For Research Use Only (RUO)
NOT FOR USE IN DIAGNOSTIC PROCEDURES

INSTRUCTIONS FOR USE

DIACHECK

Anti- ZIKA IgG, Anti- ZIKA IgM, Anti- ZIKA IgA

Indirect Enzyme Immunoassay for the detection of IgG, IgM and IgA antibodies to ZIKA Virus
In vitro use

RUO | IVD | REF ZKV-116, ZKV-216, ZKV-316

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CONTENT:

1. INTENDED USE

Indirect enzyme immunoassay for the detection of IgG, IgM and / or IgA antibodies to ZIKA Virus in human serum and plasma. The enzyme immunoassay is intended for testing individual specimens, not pooled specimens. In vitro diagnostic device for research use only RUO, only to be used by correspondingly trained laboratory personnel. The test can be processed manually or automatically. The barcode identification of each single reagent ensures their correct identification and an accurate automatic processing of the test.

2. DIAGNOSTIC RELEVANCE, DISEASE AND RECOMMENDED LITERATURE

Besides isolation and nucleic acid (PCR) detection of the infectious agent, the detection of antibodies to ZIKA virus significantly contributes to the serological diagnosis of this infection. The virus was first isolated in 1947 from a rhesus macaque monkey from the ZIKA Forest in Uganda. ZIKA virus is the causative agent of ZIKA fever. In 2015 an ongoing outbreak of the virus has turned to an epidemic thread infecting people, starting in Brazil and spreading to South and Central America and the Caribbean region and is one of the three most important mosquito (Aedes aegypti) borne viral infections in humans (besides Dengue virus and Chikungunya-virus) in tropical and subtropical regions.

The clinical spectrum ranges from asymptomatic appearance to light and possibly severe illness. Typically, 1 of 5 infected individuals show symptoms, illness starts with common signs and symptoms (e.g., fever, myalgia, head ache, pain behind the eyes, nausea, vomiting, arthralgia and a skin rash) commonly reported with the illness. High fever associated with ZIKA virus infection may last 2-4 days. Transmission of the virus to the foetus during the early stage of gestation in pregnant women has been associated with development of microcephaly. Also association of infection with development of the Guillain-Barré Syndrome has been reported. Transmission through other routes of infection may also include sexual transmission and blood transfusion.

The viral genome is detectable shortly after infection and generally before antibody development for a short period of time (5-10 days). IgM-Antibodies (and also IgA-Antibodies) are detectable after 4-6 days after onset of illness and gradually decrease over the period of a few weeks in some cases longer. Following infection high levels of IgG antibodies are detectable after 2-4 weeks of onset. Antibody cross-reactivities have been reported with Dengue- and Chikungunya virus.

Infection: ZIKA virus is mainly transmitted through infected mosquitos to humans, other routes of infection may include sexual transmission and blood transfusion. The major transmission vector being the female Aedes aegypti, the incubation period of ZIKA fever is about 4 to 7 days.

Infectious agent: ZIKA virus is a small virus that belongs to the genus Flavivirus and carries a single stranded positive sensed RNA coding for three structural proteins, C, prM, E; seven non-structural proteins, NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5; and short non-coding regions on both the 5' and 3' ends.

LITERATURE REFERENCES (Links):

1.) *ZIKA VIRUS Fact Sheet WHO* *http://www.who.int/mediacentre/factsheets/zika/en/*
2.) Zika Virus CDC http://www.cdc.gov/zika/index.html
3.) *Zika Virus Outbreak, Bahia, Brazil* *http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4593454/*
4.) Possible Association Between Zika Virus Infection and Microcephaly — Brazil, January 2015 http://www.cdc.gov/mmwr/volumes/65/wr/mm6503e2.htm
5.) Complete Coding Sequence of Zika Virus … Outbreak in 2013 http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4047448/
6.) Arboviruses: Molecular Biology, Evolution and Control http://www.horizonpress.com/arbo
7.) Molecular Virology and Control of Flaviviruses http://www.horizonpress.com/flaviviruses
8.) ZIKA Virus https://de.wikipedia.org/wiki/Zika-Virus

3. PRINCIPLE OF THE METHOD

Human IgG, IgM and / or IgA antibodies against ZIKA Virus, if present in the specimen, bind to immobilized ZIKA virus antigens on the surface of the wells of the microtiterplate, the human antibodies bound, are then detected by specific anti-human-IgG antibodies labelled to horse radish peroxidase and subsequently revealed by the substrate/chromogen colour reaction. After stopping the colour reaction the initially blue colour turns yellow and the intensity of this yellow colour is measured photometrically (extinction, absorbance, optical density (O.D.)).The intensity of the colour reaction is proportional to the corresponding antibody content

4. PACKAGE CONTENTS (for total of 96 Tests)

Nr.	DESCRIPTION		AMOUNT	VOLUME
	Instructions for use		1	
	Adhesive foils		2	
	Bag with additional bar coded labels (1 each), to identify additional vials for the preparation of ready to use reagents (Nr.5/6, 7, 8/9, 11)		1	
	Storage bag for strips		1	
1	Microplate strips, antigen coated (12 x 8 break apart wells)	Ag SORB	1	plate
2	Negative Control, human, ready to use (light green)	CONTROL -	1	0.6 ml
3	Differential Control, human, ready to use (orange)	CONTROL - / +	1	1.2 ml
4	Positive Control, human, ready to use (red)	CONTROL +	1	0.6 ml
5	Diluent buffer (blue)	BUF ….	1	20 ml
6	Additive for diluent buffer	ADD ….	1	2 ml
7	Anti-human IgG (blue)/M (yellow)/A (red) Conjugate, RTU	CONJ IgG/M/A	1	6 ml
8/9	Substrate-Chromogen (TMB) buffer solution ready to use	SUBST TMB	1	6 ml
10	Washing solution concentrate (25x conc)	WASH 25x	1	20 ml
11	Stopping solution (sulfuric acid, max. 0.2 M, corrosive)	STOP H2SO4 < 0.2M	1	15 ml

