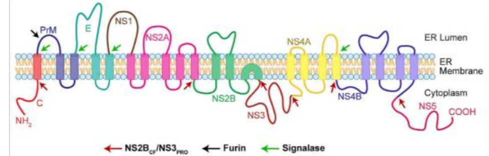
INTENDED USE

Zika Virus (ZIKV) Envelope Protein (ZENV) ELISA Kit is a sandwich immunoassay for the quantification of ZENV, recombinant or native, in cell culture, bioprocessing solutions, and or in other appropriately qualified samples. The assay is not intended for the diagnosis of Zika infection. For research use only (RUO), not for diagnosis, cure or prevention of the disease.

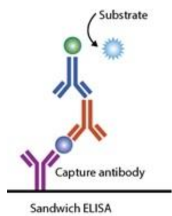
GENERAL INFORMATION

Zika virus (ZIKV), a member of the virus family Flaviviridae, transmitted by daytime-active Aedes mosquitoes, such as A. aegypti and A. albopictus. Zika virus is related to the dengue, yellow fever, Japanese encephalitis, and West Nile viruses. Like other flaviviruses, Zika virus is enveloped and icosahedral and has a non-segmented, positive-sense ss-RNA genome. There are two lineages of the Zika virus. Effective vaccines for yellow fever virus, Japanese encephalitis, and tick-borne encephalitis have been develop but there are no vaccines for Zika virus.
Zika virus genome codes for a polyprotein that is subsequently



cleaved into capsid (C), precursor membrane (prM), envelope (E), and non-structural proteins (NS). The E protein composes the majority of the virion surface and is involved with aspects of replication such as host cell binding and membrane fusion. NS1, NS3, and NS5 are large, highly-conserved proteins while the NS2A, NS2B, NS4A, and NS4B proteins are smaller, hydrophobic proteins. Like other flaviviruses, both structural and non-structural protein antibodies are detected during Zika virus infection. The member of flaviviruses share 40-60% protein sequence conservation. Moreover, vaccines have become available for JEV, YFV, and Dengue. Therefore, it is important to rule out the presence of Zika antibodies due to vaccination and/or infection from related viruses.

PRINCIPLE OF THE TEST



ZENV Envelop ELISA kit is based on the binding of ZENV in samples to two antibodies, one immobilized on the microtiter wells, and the other conjugated to biotin, which then binds to a streptavidin horseradish peroxidase (HRP) conjugate. After a washing step, chromogenic substrate is added and color is developed by the enzymatic reaction of HRP on the TMB substrate, which is directly proportional to the amount of ZENV present in the sample. Stopping Solution is added to terminate the reaction, and absorbance at 450nm is then measured using an ELISA microtiter well reader. The concentration of ZENV in samples is calculated from a standard curve of purified recombinant human ZENV of designated concentration.

Notes on sample incubation duration: For high sensitivity testing, the sample may be incubated overnight (16-20 hrs) at 2-4oC temperature. Incubation for 2-4 hours at room temp produces a little lower sensitivity (see graphs on page 5).

KIT CONTENTS

To Be Reconstituted or Diluted: Store as indicated.

Component	Instructions for Use
ZENV Standard Part No. 403003	Two (2) vials, each containing ZENV lyophilized in buffer with proteins and antimicrobial. Refrigerate lyophilized vials until used or kit lot expires.

Reconstitute 1 vial with the volume of **Working Sample Diluent** indicated on the Standard label to provide a **100 ng/ml Stock Standard**. Prepare 2-fold dilutions in the desired range, like the following; sufficient for one entire curve:

Standard	+ Diluent =	Final Concn
Stock Standard	None	100 ng/ml
320 ul of 100 ng/ml	180 ul	64 ng/ml
225 ul of 64 ng/ml	225 ul	32 ng/ml
225 ul of 32 ng/ml	225 ul	16 ng/ml
225 ul of 16 ng/ml	225 ul	8 ng/ml
225 ul of 8 ng/ml	225 ul	4 ng/ml
225 ul of 4 ng/ml	225 ul	2 ng/ml
225 ul of 2 ng/ml	225 ul	1 ng/ml
225 ul of 1 ng/ml	225 ul	0.5 ng/ml

Use the same day of testing, and/or store refrigerated for up to 2 weeks.

