Not for Sale or Distribution in the United States of America
DENGUE EARLY ELISA
Cat No. E-DEN01P

INTENDED USE
The Panbio Dengue Early ELISA is a dengue NS1 antigen capture ELISA. It is for the qualitative detection of NS1 antigen in serum, used as an aid in the clinical laboratory diagnosis of patients with clinical symptoms consistent with dengue fever. The Panbio Dengue Early ELISA should be used in conjunction with other dengue serology.

INTRODUCTION
Dengue virus is a flavivirus found largely in areas of the tropics and sub-tropics. Over half the world's population lives in regions at risk of potential dengue transmission, making dengue the most important arboviral disease in humans, in terms of morbidity and mortality[1]. There are four distinct but antigenically related serotypes of dengue viruses, and transmission is by mosquito, principally *Aedes aegypti, Aedes polynesienses* and *Aedes albopictus.*

The clinical manifestations of dengue virus infection are varied, ranging from sub-clinical through to fatal. The disease is graded according to severity as follows: non-specific febrile illness, classic dengue fever, dengue haemorrhagic fever (DHF) (grades I and II), and dengue shock syndrome (DSS) (grades III and IV)[1]. Classic dengue fever is characterised by the sudden onset of fever with two or more symptoms of: headache, retro-orbital pain, myalgia, arthralgia, rash, haemorrhagic manifestations or leukopenia[2]. A diphasic febrile course is common as is insomnia and anorexia with loss of taste or bitter taste. DHF and DSS are severe, potentially fatal complications often associated with infection by a second serotype[3].

Detection of dengue NS1 antigen by ELISA is a valuable procedure, as it allows detection of infection prior to seroconversion. NS1 antigen can be detected in serum from day 1 after onset of fever and up to day 9[4,5,6]. This compares to IgM antibodies that are not detectable until days 3-5[7,8]. Earlier diagnosis of dengue allows earlier implementation of supportive therapy and monitoring. This reduces risk of complications such as dengue haemorrhagic fever or dengue shock syndrome, especially in countries where dengue is endemic[9].

PRINCIPLE
Serum dengue NS1 antigen, when present, binds to anti-NS1 antibodies attached to the polystyrene surface of the microwell test strips (Assay Plate). Residual serum is removed from the Assay Plate by washing. HRP Conjugated Anti-NS1 MAb is added to the Assay Plate. After incubation, the microwells are washed and a colourless substrate system, tetramethylbenzidine / hydrogen peroxide (TMB/H_2O_2) is added. The substrate is hydrolysed by the enzyme and the chromogen changes to a blue colour. After stopping the reaction with acid, the TMB turns yellow. Colour development is indicative of the presence of dengue NS1 antigen in the test sample.

MATERIALS PROVIDED
1. *Anti-NS1 Antibody Coated Microwells* - **(Assay Plate)** Microwells are coated with Anti-NS1 antibodies (12 x 8 wells). Ready for use. Unused microwells should be resealed immediately and stored in the presence of a desiccant. Stable at 2-8ºC until expiry.
2. *HRP Conjugated Anti-NS1 MAb* - One bottle 15 mL **(Orange)**. Horseradish peroxidase conjugated Anti-NS1 monoclonal antibody with preservative (0.1% Proclin™). Ready for use. Stable at 2-8ºC until expiry.
3. *Wash Buffer Concentrate* - One bottle, 60 mL of 20x concentrate of phosphate buffered saline (pH 7.2-7.6) with Tween 20 and preservative (0.1% Proclin™). Crystallisation may occur at low temperatures. To correct, incubate at 37ºC until clear. Mix well. Dilute one part wash buffer concentrate with 19 parts of distilled water. Diluted buffer may be stored for one week at 2-25ºC.