5. COMPOSITION OF THE REAGENTS

Nr. 1 Microtiterplate wells, coated with ZIKA virus antigens.
Nr. 2 Negative Control: contains human IgG-, IgM- or IgA- antibodies in concentrations giving reactivities in the negative range of the test system. Reactivity range/ Index range: Index < 0.60. Buffer: 0.05 M PBS-Tween 20, pH 7.2. Preservatives:
< 0.10 % sodium azide und max. 0.03% Thiomerosal. Colour concentrate: E-104. E-132 max. 0.2 % v/v.
Nr. 3 Differential Control: contains human IgG-, IgM- or IgA-antibodies in concentrations giving reactivities in the borderline range of the test system. Reactivity range/ Absorbance range (OD-range): 0.080 - 1.000, Index = 1. Buffer: 0.05 M PBS-Tween 20, pH 7.2. Preservatives: < 0.10 % sodium azide und max. 0.03% thiomerosal. Colour concentrate: E-110 max. 0.1 % v/v.
Nr. 4 Positive Control: contains human IgG-, IgM- or IgA- antibodies in concentrations giving reactivities in the positive range of the test system. Reactivity range/ Index range: Index > 1.40. Puffer: 0.05 M PBS-Tween 20, pH 7.2. Preservatives: < 0.10 % sodium azide and max 0.03% thiomerosal. Colour concentrate: E-123 max. 0.1% v/v.
Nr. 5 Diluent buffer: 0.05 M PBS-Tween 20, pH 7.2. Preservatives: max. 0.05% thiomerosal. Colour: Bromphenolblue, max. 20 mg/Litre.
Nr. 6 Additive for diluent buffer: contains new born calf serum (NCS), Preservatives: < 0.10 sodium azide and max. 0.05% thiomerosal.
Nr. 7 Anti-human-IgG, IgM, IgA Peroxidase conjugate: Goat/sheep antibodies against human Ig-antibodies conjugated with horse radish peroxidase. Contains bovine serum ingredients max. 1.5 %. Preservatives: Preservatives: Proclin: max. 0.05 % and max.0.02% other isothiazolones, Colour: IgG, blue, IgM yellow, IgA red.
Nr. 8/9 Substrate-Chromogen buffer, ready to use
Nr. 10 Washing solution concentrate 25x conc, contains 200gr/Litre sodium chloride, 1.25 % Tween 20, max 0.01 % thiomerosal.
Nr. 11 Stopping solution: sulfuric acid (max. 1% v/v, < 0.2 M). Sulfuric acid is corrosive and must be handled cautiously.

6. MATERIAL REQUIRED BUT NOT SUPPLIED

Deionized water, graduated cylinders 1000 ml, 500 ml, 250 ml, 100 ml. Pipettes with a fixed or variable volume of 10, 100, 200 and 1000 microliters. 8-channel pipettes with variable volume of 100 or 200 microliters.
Additional vials (10 ml, 20 ml) for making the ready to use solutions.
Tubes with low protein absorption (e.g. polypropylene, polyethylene or glass) for making sample predilutions if necessary.
Incubator 37 °C +/- 1 °C (dry incubator, make sure to correctly seal the wells with the adhesive foil to prevent evaporation which may lead to erroneous results). Timer.

Washing device: using manual or automatic washing devices optimise settings according to the manufacturer's instructions for use, so that the validation criteria of the test are fulfilled.

Microtiter plate reader having a 450 nm filter, reading at the measuring wavelength (450 nm) and using a reference wavelength a filter for 620 to 650 nm is recommended.

Fully automated processing of the test with processing systems utilizing barcode identification is possible.

7. SAFETY MEASURES: Warnings, Precautions, Disposal

7.1 GLP-RULES should always be followed (GLP: Good Laboratory Practice).
7.2 The testkit is only to be used for in vitro diagnostic purposes and by professional staff only.
7.3 The use of protective laboratory clothes, protective hand gloves and also protective glasses during the actual manual procedure is recommended, Do not pipet by mouth.
7.4 All tested samples should be regarded as potentially infectious and should be handled accordingly. The controls have been derived from donations which have been tested for anti-HIV 1+2, anti-HCV and HBsAg on a single donor basis and have been found non-reactive. Nevertheless they should also be handled as potentially infectious.
Do not use heat inactivated test specimens.
7.5 Material of bovine origin used as ingredients in reagents, originate from countries known to be BSE-free at the time of purchase.
7.6 The controls and the additive for the diluent buffer contain < 0.1 % sodium azide and max. 0.05 % thiomerosal as preservatives. The dilution buffer contains max. 0.05 % thiomerosal as preservative.
7.7 Precautions to be considered using on vitro diagnostic devices containing sodium azide as preservative:
Sodium azide is poisonous, swallowing and contact with skin, eyes and mucous tissue is to be avoided. Sodium azide generates explosive azides with heavy metals like copper or lead. Disposing sodium azide-containing waste solutions, always rinse with enough water.
7.8 Precautions to be considered using in vitro diagnostic devices containing thiomerosal as preservative:
Thiomerosal is poisonous, swallowing and contact with skin, eyes and mucous tissue should be avoided. Although thiomerosal is also used in some vaccines as preservative in comparable concentrations, in vitro reagents containing thiomerosal should be handled cautiously.
7.9 Substrate Chromogen buffer, contain irritant and corrosive substances and should be handled cautiously. If contact with skin, eyes or mucous tissue occurs immediately rinse with enough water and consult a physician.
7.10 All waste solutions should be collected in adequate vessels containing disinfectants capable of inactivating human pathogenic viruses. Follow the corresponding manufacturer's instructions for use.
7.11 Disposal: follow the local safety guidelines and disposal laws and regulations for disposal.

