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Instructions for Use

The NativeAntigen COMPANY

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About the Native Antigen Company

The Native Antigen Company is accredited to ISO9001:2015 standard. We are proud to by powered by 100% renewable energy as part of our commitment to protect the environment. Should you have any comments or feedback, please get in touch, we love to hear from our customers.

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1. INTENDED USE

The Native Antigen Company (NAC) Zika Virus IgG/IgM/IgA ELISA assay is designed for the detection of Zika-specific antibodies in human serum. It is minimally cross-reactive with antibodies to Dengue virus (a closely-related flavivirus), and so can be used to distinguish human anti-Zika antibodies from other flavivirus and infectious disease antibodies, especially in epidemiological studies. Use of the assay is intended for detection of IgG in convalescent sera, although IgM/IgA are also detected earlier on in the infection cycle.

This product is FOR RESEARCH USE ONLY, and is not for use in diagnostic procedures.

2. BACKGROUND INFORMATION

Zika virus (ZIKV) is a member of the virus family Flaviviridae and the genus *Flavivirus*, transmitted by Aedes mosquitoes, such as *A. aegypti* and *A. albopictus*. Its name comes from the Zika Forest of Uganda, where the virus was first isolated in 1947. Zika virus is related to Dengue, Yellow Fever, Japanese Encephalitis, and West Nile viruses, and many antibodies (e.g. those produced as a response to infection) cross-react between these viruses. This cross-reaction causes severe problems in identifying individuals seropostive for Zika as the much wider spread background of Dengue infections cause many false positives in most standard serological ELISAs.

NAC's Zika virus antibody capture ELISA uses an in-house manufactured Zika virus-specific antigen to virtually exclude cross reactions and is thus suitable for the accurate detection of serum antibodies to Zika virus, indicating exposure. The detection of Zika specific antibodies is relevant for epidemiological studies, particularly in populations with high prevalence of Dengue and Chikungunya virus.

3. MATERIALS SUPPLIED

Component	Amount	Storage temp
Pre-coated microtitre plate	1 plate	2°C to 8°C
Negative control (NC) (Lyophilised)	1 vial	2°C to 8°C
Positive control (PC) (Lyophilised)	1 vial	2°C to 8°C
Differential control (DC) (Lyophilised)	1 vial	2°C to 8°C
Detection Reagent 1 (Lyophilised)	4 vials	2°C to 8°C
Detection Reagent 2	12 ml	2°C to 8°C
Sample diluent	60 ml	2°C to 8°C
Reagent 1 diluent	12 ml	2°C to 8°C
25X Wash buffer	100 ml	2°C to 8°C
TMB substrate	12 ml	2°C to 8°C
Stop solution	12 ml	2°C to 8°C
Plate sealers	3	--

4. STORAGE CONDITIONS

a. Store the un-opened kit at +2°C to +8°C until the expiry date on the outer box label
b. Once opened, the kit is stable for one month at +2°C to +8°C.
c. Reconstituted **Detection Reagent 1** should be used on the day of preparation.
d. 1x Wash Buffer is stable for 1 week at room temperature (+18°C to +28°C) in a sealed container.
e. Coated microwells should be used within one month of opening the pouch. After opening the plate pouch, wells must be stored in the sealed pouch with desiccant sachet at +2°C to +8°C.
f. Do not use the kit after the expiry date on the outer packaging.
g. Where multiple lots of kits have been purchased, do not combine components between kit lots, even if within shelf life.

5. MATERIALS REQUIRED BUT NOT SUPPLIED

a. Microtitre plate reader capable of absorbance measurement at 450nm and preferably capable of dual wavelength correction between 600nm and 650nm
b. Deionized water
c. Precision pipettes covering the range 5µl to 1ml
d. Microtitre plate shaker capable of 800 orbital revolutions per minute (rpm)
e. Vortex mixer
f. Disposable microcentrifuge tubes

6. WARNINGS AND PRECAUTIONS

This kit is for Research Use Only. DO NOT USE FOR DIAGNOSTIC PURPOSES. Observe the following precautions:

a. Laboratory coats and other PPE as appropriate should be worn when using the kit
b. Hands should be washed thoroughly after use
c. If handling potentially hazardous samples, use appropriate containment precautions
d. Clean up spillages and decontaminate any affected surfaces
e. Take care not to generate aerosols
f. Ventilate the work area adequately
g. Dispose of any waste and materials in accordance with local regulations

Materials of a biological origin should be handled as if capable of causing infection, using appropriate precautions and good clinical laboratory practices, even if samples have been tested for infectious agents or from a source considered safe. Decontaminate with an appropriate disinfectant and store and dispose of such materials and containers according to local regulations.

WARNING: POTENTIAL CHEMICAL HAZARD
Stop solution is 1M Hydrochloric acid, which is corrosive, causing severe damage to eyes, skin and mucous membranes, therefore wear hand, face and eye protection when handling this material. This kit contains Proclin 950 and TMB, which can be irritants to skin and mucous membranes at high concentrations. They are supplied in dilute form, and as such should present a minimal risk. In case of direct contact with any of these materials, wash the area thoroughly with water and seek medical advice.

