Operational guidance on the use of yellow fever assays in the context of surveillance
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References
Acknowledgements

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This document was developed in consultation with:


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From the Pan American Health Organization (PAHO) / WHO Regional Office of the Americas: Jairo Andres Méndez-Rico.
### ABBREVIATIONS AND ACRONYMS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<tr>
<td>BSC</td>
<td>Biosafety cabinet</td>
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<td>CDC</td>
<td>US Centers for Disease Control and Prevention</td>
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<td>CI</td>
<td>Chlorine</td>
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<tr>
<td>DEN</td>
<td>Dengue</td>
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<tr>
<td>E</td>
<td>Envelope</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>EYE</td>
<td>Eliminate Yellow Fever Epidemics</td>
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<td>EYE LTWG</td>
<td>Eliminate Yellow Fever Epidemics Laboratory Technical Working Group</td>
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<tr>
<td>GYFLaN</td>
<td>Global Yellow Fever Laboratory Network</td>
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<tr>
<td>IFU</td>
<td>Instructions for use</td>
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<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>IHR</td>
<td>International Health Regulations</td>
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<tr>
<td>LFA</td>
<td>Lateral flow assay</td>
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<tr>
<td>MAC-ELISA</td>
<td>IgM-antibody capture enzyme-linked immunosorbent assay</td>
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<tr>
<td>NAAT</td>
<td>Nucleic acid amplification test</td>
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<td>NL</td>
<td>National Laboratory</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PPE</td>
<td>Personal protective equipment</td>
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<tr>
<td>ppm</td>
<td>Parts per million</td>
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<tr>
<td>PRNT</td>
<td>Plaque-reduction neutralization test</td>
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<tr>
<td>RC</td>
<td>Regional YF Laboratory Coordinator</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RRL</td>
<td>Regional Reference Laboratory</td>
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<tr>
<td>RT-qPCR</td>
<td>Reverse transcriptase quantitative polymerase chain reaction</td>
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<tr>
<td>SOP</td>
<td>Standard operating procedure</td>
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<tr>
<td>UNICEF</td>
<td>United Nations Children's Fund</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>WN</td>
<td>West Nile</td>
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<tr>
<td>YF</td>
<td>Yellow fever</td>
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<tr>
<td>ZIK</td>
<td>Zika</td>
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The goal of immediate laboratory identification of patients suspected to be infected with yellow fever (YF) virus is to prevent YF outbreaks in YF endemic areas and at risk areas;

The recommended specimen types for laboratory confirmation of YF are blood, serum or plasma;

Laboratory confirmation of YF infection in specimens from a suspected case is ideally done using a nucleic acid amplification test (NAAT), such as reverse transcriptase-quantitative polymerase chain reaction;

If NAAT is unavailable, has generated a negative result, or if the specimen is from a convalescent patient, testing for anti-YF immunoglobulin M (IgM) is recommended;

Any individual that meets the definition of a suspected YF case should be tested in a timely manner, and as per the recommended performance indicators laid out in the WHO Surveillance Standards (6);

Specimen quality and documentation of the time of specimen collection relative to symptom onset is critical in obtaining accurate laboratory results;

A commercial NAAT, RealStar® Yellow Fever Virus RT-PCR kit 1.0 from altona, has been independently evaluated by World Health Organization (WHO) and found to be suitable for use (9,10);
Key points

- Two commercial assays for the detection of anti-YF IgM antibodies have been independently evaluated by WHO and are suitable for use:
  - The STANDARD™ Q YF IgM Test kit (a rapid diagnostic test) from SD Biosensor (13);
  - The YF MAC-HD 1.0 assay (a MAC-ELISA test) from ATCC (12).

- Confirmatory testing of YF IgM positive samples from unvaccinated patients by plaque-reduction neutralization test in a reference laboratory is required.

- Member States are requested to immediately notify WHO under the International Health Regulations 2005 of positive laboratory results, including a YF laboratory result that awaits confirmation.

- The 2023 edition of the WHO Laboratory manual for yellow fever (12) contains more comprehensive information on all aspects of this guidance document.

