

Diagnostics Evaluation Series

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Evaluation of commercially available anti-dengue virus immunoglobulin M tests

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Evaluation of commercially available anti-dengue virus immunoglobulin M tests

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Abbreviations

AFRIMS	Armed Forces Research Institute of Medical Sciences
CDC	Centers for Disease Control
CI	confidence interval
DENV	dengue virus
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
ERC	ethical review committee
GCLP	good clinical laboratory practice
HRP	horse radish peroxidase
IgG	immunoglobulin G
IgM	immunoglobulin M
MAb	monoclonal antibody
MAC-ELISA	IgM antibody capture ELISA
OD	optical density
PCR	polymerase chain reaction
PDVI	Pediatric Dengue Vaccine Initiative
QA	quality assurance
RDT	rapid diagnostic test
RFA	request for application
RNA	ribonucleic acid
SE	sensitivity
SPC	specificity
TDR	Special Programme for Research and Training in Tropical Diseases
WHO	World Health Organization

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Accessible Quality-Assured Diagnostics is a unit within the UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) aimed at promoting and facilitating the development, evaluation and deployment of diagnostic tools appropriate for use in developing countries.



Pediatric Dengue Vaccine Initiative (PDVI) was established to raise awareness and work with public and private partners in the North and South to accelerate the development and introduction of dengue vaccine(s) that are affordable and accessible to poor children in endemic countries.

Objectives

- 1** To assess the performance and operational characteristics of commercially available IgM antibody detection tests for the diagnosis of dengue.
- 2** To provide data on test performance to WHO member states
- 3** To provide advice on the inclusion of tests in the WHO Bulk Procurement Scheme

Background

The arthropod-borne flavivirus dengue virus (DENV) is found mostly in tropical and subtropical regions. Four distinct serotypes (DENV 1-4) cocirculate in many of the dengue-endemic regions of the world. Approximately 2.5 billion people live in areas at risk for acquiring dengue. Of an estimated 50 million infections annually, around 500 000 cases (of which a high proportion are children) are hospitalized with dengue haemorrhagic fever (DHF), a more severe form of the disease.

DENV infection can produce a broad spectrum of symptoms that range from mild febrile illness to severe disease. Clinical features are often nonspecific and therefore require laboratory confirmation, especially for surveillance and outbreak investigations. Virus isolation provides the most convincing evidence of infection, but facilities for culture are not always available. Detection of virus-specific RNA, by nucleic acid amplification methods such as polymerase chain reaction (PCR), provides accurate diagnosis but requires expensive reagents and equipment, laboratory infrastructure, and well-trained staff. Stringent quality control is necessary to avoid false positive results due to contamination.

Serological assays that can detect virus-specific immunoglobulin M (IgM) or immunoglobulin G (IgG) antibodies to DENV are widely available and can provide an alternative to virus isolation or PCR to support the diagnosis of dengue fever. During infection, IgM antibodies can usually be detected approximately five days after onset of fever. However, serum specimens may be negative for these antibodies if collected too early. First-time (primary) DENV infections typically have a stronger and

more specific IgM response; subsequent (secondary) infections show a weaker IgM response but a strong anti-DENV IgG response. These differing IgM response patterns to infection underscore the need to evaluate the sensitivity and specificity of commercially available tests, especially for diagnosis of secondary DENV infections. A WHO/TDR and PDVI Joint Workshop on Dengue Diagnostics and Dengue Classification/Case Management was held in Geneva in October 2004. Participants reviewed the range of diagnostic options for case management and control of dengue infection. An inventory of antigen and antibody detection tests for the diagnosis of dengue was developed by TDR. The group agreed that there is an urgent need to evaluate the performance of commercially available dengue diagnostic tests and established a PDVI-TDR working group to review priorities and develop ideal test specifications depending on whether tests are to be used for case management, epidemiological surveillance or vaccine efficacy trials. The dengue working group agreed that the highest priority is the evaluation of IgM detection tests in either a rapid test (RDT) or ELISA format.

TDR identified regional reference laboratories for Latin America and Asia. These would coordinate the activities of a network of dengue laboratories to evaluate the performance and utility of existing dengue diagnostics and related activities. This report describes the results of a laboratory-based evaluation of nine commercially available anti-DENV IgM tests, using a panel of well-characterized, archived serum specimens from persons with confirmed DENV infections and other potentially confounding infections and conditions.

1. Evaluation plan

1.1 Tests under evaluation

At the first meeting of the ad hoc expert working group for laboratory-based evaluations it was agreed that the tests included in this evaluation should have the following operational characteristics:

- identify antibody response against the four dengue serotypes
- detect both primary and secondary infection
- discriminate dengue from other flavivirus and dengue-like illnesses
- discriminate dengue from nonspecific reactivity due to systemic disorders
- work for all geographical conditions.

Manufacturers of commercially available dengue anti-DENV IgM tests that met the inclusion criteria were invited to participate. Those who accepted signed an agreement with WHO/TDR and donated the required number of tests for the evaluation. These were sent from the manufacturers to the two reference laboratories that distributed the tests to the evaluating laboratories.

Six companies, manufacturing nine tests, agreed to participate in the evaluation.

Rapid tests

- Panbio Diagnostics, Australia: Dengue Duo Cassette (DuoCassette);
- Pentax Corporation, Japan: Hapalyse Dengue-M PA Kit (HapalyseM);
- Standard Diagnostics, Republic of Korea: SD Bioline Dengue IgG/IgM (SD Bioline);
- Zephyr Biomedicals, India: Dengucheck WB (Dengucheck).

ELISAs

- Focus Diagnostics, United States of America: Dengue Fever Virus IgM Capture DxSelect™ (DxSelect);
- Omega Diagnostics Ltd., United Kingdom: PATHOZYME-DENGUE M (Pathozyme M);
- Omega Diagnostics Ltd., United Kingdom: PATHOZYME-DENGUE M CAPTURE (Pathozyme MCap);
- Panbio Diagnostics, Australia: Dengue IgM Capture ELISA (Pb IgMCap);
- Standard Diagnostics, Republic of Korea: Dengue IgM Capture ELISA (SD IgMCap).

In accordance with the terms of the confidentiality agreement signed with WHO, companies could review and comment on the data from each site and the data analyses but were unable to modify any of the conclusions. They were sent a courtesy draft of the evaluation report prior to publication.

The characteristics of the individual tests are summarized in Tables 1 & 2.

Table 1. Commercial anti-DENV IgM ELISAs evaluated

Test name	Dengue Fever Virus IgM Capture DxSelect	PATHOZYME–DENGUE M	PATHOZYME–DENGUE M CAPTURE	Dengue IgM Capture ELISA	Dengue IgM Capture ELISA
Company	Focus Diagnostics	Omega Diagnostics	Omega Diagnostics	Panbio Diagnostics	Standard Diagnostics
Country	USA	United Kingdom	United Kingdom	Australia	Republic of Korea
Method of detection	IgM capture	Indirect IgM detection	IgM capture	IgM capture	IgM capture
Format	12 strips of 8 wells	12 strips of 8 wells	12 strips of 8 wells	12 strips of 8 wells	12 strips of 8 wells
Number of tests per pack	96	96	96	96	96
Antigen	DENV 1-4	Purified DENV 2 (coated on solid phase)	DENV 1-4	Recombinant DENV 1-4	DENV 1-4
Volume of sample required	10 µL	10 µL	20 µL	10 µL	10 µL
Total incubation time (at 37 °C unless otherwise noted)	225 minutes at room temperature	120 minutes	110 minutes	130 minutes	130 minutes
Time to result	6h	4h	4h	4h	4h
Storage conditions (°C)	2–8	2–8	2–8	2–8	2–8
Additional equipment required?	Yes	Yes	Yes	Yes	Yes

Table 2. Commercial anti-DENV IgM rapid tests evaluated

Name	Dengue Duo Cassette	Hapalyse Dengue–M PA kit	SD Bioline Dengue IgG/IgM	Dengucheck–WB
Company	Panbio Diagnostics	Pentax	Standard Diagnostics	Zephyr Biomedicals
Country	Australia	Japan	Republic of Korea	India
Assay principle	Lateral flow	Particle agglutination	Lateral flow	Lateral flow
Target antibody	IgM and IgG	IgM	IgG and IgM	IgM and IgG
Format	Cassette	12 strips of 8 anti-human IgM coated microwells	Cassette	Cassette
Number of tests per pack	25	96	25	25
Antigen	Recombinant DENV 1-4	DENV 1-4	Recombinant DENV 1-4 envelope protein	Recombinant DENV (serotype not specified)
Specimen type	Serum, plasma or whole blood	Serum or plasma	Serum or plasma	Serum, plasma or whole blood
Volume of sample required	10 µL	1 µL	5 µL	5 µL
Time to result	15 minutes	90 minutes	15–20 minutes	15 minutes
Storage conditions (°C)	2–30	2–8	1–30	4–30
Additional equipment required	None	Wash bottle or automatic plate washer, micropipettes, vortex mixer	none	none

1.2 Evaluation sites

A request for applications (RFA) was posted on the TDR web site to solicit interest from laboratories to participate in the evaluation. Twenty five applications were received in response to the RFA – from the Americas (10), Asia (10), Africa (3), Australia (1) and Europe (1). A rating guide was developed to assess the applications by considering expertise and experience in performing dengue diagnostics evaluations (20%), type of laboratory (20%), range of laboratory services offered (30%) and the laboratory capacity/facility/management (30%). Nine laboratories were selected from the 25 applications taking into account geographic representation. However

two laboratories from Brazil were unable to participate in this round of evaluations, leaving the seven laboratories listed in Box 1.

The laboratories at the Centers for Disease Control and Prevention (CDC) in Puerto Rico and at Mahidol University in Thailand were selected as reference laboratories for their respective regions. In addition to performing the evaluation, they provided proficiency testing between the reference laboratories and the evaluation laboratories; assembled and validated appropriate clinical specimens to form a WHO performance panel for evaluation; and provided proficiency testing between the reference centres and the evaluation laboratories.