8. LIMITATIONS AND CAUSES OF ERROR

-It is to be considered that under certain specific laboratory working conditions adjustment of alternative incubation periods may be necessary.
- If reagents are used too cool before reaching room temperature (20 to 25 °C), a weaker colour development will occur at the end of the test run. On the other hand, if room temperature is high (appr. 30 °C or higher), a stronger colour development will occur at the end of the test run. Under these circumstances the validation criteria of the test run may not be achieved.
-Periodically check functionality of pipettes and instruments used.
-The reagents of the testkit are not to be used after its expiry date.
-Do not use heat inactivated specimens. Avoid testing contaminated samples, strong hemolytic, icteric or lipemic samples, since erroneous results may be obtained.

-To ensure the performance of the testkit storage conditions and stability of the opened and diluted reagents must be strictly respected as depicted under storage and stability.

-Reagent No. 1, antigen coated wells, No. 2, negative control, No. 3, differential control, No. 4, positive control and No. 7, conjugate solution are lot specific and are not allowed to be used together with corresponding reagents from another lot.
-Reagent No. 5, diluent buffer, No. 6, additive for diluent buffer, No.8/9 substrate chromogen buffer with substrate, No. 10, washing solution and No. 11 stopping solution are not lot specific and may be used, if necessary and respecting the corresponding expiry date, in a test run together with corresponding lot specific reagents (Nr. 1, Nr. 2, Nr. 3, Nr. 4 and Nr. 7) from another lot.

-Avoid cross contaminations during manipulations.
-Never use the same vessel for the ready to use conjugate dilution and the ready to use substrate/chromogen solution.
-Since TMB turns blue coloured upon oxidation, any contact of the reagents No. 8, No. 9 and No. 11 with heavy metals should be avoided.

Also protect TMB solutions from direct light exposure.

9. STORAGE AND STABILITY

DESCRIPTION	STORAGE	STABILITY	COMMENTS
Closed components of the test kit	2 to 8 °C	until expiry date	
Opened Microplate strips	2 to 8 °C	6 weeks	keep storage bag tightly closed avoid high humidity
Opened components No. 2, 3, 4, 5, 6, 7	2 to 8 °C	12 weeks	avoid Temperature stress and Contamination
Opened substrate buffer (No.8)	2 to 8 °C	12 weeks	avoid direct exposure to light
Specimen diluent (No.5 + No.6), ready to use	2 to 8 °C	12 weeks	prepare only the necessary volume and avoid contamination
Chromogen/Substrate solution (No.8/9), Ready to use	2 to 8 °C	12 weeks	prepare only the necessary volume and avoid direct exposure to light
Washing solution, ready to use	2 to 8 °C	12 weeks	use only a clear solution
	20 to 25 °C	max. 2 weeks	use only a clear solution
Stopping solution	2 to 8 °C	until expiry date	

10. SPECIMEN COLLECTION AND HANDLING

Plasma or serum collected by venepuncture should be tested within 2 days if stored at 2 to 8 °C after reception. Prolonged storage should be done at –20 °C or lower. Avoid repeated thawing and freezing. Samples showing particles should be centrifuged prior to be processed, to avoid possible erroneous results. Contamination should be avoided, since contaminated samples may also lead to erroneous results. Handle all samples as potentially infectious.

11. PREPARATION OF REAGENTS AND SPECIMENS

-All necessary reagents must reach room temperature (20 to 25 °C) prior to be used.
- If reagents are used too cool before reaching room temperature (20 to 25 °C), a weaker colour development will occur at the end of the test run. On the other hand, if room temperature is high (appr. 30 °C or higher), a stronger colour development will occur at the end of the test run. Under these circumstances the validation criteria of the test run may not be achieved, and corrective measures may be necessary (prolonging or shortening the incubation period)
Sample diluent (No. 5 + No. 6): 2 ml additive (No. 6) are added to 20 ml diluent buffer (No. 5). Bring only the necessary volume of the ready to use specimen diluent to room temperature.
Washing solution, ready to use: The concentrated washing solution (No. 10) is diluted 1 in 25 with deionised water (20 ml concentrate + 480 ml deionised water), use only clear solutions.
Microplate strips and wells: Take the necessary amount of strips or wells from the bag after they have reached room temperature. Place the required strips or wells firmly in the frame, make sure they are evenly arrayed in the frame. If required fill the empty plate positions with empty wells or strips (not antigen coated) according to the pipetting or washing device used to avoid overflow of fluid during pipetting or washing steps of the test run.
Not required strips or wells (antigen coated) must be transferred into the storage bag, well-sealed, avoiding humidity and reset for storage at 2 to 8 °C.
Specimens: Specimens are tested at a 1/21 dilution. Although testing of other body fluids than serum or plasma is possible, specific adjustment of the conditions is needed.
Conjugate: always prepare only the amount of conjugate needed plus =< 0.1 ml.
Chromogen-Substrate solution: always prepare only the necessary amount of chromogen-substrate solution plus =<0.1 ml.