- Guidance for the use of IgM kits must be observed;

- In laboratory settings, all manipulations of specimens originating from suspected, probable, or confirmed cases of YF should be conducted according to a risk-based approach.

- WHO can assist Member States to access testing through referral. If the need arises, Member States can contact the relevant WHO Regional YF Laboratory Coordinator.
Introduction

Yellow fever (YF) is a mosquito-borne viral disease endemic in 34 sub-Saharan African and 13 Central/South American countries. YF affects humans and non-human primates (1), with human transmission occurring when infected mosquitoes, in jungle (sylvatic), rural or urban areas, bite humans and transmit the virus. In addition, occupational transmission may potentially occur due to mishandling of infected biological specimens, in particular in non-vaccinated infection (2).

An infected bite may initially produce nonspecific febrile symptoms, and some individuals remain asymptomatic or experience mild symptoms. Severe viscerotropic disease can occur (jaundice, liver and multi-organ failure, death), with a case-fatality rate of 40-50% in persons with severe disease. The disease incidence is difficult to assess due to limitations in detection and reporting, jointly leading to overall underreporting (3).

A safe, inexpensive vaccine confers lifelong immunity (4), but low vaccine coverage and the presence of infected non-human primates in some regions mean there is an ongoing risk of YF outbreaks. Strong surveillance programmes can minimize this risk, and laboratory diagnosis of YF is integral to that effort, enabling swift identification of cases and subsequent response to mitigate disease spread.

YF virus belong to the genus Flavivirus and shares many characteristics with other closely related viruses including dengue (DEN), Zika (ZIK) and West Nile (WN) viruses. These flaviviruses have similar structural resemblances, mode of transmission, early clinical presentation, and reactions in serological tests (5). Clinical presentation of YF can be misleading and can also result in clinical misdiagnosis in favour of unrelated diseases, such as malaria, which can cause delays in diagnosis and response. Accurate and timely identification of YF is therefore critical.

The Eliminate Yellow Fever Epidemics (EYE) Strategy’s Laboratory Technical Working Group (EYE LTWG) is responsible for devising and coordinating efforts to improve YF laboratory capacity throughout affected areas with funding support from Gavi, the Vaccine Alliance (Gavi).
Purpose of the guidance and methodology of development

This guidance document is designed to assist Member States of the Global YF Laboratory Network (GYFLaN), primarily National YF Laboratories (NL), in their ongoing efforts to support laboratory surveillance and response to YF. This guidance document can be a useful resource to administrative and professional laboratory staff, clinical practitioners and public health professionals conducting arbovirus surveillance, and personnel involved in training, monitoring and supervision.

The purpose is to provide guidance on laboratory YF testing strategies with recommendations for appropriate testing methods that maximize the efficient use of resources. It complements existing WHO tools for addressing YF. Each country should tailor these strategies based on the local context and regulatory standards required for YF response and clinical laboratory testing on human specimens in the context of surveillance.

The initial draft of this document was created by WHO-HQ VPD Surveillance and Risk Assessment team in Q2 of 2023, and was further refined through a technical consultation with other experts from WHO regional and country offices, as well as with global external experts in the field of YF virus epidemiology, diagnostics and laboratory practices in Q3 of 2023. This group of individuals located across various regions are individual experts in arbovirology, in particular in the field of YF diagnostics, and are listed in the below Table 1. Peer Reviewers were Dr Amy Lambert and Christin Goodman, both renowned virologists with decades of expertise in YF diagnostics, as well as Drs Christian Malaka, Léa Gangoue, Limbaso Samson Konongoi and Emmanuel Rivalyn Nakoune-Yandoko who provided valuable end-user perspectives.
TABLE 1

List of contributing experts in the development of this operational guidance.

<table>
<thead>
<tr>
<th>Name</th>
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<th>WHO Region*</th>
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<td>Female</td>
<td>Virologist, Yellow Fever laboratory expert</td>
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* AFR: WHO African Region; AMR: WHO Region of the Americas; EUR: WHO European Region.