Component	Instructions for Use
Sample Diluent Concentrate (20x) Cat. No. SD-20T, 10ml	Dilute the entire volume, 10ml + 190ml with distilled or deionized water into a clean stock bottle. Label as **Working Sample Diluent** (**WSD**) and store at 2-8ºC until the kit lot expires or is used up.
Wash Solution Concentrate (100x) Cat. No. WB-100, 10ml	Dilute the entire volume, 10ml, to 1L with distilled or deionized water into a clean stock bottle. Label as **Working Wash Solution** and store at ambient temperature until kit is used entirely.
Anti-ZENV Detection Antibody Concentrate (100x) Part No. 403004, 0.15ml	in buffer with protein, detergents and BND as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **Working Sample Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return concentrate to 2-8ºC storage.
Streptavidin-HRP Conjugate Concentrate (100x) Part No. S-HRP100, 0.15ml	in buffer with protein, detergents and BND as stabilizers. Dilute fresh as needed; 10ul of concentrate to 1ml of **Working Sample Diluent** is sufficient for 1 8-well strip. Use within the working day and discard. Return concentrate to 2-8ºC storage.

Ready For Use: Store as indicated on labels.

Component	Part No.	Amt	Contents
Anti-Zika ENV Coated Strip Plate	403002	8-well strips (6)=48 wells	Coated with purified anti-ZENV antibodies. Return unused strips to the pouch with desiccant; re-seal and store refrigerated.
TMB Substrate	80091	12 ml	Chromogenic substrate for HRP containing TMB and peroxide.
Stop Solution	80101	12 ml	Diluted sulfuric acid.

Materials Required But Not Provided:

- Pipettors and pipettes that deliver 100ul and 1-10ml. A multi-channel pipettor is recommended.
- Disposable glass or plastic 5-15ml tubes for diluting samples and Antibody HRP Concentrate.
- Graduated cylinder to dilute Wash Concentrate; 0.2 to 1L.
- Stock bottle to store diluted Wash Solution; 200ml to 1L.
- Distilled or deionized water to dilute reagent concentrates.
- Microwell plate reader at 450 nm wavelength.

ASSAY DESIGN AND SET-UP

Sample Collection and Handling

Culture medium, bioprocessing preparations and other biological fluids may be used as samples with proper dilution to avoid solution matrix interference (See Limits of the Assay, page 6).

For all samples, clarify by centrifugation and/or filtration prior to dilution in Sample Diluent. If samples will not be assayed immediately, store refrigerated for up to a few weeks, or frozen for long-term storage.

Assay Validation

Validate the performance of the sample antigen and matrix in the assay system for recovery and parallelism (see Limits of the Assay, page 6), as follows:

Recovery – a measure of the interference of the sample matrix (diluent effect) in providing accurate quantitation of the sample ZENV relative to the Standard curve.

Prepare and run a series of dilutions of the sample antigen (concentrations that will fall within the Standard range) in Working Sample Diluent to determine the dilutions that give consistent and accurate quantitation. For most buffer solutions a minimum 5-fold sample dilution is usually sufficient.

Parallelism – dilutions of the sample should read equivalent values from the top and bottom of the Standard curve to provide good assay precision.

Prepare a dilution series of the sample antigen that gives complete recovery and falls within the full range of the Standard curve. Sample readings from the upper and lower regions of the curve should differ by less than 25%.

Plate Set-up

Bring all reagents to room temperature (18-30º C) equilibration (at least 30 minutes).

- Determine the number of wells for the assay run. Duplicates are recommended, including 10 Standard wells and 2 wells for each sample and control to be assayed.
- Remove the appropriate number of microwell strips from the pouch and return unused strips to the pouch. Reseal the pouch and store refrigerated.
- Prior to sample addition, add 200-300ul Working Wash Solution to each well and let stand for about 5 minutes.
- Aspirate or dump the liquid and pat the plate dry on a paper towel.

Assay Procedure

ALL STEPS ARE PERFORMED AT ROOM TEMPERATURE. After each reagent addition, gently tap the plate to mix the well contents prior to beginning incubation.

1. 1st Incubation **[100ul: 16 – 20 hrs (overnight) or 2 - 4 hrs; 4 washes]**

Note:
Extended sample incubation increases the sensitivity of the assay; see graphs in Calculation of Results (page 5). For overnight incubation, **cover the plate** with the Plate Sealer supplied.