Box 1. Network for laboratory-based evaluations of anti-DENV IgM tests

SOUTH-EAST ASIA

AMERICAS

Reference laboratories

Dr Sutee Yoksan
Center for Vaccine Development
Mahidol University
Bangkok, Thailand

Dr Elizabeth Hunsperger
Dengue Branch
Centers for Disease Control and
Prevention
San Juan, Puerto Rico

Evaluation laboratories

Dr Vinh Chau Nguyen
Hospital for Tropical Diseases
(Cho Quan Hospital)
Ho Chi Minh City, Viet Nam

Dr Susana Vázquez
Instituto de Medicina Tropical
"Pedro Kouri"
Havana, Cuba

Dr Philippe Buchy
Institut Pasteur in Cambodia
Phnom Penh, Cambodia

Dr Delia Enria
Instituto Nacional de Enfermedades
Virales Humanas "Dr Julio I Maiztegui"
Pergamino, Argentina

Dr Shamala Devi Sekaran
Department of Medical Microbiology
University of Malaya,
Kuala Lumpur, Malaysia

1.3 Evaluation panel

Source of sera

The network laboratories contributed specimens to the panel. The reference laboratories supplemented these samples with sera from their archives. A small number of sera for the challenge panel were also purchased from SeraCare Diagnostics (West Bridgewater, MA, USA).

Validation Methods

The CDC and the Armed Forces Research Institute of Medical Sciences (AFRIMS) IgM antibody capture (MAC) ELISAs were the reference assays used to validate specimens for the evaluation panel (Burke et al., 1982; Innis et al., 1989). All reference testing was performed at the two reference laboratories. Samples from the Americas were validated with the CDC IgM antibody capture (MAC)-ELISA at the CDC, Puerto Rico; samples from Asian sites were validated with the AFRIMS MAC-ELISA at Mahidol University, Thailand.

Panel composition

The evaluation panel comprised 350 samples selected from clinical samples submitted to the reference laboratories. Sensitivity was evaluated using 181 DENV-positive sera of different IgM titres with all four serotypes from both primary and secondary infections (except for DENV-4, see Table 3). The panel was weighted towards low and medium IgM titres in order to resemble clinical settings where secondary dengue is more common. Patients with secondary dengue usually have levels of serum IgM in the lower range (Table 3). All samples in the panel were accompanied by epidemiological data; previous flavivirus vaccine history; date of onset of symptoms; date sample was drawn; clinical diagnosis; travel history; and country of origin.

Table 3. Composition of anti-DENV IgM positive samples* in evaluation panel

DENV-positive samples			
	IgM titre	Primary	Secondary
DENV-1	Low	2	6
	Medium	11	4
	High	1	2
	Total	14	12
DENV-2	Low	2	5
	Medium	0	6
	High	1	5
	Total	3	16
DENV-3	Low	4	2
	Medium	0	4
	High	1	2
	Total	5	8
DENV-4	Low	0	3
	Medium	0	2
	High	0	2
	Total	0	7
Serotype not identified	Low	1	43
	Medium	4	33
	High	0	35
	Total	5	111
Total DENV-positive samples		27	154

Cutoffs for AFRIMS MAC-ELISA: low<2; 2≤medium≤3; high>3
Cutoffs for CDC MAC-ELISA: low≤0.5; 0.5<medium≤0.8; high> 0.8

A panel of 169 sera, validated as negative in the MAC-ELISA for the presence of anti-DENV IgM antibodies, was used to assess specificity (Table 4). The panel included sera from patients with potentially cross reactive flaviviruses (West Nile virus, yellow fever virus, St Louis encephalitis virus, Japanese encephalitis virus) and other causes of acute febrile illnesses (malaria, Lyme disease, hantavirus); sera containing interfering substances (rheumatoid factor, systemic lupus erythematosus); sera from patients with a history of dengue infection (sera positive for anti-DENV IgG but negative for anti-DENV IgM); and sera from healthy individuals living in areas non-endemic for dengue. The reference laboratory in Thailand also evaluated all nine tests against a panel of twelve sera from patients with leptospirosis. These samples were not distributed to the other six evaluation sites due to limited serum volumes. The number of positive and negative samples included in the panel was sufficient to give a point estimate for test sensitivity and specificity with an average precision of $\pm 5\%$ compared to the reference assays.

Panel preparation

Following validation at the reference laboratories, the panel samples were coded, heat inactivated, aliquoted and lyophilized. One aliquot of each sample was reconstituted and retested by the reference laboratories before being shipped to each network laboratory. The samples were coded to ensure blinded reading of the test results at the sites.

Table 4. Composition of anti-DENV IgM negative samples in evaluation panel

Serum description	n=
Anti-DENV IgM negative	
DENV positive, IgM negative	19
IgG anti-DENV positive	7
Related flavivirus IgM positives	
IgM anti-West Nile virus positive	25
IgM anti-Yellow Fever positive	4
Related flavivirus IgG positives	
IgG anti-West Nile virus positive	1
IgG anti -Yellow Fever positive	10
IgG anti-St Louis encephalitis	2
IgG anti-Japanese encephalitis virus positive	10
Febrile illness	
IgG anti-Lyme disease positive	9
Malaria	31
IgM anti-New World hantavirus positive	7
Systemic conditions	
Rheumatoid factor	6
Systemic lupus erythematosus	2
Healthy persons nonendemic areas	
Naïve	36
TOTAL NEGATIVE SERA	169

2. Site preparation

2.1 Proficiency

All sites received training in Good Clinical Laboratory Practice (GCLP) and a biobanking primer.

The reference laboratories lyophilized, reconstituted and retested a proficiency panel of sera of known IgM titres as a quality assurance (QA) measure. Samples were compiled and coded by the reference laboratories and sent to each of the evaluating laboratories. A standard operating procedure was developed to assure consistency in sample handling and testing across all sites. Since tests can be highly sensitive to storage and shipping conditions, data on the time between shipment and arrival were recorded with the test results. The evaluating laboratories performed blinded testing and returned results to the reference laboratories. All sites scored more than 90% on the proficiency panel.

2.2 Ethical considerations

Each evaluation site obtained approval from an institutional review board or ethics committee and from the WHO Research Ethics Review Committee (ERC) for performing the evaluations in accordance with the consensus evaluation protocol and for the use of unlinked archived sera in the evaluation panel.

Each site documented, to the satisfaction of the local ethics committee, the mechanism whereby all personal identifiers and patient information were unlinked from the serum specimens so that they cannot be traced to individual patients.

3. Evaluation

3.1 Before testing

Receipt of tests and samples

Each network laboratory received the identical evaluation panel and test kits of the same lot number. All laboratories followed the same procedures for handling the test kits and the samples.

1. Note date of arrival and condition of shipment.
2. Unpack tests and panel without delay when they arrive in the laboratory.
3. Store lyophilized serum samples at room temperature.
4. Transfer to, and store tests in, appropriate conditions in accordance with manufacturers' instructions in the package inserts.

Sample reconstitution

Lyophilized serum samples were not reconstituted until the laboratory was ready to start the evaluation. Once reconstituted, the sera were kept at 4 °C until the evaluation was completed. Evaluation continued without delay to avoid deterioration of the antibodies in the reconstituted samples.

3.2 Performing the tests – general procedures

All nine tests were evaluated using an identical panel of 181 reference standard anti-DENV IgM positive and 169 reference standard negative samples at all seven evaluation sites. Sites were blinded to the results of sera in the panel.

ELISA tests

Each test was performed according to the manufacturer's directions in the package insert. Specimens were tested in duplicate in the same run in order to assess the within run precision. Inter-run precision was evaluated by comparing results across evaluation sites. Results were

recorded on a standardized recording sheet retained at the sites as source data along with ELISA read-outs for future reference. Copies of the results were submitted to TDR for data analysis. The laboratories used the following general guidelines to perform the ELISA tests.

1. Withdraw the first ELISA test kit to be evaluated from storage.
2. Note lot number and expiry date, expired kits should not be used.
3. Dilute each reconstituted serum sample according to manufacturer's instructions. Only one dilution should be made.
4. Run ELISA test according to manufacturer's instructions. Run samples in the panel in duplicate. Use same dilution for both wells in order to test consistency of the wells.
5. Record results of the two wells for each sample on laboratory data collection form (ELISA).
6. Principal investigator at each site should sign off laboratory data collection forms at the end of each evaluation day.

BIOSAFETY GUIDELINES

Treat all specimens as potentially infectious

Wear protective gloves and laboratory gown while handling specimens

Do not eat, drink or smoke in the laboratory

Do not wear open-toed footwear in the laboratory

Clean up spills with appropriate disinfectants e.g. 1% bleach

Decontaminate all materials with an appropriate disinfectant

Dispose of all waste, including test kits, in a biohazard container

Rapid tests

The RDTs were performed according to the manufacturers' instructions with the exception of the recording of the results of three tests under evaluation. The tests manufactured by Panbio Diagnostics, Standard Diagnostics and Zephyr Biomedicals all detect both anti-DENV IgM and IgG in the sample. Since the current evaluation was assessing only a test's ability to detect IgM, the results of the IgG detection band were not taken into consideration.

The tests were performed by one technologist and read by two. The second technologist read the tests independently of the first and each recorded the results on different data collection forms. Only the results of the first reader were used for the statistical calculations. These forms are held at the sites as source data. The following general guidelines were used for performing the RDTs.

Technician 1

- a. Withdraw RDT under evaluation from storage.
- b. Mark test strips with the sample numbers.
- c. Perform test according to manufacturer's instructions.
- d. Record result of IgM line on Laboratory Data Collection Form (RDT, reader 1).
- e. Place test on a tray, pass to Technician 2 when a suitable number of tests have been performed. Do not show the results recorded on the form.

Technician 2

- a. Take tray containing performed RDTs from Technician 1.
- b. Read results of each test without looking at results of Technician 1.
- c. Record results of IgM line on Laboratory Data Collection Form (RDT, reader 2).

Principal investigator

At the end of each day ensure that all tests read by technician 1 have also been read and recorded independently by technician 2. Thereafter, both recording forms should be signed off by the principle investigator or designated laboratory supervisor.