All individual experts submitted a Declaration of Interest (DoI) and Confidentiality Undertakings (COU) form prior to the technical consultation. Assessment and management of conflicts of interest were performed using WHO’s Individual Expert System (EIS) on the Global Engagement Management platform, and where DoI and COU of WHO experts are reviewed and handled by the Ethics Team ( ethicsoffice@who.int ). Following this assessment, only experts vetted by the Ethics Team were invited for the consultation.
YF TESTING IN THE CONTEXT OF SURVEILLANCE

The minimal standard design for YF surveillance is comprised of a nationwide, passive, facility-based, case-based surveillance intervention where laboratory testing is performed for all suspected cases as per epidemiological setting. YF surveillance for human cases should take place among all age groups (6). Laboratory testing of clinical specimens and results reporting for clinically suspected cases must be timely and accurate for early detection of outbreaks. All cases of acute fever and jaundice in high-risk areas should undergo laboratory confirmation, with positive results triggering the activation of an appropriate outbreak response.

Scenarios and individuals that warrant YF testing include:

- Routine YF surveillance intervention where:
  - All suspected cases of YF living in or visiting a YF endemic region must be investigated in districts where active YF virus circulation has not yet been confirmed;
  - All cases of acute fever and jaundice in high risk areas when there is not an outbreak should include laboratory confirmation to confirm the diagnosis;

- Testing during a confirmed outbreak:
  - All suspected case occurring during a documented YF outbreak should ideally also be tested. However, in order to avoid over burdening of the laboratory, if the laboratory has reached maximal capacity, priority should be given to testing specimens from those areas where local transmission has not yet been confirmed;
  - It is not essential to perform serology testing to differentiate yellow fever and other flaviviruses on specimens where local transmission of YF has already been confirmed.

- Outbreak or a cluster of illnesses of unknown cause with clinical similarities to YF;

- Unvaccinated travellers with illness consistent with YF symptoms returning from a YF endemic area or with ongoing YF transmission;

- Suspected laboratory-acquired infection.

The decision to test should also be based on both clinical and epidemiological factors. A suspected case is any person with acute onset of fever, with jaundice appearing within 14 days of onset of the first symptoms. This is a sensitive but not specific definition. Definitions for probable, confirmed, and discarded cases are available in the WHO Surveillance Standards for Yellow Fever (6).
QUALITY OF LABORATORY RESULTS STARTS AT THE PREANALYTICAL PHASE

The quality of YF test results is determined by all personnel involved at every stage of the testing procedure; from the investigation request, patient identification, sample collection, handling, and transportation processes, to the actual analytical (testing) phase. A series of pre-examination procedures throughout the preanalytical, analytical and the postanalytical phases play a critical role in ensuring quality-assured laboratory results are delivered.

Pre-examination procedures related to the preanalytical phase include:

- ensuring adequate indication for testing;
- the use of a standardized request form;
- ensuring traceability of a collected specimen to an identified patient with correct documentation and labelling of specimen;
- ensuring all specimen and clinical information critically required to inform testing (e.g. YF vaccination history, date of specimen collection, date of symptom onset, etc.) is documented and accompany the specimen
- ensuring that specific specimen collection guidance and policies are in place to include details on appropriate specimen type, the minimum volume sufficient for all possible test procedures, and measures to ensure specimen integrity and prevent its deterioration (e.g., hemolysis);
- proper transitional storage of specimens until transport to laboratory;
- arranging expeditious transport to preserve specimen integrity;
- monitoring of samples in transit;
- recording the receipt of samples;
- processing of urgent samples and policies for rejection of samples.
ROLE OF THE SURVEILLANCE PROGRAMME AND NATIONAL LABORATORY IN SPECIMEN COLLECTION

The surveillance programme must be familiar and implement appropriate specimen collection and handling procedures, and requirements to ensure the best possible YF testing conditions to obtain an accurate result, as well as establish excellent communication channels with the NL. While the NL is not usually directly responsible for obtaining the specimens for YF testing, it can benefit for the NL to take part in organizing and/or facilitating trainings for health workers, at facility level, in the collection, handling, and referral of specimens from suspected cases of YF. Minimally, NL staff must have well-established communication with the health centres, epidemiologists, and clinicians who obtain the specimens, and to convey the complete sets of requirements needed to ensure quality assured testing of the specimen. The latter require the NL staff to master all the details underlying each of the requirements. These include knowledge on appropriate specimen type, volume requirement needs for the NL and potential confirmatory testing at the Regional Reference Laboratory (RRL), packaging and handling procedures for domestic and international transportation, and the minimum required case information.