12 TEST PROCEDURE

The test should only be performed by properly trained professional laboratory staff.
All reagents must have reached room temperature (20 to 25 °C) prior to start performing the test run.
Incubation periods: Specimens and controls 30 min. at 37 °C, Conjugate 30 min. at 37°C and TMB-Chromogen / Substrate solution 10 to 20 min. at room temperature (20 to 25 °C). Adaptation of incubation periods to specific internal laboratory needs is possible, according to GLP they should be validated.

12.1 TEST PROCEDURE (summary)

PREPARATION OF REAGENTS / PREPARATION OF TESTPROTOCOL

SPECIMEN DILUTION: 1/21 dilution
Performing multiple testing of specimens a corresponding volume is prepared in a separate tube or plate:
0.050 ml OF THE SPECIMEN DILUTION ARE PIPETTED

Pipetting specimens directly in the plate dispense first 0.200 ml of specimen diluent and then add 0.010 ml specimen
0.200 ml OF SPECIMEN DILUENT + **0.010 ml** SPECIMEN

0.050 ml OF THE CONTROLS ARE PIPETTED

INCUBATION OF SPECIMENDILUTIONS AND CONTROLS 60 min. at 37 °C

WASH 4x

PIPETTING OF THE CONJUGATE (0.050 ml)

INCUBATION WITH CONJUGATE 30 min. at 37°C

WASH 4x

PIPETTING OF THE TMB-CHROMOGEN-SUBSTRATE SOLUTION (0.050 ml)

INCUBATION WITH TMB-CHROMOGEN-SUBSTRATE SOLUTION
(10 …20 min at room temperature, ca. 20…25 °C)

PIPETTING OF THE STOPPING SOLUTION (0.100 ml)

PHOTOMETRIC MEASUREMENT at 450nm (Ref: 630 nm)

TESTVALIDATION / EVALUATION

12.2 TESTPROCEDURE (manual)

1. **Preparation:** Before starting with the test make sure that each single reagent has reached room temperature ca. 20 to 25 °C. Check each single reagent to be use for its identity, verify the sequence of dilution and pipetting. Processing more than one strip it is recommended to identify each single strip (e.g.: 1,2,3 etc.).

2. **Test protocol:** Prepare a test protocol according to the specimen identification numbers for the dilution and pipetting sequences of the specimens and the controls to be tested. One well is assigned to the negative control, two wells to the differential control and one well to the positive control. If necessary more controls may be scheduled.

3. **SPECIMEN PREPARATION, DILUTION, PIPETTING OF SPECIMENS AND CONTROLS, INCUBATION:**

   Specimens, liquid serum or plasma, are prepared to be tested at a **1/21** dilution.
   Performing multiple testing of specimens a corresponding dilution volume is prepared in a separate tube or plate, then **0.050 ml** of the diluted specimen is pipetted into the corresponding well according to the pipetting protocol (it is recommended to pipette in replicates at the beginning to establish the own pipetting accuracy and reproducibility).
   Are the specimens directly diluted into the wells then **0.200 ml** specimen diluent are dispensed first into each well and then **0.010** ml of each specimen are added into the corresponding wells, mix well (it is recommended to pipette in replicates at the beginning to establish the own pipetting accuracy and reproducibility).
   In addition **0.050 ml** of each control are pipetted into their corresponding wells.
   Pipetting longer series of specimens it is recommended to pipette the controls after reaching half of the series to compensate for pipetting delays. The corresponding controls may also be pipetted at the beginning and at the end of a longer series, for evaluation the mean of the corresponding results is used.
   After finishing pipetting shake gently the plate, cover it with the adhesive foil and incubate for 60 min. +/- 2 min. at 37 °C +/- 1°C.

4. **Preparation:** Before finishing the first incubation (specimen incubation) period prepare the necessary volume of conjugate solution. Make sure that the solution has reached room temperature (20 to 25 °C).
   For 8 wells 0.8 + 0.1 = 0.9 ml conjugate are needed.

5. **Wash step 1 :** Remove the specimen dilutions and controls form the wells, add 0.300 ml washing solution to each well, wait 1 min. Repeat the sequence removal of the washing solution, adding washing solution and waiting 1 min. 3 times, totally 4 washing cycles. After last removal of washing solution, make sure the wells have been completely voided, eventually tap the plate upside down on absorbent paper.

6. Conjugate: **0.050 ml** of the anti-human IgG-, IgM- or IgA-PO- conjugate solution are pipetted in all wells. After finishing pipetting, cover it with the adhesive foil and incubate for 30 min. +/- 2 min. at 37 °C +/- 1°C.

7. PREPARATION: Before finishing the second incubation (conjugate incubation) prepare the corresponding volume of TMB-Chromogen/substrate solution and keep it in the dark until use. Make sure the solution has reached room temperature (20 to 25 °C).