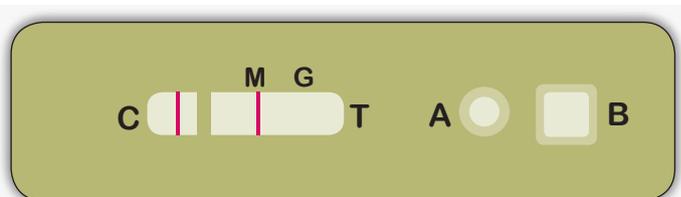
3.3 Performing the tests — specific procedures

The following pages contain an illustrated summary of the test procedure for each of the tests covered in this report. For full details and any questions please refer to the product insert for each test kit.

Dengue Duo Cassette (Panbio Diagnostics)

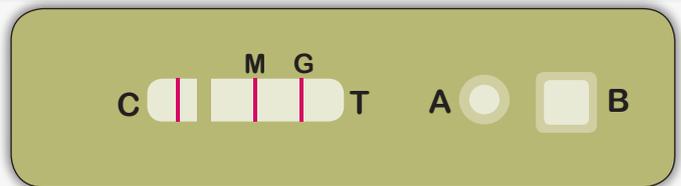
Outline of procedure

1. Add 10 μL of whole blood, serum or plasma to circular well using a micropipette or the MicroSafe[®] pipette provided.
2. Allow sample to absorb entirely into specimen pad within circular well.
3. Hold buffer bottle vertically and 1 cm above square well.
4. Add 2 drops of buffer to square well at base of cassette.
5. Read result exactly 15 minutes after adding buffer to cassette.
6. Any trace of a pink line in test area indicates a positive result.
7. Any results read outside 15 minutes should be considered **invalid** and must be repeated.



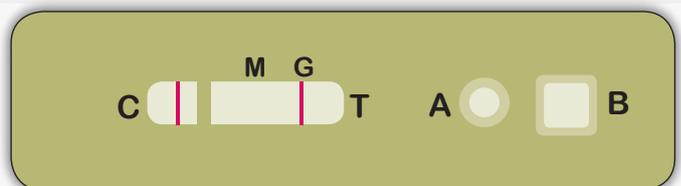
IgM positive

Pink bands appear in the IgM and Control regions.



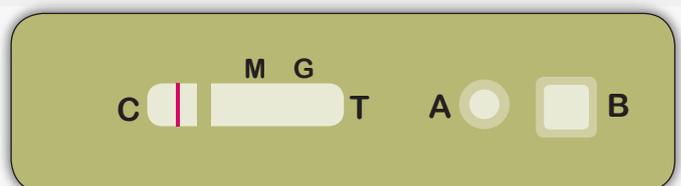
IgM and IgG positive

Pink bands appear in the IgM, IgG and Control regions.



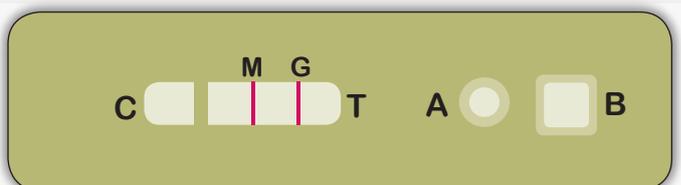
IgG positive

Pink bands appear in the IgG and Control regions.



Negative

A pink band appears in the Control region only.



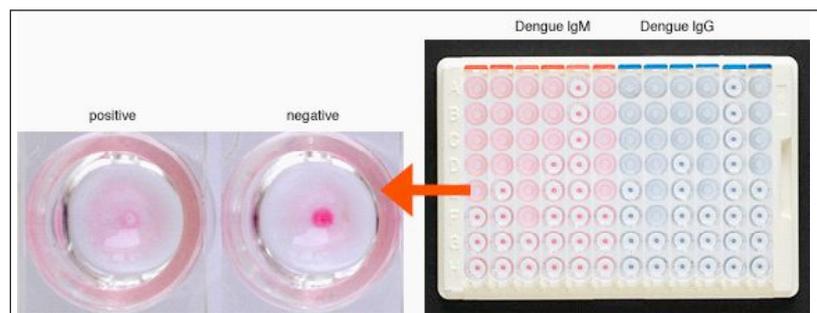
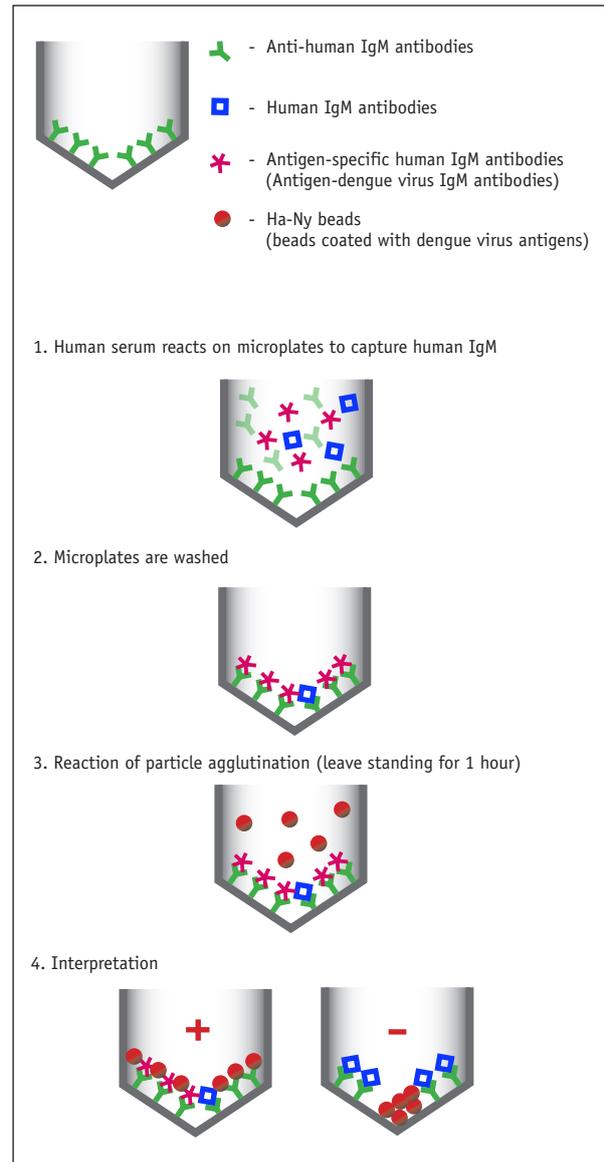
Invalid

No pink band appears in the Control region.

Hapalyse Dengue-M PA kit (Pentax Corporation)

Outline of procedure

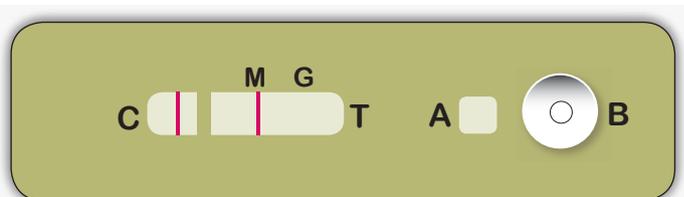
1. Bring reagents to room temperature. Reconstitute freeze-dry antigen-coated beads. Make 1x wash buffer.
2. Dilute serum samples into appropriate dilution.
3. Wash wells with 1x wash buffer, three times.
4. Add diluted serum samples to the wells.
5. Incubate wells for 30 minutes at room temperature.
6. Wash wells with 1x wash buffer, three times.
7. Add 100 μ L of Ha-Ny bead slurry to the wells.
8. Incubate wells for 1 hour at room temperature.
9. Observe beads' agglutination patterns.



SD Bioline Dengue IgG/IgM (Standard Diagnostics)

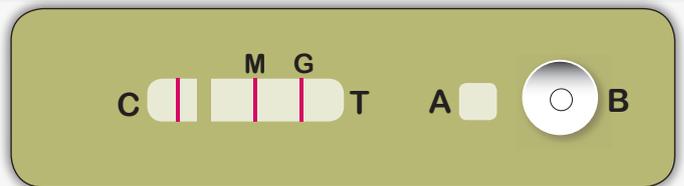
Outline of procedure

1. Remove test device from foil pouch. Place on flat, dry surface.
2. Add 5 μL of serum or plasma with a 5 μL capillary pipette into the square sample well.
3. Add 3–4 drops (about 90–120 μL) of assay diluent to the round-shaped well.
4. Interpret test results at 15–20 minutes.



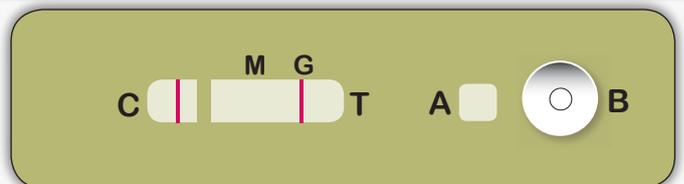
IgM positive

Pink bands appear in the IgM and Control regions.



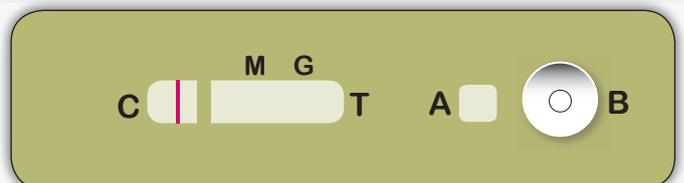
IgM and IgG positive

Pink bands appear in the IgM, IgG and Control regions.



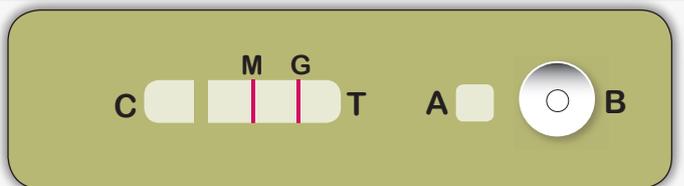
IgG positive

Pink bands appear in the IgG and Control regions.