- Add 100ul of standards, samples and controls each to pre-determined wells.
- Tap the plate gently to mix reagents and incubate for **2-4 h** or **overnight** (apply the plate sealer to cover the strips).
- Wash wells 4 times and pat dry on fresh paper towels. As an alternative, an automatic plate washer may be used. Improper washes may lead to falsely elevated signals and poor reproducibility.

2. 2nd Incubation **[100ul – 60 min; 4 washes]**

- Add 100ul of diluted biotin-Detection Antibody to each well.
- Shake gently for 5-10 seconds and Incubate for **60** minutes.
- Wash wells 4 times as in step 1.

3. 3rd Incubation **[100ul – 30 min; 5 washes]**

- Add 100ul of diluted Streptavidin-HRP Conj. to each well.
- Shake gently for 5-10 seconds and Incubate for 30 minutes.
- Wash wells 5 times as in step 1.

4. Substrate Incubation **[100ul – 15 min]**

- Add 100ul TMB Substrate to each well. Shake gently for 5-10 seconds. The liquid in the wells will begin to turn blue.
- Incubate for 15 minutes in the dark, e.g., place in a drawer or closet.

5. Stop Step **[Stop: 100ul]**

- Add 100ul of Stop Solution to each well.
- Tap gently to mix. The enzyme reaction will stop; liquid in the wells will turn yellow.

6. Absorbance Reading

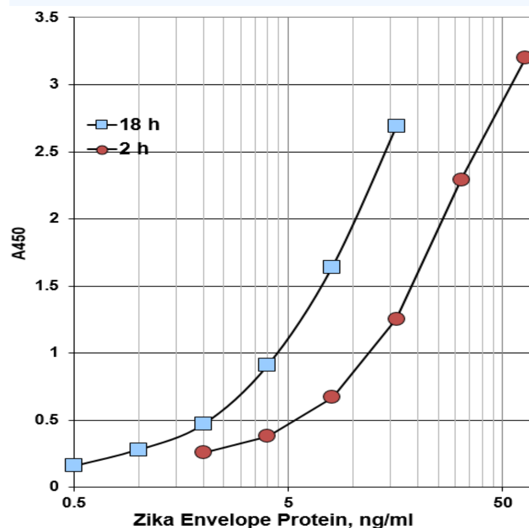
- Use any commercially available microplate reader capable of reading at 450nm wavelength. Use a program suitable for obtaining OD readings, and data calculations if available.
- Read absorbance of the entire plate at 450nm using a single wavelength within 30 minutes after Stop Solution addition. If available, program to subtract OD at 630nm to normalize well background.

CALCULATION OF RESULTS

1. The results may be calculated using any immunoassay software package. The four-parameter curve-fit is recommended. If software is not available, ZENV concentrations may be determined as follows:
2. Calculate the mean OD of duplicate samples.
3. On graph paper plot the mean OD of the standards (y-axis) against the concentration (ng/ml) of ZENV (x-axis). Draw the best fit curve through these points to construct the standard curve. A point-to-point construction is most common and reliable.
4. The ZENV concentrations in unknown samples and controls can be determined by interpolation from the standard curve.
5. Multiply the values obtained for the samples by the dilution factor of each sample.
6. Samples producing signals higher than the 16 or 64 ng/ml standard should be further diluted and re-assayed.

Typical Results:

Wells	Calibrators & Samples	Sample Incubation 18 h	2 h
		A450	
	Diluent Blank	0.04	0.09
A1, A2	0.5 ng/ml **Standard**	0.16	-
B1, B2	1 ng/ml **Standard**	0.28	-
C1, C2	2 ng/ml **Standard**	0.47	0.26
D1, D2	4 ng/ml **Standard**	0.91	0.38
E1, E2	8 ng/ml **Standard**	1.64	0.67
F1, F2	16 ng/ml **Standard**	2.69	1.26
G1, G2	32 ng/ml **Standard**	-	2.29
H1, H2	64 ng/ml **Standard**	-	3.18



PERFORMANCE CHARACTERISTICS

Sensitivity

Overnight ELISA has sensitivity of ~0.25 ng/ml and the 2 hrs incubation ~1 ng/ml.