SAFETY

Standard operating procedures (SOPs) for specimen collection that include safety measures must be implemented. All specimens collected for laboratory investigations should be regarded as potentially infectious. Measures should be taken to minimize the risk of clinical and laboratory transmission when collecting and handling clinical specimens from suspected cases of viral haemorrhagic fever, including suspected cases of YF. Appropriate personal protective equipment (PPE) including eye covering and gloves, and disinfection practices must be used. All needles and syringes should be disposed of appropriately. YF vaccination of health workers in contact with patients from endemic areas for YF is often included in local policies. Further guidance on this can be found here (https://www.who.int/publications/i/item/9789240052154).

SPECIMEN INFORMATION

A completed specimen submission form must be obtained for each specimen to ensure appropriate testing and correct case classification. Information critical to the eventual interpretation of YF testing results must include at a minimum:
- symptoms
- date of onset of symptoms;
- date of specimen collection;
- specimen type;
- place of residence;
- recent travel history, including dates;
- any history of YF vaccination, including date of vaccination if available. If vaccination history is unavailable and cannot be ascertained by any means, this should be noted on the form, so that further follow-up is not attempted;
- the inclusion of results of malaria testing and tests for other etiologic agents is highly desirable.
SPECIMEN TYPES

- **Serum** is the recommended sample type for YF testing. It is the most versatile patient specimen and can be used in molecular (Reverse transcription quantitative polymerase chain reaction [RT-qPCR]) and serological (enzyme-linked immunosorbent assay [ELISA]; immunofluorescence assay; lateral flow assay [LFA]; and plaque-reduction neutralization test [PRNT]) testing;

- **Plasma** can be used for RT-qPCR and some serological applications;

- **Whole blood** can be used for RT-qPCR and some lateral flow applications but has not been validated for use by WHO in commercial assays. Whole blood should not be collected in heparinized tubes and should not be used in ELISA-based assays;

- **Liver, kidney or other tissue samples** obtained postmortem from deceased subjects (humans or non-human primates) with suspected infection, which can be used for histopathology and histochemistry (ideally both 1 cm³ fresh-frozen and 10% formalin-fixed) in addition to blood obtained by cardiac puncture, which can be tested by RT-qPCR.

TIMING OF SPECIMEN COLLECTION

A blood sample should be collected on first contact with every suspected YF case even in the absence of an declared outbreak in the area – not waiting for the ideal window or a second sample – and within 14 days of symptom onset. In some cases, a second specimen will be requested following initial testing.

COLLECTING THE SPECIMEN

The recommended specimen type for laboratory confirmation of YF in non-fatal cases is serum. Serum should be separated from whole blood promptly to prevent false negative results in IgM assays. Blood should be collected by venepuncture into a sterile red-top tube (plain non-barrier, no preservative, no anticoagulant, with clot activator) or ideally into a serum separator tube with clot activator, without anticoagulant. Staff should be trained in practices to avoid haemolysis of the blood to avoid interference with the tests.

VOLUME

A strict minimum of 1 ml of serum is needed to confirm YF (2 ml is ideal). To obtain this volume of serum, 2.5-5 ml of blood should be drawn from adults; collection of 1-2 ml of blood is acceptable from infants. If it is not possible to separate the serum at the collection site (as described below in Processing of blood specimens), a strict minimum of 5 ml of blood must be drawn.
PROCESSING OF BLOOD SPECIMENS

To avoid haemolysis and test interference, the serum must not remain in contact with a blood clot longer than the recommended times mentioned below.

If a centrifuge is available at or near the collection site: The serum separator or regular stoppered tube containing the blood should be placed in an upright position at room temperature immediately following collection for 30-60 minutes to allow the blood to clot. After the blood has clotted, centrifuge the tube at 1000 x g for 10 minutes to separate the serum from the clot. If a serum separator tube was used, carefully transfer the serum into a sterile, freezer compatible screw-capped vial (cryovial), labelled with the patient ID, specimen type, and date of collection. If a serum separator was not used, carefully draw off the serum without disturbing the red blood cells.