Negative

A pink band appears in the Control region only.



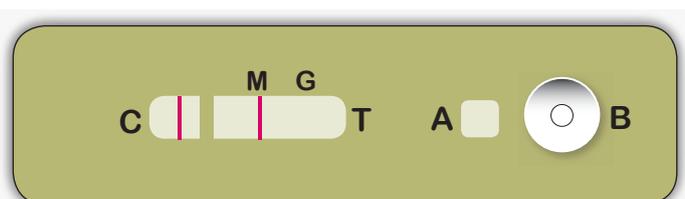
Invalid

No pink band appears in the Control region.

Dengucheck-WB (Zephyr Biomedicals)

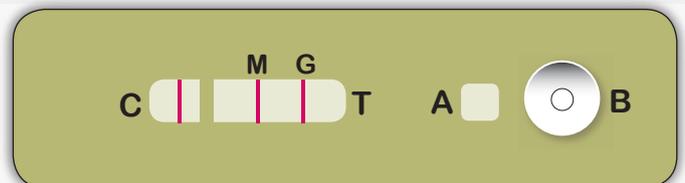
Outline of procedure

1. Bring kit components to room temperature before testing.
2. Open pouch and retrieve test device. Once opened, device must be used immediately.
3. Label test device appropriately.
4. Use sample dropper provided to add one drop of serum/plasma/whole blood to sample port (A).
5. Add five drops of sample running buffer to reagent port (B).
6. Read test results after exactly 15 minutes.



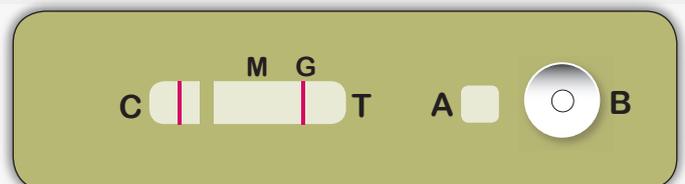
IgM positive

Pink bands appear in the IgM and Control regions.



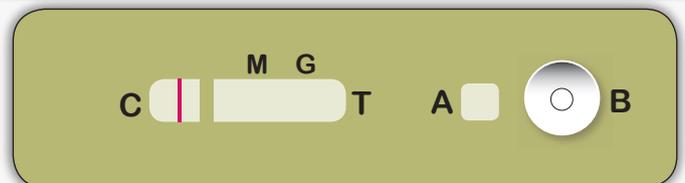
IgM and IgG positive

Pink bands appear in the IgM, IgG and Control regions.



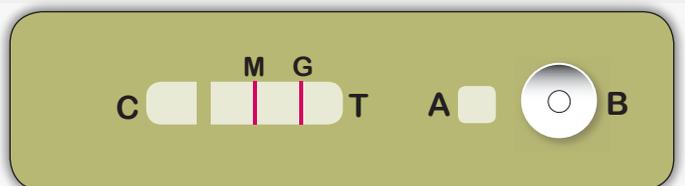
IgG positive

Pink bands appear in the IgG and Control regions.



Negative

A pink band appears in the Control region only.



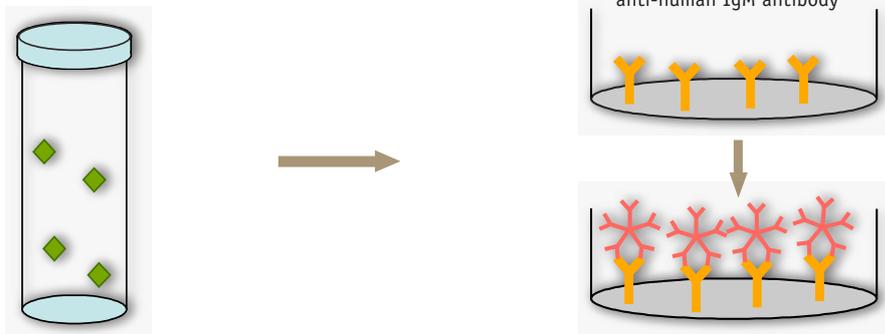
Invalid

No pink band appears in the Control region.

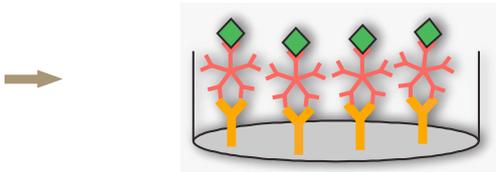
Dengue Fever Virus IgM Capture DxSelect (Focus Diagnostics)

The Focus Diagnostics Dengue Fever Virus IgM Capture DxSelect™ may be used in two ways. The classical CDC protocol uses an overnight capture antigen incubation step. Alternatively, the capture antigen incubation step can be shortened to two hours at room temperature.

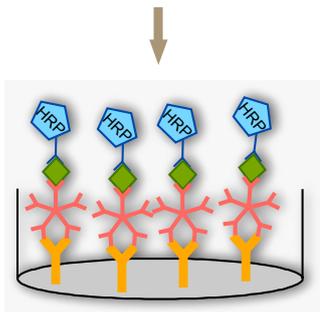
Outline of procedure



1. Prepare antigen solution.
2. Fill wells with 1x buffer solution and allow to soak for 5 minutes. Decant (or aspirate) the antigen wells.
3. Dispense 100 μ L of the sample diluent into the blank wells and 100 μ L of each diluted specimen, control or calibrator into the appropriate wells.
4. Incubate for 60 ± 1 minutes at room temperature (20–25 °C).
5. Empty contents of the wells.
6. Wash wells with 1x wash buffer three times; allow last wash to soak for 5 minutes before decanting or aspirating.



7. Use a 100 μL 8- or 12-channel pipettor to add prepared (see Step 1) 100 μL antigen solution to all wells.
8. Cover plates with sealing tape and incubate for 2 hours at room temperature (20–25 $^{\circ}\text{C}$).
9. Repeat steps 5 to 6.

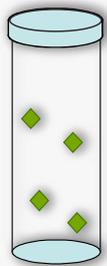


10. Use a 100 μL 8- or 12-channel pipettor to add 100 μL of IgM conjugate to all wells.
11. Incubate for 30 ± 1 minutes at room temperature (20–25 $^{\circ}\text{C}$).
12. Repeat steps 5 to 6.
13. Use a 100 μL 8- or 12-channel pipettor to add 100 μL of substrate reagent to all wells. Begin incubation timing when substrate reagent is added to the first well.
14. Incubate for 10 ± 1 minutes at room temperature (20–25 $^{\circ}\text{C}$).

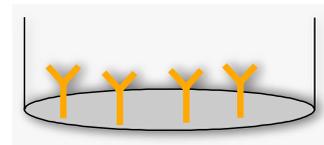
- 
15. Use a 100 μL 8- or 12-channel pipettor to stop reaction by adding 100 μL of stop reagent to all wells.
 16. Measure absorbance of each well at 450 nm within 1 hour of stopping the assay.

Dengue IgM Capture ELISA (Panbio Diagnostics)

Outline of procedure

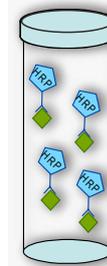


1. Add 10 μL of antigen in 2.5 mL of antigen-diluent and mix. Unused concentrated antigen should be stored at 2–8 $^{\circ}\text{C}$.
2. Remove required volume of diluted antigen and mix with an equal volume of MAb tracer in a separate glass vial or test tube. DISCARD UNUSED DILUTED ANTIGEN.

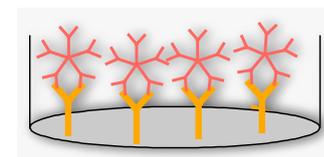


3. Add 100 μL of diluted samples and controls to assay plate.

- 4a. Incubate 1 hour at 20–25 $^{\circ}\text{C}$

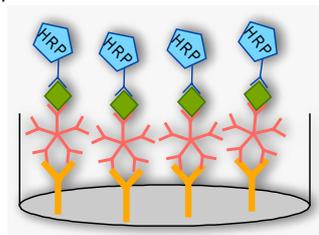


- 4b. Cover plate and incubate 1 hour at 37 $^{\circ}\text{C} \pm 1^{\circ}\text{C}$



5. Wash the assay plate x 6. After gentle rotation to mix the antigen-MAb solution, transfer 100 μL per well to the assay plate.

6. Cover plate and incubate 1 hour at 37 $^{\circ}\text{C} \pm 1^{\circ}\text{C}$

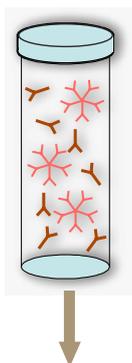


7. Wash the assay plate x 6. After the final wash, add 100 μL TMB per well and incubate at 20–25 $^{\circ}\text{C}$ for 10 minutes. Stop the reaction with 100 μL Stop Solution and read at 450 nm (Reference 600–650 nm).

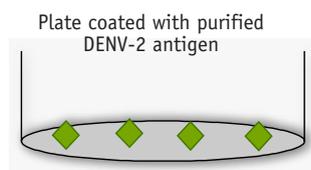
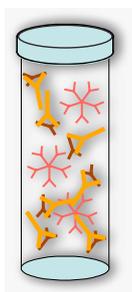
Pathozyme-Dengue M (Omega Diagnostics Ltd.)

Outline of procedure

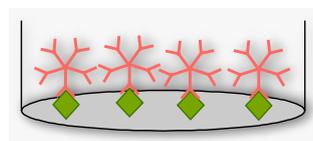
1. Dilute test sera 1:20 by adding 10 μL of serum to 200 μL of serum diluent.



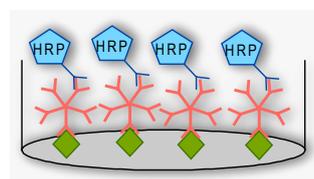
2. Add 25 μL of IgG absorb to 35 μL of diluted sera and 35 μL of working strength low positive control. Prepare the low positive control in duplicate. Mix well and leave for 15 minutes at 37 $^{\circ}\text{C}$.



