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DENGUE IgG INDIRECT ELISA

Cat No. E-DEN01G

INTENDED USE
The Panbio Dengue IgG Indirect ELISA is for the qualitative detection of IgG antibodies to dengue antigen serotypes (1, 2, 3 and 4) in serum, as an aid in the clinical laboratory diagnosis of patients with clinical symptoms and past exposure consistent with dengue fever. It may also be used to distinguish between primary and secondary dengue infection.

SUMMARY AND EXPLANATION
The dengue group of arboviruses is transmitted by mosquito, principally *Aedes aegypti* and *Aedes albopictus*. Dengue infection, associated with febrile disease, is characterised by the sudden onset of fever, intense headache, myalgia, arthralgia and rash. Severe complications of dengue infection include dengue haemorrhagic fever and dengue shock syndrome. An increased prevalence of such complications is associated with secondary infection with a different dengue serotype.

Detection of specific antibody of the IgG class to the four dengue serotypes, by ELISA, is valuable for the diagnosis of previous exposure to dengue. The presence of rising levels of IgG in paired sera is suggestive of active dengue infection. Traditionally, haemagglutination-inhibition (HAI) titres have been used to classify infections as primary or secondary. The current definition depends upon an assay of paired serum specimens separated in time by at least 7 days, though any acute specimen with a HAI titre ≥1:2560 is defined as coming from a patient with secondary flavivirus infection[1]. Similarly, a value of > 4 times the cut-off serum value in the Panbio Indirect ELISA can be used to distinguish between primary and secondary dengue infection.

PRINCIPLE
Serum antibodies, when present, combine with a combination of the dengue antigens attached to the polystyrene surface of the microwell test strips. Residual serum is removed by washing and peroxidase conjugated anti-human IgG is added. 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 becomes yellow. Colour development is indicative of the presence of dengue IgG antibodies in the test sample.

MATERIALS PROVIDED
1. *Dengue Antigen (Serotypes 1, 2, 3, 4) Coated Microwells* - (12x8 wells). Ready for use. Unused microwells should be resealed immediately and stored in the presence of the desiccant. Stable at 2-8ºC until expiry.
2. *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 2-25ºC.
3. *Serum Diluent* - Two bottles, 50 mL **(Pink)**. Ready for use. Tris buffered saline (pH 7.2-7.6) with preservatives (0.1% Proclin™) and additives. Stable at 2-8ºC until expiry.
4. *HRP Conjugated Anti-Human IgG* - One bottle, 15 mL **(Green)**. Ready for use. Horseradish peroxidase conjugated sheep anti-human IgG with preservative (0.1% Proclin™) and protein stabilisers. 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 Serum* - One **Red**-capped vial, 200 µL human serum (contains 0.1% sodium azide and 0.005% gentamycin sulphate). Stable at 2-8ºC until expiry.

7. *Calibrator Serum* - One **Yellow**-capped vial, 400 µL human serum (contains 0.1% sodium azide and 0.005% gentamycin sulphate). Stable at 2-8ºC until expiry.
8. *Negative Control Serum* - One **Green**-capped vial, 200 µL human serum (contains 0.1% sodium azide and 0.005% gentamycin sulphate). Stable at 2-8ºC until expiry.
9. *Stop Solution* - One bottle, 15 mL. Ready for use. 1M Phosphoric acid. Stable at 2-25ºC until expiry.

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(Xn - Harmful) Control and Calibrator Sera 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 microtitre plate for serum dilutions

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-coated wells 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 the Biosafety Level 2[2].

(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-A4, 1998).

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-A2, 1999).

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.

ELISA PROCEDURE
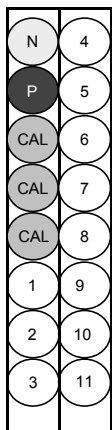
(i) Remove the required number of microwells from the foil sachet and insert into strip holder. Five microwells are required for Negative Control, Positive Control and Calibrator in triplicate. Ensure the remaining unused microwells are sealed tightly in the foil sachet.