If a centrifuge is not available at or near the collection site: Refrigerate the blood specimen at 4-8°C immediately following collection. Serum should be carefully removed from the clotted red blood cells about 6 hours if a serum separator tube was used; blood drawn in a plain tube will require longer, but serum should be removed no longer than 24 hours following collection.

STORAGE OF SERUM

Serum specimens should be transported to the laboratory within 48 hours. If the serum specimen is not transported within 24 hours of collection, store the separated serum at 4-8°C until transport. Overall storage at 4-8°C, including at the testing site should be no longer than 7 days. Specimens should be frozen at or below -20°C if stored for longer in a non-frost-free freezer.

SPECIMENS FROM FATAL CASES

Liver or kidney specimens collected at postmortem from patients (and non-human primates in the Region of the Americas), along with blood from cardiac puncture can improve the outcome of YF surveillance (6). Fresh tissue approximately 1 cm³ can be frozen at -80°C and sent directly to a reference laboratory on dry ice, or fixed in 10% buffered formalin and transported at room temperature. Tissue samples that will only be tested by RT-qPCR can be stored in an RNA stabilization solution and shipped at room temperature. Non-human primate specimen testing should be coordinated by the Pan American Health Organization YF Regional Coordinator (RC).
Successful shipments of specimens require advanced planning and communication between the sender, carrier, and receiver. Specimen shipping requirements may vary by country and international regulations (7). All specimens included in the shipment must be properly labelled and should be accompanied with the specimen submission form, in addition to all other documentation and forms that might be required by national programmes.

**TIMING OF SHIPMENT**

The specimen should be shipped to the NL within 48 hours of collection.

**SHIPPING TEMPERATURE**

Serum or other samples that are being transported within 24 hours of collection may be shipped without cold packs providing the ambient temperature is no greater than 20°C. As this is rarely the case in YF endemic countries, it is advisable to store specimen in the refrigerator and to organize prompt pick-up for transport referral. Before the serum can be separated by centrifugation, whole blood can be stored up to 6 hours at 20-25°C, and up to 24 hours when stored at 4-8°C. Whole blood cannot be frozen. Serum should be stored at 4-8°C until shipment and for a maximum of 7 days. Beyond this period it is advisable to store the serum at -20°C or colder. For specimens that have been refrigerated, cold packs or wet ice should be included in the package. Frozen specimens should remain frozen throughout transit by shipping with frozen cold packs in an insulated container. If the transit interval is greater than 3 days, the use of dry ice is advisable, if available.

**WITHIN-COUNTRY SHIPMENTS**

Within-country shipments must meet all national requirements. The NLs should work closely with domestic carriers to ensure appropriate packaging (triple packaging system), documentation and labelling.
INTERNATIONAL SHIPMENTS FOR REFERRED SPECIMENS

If a specimen meets the requirements for referral to a RRL or other reference laboratory, which includes a strict minimum of 0.5 ml of serum (ideally ≥1 ml), NLs in the WHO African Regional Office and Eastern Mediterranean Regional Office regions should use the standardized procedure and EYE.ops booking form. All transport costs and shipping materials are covered by EYE.ops, which is the transportation mechanism set up by the EYE strategy to facilitate and expedite international shipping of specimens between NL and reference laboratories. Specimens can be used for diagnosis or for quality control review.

The EYE.ops shipping procedure consists of three phases:

1. NL contacts EYE.ops using the instruction provided in the EYE.ops booking form: https://tinyurl.com/eyeopsbookingform
2. EYE.ops and/or their shipping logistics partner acknowledges and clears the request within 24 hours;
3. EYE.ops arranges for transport of the specimen(s) from the NL using a WHO pre-determined partner courier. The video https://youtu.be/iBEAmwcbxn0 contains all details of the process.

The use of the WHO pre-determined partner courier for referred specimens is not mandatory when other well-established methods are available, or for laboratories not supported by EYE.ops.