3. Dispense 50 μL of negative control, sera and low positive control made up in step 2 to each well.
4. Shake gently for 5 seconds. Incubate for 60 minutes at 37 $^{\circ}\text{C}$.
5. Discard well contents and wash three times.



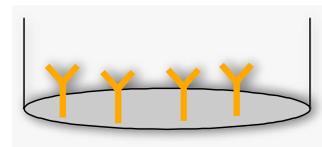
6. Dispense 50 μL of anti-human HRP-IgM conjugate to each well. Shake gently for 5 seconds.
7. Incubate for 30 minutes at 37 $^{\circ}\text{C}$.



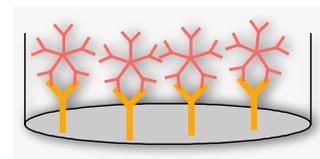
8. Repeat step 5.
9. Dispense 100 μL of substrate to each well. Shake gently for 5 seconds.
10. Incubate in the dark for 15 minutes at room temperature (20–30 $^{\circ}\text{C}$).
11. Dispense 100 μL of stop solution to each well.
12. Read the optical density (OD) using an EIA reader with a 450 nm filter.

Pathozyme-Dengue M Capture (Omega Diagnostics Ltd.)

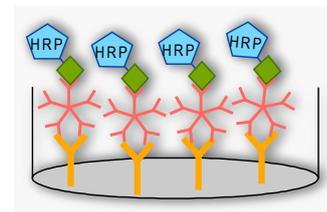
Outline of procedure



1. Dilute test sera 1:50 by adding 20 μL of serum to 1000 μL of serum diluent.
2. Dispense 100 μL of negative control, sera and low positive control to a well. Shake gently for 5 seconds.
3. Incubate for 60 minutes at 37 $^{\circ}\text{C}$.
4. Discard well contents and wash five times



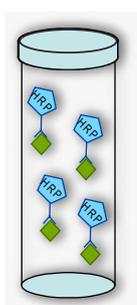
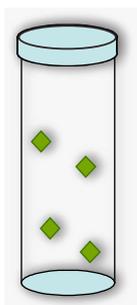
5. Dispense 100 μL of working strength conjugate to each well. Shake gently for 5 seconds.
6. Incubate for 30 minutes at 37 $^{\circ}\text{C}$.



7. Repeat step 4.
8. Dispense 100 μL of substrate to each well. Shake gently for 5 seconds.
9. Incubate in the dark for 20 minutes at 37 $^{\circ}\text{C}$.
10. Dispense 100 μL of stop solution to each well.
11. Read the OD using an EIA reader with a 450 nm filter.

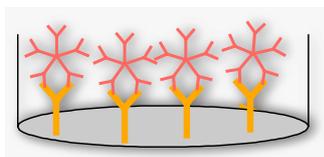
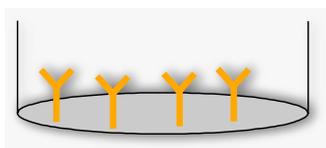
SD Dengue IgM Capture ELISA (Standard Diagnostics)

Outline of procedure



1. Dilute the Dengue Antigen 1/25 with the Conjugate Diluent.
2. Dilute the Anti-Dengue horse radish peroxidase (HRP) Conjugate 1/101 with the diluted Dengue Antigen of above 1.

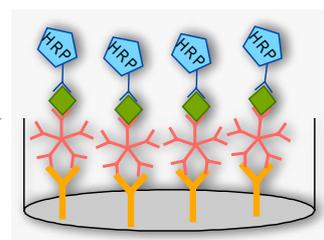
- 4a. Incubate at room temperature (16–30°C) for 60 minutes.



3. Pipette 100 µl of diluted controls and patient samples into microplate wells.

- 4b. Incubate at 37± °C for 60 minutes.

5. Wash the well 5 times with 350 µl of diluted Washing Solution. Pipette 100 µl of diluted Anti-Dengue HRP conjugate solution into the appropriate microplate wells.



6. Incubate the wells at 37± °C for 60 minutes.



7. After final wash, pipette 100 µl colour developer into each well and incubate for 10 minutes at room temperature (15–30 °C).



8. Stop the reaction with 100 µl of Stopping Solution and read at 450 nm.

Handling of indeterminate results

Results that were not clearly positive were recorded as indeterminate. The test was repeated if sufficient test kits were available. Otherwise, indeterminate results were recorded as negative.

Test reproducibility

The performance of the tests is dependent not only on sensitivity and specificity but also reliability of performance. This reliability can be measured by assessing the reproducibility of tests. Two types of measurements were made to evaluate reproducibility.

1. Lot-to-lot reproducibility. Will the test give the same results with tests of different manufacturing lots using the same specimens? Lot-to-lot testing was performed with a 25 sample subset of the panel at each reference laboratory. Five companies provided a second production lot for lot-to-lot reproducibility testing.
2. Operator-to-operator reproducibility (RDTs only). Will the test give the same results on the same specimen if it is performed by two different operators?

Reproducibility testing was performed only at the two reference laboratories.

Assessing operational characteristics

Having tested the specimens from the evaluation panel the technicians assessed each RDT for the following operational characteristics.

1. Clarity of kit instructions (maximum score 3)
2. Technical complexity or ease of use (maximum score 3)
3. Ease of interpretation of results (maximum score 3)

Tests that do not require any additional equipment or supplies received an additional point.

The highest possible score is 10. This indicates that the test has user friendly operational characteristics.

Data analysis

The results from reader 1 were compared to those from the reference standard testing. Sensitivity and specificity compared to the reference standard were calculated for each site. A kappa value was used to estimate overall performance by determining the combined agreement of sensitivity and specificity for all sites against the reference standard results. A kappa value of 0.8 or more indicates excellent agreement with the reference test. The site-to-site variability of the evaluation was summed up with the Breslow-Day test for homogeneity of odds ratios. The observed variations between sites are statistically significant when the homogeneity (p-value) is <0.05 .

4. Results

The results of the evaluation of ELISA tests and the rapid tests are presented in Tables 5 and 6. The sensitivity and specificity of the tests for each site were calculated using the reference laboratory results as a reference standard. The overall performance of the tests (kappa values) and the variation in test results between sites (homogeneity of kappa) were also calculated and are shown in the tables.

Table 5. Sensitivity and specificity: ELISA tests

Site	DxSelect		Pathozyme M		Pathozyme MCap		Pb IgMCap		SD IgMCap	
	SE	SPC								
Thailand	99.4	85.8	55.8	87	56.4	98.2	98.9	84.6	97.8	88.2
Cambodia	97.8	85.6	64.1	89.2	64.1	96.4	98.3	85	98.3	89.8
Malaysia	99.4	66.9	59.1	87	61.3	99.4	99.4	85.6	97.8	87.6
Viet Nam	95	90.3	60.2	84.3	61.9	97.8	98.3	85.1	98.3	82.1
Puerto Rico	99.4	74	70.7	68	72.9	97.6	99.4	81.6	93.9	85.8
Argentina	99.4	78.1	59.6	88.2	61.2	98.2	98.9	86.4	98.3	87
Cuba	99.4	81.1	60.8	88.2	58	97	100	82.8	98.9	85.2
All sites	98.6	79.9	61.5	84.6	62.3	97.8	99	84.4	97.6	86.6
CI₉₅	98.0, 99.2	77.6, 82.2	58.8, 64.2	82.5, 86.7	59.6, 65.0	97.0, 98.6	98.4, 99.5	82.3, 86.5	96.8, 98.4	84.6, 88.6
Homogeneity of kappa		0.48		0.11		0.60		0.73		0.11
Kappa	0.81		0.46		0.59		0.84		0.85	
CI₉₅	0.78/ 0.83		0.42/ 0.49		0.56/ 0.62		0.82/ 0.86		0.83/ 0.87	

SE – sensitivity; SPC – specificity; CI95 – 95% confidence interval)

Table 6. Sensitivity and specificity: rapid tests

Site	DuoCassette		HapalyseM		SD Bioline		Denguecheck	
	SE	SPC	SE	SPC	SE	SPC	SE	SPC
Thailand	65.2	98.2	98.9	84	64.1	92.3	23.2	82.8
Cambodia	85.6	90.4	97.8	75.4	72.9	86.8	26	82.6
Malaysia	66.3	92.3	96.1	74	49.7	88.2	14.4	91.7
Viet Nam	83.3	91.8	97.8	77.5	63.9	88.1	18.2	91.8
Puerto Rico	74.6	91.1	99.4	77.5	47	91.7	24.9	85.2
Argentina	84.8	82.8	98.9	79.9	59	92.3	13.5	90.5
Cuba	84.5	87.6	95	68	69.6	89.9	23.2	83.4
All sites	77.8	90.6	97.7	76.6	60.9	90	20.5	86.7
	75.5, 80.1	88.9, 92.3	96.9, 98.5	74.1, 79.0	58.2, 63.6	88.3, 91.7	18.3, 22.7	84.7, 88.7
Homogeneity of kappa (p-values)	0.1485		0.0024		0.0858		0.9229	
kappa	0.68		Not applicable*		0.5		0.07	
CI95	0.66/ 0.71				0.47/ 0.54		0.04/ 0.10	

SE – sensitivity; SPC – specificity; CI95 – 95% confidence interval

* The kappa values are not homogeneous therefore a kappa for all sites is not appropriate

4.1 ELISAs

Sensitivity

Three of the five tested ELISAs showed high sensitivity in comparison with the reference test – Pb IgM Cap (99.0%), DxSelect (98.6%) and SD IgM Cap (97.6%) (Table 5). This high degree of sensitivity was not shown by the two