7. REAGENT PREPARATION

Allow all reagents to warm to ambient temperature for 30 minutes before using the kit. Mix liquid reagents by gentle inversion or on a rocking roller mixer prior to use.

Wash buffer: Dilute the 25x wash buffer 1 in 25 with purified laboratory grade water to make 1x wash buffer.

Microtitre strips: Select the number of strips required for the controls and samples to be run. Store all unused strips sealed in the foil pouch with the supplied silica gel sachet to protect them from moisture.

Negative, Positive and Differential controls: Reconstitute the NC and PC using 500µl of **Sample Diluent**. Reconsitute the DC in **Sample Diluent** using the volume indicated on the label. Allow the controls to stand for 10 minutes. Vortex before use. Dilute each control 1 in 101 (for example, 5µl plus 500µl) in **Sample Diluent**, mix thoroughly before adding it to the microwells.

Samples: Samples that show a precipitate should be centrifuged to remove the debris before use (avoid any floating lipid; highly lipaemic samples interfere with the assay). All the samples are diluted 1 in 101 in **Sample Diluent**; ensure that the diluted samples are thoroughly mixed.

Detection Reagent 1: Reconstitute by adding 3ml of Reagent 1 diluent to

one lyophilised bottle, vortex and allow it to stand for 10 minutes. *Vortex again just before addition to the plate.*

8. SAMPLE DETAILS

Human-derived samples should be handled as follows:

- Routine precautions for venipuncture should be followed when collecting blood samples.
- Allow time for the blood to fully clot.
- Within 3 hours of collection, centrifuge to separate serum, and collect at least 500µl of sample without cells in a securely capped sample tube.
- Samples not tested on day of preparation must be frozen at -20°C or colder for storage or shipment.
- This assay has only been evaluated on human serum.
- Avoid repeated freeze/thaw cycles, no more than 3 times.

9. ASSAY PROTOCOL

1. Bring all the reagents and samples to room temperature. Prepare a plate plan to indicate the position of 6 control wells (2xNC, 2xPC, 2xDC) and all samples in duplicate on the plate.
2. Prepare wash buffer solution (Refer to section 7)
3. Prepare the NC, PC, DC and samples to be tested (refer to section 7)
4. Add 100µl of the diluted controls and diluted samples (in **Sample Diluent**) in separate duplicate wells as indicated by the plate plan. Cut an appropriate amount of plate sealer, cover the plate and incubate on a shaker set to 800rpm for 30 minutes at ambient (18°C to 28°C) temperature.
5. 10 minutes before the first incubation is due to end, reconstitute the **Detection reagent 1** (refer to section 7)**.**
6. Aspirate the plate contents, taking any safety precautions as required by sample type. Fill each well with 300µl of 1x wash buffer and perform 3 wash/empty cycles, then invert the plate onto absorbent paper and pat away any excess wash buffer.
7. Add 100µl of the **Detection reagent 1** to every well used, apply the

plate sealer and incubate the plate on a shaker set to 800rpm for 30 minutes at ambient (18°C to 28°C) temperature.

8. Aspirate the plate contents, taking any safety precautions as required by sample type. Fill each well with 300µl of 1x wash buffer and perform **5** wash/empty cycles, then invert the plate onto an absorbent paper and pat away any excess wash buffer.
9. Add 100µl of the **Detection reagent 2** to every well used, apply the plate sealer and incubate the plate on a shaker set to 800rpm for 30 minutes at ambient (18°C to 28°C) temperature.
10. Repeat the washing step as described in step 8.
11. Add 100µl of TMB substrate to every well used. Cover the plate with **a new plate sealer** and incubate the plate for 15 minutes on a plate shaker set to 800rpm at ambient (18°C to 28°C) temperature. The plate should be protected from direct sunlight.
12. After 15 minutes, add 100µl of stop solution to every well used. Read the plate at 450nm or 450/620nm using an ELISA plate reader within **15 minutes** of stopping the reaction.

10. RESULT VALIDITY

In order for the assay to be considered valid, the following criteria must be met:

- Negative control : The mean absorbance value of negative controls must be less than the mean differential control value
- Differential control : The mean absorbance value should be between 0.070 and 0.900 inclusive
- Positive control : The mean absorbance value of positive controls must be higher than the mean differential control value

If these criteria are not met, the test is not valid and must be repeated

11. RESULT CALCULATIONS AND INTERPRETATION

Calculate the Ratio Value (RV) for each sample by dividing the mean Optical Density (OD) value of each sample by the mean OD of the differential control:

Ratio Value = Mean OD of sample / Mean OD of differential control

The results should then be interpreted as follows:

Ratio	Interpretation
< 0.25	Negative for Zika Antibodies
≥ 0.25 but ≤ 0.4	Indeterminate
> 0.4	Positive for Zika Antibodies

Indeterminate samples are to be considered positive for flavivirus antibodies, but may either be low levels of Zika antibodies, or very high levels of related flavivirus antibodies most likely Dengue.