4. *Serum Diluent 3* - Two bottles, 22 mL **(Brown)**. Ready for use. Tris buffered saline (pH 7.2-7.6) with preservatives (0.1% Proclin™) and additives. Stable at 2-8ºC until expiry.
5. *Tetramethylbenzidine TMB* - One bottle, 15 mL. Ready for use. A mixture of 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide in a citric-acid citrate buffer (pH 3.5-3.8). Stable at 2-8ºC until expiry.
6. *Positive Control* - One **Purple**-capped vial, 400 µL recombinant antigen (contains 0.1% Proclin™ and 50 µg/mL gentamycin sulphate). Stable at 2-8ºC until expiry.
7. *Calibrator* - One **Orange**-capped vial, 600 µL recombinant antigen (contains 0.1% Proclin™ and 50 µg/mL gentamycin sulphate). Stable at 2-8ºC until expiry.
8. *Negative Control* - One **White**-capped vial, 400 µL human serum (contains 0.1% Proclin™, 50 µg/mL gentamycin sulphate and 0.1% sodium azide). Stable at 2-8ºC until expiry.
9. *Stop Solution* - One bottle, 15 mL. Ready to use. 1M Phosphoric acid. Stable at 2-25ºC until expiry.

Proclin™ 300 is a registered trademark of Rohm and Haas Company.

X **(Xn - Harmful) Control Safety Precaution:**
Concentration of sodium azide in these components is classified as harmful and subject to the following risk phrases (R22, R32) "Harmful if swallowed" and "Contact with acids liberates very toxic gas."

ADDITIONAL MATERIALS REQUIRED BUT NOT PROVIDED
(1) Accurate adjustable micropipettors with disposable pipette tips (5-1000 µL capacity).
(2) Deionised water
(3) Microplate washing system
(4) Microplate reader with 450 nm filter
(5) Timer
(6) Graduated cylinder
(7) Flask
(8) Test tubes or microplate for dilutions
(9) Disposable serological pipettes and pipette aids

PRECAUTIONS
FOR *IN VITRO* DIAGNOSTIC USE
(i) All human source material used in the preparation of controls has been tested for antibody to human immunodeficiency virus 1 & 2 (HIV 1&2), hepatitis C (HCV) as well as hepatitis B surface antigen and found to be negative. However no test method can offer complete assurance and all human controls and antigen should be handled as potentially infectious material. The Centers for Disease Control and Prevention and the National Institutes of Health recommend that potentially infectious agents be handled at Biosafety Level 2[10].
(ii) This test should be performed on serum only. The use of whole blood, plasma or other specimen matrix has not been established.
(iii) Icteric or lipaemic sera, or sera exhibiting haemolysis or microbial growth should not be used.
(iv) Do not heat-inactivate sera.
(v) All reagents must be equilibrated to room temperature (20-25ºC) before commencing the assay. The assay will be affected by temperature changes. Do not remove microwells from closed bag until they have reached room temperature (20-25ºC).
(vi) Dispense reagents directly from bottles using clean pipette tips. Transferring reagents may result in contamination.
(vii) Unused microwells should be resealed immediately and stored in the presence of desiccant. Failure to do this may cause erroneous results.

(viii) Substrate System:
   (a) As TMB is susceptible to contamination from metal ions, do not allow the substrate system to come into contact with metal surfaces.
   (b) Avoid prolonged exposure to direct light.
   (c) Some detergents may interfere with the performance of the TMB.
   (d) The TMB may have a faint blue colour. This will not affect the activity of the substrate or the results of the assay.

⚠ **WARNING**

(ix) Some kit components contain sodium azide, which may react with lead or copper plumbing to form highly explosive metal azide compounds. When disposing of these reagents through plumbing fixtures, flush with a large volume of water to prevent azide build-up in drains.

(x) Sodium azide inhibits conjugate activity. Clean pipette tips must be used for the conjugate addition so that sodium azide is not carried over from other reagents.

FOR FURTHER SAFETY INFORMATION PLEASE REFER TO THE MATERIAL SAFETY DATA SHEET (MSDS) AVAILABLE FROM PANBIO.