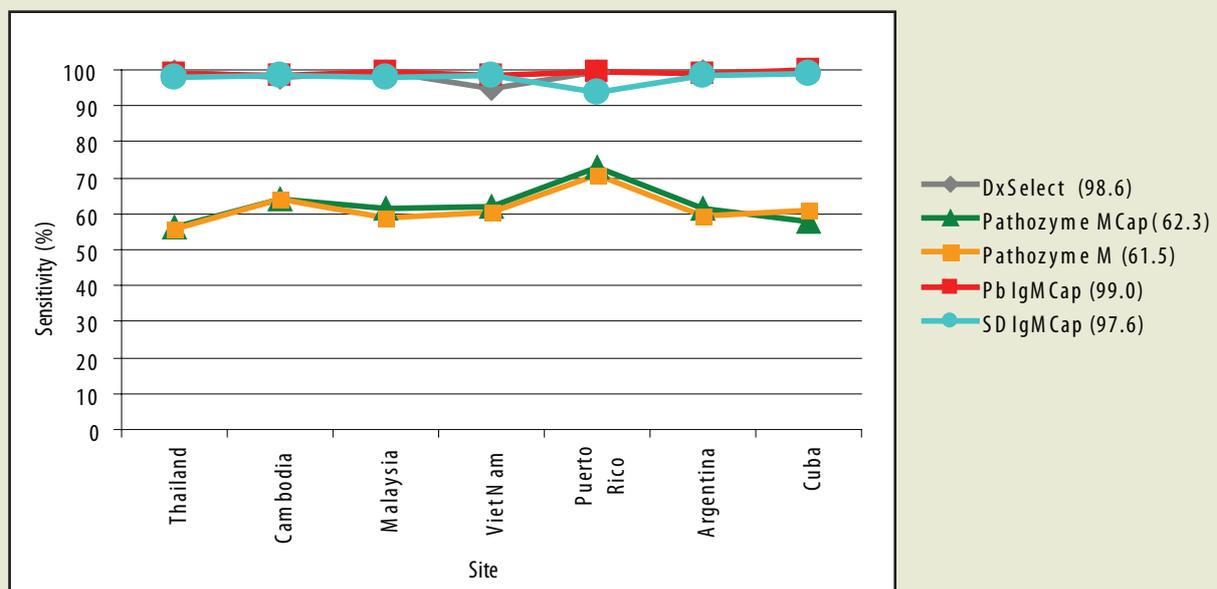
Omega tests – Pathozyme M Cap (62.3%) and Pathozyme M (61.5%). All differences are statistically significant except those between Pb IgM Cap and DxSelect and between the two Omega tests (Table 7).

There was no significant differences in the sensitivity of the 5 ELISAs reported by each site (Fig. 1).

Table 7. Comparative sensitivity (%) of performance: ELISA tests

	DxSelect (98.6%)	Pathozyme M Cap (62.3%)	Pathozyme M (61.5%)	Pb IgM Cap (99.0%)
DxSelect (98.6%)				
Pathozyme M Cap (62.3%)	<0.0001			
Pathozyme M (61.5%)	<0.0001	0.6432		
Pb IgM Cap (99.0%)	0.2008	<0.0001	<0.0001	
SD IgM Cap (97.6%)	0.0339	<0.0001	<0.0001	0.0007

Fig. 1. Sensitivity of ELISA test: comparison between seven evaluation laboratories



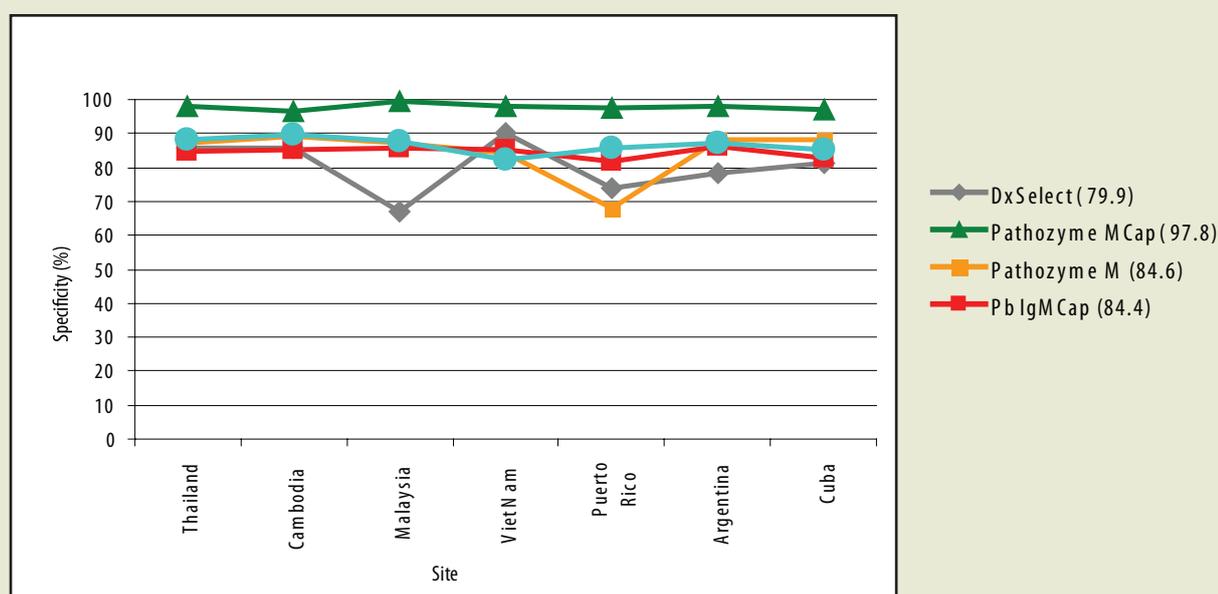
Specificity

The highest overall specificity was recorded for Pathozyme MCap (97.8%) (Table 8). The other four tests showed specificities in the 80–90% range. The differences in specificity were statistically significant between all tests except between Pb IgMCap and Pathozyme M and between Pb IgMCap and SD IgMCap (Table 8). Differences between sites are not statistically significant (Fig. 2).

Table 8. Comparative specificity (%) of performance: ELISA tests

	Focus C-E (79.9%)	Pathozyme MCap (97.8%)	Pathozyme M (84.6%)	Pb IgMCap (84.4%)
DxSelect (79.9%)				
Pathozyme MCap (97.8%)	0.0159			
Pathozyme M (84.6%)	0.0002	<0.0001		
Pb IgMCap (84.4%)	0.0005	<0.0001	0.951	
SD IgMCap (86.6%)	<0.0001	<0.0001	0.0339	0.0851

Fig. 2. Specificity of ELISA test: comparison between seven evaluation laboratories



Overall performance

DxSelect, Pb IgM_{Cap} and SD IgM_{Cap} showed excellent overall agreement with the reference assay results: their kappa values ranged from 0.81 to 0.85 (Table 5). The two Omega IgM kits showed low agreement with the reference test results with kappa values of 0.46 and 0.59 (Table 5). There were no significant site-to-site differences for any of the IgM ELISA assays (homogeneity $p > 0.05$ for all tests).

4.2 Rapid tests

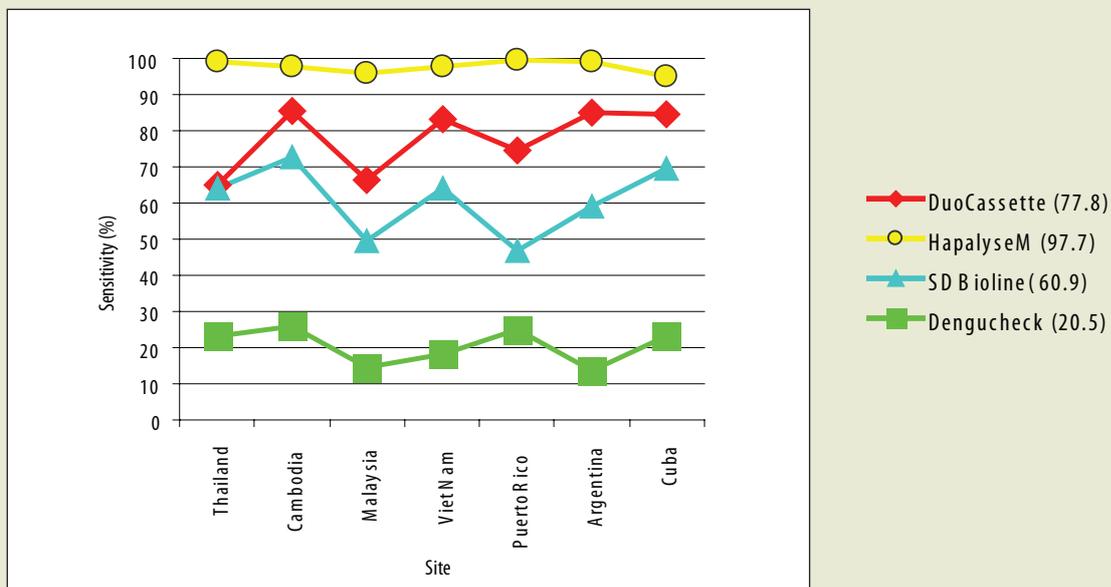
Sensitivity

Overall, the RDTs showed lower agreement with the reference standard assays for both sensitivity and specificity than the ELISA-based tests. However, HapalyseM's 97.7% sensitivity (Table 6, Fig. 2) is comparable to the three high-performing ELISA microplate tests. The remaining

Table 9. Comparative sensitivity (%) of performance: rapid tests

	DuoCassette (77.8%)	HapalyseM (97.7%)	SD Bioline (60.9%)
DuoCassette (77.8%)			
HapalyseM (97.7%)	<0.0001		
SD Bioline (60.9%)	<0.0001	<0.0001	
Dengucheck (20.5%)	<0.0001	<0.0001	<0.0001

Fig. 3. Sensitivity of rapid tests: comparison between seven evaluation laboratories



tests all show sensitivities of <80%, with Dengucheck the least sensitive (20.5%). Differences in sensitivity are statistically significant for all comparisons (Table 9). Apparent variations among the sites – especially for DuoCassette and SD Bioline (Fig. 3) – are not statistically significant.

