction mixture is measured by plate reader at 450nm and 405nm. The higher the reading is, the higher concentration of GAD autoantibodies. Low concentration of GAD autoantibodies (<18U/ml) is recommended to be read off the 450nm calibration curve, while high value of GAD autoantibodies to be read off 405nm standard curve. The measuring interval is 5-2000U/ml (units are NIBSC 97/550)

[Storage and preparation of test serum samples]

Test samples are suggested to be assayed immediately after separation of serum, or preferably stored at -20^{0}C or below in aliquots. Duplicate test is recommended therefore 50µl

is sufficient for each aliquot (25µl per test). Lipemic or hemolyzed sera, as well as plasma samples are not recommended. When required, vortex test serum samples at room temperature to ensure homogeneity. Then centrifuge samples at 10-15,000 rpm for 5 minutes prior to assay to remove particulates. Please do not omit this centrifugation step if samples are cloudy and containing particles.

[Symbols]

	Manufacturer		Biohazard
	Expiry date		Consult Instruction
	Lot number		Store
	Catalog number		Caution
	*In Vitro* Diagnostic Device		Positive control
	Negative control		

[Materials required and not supplied]

Pipettes capable to dispense 1000µl, 100µl and 25µl.
Multi-channel pipettes
Plate shaker capable of shaking at 500rpm.
96-well plate reader capable of absorbance measurement at 450nm and 405nm
Distilled water

[Reagent and materials supplied in IMD GAD Autoantibody ELISA kit]

A	GAD protein coated ELISA plate	12 strips of 8 wells (96 wells in total) in a frame and sealed in a foil bag. Make sure test strips are firmly fitted into the provided frame before use. Equilibrate test strips to room temperature before use. After opening, seal unused strips in the original self-seal foil bag with desiccant. Store the re-sealed foil bag at 2-8C for up to 16 weeks.
B 1-7	Calibrators	0.3ml x 7 5, 10, 18, 35, 120, 250, 2000 (U/ml) (Units are WHO standard NIBSC 97/550) Ready to use
C	Positive control	0.3 ml x 1 Ready to use
D	Negative control	0.3 ml x 1 Ready to use

E	GAD-Biotin	3 vials Lyophilized
F	Reconstitution buffer	15ml x 1 Pale yellow Ready-to-use for reconstitution of GAD-Biotin.
G	20 x Streptavidin horseradish peroxidase (STV-HRP)	0.7ml x 1 Dilute 1 in 20 with dilution buffer (H). For example, 0.5 mL (G) + 9.5 mL (H).
H	Dilution buffer (For STV-HRP)	15ml x 1 Ready to use
I	Substrate solution (3,3′,5,5′-Tetramethylbenzidine, TMB)	Ready to use Equilibrate to room temperature for 15 minutes before use
J	Stop solution	15ml x 1 2M H_2SO_4 Ready to use
K	10X Wash buffer	50ml x 1 Dilute with distilled water (1:10)

[Assay procedure]

PREPARATION OF REAGENTS

1. 1X wash buffer.
Prepare 1×Wash buffer by mixing the 10×Wash buffer (50 ml) with 450 ml of distilled water or deionized water. If precipitates are observed in the 10× Wash buffer bottle, warm the bottle in a 37°C water bath until the precipitates disappear. The 1×Wash buffer may be stored at 2-8°C for up to one month.

2. 1x detection GAD-Biotin solution
Reconstitute each vial of lyophilized GAD-Biotin with 4.5ml IMD reconstitution buffer (F). Return the reconstituted detection solution to 2-8°C immediately. The detection solution can be stored at 4^0C for up to 3 days after reconstitution or stored at -80^0C or -20^0C for long-term use in aliquots (< 3 freeze/thaw cycles).

3. 1x STV-HRP solution
Dilute 1 in 20 with dilution buffer (H). For example, 0.5 mL (G) + 9.5 mL (H). The 1×STV-HRP buffer may be stored at 2-8°C for up to one month.

Please pre-balance all the reagent to room temperature (20-25°C) for at least 30 minutes before use.

Step 1	Pipette 25µl of test serum samples, calibrators (B1-7) and controls (C and D) into respective plate wells in duplicate. Leave at least one well for blank.
Step 2	Cover the frame and shake the wells at room temperature at 500 rpm for 1 hour on a plate shaker.