8. Wash step 2 : Remove the conjugate solution form the wells, add 0.300 ml washing solution to each well, wait 1 min. Repeat the sequence removal of the washing solution, adding washing solution and waiting 1 min. 3 times, totally 4 washing cycles. After last removal of washing solution, make sure the wells have been completely voided, eventually tap the plate upside down on absorbent paper.
Wipe carefully the bottom of the strips from outside with absorbent paper, to remove all possible liquid residues that could interfere with photometric reading.

9. TMB-Chromogen/ Substrate Incubation: **0.050 ml** of the TMB-Chromogen/substrate solution (No. 8/9) are pipetted in all wells. After finishing pipetting, incubate the plate for 15 +/- 5 min. at room temperature (20 to 25°C) avoiding exposure to light (dark chamber, dark box, a closed drawer).
Reactive specimens develop a blue colour.

10. Reaction stop: After finishing the TMB-Chromogen/substrate incubation add **0.100 ml** Stop solution to all wells. Reactive specimens turn from blue to yellow.

11. Photometric Reading: Photometric reading should be done within 10 to 20 minutes after stopping the colour reaction with a microtiter plate photometer at 450 nm (if possible with the reference wavelength set at 630 nm) Blanking is done against air.

12. Validation: According to the validation criteria (see under Validation).

13. Evaluation: According to the evaluation criteria (see under Evaluation).

12.3 TEST PROCESSING WITH AUTOMATIC DEVICES

Test processing with automatic devices may be carried out according to the assay definition programs of the automatic device in use (e.g.: BEP® 2000, EtiMax3000, Evolis and Quickstep among others).
The assay definition program allows the bar code identification of the reagents and of the specimens and their sequential process assignment for the entire process of the test.

After defining the jobs to be done a list of the corresponding reagents needed is generated .including the necessary reagent volumes and their corresponding containers (Specimen diluent, controls, conjugate, TMB-Chromogen/Substrate solution, stop solution and wash solution).

For each single reagent needed the minimal calculated quantities have to be present in the corresponding amounts and in the corresponding bar coded vials to be processed.

The barcoded reagent vials prepared are placed in the corresponding reagents' rack for processing.
The racks containing the barcoded specimens and the racks containing the barcoded reagents can then be introduced in the processing area. During introduction of the racks barcode reading is effected and the position of each barcoded reagent and specimen registered.

After verifying the necessary amounts of reagents the plate to be processed is requested (it is possible to align a variety of different tests, the only requirement is that the different assays to be processed must have all the same single incubation periods for each incubation).

Before inserting the plate in the plate holder make sure that besides the wells needed to be processed in their corresponding positions, empty positions are filled with empty wells to prevent overflow of washing solution in the washing chamber.
After inserting the plate it is brought to the pipetting area and the assay/assays job/jobs is/are started.

The job is processed in the following way:

1. Dispensing specimen diluent (0.200 ml) in the wells assigned for the specimens.
2. Dispensing specimens in the wells assigned for specimens (0.010 ml in 0.200 ml, 1:21 dilution).
3. Dispensing the controls in the wells assigned for the controls (0.050 ml).
4. Incubation of the specimens and controls: 30 min. +/-1 min at 37 +/- 1°C.

5. Washing
6. Dispensing the conjugate (0.050 ml).
7. Incubation with conjugate: 30 min. +/- 1 min at 37 +/- 1°C.
8. Washing
9. Dispensing the TMB-Chromogen / Substrate solution (dispense a total volume of 0.050 ml).
10. Incubation with TMB-Chromogen / Substrate solution: 15 min. +/- 1 min at room temperature (20 to 25 °C).
11. Stop the reaction by adding stopping solution (0.100 ml).
12. Photometric reading in integrated photometer.
13. Results may be printed out or further transferred online. The corresponding protocols include validated and evaluated Results.

13. VALIDATION OF THE TEST, CORRECTIVE MEASURES, GENERAL CONSIDERATIONS

Validation:
Results obtained in absorbance units (extinction units, O.D. units) for the controls are used if the values of the differential control are higher than 0.080 and lower than 1.000 (optimally between 0.200 and 0.600) and the deviation of the values obtained for the differential control falls within +/- 20 % of the mean value. Additionally the corresponding index value of the negative control must be < 0.6 and the corresponding index value of the positive control must be > 1.4.
These criteria apply to all our systems.

$$\text{Index value of the controls} = \frac{\text{absorption at 450nm of the corresponding control}}{\text{Mean absorption at 450nm of the differential control}}$$

Example of a validation: mean value of the absorption of the differential control 1.value: 0.280, 2. value: 0.320

Controls	O.D.- value 450 nm	Mean value: 0.300 Index
Absorption (O.D. value) of the negative control	(Nr. 2) 0.100	0.100 / 0.300 = 0.333
Absorption (O.D. value) of the differential control	(Nr. 3) 0.280	0.280 / 0.300 = 0.933
Absorption (O.D. value) of the differential control	(Nr. 3) 0.320	0.320 / 0.300 = 1.067
Absorption (O.D. value) of the positive control	(Nr. 4) 0.600	0.600 / 0.300 = 2.000

Are the values obtained within the range of the validation criteria, then the test run is valid, and evaluation can be performed. If the validation criteria are not met, then the test is not valid and must be repeated.