(ii) Using suitable test tubes or a microtitre plate, dilute the Positive Control (P), Negative Control (N), Calibrator (CAL), and patient samples.

    (a) To 10 µL serum add 1000 µL of serum diluent. Mix well. **Alternatively,**

    (b) To 10 µL serum add 90 µL of serum diluent. Take 20 µL of the diluted serum and add 180 µL serum diluent. Mix well.

(iii) Pipette 100 µL of diluted patient sample, controls and calibrator sera into their respective microwells.

(iv) Cover plate and incubate 30 minutes at 37ºC ± 1ºC.

(v) Wash six (6) times with diluted wash buffer (refer to washing procedure below).

(vi) Pipette 100 µL HRP conjugated anti-human IgG to each well.

(vii) Cover plate and incubate for 30 for minutes at 37ºC ± 1ºC.

(viii) Wash six (6) times with diluted wash buffer (refer to washing procedure below).

(ix) Pipette 100 µL TMB into each well.

(x) Incubate 10 minutes at room temperature (20-25ºC), timing from the first addition. A blue colour will develop.

(xi) 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.

(xii) 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.

CALCULATIONS

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

(1) Calculate the average absorbance of the triplicates of the Calibrator 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

QUALITY CONTROL

Each kit contains Calibrator, Positive and Negative Control sera. Acceptable values for these sera 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.

INTERPRETATION OF RESULTS

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

RESULT	INTERPRETATION
Negative	No evidence of a past dengue infection. If a recent dengue infection is suspected this can be confirmed by testing a further specimen 7-14 days later.
Equivocal	Suggest samples should be re-tested. If specimen remains equivocal following repeat testing then the specimen may be tested by an alternate method or another patient specimen obtained and tested.
Positive	Presence of detectable IgG antibodies indicates evidence of past or recent dengue infection.

The following is a recommended method for reporting the results obtained: "The following results were obtained with the Panbio Dengue IgG Indirect 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 antibody present." The result should be reported as positive, negative or equivocal and not as a numerical value.

EXPECTED VALUES AND 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. 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 (dengue 1, dengue 2, dengue 3, dengue 4, Japanese encephalitis, West Nile virus, Murray Valley encephalitis, etc) is common at the level of IgG[5,6,7]. Trials in South East Asia have shown that 90% of patients with Japanese encephalitis (n=20) had an IgG Indirect result of > 10 Panbio Units, and 25% of these patients demonstrated a result >40 Panbio Units.
4. The cut-off has been determined using a local population. Population seroepidemiology may vary over time in different geographical regions. Consequently, the cut-off may require adjustment based on local studies.
5. The performance characteristics have not been established for visual result determination.
6. All sera indicating acute infection by the ELISA screening test should be referred to a reference laboratory for confirmation of positivity and epidemiological recording.

PERFORMANCE CHARACTERISTICS

Site 1:

Clinical trials at an army base in Bangkok and an Australian university showed excellent correlation between the Panbio IgG Indirect ELISA and in-house haemagglutination-inhibition (HAI) assays. In the Australian university trial, 977 of 997 sera (98%) showed agreement in the two assays, while in the army trial an IgG result of 40 Panbio Units correlated with a HAI titre of 1:2560, the cut-off used by the army to distinguish between primary and secondary dengue[3]. Consequently, a result of 40 Panbio Units in the Panbio IgG Indirect ELISA could be used to distinguish between primary and secondary dengue, as no patients with primary dengue (n=30) and the majority with secondary dengue (46/48 or 96%) showed a result >40 Panbio Units.

Site 2:

386 retrospective sera characterised by Haemagglutination Inhibition (HAI) and ELISA methods were tested on the Panbio Dengue IgG Indirect ELISA. The sera included samples from the following groups: 108 seronegative samples, 84 primary samples, 94 secondary samples and 100 endemic samples. These sera were tested on the Panbio Dengue IgG Indirect ELISA and the results were compared relative to the dengue serological status to determine the sensitivity, specificity, and agreement of the assay. The data is summarised in Table 1.