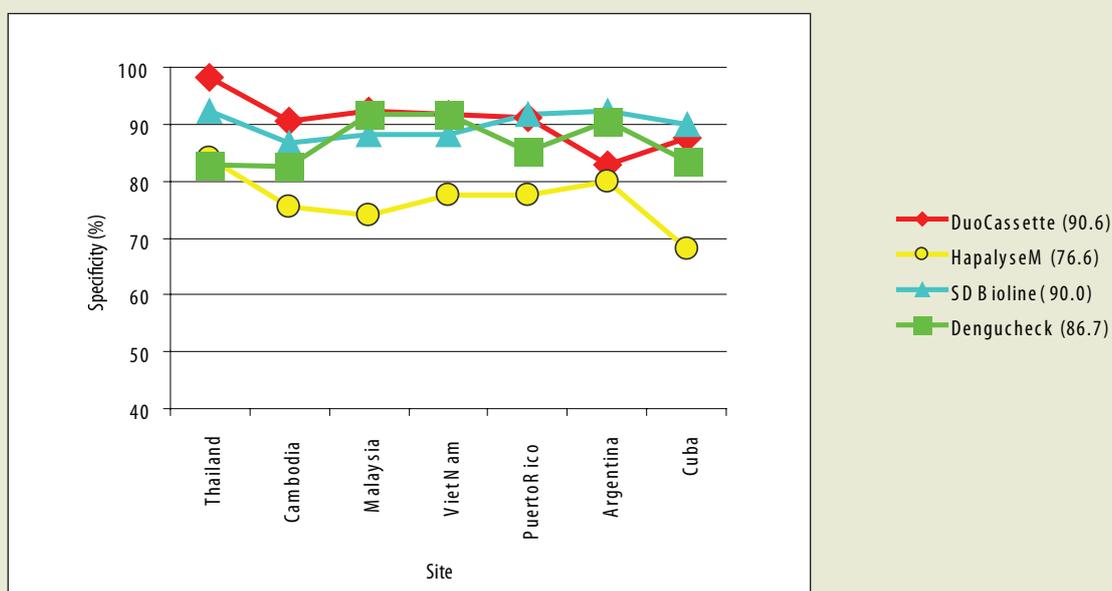
Specificity

The specificity of the RDTs is equivalent to the ELISA tests except for the HapalyseM: this shows the lowest specificity of all tests evaluated (76.6%) (Table 6). Differences in specificity were statistically significant for all comparisons except that between SD Bioline and DuoCassette ($p>0.05$) (Table 10). The specificities reported by the sites were not statistically different from each other (Fig. 4).

Table 10. Comparative specificity (%) of performance: rapid tests

	DuoCassette (90.6%)	HapalyseM (76.6%)	SD Bioline (90.0%)
DuoCassette (90.6%)			
HapalyseM (76.6%)	<0.0001		
SD Bioline (90.0%)	0.6008	<0.0001	
Dengucheck (86.7%)	0.0028	<0.0001	0.0094

Fig. 4. Specificity of rapid tests: comparison between seven evaluation laboratories



Overall performance

The kappa values for the RDTs showed that none of the tests had good agreement with the reference assays (Table 6). HapalyseM showed the highest overall performance (kappa value 0.64–0.83 for the seven sites). Dengucheck had the lowest agreement with the reference standard (kappa=0.07). HapalyseM was the only RDT that did not perform homogeneously over the sites ($p=0.0024$). However, it was the only RDT that did not vary significantly between readers (data not shown).

4.3 Cross-reactions

The panel of challenge samples consisted of 114 sera from patients with 11 different conditions or potentially cross-reactive flaviviruses. All assays were tested against the samples and the false positive rate was calculated as the percentage of positive results of the total number of testings performed, i.e. RDTs were read twice at each of the seven sites. Consequently, fourteen was multiplied by the number of samples for a specific disorder. The results from the two wells used for each sample in the ELISA assays were treated similarly (Fig 5).

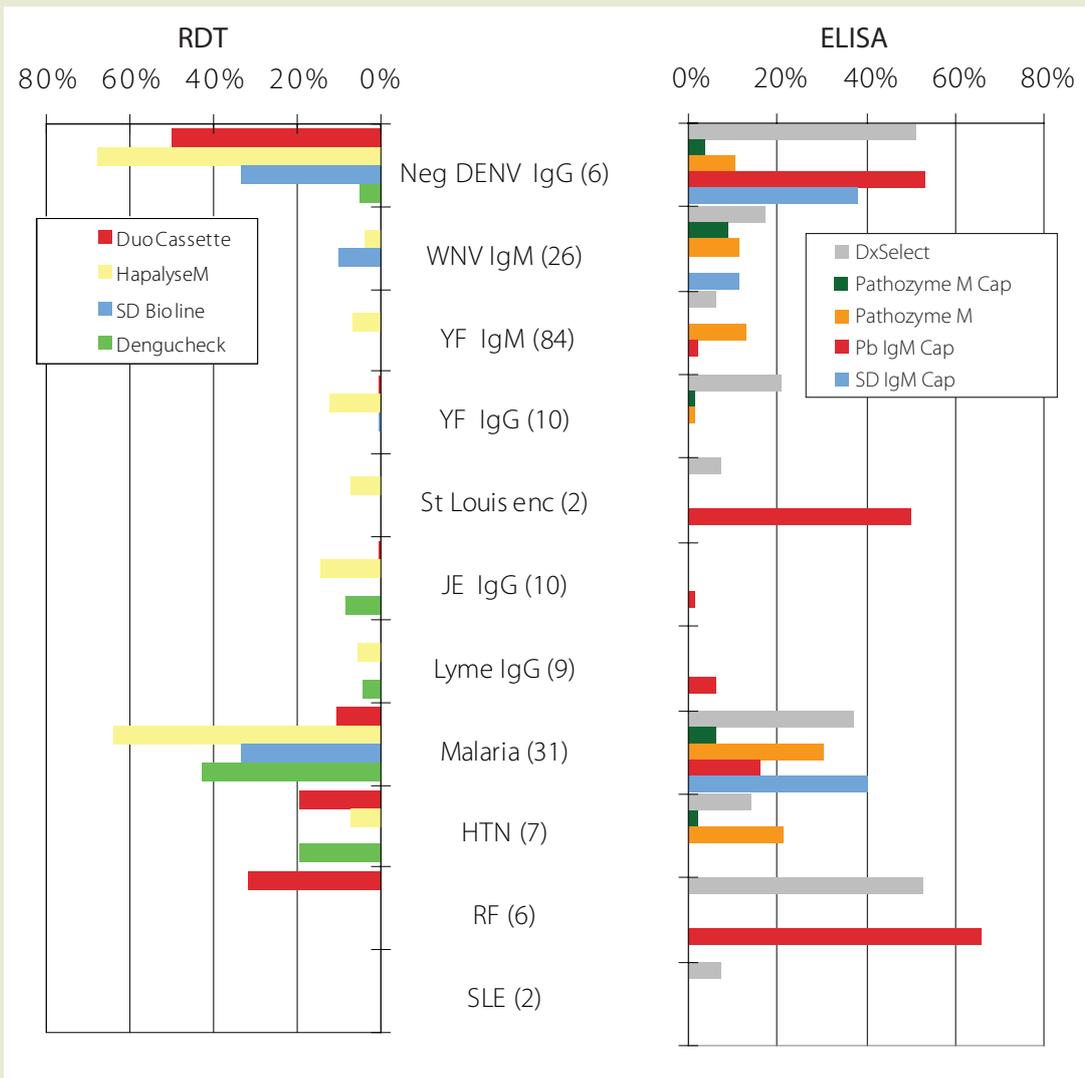
ELISA

Malaria, rheumatoid arthritis and anti-DENV IgG caused most false positive test results. Pb IgMCap showed high rates of cross-reactivity to the anti-DENV IgG (53.6%) and the rheumatoid arthritis samples (65.8%). DxSelect cross-reacted with the anti-DENV IgG (51.2%), rheumatoid arthritis (52.6%) and malaria samples (37.0%). SD IgMCap gave false positives in 38.1% of the anti-DENV IgG and 40.3% of the malaria results.

Rapid tests

Malaria and anti-DENV IgG samples also caused the highest false positive rates in the RDTs. The Pentax test (the only agglutination test) cross-reacted with the malaria samples in 69.9% of the results; Dengucheck gave 43% false positive results and SD Bioline gave 33%. The anti-DENV IgG samples showed false positive results from HapalyseM (68%), DuoCassette (50%) and SD Bioline (33.3%). DuoCassette also cross-reacted in 31.6% of the rheumatoid arthritis test results.

Fig. 5. False positive rate (%) for rapid tests and ELISAs



The number of samples of each disorder tested in parentheses. Where not indicated the immunoglobulin status is not known. Neg DENV IgG: anti-DENV IgM negative and IgG positive; WNV IgM: anti-West Nile virus IgM positive; YF IgM: anti-Yellow Fever IgM positive; YF IgG: anti-Yellow Fever IgG positive; St Louis Enc: Saint Louis encephalitis; JE IgG: anti-Japanese encephalitis IgG positive; Lyme IgG: anti-Lyme disease IgG positive; HTN: anti-New World hantavirus IgM positive; RF: rheumatoid factor; SLE: systemic lupus erythematosus.

4.4 Test reproducibility and user-friendliness

Lot-to-lot testing

Two assessments were performed in order to test reproducibility. A lot-to-lot comparison was made with two different batches of seven of the tests (Table 11). Both SD Bioline and HapalyseM scored 100% agreement between the two lots. DuoCassette and Pathozyme MCap had an

acceptable kappa of 0.78 while Pathozyme M, Pb IgM_{Cap} and Dengucheck scored lower.

Reader-to-reader variation

The reader-to-reader reproducibility of the RDTs was also assessed (Table 12). All tests performed well with high kappa values for DuoCassette and HapalyseM (over 0.8) and SD Bioline and Dengucheck (just under 0.8).