Corrective measures:

Before repeating the test, the following possible corrective measures should be considered:

1) Example 1: obtaining too high an absorbance value e.g. 1.6 for the differential control, a correction factor of 0.5 can be applied to all values and the test may be revalidated. This revalidation of the test run only applies if the criteria for the Index-value of the positive control (>1.4) and for the negative control (<0.6) also apply.
Alternatively if some sample values are above absorbance 3.0 (OVER), a dilution by factor 2 (dispense additionally 0.2 ml stopping solution to each of the stopped wells mix well and then withdraw 0.2 ml from each of them, 1 in 2 dilution) can be performed on all samples to bring 'OVER' values in the measuring range of the photometer (measuring range of the photometer should be from 0 to 3).

2) Example 2: obtaining too low an absorbance value for the differential control (not under 0.060) a factor of 2 may be applied and the test run revalidated. This revalidation of the test run only applies if the criteria for the Index-value of the positive control (>1.4) and for the negative control (<0.6) also apply.
Alternatively performing the next test run the reaction time can be extended from e.g. 10 min to 20 min or to 30 min.

Should this possible corrective measures not lead to acceptable results, then the test run has to be repeated.

General considerations on peroxidase reactivity:

1) The peroxidase reaction in our systems is initially practically linear with time and starts levelling off slowly after about 10 to 20 min.
2) Therefore after a reaction time of 20 min the absorbance value of a particular sample will be approximately 2x the value after 10 min. reaction time.

3) This means practically that stopping the reaction of a given sample after 10 min. giving an absorbance value of 0.8, stopping after 20 min. will result in an absorbance value of approximately 1.6.
4) Due to the fact that the course of the reaction is practically linear during the first 10 to 20 min. for all reactivities, low and high, the proportions of the different reactivities to each other remain the same.

5) Incubations at room temperature lead to higher absorbance values at 30…35°C than at 20…25°C during the same time period (approximately 2x higher). According to our test procedure the reaction time for the Chromogen/Substrate incubation is set at 15 min. +- 5 min. for the manual procedure, this means between 10 and 20 min. reaction time.
5) It is to be considered that the reactivity of the conjugate gradually slowly decreases with time, therefore reactivities are set relatively high at the beginning to assure that the validation criteria apply over the entire stability period claimed.
6) Due to these facts it is possible to introduce a corrective factor in case that the absorbance values obtained for the differential control surpasses the upper limit value or remains under the lower limit value, not fulfilling the validation criteria, as specified under corrective measures.

14. EVALUATION

Evaluation of test results can be performed if the validation criteria apply. Evaluation of the results for each specimen is done after calculating the Index value for each single specimen. Calculation of the index value corresponds to a normalization of the results against the value obtained for the differential control in each single test run and may be assigned as a 'test reference value'. The Index value is obtained by dividing the absorption value (extinction, O.D. value) of each single specimen by the mean value of the differential control.

$$\text{Index} = \frac{\text{Absorption at 450 nm of a specimen}}{\text{Mean Absorption at 450 nm of the differential control}} \quad \text{(Index of a specimen)}$$

Index values (Test reference values) higher than 1.00 are scored reactive and indicate a presence of IgG antibodies, Index values lower than 0.90 are scored non-reactive and indicate an absence of IgG antibodies. Index values between 0.90 and 1.00 are scored questionable. For weakly reactive results it is recommended to consider a confirmatory test or to request a second specimen 10 to 14 days later to be tested in the same test run with the first specimen.

Example of a qualitative evaluation

Qualitative evaluation is done according to the reactivity of the differential control. All specimens giving Index values higher than that of the differential control are considered as reactive and all giving lower Index values are considered as non-reactive. The entire Index range may be divided in ranges with increasing reactivity and to these ranges a diagnostic meaning may be assigned. The higher the reactivity the higher the diagnostic meaning.

Mean value of the differential control: 1. value: O.D. 0.280, 2.value: O.D. 0.320, Mean value: O.D. 0.300

Specimen O.D. 450 nm	Index/ Test reference value	Index Range	Evaluation Ranges
Spec. No. 1.....0.080	0.080 / 0.300 = 0.266	< 0.900	non-reactive
Spec. No. 2.....0.280	0.280 / 0.300 = 0.933	0.900-1.000	border line
Spec. No. 3.....0.350	0.350 / 0.300 = 1.167	1.000-1.500	weakly reactive
Spec. No. 4.....0.500	0.500 / 0.300 = 1.667	1.500-2.000	reactive
Spec. No. 5.....0.700	0.700 / 0.300 = 2.333	2.000-3.000	highly reactive
Spec. No. 6.....1.000	1.000 / 0.300 = 3.333	3.000-5.000	very highly reactive

Example of a quantitative evaluation after introduction of relative units

For clinical reports quantitative results in relative units are usually requested to better assess and assign the results obtained. For this purpose the simplest way is to multiply the Index value with a simple factor and assign the new range of values a new range of units. It is to be considered that these relative units are also based on a logarithmic scale.