For international transport, cultures or specimens known to contain live infectious substances affecting humans should be transported as Category A, UN2814, and all other diagnostic samples are transported as Category B, UN3373.
HANDLING OF SPECIMENS AND OCCUPATIONAL HEALTH

Each laboratory should develop specific SOPs for opening packages and logging receipt of specimens according to the respective institutional risk assessment.

- Laboratory personnel should wear appropriate PPE;
- Staff should be vaccinated for YF, unless otherwise is specifically mentioned in national guidelines;
- Unpacking and accessioning is ideally done with two persons – one to manipulate the specimens and one to record the information;
- Follow-up specimens should be associated with the original specimen in the accessioning process;
- Specimens from patients with suspected YF infection should be considered infectious and must be manipulated (unpacking, transferring, aliquoting, testing) and accessioned in a functioning Class II biosafety cabinet (BSC), prior to sample inactivation – e.g., 56°C for 30 minutes or through RNA lysis as part of molecular testing procedure. Properly inactivated specimens do not require a BSC but must be handled according to good laboratory safety practices;
- Where the use of a centrifuge is required for a procedure, safety cups or sealed rotors should be used;
- Disinfectants, such as 70% ethanol, should be used to wipe down surfaces and a solution of sodium hypochlorite containing 5000 ppm Cl (corresponding to a freshly prepared 1:10 dilution of typical household bleach containing 5% sodium hypochlorite) should be available in case of spills. All potentially contaminated materials should be placed inside biohazardous discard containers that are lined with leak-proof bags.

CASE INFORMATION

It is incumbent upon the laboratory to make sure the minimum required case information is received with the specimen, and to follow-up with the clinic if it is not.
STORAGE OF SPECIMENS

Following receipt and accessioning, specimens should ideally be stored at 4-8°C, and tested as soon as possible, with the exception of specimens that arrive frozen to the laboratory. Such specimens should remain frozen at -20°C or preferably at -70°C in a non-frost-free freezer, according to the SOP for the specimen type. Specimens stored at 4-8°C that cannot be tested within a week, should be aliquoted (depending on use) and immediately (ideally within 72 hours from sample collection) frozen at -20°C or below. Repeated cycles of freezing and thawing should be avoided especially in specimens that will be tested by RT-qPCR, to prevent degradation of the RNA.
YF testing algorithms developed for Africa and the Region of the Americas have been harmonized to encompass regional needs. The testing sequence and result interpretations have been standardized. The algorithms are used when triaging specimens for testing, with attention being paid to the superscript notes.

Testing methods and interpretation are largely dictated by the dynamics of YF infection as shown in Figure 1.

**FIGURE 1.** Sensitivities of molecular (RT-qPCR) and serologic (IgM) assays according to the number of days after onset of symptoms.

The sequence of YF testing used in routine surveillance settings (e.g., investigation of suspected cases from a location without a current YF outbreak) follows the Yellow Fever Testing Algorithm for Routine Surveillance in Figure 2.
A suspected YF case is any person with acute onset of fever, with jaundice appearing within 14 days of onset of the first symptoms. Clinical suspicion of YF may be made prior to the appearance of jaundice and is based on other clinically compatible symptoms such as fever, headache, stiffness, nausea, vomiting, and fatigue and on epidemiologic factors. Assessment of YF vaccination history, malaria testing history, travel history, and history of contact with a known YF case (if relevant) should be recorded and taken into consideration when interpreting test results. 

1. A suspected YF case is any person with acute onset of fever, with jaundice appearing within 14 days of onset of the first symptoms. Clinical suspicion of YF may be made prior to the appearance of jaundice and is based on other clinically compatible symptoms such as fever, headache, stiffness, nausea, vomiting, and fatigue and on epidemiologic factors. Assessment of YF vaccination history, malaria testing history, travel history, and history of contact with a known YF case (if relevant) should be recorded and taken into consideration when interpreting test results.

2. Applicable to RT-qPCR testing and IgM RDT only. EDTA blood tubes should ideally be used. Avoid using sodium heparin tubes as this interferes with PCR.