SPECIMEN COLLECTION AND PREPARATION

Blood obtained by venipuncture should be allowed to clot at room temperature (20-25ºC) and then centrifuged according to the Clinical and Laboratory Standards Institute (CLSI) (Approved Standard - Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture, H3-A5, 2003).

The serum should be separated as soon as possible and refrigerated (2-8ºC) or stored frozen (-20ºC) or colder if not tested within two days. Self-defrosting freezers are not recommended for storage. The use of icteric sera or sera exhibiting haemolysis, lipaemia or microbial growth is not recommended. The CLSI provides recommendations for storing blood specimens, (Approved Standard - Procedures for the Handling and Processing of Blood Specimens, H18-A3, 2004).

TEST PROCEDURE

Note: Ensure all reagents are equilibrated to room temperature (20-25ºC) before commencing assay. Performing the assay outside the time and temperature ranges provided may produce invalid results. Assays not falling within the established time and temperature ranges must be repeated.

Control and Sample Predilution
(i) Remove the required number of microwells from the foil sachet and insert into strip holder. Five microwells are required for Positive Control (P), Negative Control (N) and Calibrator (CAL) in triplicate. Ensure the remaining unused microwells are resealed tightly in the foil sachet in the presence of desiccant.
(ii) Using suitable test tubes or a microtitre plate, dilute the Positive Control, Negative Control, Calibrator, and patient samples. Add 135 µL Serum Diluent to 15 µL of sample. Mix well. **The final dilution of the sample is 1 in 10.**

⚠ **CAUTION**
   Do not vortex controls. Controls contain glycerol. Ensure proper mixing of diluted controls. Mix well by inversion or gentle pipetting. Vortexing is not effective.

ELISA PROCEDURE
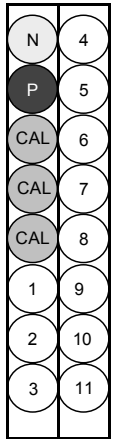
(i) Pipette 100 µL diluted test samples and controls into their respective microwells.
(ii) Cover plate and incubate for 1 hour at 37ºC±1ºC.
(iii) Wash six (6) times with diluted Wash Buffer (refer to washing procedure below).
(iv) Pipette 100 µL HRP Conjugated Anti-NS1 MAb into each well.
(v) Cover plate and incubate for 1 hour at 37ºC±1ºC.
(vi) Wash six (6) times with diluted Wash Buffer (refer to washing procedure below).
(vii) Pipette 100 µL of TMB into each well.
(viii) Incubate for 10 minutes at room temperature (20-25ºC), timing from the first addition. A blue colour will develop.

(ix) Pipette 100 µL of Stop Solution to all wells in the same sequence and timing as the TMB addition. Mix well. The blue colour will change to yellow.
(x) Within 30 minutes read the absorbance of each well at a wavelength of 450 nm with a reference filter of 600-650 nm.
Note: If a dual wavelength spectrophotometer is available, set the reference filter between 600-650 nm. Reading the microwells at 450 nm without a reference filter may result in higher absorbance values due to background.

WASHING PROCEDURE

Efficient washing to remove uncomplexed sample or components is a critical requirement of the ELISA procedure.

A. Automated Plate Washer
(1) Completely aspirate all wells.
(2) Fill all wells to rim (350 µL) during wash cycle.
(3) On completion of 6 washes, invert plate and tap firmly on absorbent paper towel to ensure all wash buffer is removed.
(4) Automated plate washers must be well maintained to ensure efficient washing. Manufacturer's cleaning instructions should be followed at all times.
B. Manual Washing
(1) Discard contents of plate in appropriate waste container.
(2) Fill wells with wash buffer using a suitable squeeze bottle. Avoid bubbling of wash buffer as this may reduce wash efficiency. Discard wash buffer from wells immediately.
(3) Refill wells with wash buffer and discard immediately.
(4) Repeat step (3) another four times. This will make a total of six washes with wash buffer.
(5) After the final wash, discard contents of wells and tap the plate on absorbent paper towel to ensure all wash buffer is removed.