Step 3	Discard the content and tap the plate on a clean paper towel to remove residual solution in each well. Add 300 µl of 1×Wash buffer to each well and incubate for 1 minute. Discard the 1×Wash buffer and tap the plate on a clean paper towel to remove residual wash buffer. Repeat the wash step for a total 3 washes.
Step 4	Add 100 µl of reconstituted GAD-Biotin to each well.
Step 5	Cover the frame and shake the wells at room temperature at 500 rpm for 1 hour on a plate shaker.
Step 6	Wash each well 3 times as described in step 3.
Step 7	Pipette 100 µl of 1x STV-HRP solution to each well
Step 8	Cover the frame and shake the wells at room temperature at 500 rpm for 20 minutes on a plate shaker.
Step 9	Wash each well 4 times as described in step 3.
Step 10	Add 100 µl of Substrate solution (I) to each well, incubate at room temperature for 15 minutes. **Protect from light**.
Step 11	Add 100 µl of Stop solution (J) to each well, gently tap the plate frame for a few seconds to ensure thorough mixing.
Step 12	Measure absorbance of each well at 450 nm and/or 405nm immediately.

[Data Analysis]

1. Subtract absorbance of blank from that of standards and samples.

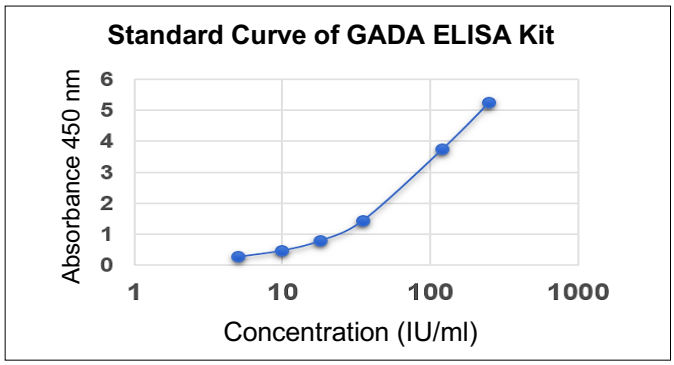
2. A calibration curve can be established by plotting calibrator concentration on x-axis (log-scale) against the absorbance of the calibrators on the y-axis (linear scale). The best fit line can be generated with any curve-fitting software by regression analysis. Any curve of 4-parameter or log-lin curve fitting can be used for calculation.

3. The GAD autoantibody concentrations in sera can be read off the calibration curve. Negative control can be assigned a value of 0.5U/ml to assist the statistical software to process the data analysis. Samples with high GAD autoantibody concentrations can be diluted with GAD autoantibody negative serum or the kit negative control Some sera will not dilute in a linear way according to the kit calibrators (standardised against NIBSC 97/550). Most test sera will have values below 250 U/mL and the 2000 U/mL calibrator need not always be included.

[Typical result] (Example only, not for calculation of actual results)

Calibrator*	Concentration (U/ml)	OD450	OD405
1	**2000**	**5.160**	**1.551**
2	**250**	**4.345**	**1.306**
3	**120**	**3.732**	**1.0865**
4	**35**	**1.445**	**0.439**
5	**18**	**0.794**	**0.2565**
6	**10**	**0.4785**	**0.168**
7	**5**	**0.276**	**0.1125**

Nnegative Control	**0**	**0.096**	**0.0655**
Positive Control	**15.18**	**0.746**	**0.235**



[Assay cut-off value]

< 5 U/ml	**Negative**
≥ 5U/ml	**Positive**

This cut-off value has been validated at IMD. However, it is recommended that each laboratory should establish its own normal and pathological reference range for GAD autoantibody level. Furthermore, it is also recommended that each laboratory should include its own panel of control samples in the assay.

[Clinical evaluation]

Clinical Evaluation		
Sensitivity	**92.5% (n=80)**	
Specificity	**95% (n=150)**	
Inter Assay Precision		
Sample	**U/ml (n=20)**	**CV**
1	**120**	**5.75%**
2	**18**	**6.66%**
3	**5**	**5.26%**
Intra Assay Precision		
Sample	**U/ml (n=20)**	**CV**
1	**120**	**6.61%**
2	**18**	**4.63%**
3	**5**	**5.65%**

[Reference]

Stenström, Gunnar, et al. "Latent autoimmune diabetes in adults: definition, prevalence, β-cell function, and treatment." *Diabetes* 54.suppl 2 (2005): S68-S72

Schmidli, Robert S., Peter G. Colman, and Ezio Bonifacio. "Disease sensitivity and specificity of 52 assays for glutamic acid decarboxylase antibodies: the Second International GADAB Workshop." *Diabetes* 44.6 (1995): 636-640.

Moffett, Ashley, et al. "Variation of maternal KIR and fetal HLA-C genes in reproductive failure: too early for clinical intervention." *Reproductive biomedicine online* 33.6 (2016): 763-769.