



Assay Protocol KRONUS® GADAb ELISA

1.0 Purpose

The purpose of this procedure is to define the protocol for the use of the modified KRONUS®, GAD Autoantibody ELISA kit.

2.0 Application/Scope

This procedure shall be applied to all autoantibody screening undertaken by screening labs in partnership with the nPOD project.

3.0 Definitions

GADA: Glutamic acid decarboxylase antibody. Glutamic acid decarboxylase (GAD) is an enzyme which is present in the islet cells of the pancreas. Antibodies to GAD are found in a high percentage of patients with type 1 diabetes at the time of diagnosis.

4.0 References

- 4.1 Eisenbarth, G.S. & McCulloch, D.K. (2008). *Pathogenesis of type 1 diabetes mellitus*. Retrieved from www.uptodate.com on July 14, 2008.

5.0 Associated SOPs

- 5.1 Shipping and Handling
- 5.2 New Case Protocol

6.0 Responsibilities

- 6.1 nPOD staff
 - 6.1.1 Coordinate shipment of KRONUS® kits to designated laboratories directly from KRONUS®, Inc.
 - 6.1.2 Assist with training of designated laboratory personnel.
- 6.2 Screening Laboratories
 - 6.2.1 Conduct autoantibody screening.
 - 6.2.2 Contact nPOD executive director when an autoantibody positive, consented donor has been identified.
 - 6.2.3 Report results to nPOD via nPOD online database.

7.0 Materials and Equipment

- 7.1 KRONUS GAD autoantibody ELISA kit.
- 7.2 Microtiter plate reader (with proper filter) set at 450nm or 405nm.
- 7.3 Pipettors calibrated to 25 and 100 μ L.
- 7.4 Means of measuring out various volumes to reconstitute and dilute reagents supplied.
- 7.5 Deionized or distilled water.
- 7.6 ELISA plate shaker or orbital rotator.
- 7.7 Wash bottles or automatic plate washing system.

8.0 Procedure

- 8.1 Calculate the number of individual ELISA plate wells needed for the assay. Allow all the reagents supplied, including the pouch containing the plate wells, to reach room temperature (at least 30 min). Remove the number of strip wells required and fit them firmly into the frame provided. Kit calibrators and positive control (duplicate wells for each) must be included in each assay run. Calculate the volume of SA-POD needed for the assay (3 below).
- 8.2 Pipette 25 μ L of calibrators (in duplicate) into suitable wells of the coated strips and similarly for the serum samples and kit positive and negative controls.
- 8.3 Cover the frame and incubate on an ELISA plate shaker (shaking at 500 rpm) at room temperature for 1 hour. During this 1 hour incubation, reconstitute the GAD65-biotin, dilute the required amount of streptavidin-peroxidase and dilute the concentrated wash.
- 8.4 After the first incubation period, aspirate or shake out the samples from the wells and wash each well with dilute washing solution three times. After the final wash, remove any excess liquid by gently tapping the inverted plate on an absorbent material.
- 8.5 Pipette 100 μ L of reconstituted GAD65-biotin into each well (a repeating Eppendorf type pipette is preferred). Cover the frame and incubate on an ELISA plate shaker (shaking at 500rpm) at room temperature for 1 hour.
- 8.6 Repeat the washing procedure as described in step 4, inverting and tapping the plate gently after the final wash.
- 8.7 Pipette 100 μ L of reconstituted SA-POD into each well (a repeating Eppendorf type pipette is preferred). Cover the frame and incubate on an ELISA plate shaker (shaking at 500 rpm) at room temperature for 20 minutes.
- 8.8 After this incubation, aspirate or shake out the contents of the wells and wash each well with dilute washing solution three times and once with deionized

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water (water wash step is not required if an automated plate washer is used). Invert and tap as before.

- 8.9 Pipette 100 μ L of peroxidase substrate (TMB) into each well (a repeating Eppendorf type pipette is preferred), cover the frame and incubate for 20 minutes at room temperature in the dark without shaking during which time a blue color will develop.
- 8.10 Stop the substrate reaction by addition of 100 μ L of stop solution to each well (a repeating Eppendorf type pipette is preferred). Shake the plates for 5 seconds at about 500 shakes per minute. This will cause the blue color to turn yellow. It is important to ensure that the substrate incubation time (i.e time from addition of substrate to addition of stop solution) is the same for each well.
- 8.11 Within 5 min after the addition of stop solution to the plate wells, read their absorbance at 405 nm and then again at 450 nm using an ELISA plate reader blanked against a well containing 100 μ L of substrate plus 100 μ L of stop solution. Plot a calibration curve with absorbance at 450 nm or 405 nm on the vertical axis and calibrator concentration on a log₁₀ scale on the horizontal axis and read off the concentration of GAD65 antibody in the test sera and positive control.
- 8.12 For purposes of nPOD, a reading greater than 5 IU/ml will be considered positive. Should this value change, all participants will be alerted individually and this document will be updated.

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