QUALITY CONTROL

Each kit contains Calibrator, Positive and Negative Controls. Acceptable values for these controls are found on the accompanying specification sheet. The negative and positive controls are intended to monitor for substantial reagent failure. The positive control will not ensure precision at the assay cut-off. The test is invalid and should be repeated if the absorbance readings of either the controls or the calibrator do not meet the specifications. If the test is invalid, patient results cannot be reported.

Quality Control (QC) requirements must be performed in conformance with local, state, and/or federal regulations or accreditation requirements and your laboratory's standard QC procedures.

It is recommended that the user refer to CLSI C24-A and 42 CFR 493.1202(c) for guidance on appropriate QC practices.

CALCULATIONS

> **IMPORTANT NOTE: The calibration factor is batch specific and is detailed in the specification sheet. Obtain the calibration factor value before commencing calculations.**

(1) Calculate the average absorbance of the Calibrator triplicates and multiply by the calibration factor. This is the Cut-off Value.
(2) An index value can be calculated by dividing the sample absorbance by the Cut-off Value (calculated in step (1) above).
Alternatively,
(3) Panbio Units can be calculated by multiplying the index value (calculated in step (2) above) by 10.

Index Value = $\dfrac{\text{Sample Absorbance}}{\text{Cut-off Value}}$

   Example: Sample A Absorbance = 0.949
             Sample B Absorbance = 0.070

             Mean absorbance of Calibrator = 0.802
             Calibration Factor = 0.62
             Cut-off Value = 0.802 x 0.62 =0.497
   Sample A (0.949/0.497) = 1.91 Index Value
   Sample B (0.070/0.497) = 0.14 Index Value

Panbio Units = Index Value X 10
   Sample A 1.91 X 10 = 19.1 Panbio Units
   Sample B 0.14 X 10 = 1.4 Panbio Units

INTERPRETATION OF RESULTS

The cut-off has been determined using endemic and non-endemic negative populations. The cut-off was determined using a population from Australia, Honduras and Thailand. This population consisted of 43 characterised NS1 positive samples, 144 negative samples from non-endemic blood donors and 69 negative samples from individuals residing in dengue endemic regions.

Diagnosis of Dengue Infection: The Panbio Dengue Early ELISA assesses the presence of dengue NS1 antigen in a patient's serum. A positive result (>11 Panbio Units) is indicative of either an active primary or secondary dengue infection. If differentiation between primary and secondary infection is required, the Panbio Dengue Duo IgM Capture and IgG Capture ELISA (E-DEN01D) should be used.

INDEX	PANBIO UNITS	RESULT
<0.9	<9	Negative
0.9 – 1.1	9 - 11	Equivocal
>1.1	>11	Positive

RESULT	INTERPRETATION
Negative	No detectable dengue NS1 antigen. The result does not rule out dengue infection. An additional sample should be tested with serology in 7-14 days.
Equivocal	Equivocal samples should be repeated. Samples that remain equivocal after repeat testing should be repeated by an alternative method or another sample should be collected.
Positive	Presence of detectable dengue NS1 antigen. Dengue serology assays should be performed on follow-up samples to confirm dengue infection.

The following is a recommended way of reporting the results obtained: "The following results were obtained with the Panbio Dengue Early ELISA. Values obtained with different methods may not be used interchangeably. The magnitude of the measured result, above the cut-off, is not indicative of the total amount of antigen present." The result should be reported as positive, negative or equivocal, and not as a numerical value.

TEST LIMITATIONS

1. The clinical diagnosis must be interpreted with clinical signs and symptoms of the patient. The results from this kit are not by themselves diagnostic and should be considered in association with other clinical data and patient symptoms.

2. Screening of the general population should not be performed. The positive predictive value depends on the likelihood of the virus being present. Testing should only be performed on patients with clinical symptoms or when exposure is suspected.

3. Serological cross-reactivity across the flavivirus group is common (i.e. between dengue 1, 2, 3 & 4, Murray Valley encephalitis, Japanese encephalitis, Yellow fever and West Nile viruses). These diseases must be excluded before confirmation of diagnosis.