3. If the IgM results are uninterpretable (UI), due to a high background or potential laboratory factors, consider repeating the test, and repeat a 2nd specimen if repeat result is still UI. If testing is not available to be UI, treat as negatives. If a rapid IgM LEIA test is used, serial testing including a confirmatory test of a MAC-ELISA based assay and communication of preliminary results might be required; please refer to the Operational guidance on retesting) to a RRL is advisable. If a repeated equivocal test result remains equivocal upon re-testing a second sample may be requested and tested. If the second sample repeat as equivocal both samples should be sent to the RRL.

4. A positive IgM result alone is not confirmatory but considered presumptive evidence of infection. Additional clinical and epidemiological criteria, such as history of vaccination, must be used for the final interpretation of the results and classification of the suspected YF case.

5. Age-appropriate paired sample set to demonstrate seroconversion should be performed in an RRL.

6. In the case where a MAC-ELISA method is used, an equivocal result is when a valid result falls within the range between a negative or positive result. Refer to the test instruction for the indicated range of equivocality for this specific test. Equivocals do not refer to an UI test result, e.g., equivocal is not due to presence of factors that cause non-specific background reactions.

7. In the case of a first equivocal test result, the test can be repeated based on the type of IgM assay used. Consider retesting should be taken into account and direct referral of specimen with equivocal results (without repeating) to a RRL is advisable. If a second equivocal test result remains equivocal upon re-testing a second sample may be requested and tested. If the second sample repeat as equivocal both samples should be sent to the RRL.

8. A positive IgM result alone is not confirmatory but considered presumptive evidence of infection. Additional clinical and epidemiological criteria, such as history of vaccination, must be used for the final interpretation of the results and classification of the suspected YF case.

9. Do not delay reporting of the inconclusive result. With an inconclusive result, infection cannot be ruled out, though it is less likely if molecular testing is negative. Whenever possible a second sample taken ≥10 days post onset of illness should be requested and tested to account for possible seroconversion.

10. Fresh and fixed tissue samples (≥ 1cm³) should be collected (liver and kidney tissue should always be collected; other clinically compatible symptoms such as fever, headache, stiffness, nausea, vomiting, and fatigue and on epidemiologic factors. Assessment of YF vaccination history, malaria testing history, travel history, and history of contact with a known YF case (if relevant) should be recorded and taken into consideration when interpreting test results.

11. With a confirmatory test result, in cases where no YF virus circulation has been recently described, differential neutralization testing with flaviviruses endemic in the area of exposure or neutralization testing of a positive serum sample could be performed in an RRL.

12. Final interpretation to be reported and advice on conclusion should occur after all testing is complete (e.g., malaria, differential IgM and PRNT for other flaviviruses).

13. Haemorrhagic fever case collection must always be done. For fatal cases, RT-qPCR should be performed on all available samples, independent of the collection date. If the laboratory has capacity to only test by RT-qPCR or IgM serology, all samples at any number of days post-onset should be tested with the assay.

14. For cases with no history of vaccination, vaccination history unknown, or vaccinated ≥14 days before onset of illness. In areas where YF virus infections have not been reported recently, immediate confirmatory testing at a Regional Reference Laboratory (RRL) is required for such cases (need to specifically request to RRL when referring specimen).

15. In the case when a MAC-ELISA method is used, an equivocal result is when a valid result falls within the range between a negative or positive result. Refer to the test instruction for the indicated range of equivocality for this specific test. Equivocals do not refer to an UI test result, e.g., equivocal is not due to presence of factors that cause non-specific background reactions.
KEY POINTS WHEN TESTING FOR YF IN BOTH ROUTINE SURVEILLANCE AND DURING A CONFIRMED OUTBREAK

- For fatal cases, test fresh tissues with RT-qPCR, and fixed tissues with immunohistochemistry. Positive results confirm YF, while negative results exclude YF as the infecting agent;

- In both fatal and non-fatal cases, serum, plasma or whole blood specimens collected ≤14 days after symptom onset should be first tested by YF RT-qPCR. Positive results are reported as confirmed YF infections and no further testing is required. If YF RNA is not detected, infection is not ruled out as RNA may be below the limit of detection or the timing of infection may not have been reported accurately. False negative results can occur due to poor quality or mishandling of specimens, shipping issues, or technical reasons. Serum and plasma specimens yielding negative molecular results are reflexed to IgM testing.