Table 11. Test reproducibility: lot-to-lot reproducibility of rapid tests and ELISAs

Parameter	RDT				ELISA		
	Dengucheck*	HapalyseM*	SD Bioline*	DuoCassette**	Pathozyme M _{Cap} *	Pathozyme M*	Pb IgM _{Cap} **
Agreement	19/25	25/25	25/25	115/129	24/25	21/25	106/129
Percentage	76	100	100	89.1	96	84	82.2
McNemar's for correlated proportion	p=0.014	Ne	Ne	p=0.1088	p=0.3173	p=0.0455	p=0.0012
Kappa	0.39	Ne	Ne	0.78	0.78	0.66	0.37

* Tested at CDC, Puerto Rico

** Tested at Mahidol University, Thailand

Table 12. Test reproducibility: reader-to-reader reproducibility of rapid tests*

Test	Kappa (95% C.I.)		Homogeneity of kappa
DuoCassette	0.82	+/- 0.03	0.0002
HapalyseM	0.81	+/- 0.04	0.0693
SD Bioline	0.78	+/- 0.03	<0.0001
Dengucheck	0.75	+/- 0.04	<0.0001

* Each test was read and recorded independently by two operators

User-friendliness

The technicians were asked to score (0-3) the user-friendliness of the tests (Table 13). In addition to the points awarded for each parameter, one additional point was given to tests that did not require extra equipment (e.g. micropipettes or plate readers). Among the RDTs, DuoCassette scored highest with a mean score of 7.4;

Pb IgM_{Cap} scored highest among the ELISA tests, with a mean of 6.4.

The laboratories could also comment on the tests. The main problems are summarized in Table 14 – all four RDTs were described as difficult to interpret and the ELISAs were described as laborious or time-consuming.

Table 13. Operational characteristics of evaluated tests*

Test	RDT				ELISA				
	DuoCassette	HapalyseM	SD Bioline	Dengucheck	DxSelect	Pathozyme M _{Cap}	Pathozyme M	Pb IgM _{Cap}	SD IgM _{Cap}
Clarity of kit instructions	2.4	2.2	2.2	2.2	1.6	2	1.6	2.2	1.8
Technical complexity	2.8	1.6	2.8	2.8	1.6	1.8	1.4	2	2
Ease of interpretation of results	1.2	1.4	1	0.8	1.8	2.2	2.2	2.2	2.4
Equipment required but not provided	1	0	1	1	0	0	0	0	0
TOTAL SCORE	7.4	5.2	7	6.8	5	6	5.2	6.4	6.2

* The numbers represent the mean value scored by five of the seven evaluation sites. Sites could score from 0 to 3, where 0 is the lowest score and 3 the highest.

Table 14. Selected comments from evaluation sites: user-friendliness of tests

RDTs				
DuoCassette	HapalyseM	SD Bioline	Dengucheck	
Weak bands Interpretation difficult	Need more than 1½ hours Could be false positives (bottom not well-defined)	Weak bands	Weak bands Some background Instructions not clear	
ELISAs				
DxSelect	Pathozyme M _{Cap}	Pathozyme M	Pb IgM _{Cap}	SD IgM _{Cap}
Complicated technique (wash) High OD Long incubation period Instructions not clear	Laborious Instructions not clear	Insufficient conjugate Need extra step in dilution	Dilution laborious Standard cut-off sometimes high	Dilution laborious

Conclusion

The PDVI-WHO/TDR network was established to provide independent quality evaluations of dengue tests and advice on the selection and utility of dengue IgM tests according to performance and local conditions. PDVI and WHO/TDR do not certify or recommend any specific product but make data on performance and utility of diagnostic tests available to all United Nations Member States and on their web sites.

This laboratory-based evaluation used a specially designed serum panel, collected from DENV endemic countries in Asia and the Americas, to determine the performance of nine commercially available anti-DENV IgM tests. A sample size of 350 sera is adequately powered to give a precision of $\pm 5\%$ around the point estimates of sensitivity and specificity of each test compared to the reference assays. All four DENV serotypes were represented in the panel although not in equal proportions. The small number of DENV-4 sera reflects its rarity in most regions. The panel contained high, medium and low concentrations of anti-DENV IgM with a weight towards low and medium levels. This allowed better discrimination of comparative sensitivity of the tests under evaluation. The panel contained appropriate specimens to determine test specificity against pathogens that often cocirculate with DENV. The panel also included specimens positive for IgM or IgG against other flaviviruses that may cross-react with DENV. Field trials are required to determine the performance and utility of these tests in a local context.

The three ELISA kits performing at an acceptable level (DxSelect, Pb IgMCap, SD IgMCap) showed strong agreement with reference standards and were consistent across all evaluation sites. Of concern are false-positive results shown by some tests on sera that were all anti-DENV IgM negative but malaria positive, anti-DENV IgG positive or rheumatoid factor positive.

The reference laboratory at Mahidol University also tested all kits against 12 sera from patients with leptospirosis. Pb IgMCap showed cross-reactivity with 58%; DxSelect showed cross-reactivity with 25%. HapalyseM, the agglu-

tionation test from Pentax, showed high sensitivity (and specificity that reached almost 80%) but showed 67% false positivity towards the malaria samples. Hence, the test is not useful in settings where dengue and malaria are endemic. Further studies are needed to elucidate the cause of this cross-reactivity.

Among the RDTs the three lateral flow tests did not show an acceptable performance against the reference standard assays. DuoCassette reached the highest level of performance with a sensitivity of 77.8% (kappa value 0.68). SD Bioline and Denguecheck showed values well below this level. Taking into consideration the ease of use of the lateral flow tests, it can be argued that tests with a sensitivity of around 80% can be useful in monitoring outbreaks of dengue.

Denguecheck and Pb IgMCap did not have acceptable lot-to-lot reproducibility – kappa values of 0.39 and 0.37, respectively. This low level of agreement between lots may be linked to the manufacturing process and reagents used and should be addressed by the manufacturers.

Technicians were asked to score tests' user-friendliness. The three lateral flow RDTs received higher scores than the ELISAs or the Pentax (agglutination) test as they are simple to perform, involve only a few steps and require no extra equipment or supplies. All the technicians commented that the RDTs showed weak bands but this did not result in reader-to-reader variability.

This evaluation has several limitations. Test performance was compared with reference laboratory assay results. These may be less sensitive than the commercial assays and cause some results to be misclassified as false positives. Specificity of the tests may be higher in real-life settings because not all potential causes of false-positive results will be present. The panel consisted of a high proportion of specimens from persons with secondary DENV infections and therefore was weighted towards higher anti-DENV IgG and lower anti-DENV IgM levels. However, this reflects the situation in most dengue-

endemic countries and therefore tests that performed well against this panel could be expected to perform well in these diagnostic settings. The evaluation could not comprehensively assess whether the kits can detect primary infections of all four virus serotypes as all four DENV types were not represented equally in the panel. The number of samples in the different disease categories was not large enough to draw statistically significant conclusions. However, the results give an indication of what further analyses are necessary to determine the usefulness of a specific test, taking account of local epidemiology.

The IgM assays used widely in dengue-endemic countries should not be used as a confirmatory test for current illness. The IgM antibody persists on average for about 60 days and therefore its presence simply means that the person from whom the sample is taken has had a dengue infection at some time in the past two to three months. Limitations of anti-DENV IgM tests include their inability to identify the infecting DENV type and potential cross-reactivity with antibodies to other flaviviruses. However, IgM tests can be useful for surveillance and can support diagnosis of DENV infection in consideration with clinical symptoms, medical history and other epidemiological information.

Selection of tests for procurement

A number of factors should be considered before choosing which test to procure. The factors considered may vary according to whether the tests are to be used to support dengue case detection, management, surveillance or disease control.

- Performance is an important factor. A highly sensitive test is required to support case detection. The specificity of the test is of lesser importance as there are few adverse effects from overtreatment of dengue fever (since no specific anti-dengue drugs are available). A strong cross reactivity to a specific disease or condition may be acceptable if there is low prevalence of the disease or condition in the setting in which the test will be used.
- Ease of use is important when a test is used in field settings with no, or variable, access to electricity. Personnel training and the workload of the clinic should also be considered.
- Test stability can be crucial in settings with extreme temperatures. Even if good storage conditions are available in the laboratory, tests may be exposed to high temperatures during transportation if no proper cold chain is in place.
- Climate factors, e.g. very high humidity, can directly influence test performance.
- Long shelf-life can be of high importance in remote settings with poor resources. This reduces the pressure on the supply chain as well as the risk of having to discard unused tests that have expired.
- Price often has a strong impact on test selection. Companies with tests that show an acceptable performance are invited to submit a tender for inclusion of their test in the WHO Bulk Procurement Scheme. This scheme has been developed to enable UN member states to have access to quality-assured medicines, vaccines and diagnostics at negotiated pricing.

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Annex 1. Operational characteristics form

Name of test:

Manufacturer:

Date of evaluation:

1. Clarity of kit instructions

- difficult to follow 0
- fairly clear 1
- very clear 2
- excellent 3

2. Technical complexity

complex 0

If yes, why? (check all that apply)

- Small volumes
- Multiple steps
- Short time intervals between steps
- Test difficult to manipulate
- No space for labelling
- Incomplete migration of samples
- Other:

- fairly easy 1
- very easy 2
- excellent 3

3. Ease of interpretation of results

difficult 0

If yes, why? (check all that apply)

- Signal intensity low or diffuse
- Signal colour variation

fairly easy 1

very easy 2

unambiguous 3

4. Equipment required but not provided e.g. micropipette

yes 0

no 1

If no, what is required?

.....

Comments:

.....

.....

.....

.....

Annex 2A. Laboratory data collection form (ELISA)

Name of test:

Manufacturer:

Date of evaluation:

LOT number:

Expiry date:

Date of sample reconstitution:

Dilution:.....V buffer:.....V sample:.....Dilution factor.....Cut off:.....

Row number	Serum ID (or control)	Date of testing	Test Results				Daily signature of PI
			Well 1		Well 2		
			OD	Result	OD	Result	
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							
11							
12							
13							
14							
15							

Annex 2B. Laboratory data collection form (Rapid Test)

Name of test:

Manufacturer:

Date of evaluation:

LOT number:

Expiry date:

Date of sample reconstitution:

Row number	Serum ID (or control)	Date of testing	Test Results	Daily signature of PI
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				

Annex 3. Contact details for dengue diagnostic evaluations

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