Example: multiplying the Index values of the specimens in above table by 10 gives the new unit values (logarithmic scale):
Relationship between O.D.values, Index values and unit values for the above mentioned results

	Spec. No.1	Spec. No.2	Spec. No.3	Spec. No.4	Spec. No.5	Spec. No.6
O.D.values:	0.080	0.280	0.350	0.500	0.700	1.000
Index values:	0.266	0.933	1.167	1.667	2.333	3.333
Units values:	2.66	9.33	11.67	16.67	23.33	33.33

Further mathematical evaluation methods of the results, like using a standard curve (with serum dilutions) as a reference, or with the help of the <one point quantification> are also possible. However it has to be kept in mind that all these additional evaluation methods use one common basic operation: calculating a reference value of the basic reactivity with at least one standard before further mathematical transformation (logarithmic, exponential, polynomial, 4 PL Model, etc.) is done to obtain the corresponding relative units.

The scales of the relative units found are also divided in reactivity ranges with increasing reactivity that can be related to an increasing probability of a diagnostic indication
In principle however all these evaluation methods operate with the same originally measured values (absorption, extinction, O.D. value) and corresponding differentiating reactivity ranges.

15. INTERPRETATION OF RESULTS

The probability to assign a diagnostic significance to a given reactivity increases with increasing absorption value, or increasing Index value or increasing value of relative units.

EXAMPLE:

Specimen O.D. 450 nm	Index	Index Range	Relative Units (e.g.: Index x 10)	Relative Units Range	Evaluation Ranges	Diagnostic Significance
Spec. No. 1.....0.080	0.266	< 0.900	2.66	< 9	non-reactive	- - -
Spec. No. 2.....0.280	0.933	0.900-1.000	9.33	9 –10	border line	- - / +
Spec. No. 3.....0.350	1.167	1.000-1.500	11.67	10 –15	weakly reactive	- / + +
Spec. No. 4.....0.500	1.667	1.500-2.000	16.67	15 –20	reactive	+ + +
Spec. No. 5.....0.700	2.333	2.000-3.000	23.33	20 –30	highly reactive	+ + + +
Spec. No. 6.....1.000	3.333	>3.000	33.33	>30	very highly reactive	+ + + + +

In general the presence of IgG antibodies indicates a past infection or vaccination. The detection of IgG antibodies during the course of an infection may indicate a current infection, if the results of a parallel determination of two specimens from the same patient, taken 10 to 14 days apart, indicate a seroconversion (conversion from negative to positive).
It is to be considered that in the early stage of a seroconversion the results obtained may still fall under the values of the differential control.

The detection of IgM antibodies during the course of an infection generally indicate a current infection.
It is to be considered that in the early stage of a seroconversion (conversion from negative to positive) the results obtained may still fall under the values of the differential control.

Borderline and weakly reactive results should be retested together with an additional sample drawn 10 to 20 days apart. If no differences in reactivity are detected no evidence for a current infection may be assigned, if clear increments in reactivity are detected, support for a current infection may be indicated.

Very high IgG reactivities may indicate the peak of the acute phase of a current infection.
Positive results with very high IgM reactivities correspond with high probability to the acute phase of a current infection.
Since in the early phase of seroconversion IgM antibodies may not yet be detectable, it is recommended to test at least two specimens drawn 10 to 14 days apart to better assess reactivity increments during the course of seroconversion.
Detection of seroconversion or increasing reactivities indicate current or recent infection.

The simultaneous detection of IgA- and a seroconversion for IgG-antibodies very strongly support a current infection.

Interpretation of serological results should always only be done together with clinical data.
Caution is recommended due to the reported flavivirus induced antibody cross reactivity

http://www.cdc.gov/mmwr/volumes/65/wr/mm6521e1.htm

Testing for IgM-antibodies rheuma-factor (RF) interferences may generate false positive results; testing for rheuma-factor, and if positive, retesting of positive specimens after RF-absorption is highly recommended.

16. EXPECTED RESULTS: REPRODUCIBILITY, PERFORMANCE CHARACTERISTICS

REPRODUCIBILITY:

Reproducibility of the results of the controls and test specimens in our test systems is calculated according to the mean of the index value (MW), the standard deviation (SA) and the variation coefficient (VK) (minimum n=4).

$$\text{Coefficient of variation (VK)} = \frac{\text{Standard deviation (SA)}}{\text{Mean (MW)}} \times 100$$

Repeated determinations of the same samples (n >= 4) in our test systems allow to define the following ranges for the coefficient of variation of a given index value:

Intraassay coefficient of variation of a given index value of a sample should be less than +- 10 %, and not greater than +- 20 %, should this occur, so it is mandatory to review the test conditions and working techniques.

Interassay coefficient of variation of a given index value of a sample should be less than +- 10 %, and not greater than +- 25 %, should this occur, so it is mandatory to review the test conditions and working techniques.

PERFORMANCE CHARACTERISICS:

Generally the prevalence of IgG antibodies in a population depends on the incidence of infection in the various subpopulations at different age groups., incidence is also dependent on the mobility of these subpopulations, on the availability of adequate vaccines, on the geographical location and last but not least on the locally given socio-economic conditions.Generally IgM- and IgA antibodies are only detected during the acute phase of infection mostly at high levels

Reference population

The expected values for IgG antibody reactivity as adjusted in our systems correspond to the expected distribution of negative and positive samples in a Swiss blood donor population.

Depending on the infectious agent the proportion of positive samples for IgG may vary between 0…10%, 10…30%, 30…50%, 50…70% and 70…90% or more.

The proportion of IgG positive samples for ZIKA virus is adjusted between approximately 1-3 % in our blood donor population.

Fig. 1 Distribution of reactivity in a blood donor population.