4. The performance characteristics have not been established for visual result determination.

5. All sera demonstrating a positive result by the Panbio Dengue Early ELISA test should be referred to a reference laboratory for confirmation of IgM positivity and epidemiological recording.

6. The Panbio Dengue Duo IgM Capture and IgG Capture ELISA (E-DEN01D) is most convenient for IgM and IgG determination. It uses the capture method for both the IgG and the IgM ELISA, so both IgM and IgG are determined using a common method and common serum dilution. It can also be used for presumptive differentiation between primary and secondary dengue infection.

EXPECTED VALUES

The dengue NS1 antigen is only detected in patient serum early in the course of disease, between days 1-9 after onset of clinical signs. Once anti-NS1 IgG antibodies are produced (generally corresponding to defervescence) NS1 is no longer detectable in serum. The Panbio Dengue Early ELISA is therefore a marker of acute active infection only. Outside of this window, dengue infection must be diagnosed using alternative serological assays.

Primary dengue infection is characterised by the presence of significant or rising levels of IgM 3-5 days after the onset of infection, which can persist for 3-5 months. Secondary infection is characterised by elevation of specific IgG 1-2 days after the onset of infection and in the majority of cases (>70%) is accompanied by elevation of IgM. In early infections and some secondary infections detectable levels of IgM antibodies may be low. Some patients may not produce detectable levels of antibody within the first seven to ten days after infection. The Panbio Dengue Early ELISA however, helps detect early presence of antigen in serum. Where symptoms persist, it is recommended that patients be re-tested serologically seven days after the first specimen.

PERFORMANCE CHARACTERISTICS

Two hundred and fifty six (256) retrospective sera from individuals of various ages and both genders were tested on the Panbio Dengue Early ELISA as part of an in-house study. The sera included samples from the following groups: 69 endemic negative specimens from Thailand, 144 non-endemic negative specimens from Australia and the USA and 43 positive specimens from patients in Honduras and Thailand with early dengue infection. These samples were masked and tested on Panbio Dengue Early ELISA. The results were compared as the positive and negative agreement to a commercially available dengue NS1 antigen detection assay. The data is summarised in Table 1.

Table 1
Sensitivity and Specificity of Panbio Dengue Early ELISA

Dengue Status	Positive	Equivocal[a]	Negative	Total
Positive (+) by commercial ELISA	39	0	4	43
Endemic Negative Normal Patients (-) by IgG/IgM ELISA	0	1	68	69
Non-endemic Negative Normal Patients (-) by IgG/IgM ELISA	0	0	144	144
Total	**39**	**1**	**216**	**256**

                                                          95% CI*
Patients with early dengue infections
Positive Presumptive
Agreement   = 39/43   = 90.7%   77.9 – 97.4%

Normal Patients (Endemic and Non-Endemic)
Negative Presumptive
Agreement[b]   = 212/212   = 100.0%   98.3 – 100.0%

Total Agreement   = 251/255   = 98.4%   96.0 – 99.6%

*Confidence Interval.
[a] Retesting of equivocal sample was not conducted.
[b] Equivocal not included as retesting was not conducted.

REPRODUCIBILITY

The reproducibility of the Panbio Dengue Early ELISA was established by testing 8 samples three times each on three kit batch numbers on three different days. Within-run, between day, between batch and total precision were estimated by analysis of variance (ANOVA Type II) and are presented in Table 2.