Specificity

The antibodies used in this kit are specific for the Zika envelope protein, and do not cross-react with other Zika proteins, nor the envelope antigens from related flaviviruses (e.g., Dengue, West Nile, JEV).

Precision

Samples containing low, medium and high concentrations of ZENV were assayed as duplicates in multiple assays (n=5), using separately prepared standard curves and an 18 h initial incubation time, to obtain between-assay reproducibility. Coefficients of variation were calculated for the concentrations using a point-to-point curve-fitting program.
ZENV concentrations were measured with good between-assay (4.6 to 9.3 %CV) reproducibility.

Sample	ZENV ng/ml	Inter-assay %CV
High Concn	11.1	**9.3**
Medium Concn	5.7	**8.5**
Low Concn	2.7	**4.6**

Recovery

Serum

Recovery (2h assay)
Purified ZENV was spiked into a human, monkey and mouse serum sample, each diluted 1:10. Observed assay values compared to expected values ranged from **8.8** to **0%**, indicating poor recovery of ZENV in serum diluted to 10%.

Culture Medium

Linearity of Dilution and Recovery (18h assay)
ZENV was spiked into Sample Diluent with 10% Neonatal Bovine Serum at 4 levels, 2-16 ng/ml. The mean recovery ranged from **89** to **113%**, demonstrating linear dilution and equivalent quantification across the standard range.

LIMITS OF THE ASSAY

1. Detection and/or quantitation of ZENV in serum or plasma has not been demonstrated. Incomplete recovery of spiked ZENV in human, mouse and monkey serum (see above) suggests some alteration of ZENV in serum/plasma that alters the specific interaction of ZENV with the assay's capture and/or detection antibodies.
2. ZENV that is incomplete in sequence (truncated) or is aggregated and/or associated with other biomolecules may not produce dilution curves **parallel** with the Standard curve. For cases of non-parallelism, it may be useful to establish an alternative Standard curve using the altered ZENV preparation.
3. The 100 ng/ml Standard can be diluted to suit any incubation conditions the experiment may require.

QUALITY CONTROL

Reagents Accurate and reproducible assay results rely on proper storage, handling and control of reagent and sample temperature. Store all reagents as indicated, and warm to room temperature only those to be used in the assay. Shelf-life of the critical reagents and samples will diminish with extended exposure to non-refrigeration, resulting in inaccurate assay results. All solutions should be clear. Cloudiness or particulates are indications of reagent contamination or instability and may interfere with proper performance of the assay. Do not use.

Standard Curve The signal generated by the standards should be continuously increasing in OD from the lowest Standard to the highest Standard, with a difference greater than 1.2 OD. Non-uniform or low signals may indicate problems with technique, protocol directions and/or reagent preparation, use or stability. Do not rely on results generated from an assay with these issues.

Technique Accurate and reproducible assay results rely on good lab technique regarding pipetting, plate washing and handling of samples and reagents.

Equipment Precision of results relies on uniform and effective washing techniques; an automatic washer may be used. ELISA reader and pipettes should be properly calibrated.

STORAGE AND STABILITY

The microtiter well plate and all other reagents, if unopened, are stable at 2-8° C until the expiration date printed on the box label. Stabilities of the working solutions are indicated under Reagent Preparation.

PRECAUTIONS AND SAFETY INSTRUCTIONS

Standards, Sample Diluent, and Antibody HRP contain bromonitrodioxane (BND: 0.05%, w/v). Stop Solution contains 1% sulfuric acid. Follow good laboratory practices, and avoid ingestion or contact of any reagent with skin, eyes or mucous membranes. All reagents may be disposed of down a drain with copious amounts of water. MSDS for TMB, sulfuric acid and BND can be requested or obtained from the ADI website: http://4adi.com/commerce/info/showpage.jsp?page_id=1060&category_id=2430&visit=10

Instruction Manual No. RV-403001-ENV-48

Zika Virus (ZIKV) Envelope Protein (ZENV) ELISA Kit for vaccine testing

Cat. No. RV-403001-ENV-48, 48 Tests

For Quantitation of ZENV in vaccines, purified proteins and biological buffers

For research use only (RUO), not for diagnosis, cure or prevention of the disease.

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