Blood donors CH (group 1) non endemic region negative population(n=84)	Reactive % n/N
IgG Reactive 2 (out of 84) 2.38 % 'false positive'	2.38 2/84 'Specificity' 97.62 %
IgM Reactive 2 (out of 84) 2.38 % 'false positive'	Reactive 2.38 2/84 'Specificity' 97.62 %

Legend: n / N: number of positive samples / total number of tested samples, n. d.: not done.

Fig. 2. Distribution of reactivity in an endemic population with suspected outbreak.

Endemic population (group 2) from a region With suspected outbreak (n=275)	Reactive % n/N
Suspected reactive population (n=275) **IgG** non-reactive 16, reactive 259,	Reactive 94.18 259/275 94.18 % found reactive
Suspected reactive population (n=275) **IgM** non-reactive 228, reactive 47	17.09 259/275 17.09 % found reactive

Legend: n / N: number of positive samples / total number of tested samples, n. d.: not done.

Fig. 3. Reactivity of suspected Dengue IgM positive specimens (Reference commercial Dengue IgM Test)..

Suspected Dengue IgM pos population (group 3) (n=16)	Reactive % n/N
Suspected reactive population (n=16) **IgG** non-reactive 1, reactive 15,	Reactive 93.75 15/16 93.75 % found reactive
Suspected reactive population (n=16) **IgM** non-reactive 14, reactive 2	12.5 2/16 12.5 % found reactive

Legend: n / N: number of positive samples / total number of tested samples, n. d.: not done.

Fig. 4. Reactivity of a suspected Dengue study population..

Suspected Dengue study population (group 4) (n=29)	Reactive % n/N
Suspected Dengue study population (n=29) **IgG** non-reactive 0, reactive 29,	Reactive 100 29/29 100 % found reactive
Suspected reactive population (n=29) **IgM** non-reactive 22 , reactive 7	24.14 7/22 24.14 % found reactive

Legend: n / N: number of positive samples / total number of tested samples, n. d.: not done.

Relative specificity:

As depicted in Fig. 1. A relative specificity of 97.62 % was obtained with a blood donor population from a non-endemic region for IgG and IgM.
As depicted in Fig 3 12.5% from a suspected Dengue IgM positive population (n=16) reacted with the Diacheck ELISA IgM Test. In this populatio93.75 % reacted positive.

INDIVIDUAL RESULTS
Fig. 5 Results with patient samples with Zikavirus-infection related symptoms.

Patient JML ID		JMLID 1	JMLID 2	JMLID 3	JMLID 4	JMLID 5	JMLID 6
Date of 1st Symptoms (Zika)		12.12.2015	14.12.2015	15.12.2015	14.12.2015	14.12.2015	25.02.2016
Date of sample collection		18.12.2015	18.12.2015	19.02.2016	20.02.2016	22.02.2016	27.02.2016
Samples from the same donor		collected	18.12.2015	collected	20.02.2016		
Days after 1st symptoms		6 days	4 days	66 days	68 days	70 days	2 days
in house	PCR CT	**33.3**	**33.04**	n/a	n/a	n/a	n/a
Commercial CE	ELISA IgG	**2.21**	**1.34**	**4.69**	**3.11**	**4.43**	**0.76**
Commercial CE	ELISA IgM	**0.07**	**0.38**	**0.3**	**0.26**	**0.81**	**0.13**
Diacheck RUO	ELISA IgG	**4.697**	**2.423**	**5.299**	**4.521**	**5.218**	**2.5**
Diacheck RUO	ELISA IgM	0.146	**6.354**	0.083	**6.708**	**4.396**	0.688
	IF Ab	n/a	n/a	n/a	n/a	n/a	n/a
	other	n/a	n/a	n/a	n/a	n/a	n/a
ZYKA SYMPTOMS		yes	yes	Yes	yes	yes	yes
Date of 1st Guillan-Barré Symptoms							
Fever		yes	yes	Yes	yes	yes	yes
Skin rash		yes	yes	Yes	yes	yes	yes
Joint pain		yes		Yes			yes
Myalgia		yes	yes	Yes	yes	yes	yes
Conjunctivitis		yes	yes	Yes	yes	yes	yes
Eye pain		yes	yes	Yes	yes	yes	yes
Headache/Cephalgia			yes	Yes	yes	yes	
Diarrhea			yes		yes	yes	
Patient developed GB Syndrom		no	no	No	no	no	no

Samples JMLID 2 and JMLID 4 were collected from the same donor (patient) 2 months apart.

The IgG values (Index / ratio) in the first sample are already positive with both test systems and in the second sample a higher IgG reactivity is detected, indicating a possible ongoing seroconversion.

The IgM value (Index / ratio) of the first sample is already positive with the DIACHEK Test, whereas it is still negative in the Commercial CE Test, in the second sample the IgM value of the DIACHECK Test is still positive, whereas the Commercial CE Test is again negative. The IgM peak may have been missed due to the collection window of 2 months (62 days) between these two samples. In these 2 samples no IgA reactivity could be detected.

Further The DIACHECK Test recognizes IgM reactivity in Sample JMLID 5, whereas the Commercial CE Test does not.

Relative sensitivity

A relative sensitivity including the study of several seroconversions still has to be completed.

When comparing different ELISA test systems one should always bear in mind that, obtained results, very much depend on the composition of the tested sample population and also on the characteristics of the antigen preparation used, therefore these results are only indicative for the population of samples used for this comparative testing.