Table 2
Panbio Dengue Early ELISA
Precision Measures (Using Index Value*)

Sample	n	*Mean	Within		Between Day		Between Batch		Total	
			*SD	CV	*SD	CV	*SD	CV	*SD	CV
Positive	27	2.25	0.11	5.0%	0.03	1.2%	0.36	16.1%	0.32	14.3%
Calibrator	27	1.28	0.05	4.0%	0.00	0.0%	0.05	3.7%	0.06	4.9%
Negative	27	0.63	0.06	8.9%	0.00	0.0%	0.04	6.5%	0.07	10.4%
#1	27	5.34	0.24	4.4%	0.08	1.5%	0.48	9.0%	0.47	8.8%
#2	27	5.72	0.22	3.8%	0.08	1.4%	0.57	9.9%	0.52	9.1%
#3	27	2.51	0.15	5.8%	0.00	0.0%	0.22	8.7%	0.23	9.2%
#4	27	6.64	0.39	5.9%	0.31	4.7%	0.53	8.0%	0.65	9.8%
#5	27	1.79	0.13	7.3%	0.07	3.7%	0.20	11.2%	0.22	12.2%
#6	27	1.53	0.12	8.1%	0.00	0.2%	0.11	7.5%	0.16	10.2%
#7	27	0.49	0.05	10.4%	0.00	0.0%	0.05	10.0%	0.06	13.0%
#8	27	0.52	0.09	17.1%	0.00	0.0%	0.03	5.6%	0.09	17.4%

All values are calculated from Index values (Cut-off using OD)
SD= Standard Deviation; CV= Coefficient of Variation

Note: Standard Deviation results have been rounded to two decimal places for tabulation purposes.

*Index value is calculated by dividing the sample absorbance by the cut-off value.

CROSS-REACTIVITY

A panel of 95 specimens from patients with confirmed diseases other than dengue was tested to establish the analytical specificity of the Panbio Dengue Early ELISA. The specimens were from patients with diseases that have potential for cross-reactivity. Each of the specimens included in the study was characterised with respect to disease diagnosis prior to analysis with the Panbio Dengue Early ELISA. Minimal cross-reactivity was observed across 95 specimens. Refer to Table 3 for a summary of results.

Table 3
Cross-reactivity Analysis-
Panbio Dengue Early ELISA

Disease Type*	Total Specimens	Positive Result
Epstein-Barr virus	14	0/14
Malaria	10	0/10
Anti-nuclear antibody	12	0/12
Rheumatoid factor	11	1/11
Hepatitis A	10	0/10
Leptospirosis	13	0/13
West Nile virus	10	0/10
Scrub typhus	6	0/6
Japanese encephalitis	9	1/9
Total	**95**	**2/95**

*Characterisation based on serology.

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DENGUE EARLY ELISA
E-DEN01P

> ⚠️**CAUTION**:
> **Do not vortex controls. Mix well by inversion or gentle pipetting.**

◆ — Antigen

1. Add 135 µL of Serum Diluent to 15 µL of each sample and the controls. Final dilution is 1 in 10.

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2. Add 100 µL of diluted samples and controls to assay plate.

ASSAY PLATE

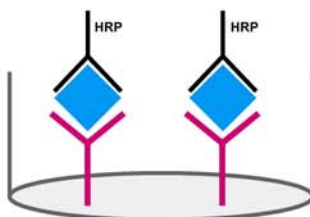Antigen
α-NS1 antibody

⬇
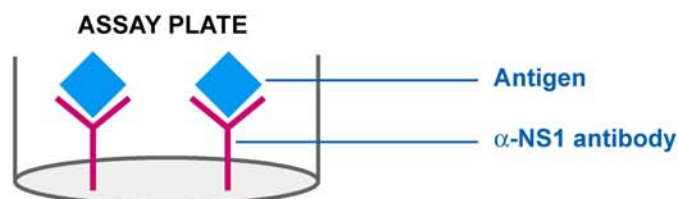
3. Cover plate and incubate for 1 hour at 37°C ± 1°C.

⬇

4. Wash the assay plate six times (x 6).

⬇

5. Add 100 µL HRP Conjugated Anti-NS1 MAb into each well on the assay plate.

HRP   HRP

⬇

6. Cover plate and incubate for 1 hour at 37°C ± 1°C.

⬇

7. Wash the assay plate six times (x 6). After the final wash, add 100 µL TMB per well and incubate at room temperature (20 - 25°C) for 10 minutes. Stop the reaction with 100 µL Stop Solution and read at 450 nm (Reference 600 - 650 nm).