12. EXAMPLE DATA

Below is an example of results obtained from three different samples:

	Mean O.D	Ratio value	Interpretation
DC	0.529		
Sample 1	0.056	0.056/0.529 = 0.11	Negative for Zika Antibodies
Sample 2	0.203	0.203/0.529 = 0.38	Indeterminate
Sample 3	1.006	1.006/0.529 = 1.90	Positive for Zika Antibodies

13. INTER-ASSAY REPRODUCIBILITY

3 control samples were run on 3 plates from 3 kit lots.

Control samples	Ratio Value	CV%
High	4.29	10.5
Mid	2.48	8.3
Low	1.61	8.1

14. INTRA-ASSAY REPRODUCIBILITY

3 control samples were run in sets of 10 replicates across 3 kit batches on a single occasion. Average intra-assay variability is shown below.

Control samples	CV%
High	1.4
Mid	2.5
Low	3.7

15. ASSAY DRIFT

Sets of high, medium and low samples were run in duplicates and added to the plate at 5 minute intervals. Using least squares regression, the time at which the RV would vary by +/- 10% of the intercept value was calculated. In all cases the time at which the RV would reach +/- 10% was greater than 20 minutes.

16. SENSITIVITY AND SPECIFICITY

The following clinical samples of disease state sera/plasma were tested in this assay using the standard protocol. Of the Zika patient sera, 39 were commercially sourced and tested in house, 10 were field tested by a third party in Brazil, and 12 were UK returning travellers tested by a third party. Of the Dengue and Negative Brazilian sera, 118 were tested in Brazil by a third party.

Zika patient sera				
Total	Positive	Indeterminate	Negative	Sensitivity
62	56	2	4	90.3%
Dengue patient sera and plasma				
Total	Positive	Indeterminate	Negative	Specificity
78	5	7	66	93.6%
Chikungunya patient sera				
Total	Positive	Indeterminate	Negative	Specificity
12	0	1	11	100%
West Nile Virus patient sera				
Total	Positive	Indeterminate	Negative	Specificity
10	0	0	10	100%
Negative Brazilian patient sera (~90% seropositive for DENV)				
Total	Positive	Indeterminate	Negative	Specificity
90	11	5	74	87.8%
Negative UK patient sera				
Total	Positive	Indeterminate	Negative	Specificity
13	0	0	13	100%
Total non-Zika sera				
Total	Positive	Indeterminate	Negative	Overall Specificity
203	16	13	174	92.1%

For the purposes of the above calculations, indeterminate results were taken as negative.

17. COMPETITOR ANALYSIS

144 of the above Dengue-positive and Negative Brazillian (90% DENV positive) clinical sera that were known to be Zika negative due to location and date of collection were tested alongside a competitor Zika IgG ELISA assay. 116 of the samples were performed in Brazil by a third party.

	Competitor E	NAC
Positive	44	14
Indeterminate	14	11
Negative	86	119
Specificity	**69.4%**	**90.3%**

The Native Antigen Company (NAC) ELISA was far less cross-reactive than the competitor assay.

18. INTERFERING SUBSTANCES

Serum samples were spiked with common interfering substances and evaluated in comparison with un-spiked material.

Substance	Amount	Result
Haemoglobin	2 mg/mL	No interference
Bilirubin	1 mg/mL	No interference
Rheumatoid factor	54 IU/ml	No interference
Triolein	45.5 mg/ml	No interference

Problems with lipaemia can arise in non-fresh freeze/thawed samples. To avoid false positives, centrifuge the sample and avoid the lipid layer and pellet.

19. TROUBLESHOOTING

The following table may be used to troubleshoot the assay.

Issue	Possible Cause	Solution
Low signal	Incubation time to short	Repeat
	Precipitate can form in wells upon substrate addition when concentration of target is too high	Increase dilution factor of sample
	Increase dilution factor of sample	Detection may be reduced or absent in untested sample types
	Sample prepared incorrectly	Ensure proper sample preparation/dilution
Large CV	Bubbles in wells	Ensure no bubbles present prior to reading plate
	All wells not washed equally/thoroughly	Check that all ports of plate washer are unobstructed/wash wells as recommended
	Incomplete reagent mixing	Ensure all reagents/master mixes are mixed thoroughly
	Inconsistent pipetting	Use calibrated pipettes & ensure accurate pipetting
	Inconsistent sample preparation or storage	Ensure consistent sample preparation and optimal sample storage conditions (e.g. minimize freeze/thaws cycles)

High background	Wells are insufficiently washed	Wash wells as per protocol recommendations
	Contaminated wash buffer	Make fresh wash buffer
	Waiting too long to read plate after adding stop solution	Read plate immediately after adding stop solution
Low sensitivity	Improper storage of ELISA kit	Store all reagents as recommended. Please note all reagents may not have identical storage requirements.
	Using incompatible sample type (e.g. Serum vs. cell extract)	Detection may be reduced or absent in untested sample types

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