Sera characterised by HAI at a university in Malaysia were further analysed to determine the Panbio Dengue IgG ELISA ability to distinguish between primary and secondary infection in sera identified as positive in the ELISA diagnosis. Sera were characterised by the university using a HAI titre ≥ 1:1280, based on WHO criteria[4], to distinguish between primary and secondary infection. Panbio Units of >40 were used to detect secondary infection and Panbio Units between >11-40 were used to identify primary infection. The results are summarised in Table 2.

Table 1
Serological Sensitivity and Specificity of
Panbio ELISA versus Dengue Status

Dengue Status	Panbio ELISA			
	Positive	Equivocal[a]	Negative	Total
Seronegative	0	0	108	108
Primary	28	5	51	84
Secondary	92	0	2	94
Endemic	62	1	37	100
Total	182	6	198	386

			95% CI*
Serological Sensitivity (Secondary)	= 92/94	= 97.9%	92.5 – 99.7%
Serological Sensitivity (Endemic)	= 62/100	= 62.0%	51.8 – 71.5%
Serological Specificity (Negatives)	= 108/108	= 100.0%	96.6 – 100.0%
Primary Serological Agreement	= 262/302	= 86.8%	82.9 – 90.6%

[a]Retesting of equivocal samples was not conducted, as the samples were unavailable.
*Confidence Interval

Table 2
Serological Diagnosis of
Panbio ELISA Positivity versus Dengue Status

Dengue Status	Panbio ELISA			
	PBU 9-11 (Equivocal)	PBU >11-40	PBU >40	Total Positive
Primary Titre <640	3	16	4	23
Secondary Titre ≥1280	0	2	90	92
Total	3	18	94	115

PBU = Panbio Units

REPRODUCIBILITY

The reproducibility of the Panbio Dengue IgG Indirect ELISA kit was determined by testing 8 sera 3 times each on three Panbio 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 3.

Table 3
Panbio Dengue IgG Indirect Study
Precision Measures (Using Index Value*)

Sample	n	*Mean	Within		Between Day		Between Batch		Total	
			*SD	CV	*SD	CV	*SD	CV	*SD	CV
#1	27	3.51	0.28	7.9%	0.00	0.0%	0.45	12.8%	0.46	13.2%
#2	27	5.33	0.24	4.5%	0.10	1.9%	0.79	14.8%	0.70	13.2%
#3	27	2.79	0.17	6.0%	0.00	0.0%	0.36	13.0%	0.34	12.3%
#4	27	1.74	0.11	6.1%	0.04	2.3%	0.34	19.3%	0.30	17.3%
#5	27	1.41	0.13	8.9%	0.05	3.9%	0.12	8.3%	0.17	11.7%
#6	27	1.71	0.17	9.9%	0.03	2.0%	0.08	4.5%	0.18	10.8%
#7	27	0.68	0.17	20.8%	0.08	11.9%	0.00	0.0%	0.16	23.0%
#8	27	0.70	0.05	6.4%	0.08	10.7%	0.04	5.8%	0.08	12.0%

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 63 specimens from patients with confirmed diseases other than dengue fever was tested to establish the analytical specificity of the Panbio Dengue IgG Indirect ELISA. The specimens were from patients with diseases that have the 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 IgG Indirect ELISA. No cross-reactivity was observed for any of the 63 specimens tested from the disease panel. Refer to Table 4 for a summary of the results.

Table 4
Cross-reactivity Data –
Panbio Dengue IgG Indirect ELISA

Disease Type	Total Specimens	Positive Result
Rubella	8	(0/8)
Barmah Forest virus	10	(0/10)
Scrub typhus	10	(0/10)
Anti-nuclear antibody	10	(0/10)
Rheumatoid factor	10	(0/10)
Ross River virus	10	(0/10)
West Nile virus	5	(2/5)
Total	**63**	**(2/63)**

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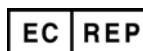
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