- Serum and plasma specimens collected >14 days after symptom onset are tested using IgM methods. Additionally, specimens with negative nucleic acid amplification test (NAAT) results or from regions where NAAT is unavailable should be tested for YF IgM;

- YF IgM positive results from persons with no or uncertain history of YF vaccination are not confirmed. They indicate presumptive evidence of acute YF infection or YF vaccination. An IgM positive test result may represent:
  - nonspecific reactivity;
  - cross-reactivity to another flavivirus;
  - residual IgM positivity from a previous YF virus infection;
  - or recent YFV vaccination.

- Differential and confirmatory testing of YF IgM positive and equivocal specimens at the RRL changes the presumptive status to confirmed or excluded. Vaccination history is critical in determining the interpretation and the need for confirmatory testing at the RRL due to the persistence of vaccine-induced IgM. PRNT typically includes YF and other flaviviruses. Successful YF virus confirmation by PRNT is usually limited to primary YF virus infections because non-differentiated flavivirus results occur due to previous exposure to other flaviviruses;

- Negative IgM results from a jaundiced or haemorrhagic case, or from a specimen drawn from a suspected case >7 days after symptom onset, excludes YF as the infecting agent. Negative IgM results are considered inconclusive if the specimen was taken ≤7 days after symptom onset as seroconversion may not have occurred yet. In this case, efforts should be made to obtain a second specimen >10 days post-onset.
TESTING DURING AN OUTBREAK

The sequence of testing used during an outbreak follows the Yellow Fever Testing Algorithm during Outbreak shown in Figure 3. The initial order of testing differs from routine surveillance. Here, the dynamics of infection are not taken into consideration. Serum, plasma, or whole blood specimens from all fatal and non-fatal cases are first tested by RT-qPCR, regardless of date after onset of symptoms. If results are positive, the result is considered confirmed, and if negative, specimens are reflexed to IgM testing. Interpretations of IgM testing considers the dates after symptom onset similarly to routine surveillance and final case interpretation must consider clinical presentation and epidemiological context. Note that fresh and fixed tissue from fatal cases is treated the same as in routine surveillance.
A suspected YF case is any person with acute onset of fever, with jaundice appearing within 14 days of onset of the first symptoms. Clinical suspicion of YF may be made prior to the appearance of jaundice and is based on other clinically compatible symptoms such as fever, headache, myalgia, arthralgia, vomiting, and urticaria and on the epidemiologic context. A history of contact with a known YF case (if relevant) should be recorded and taken into consideration when interpreting test results.

2. Applicable to RT-qPCR testing and IgM RDT only. EDTA blood tubes should ideally be used. Avoid using sodium heparin tubes as interfering with PCR.

3. Whenever available, RT-qPCR should be the first-line test during an outbreak, irrespective of the number of days since symptom onset. Therefore, RT-qPCR might be reported in all samples collected as a primary test. A positive result in these samples will confirm YF infection. Whereas a negative result would not exclude the possibility of a YF infection. Samples with negative RT-qPCR results should be referred for IgM testing regardless of the day post-onset of illness that they were collected as a negative molecular result does not rule out YF and serology should be done. In case of non-specificity of the typical blood-based specimens, RT-qPCR on alternative specimens such as saliva, urine, and sputum could be useful to support case confirmation. For fatal cases, RT-qPCR should be performed on all available samples, independent of the collection date. If the laboratory has capacity to only test by RT-qPCR or IgM serology, all samples at any number of days post-onset of illness should be tested with the available assay.

4. For cases with no history of vaccination, vaccination, history unknown, or vaccinated >14 days before onset of illness.

5. If IgM results are uninterpretable (UI) due to high background and/or potential inhibitory factors, consider repeating the test, and request a 2nd specimen if repeat result is also UI. If testing is not continuous to be UI, must be negative. If a rapid IgM LEISA test is used, serial testing including a confirmation round of a MAC-ELISA based assay and communications of preliminary results might be required; please refer to the Operational guidance on the use of YF test for more details.

6. If the case where a MAC-ELISA method is used, an equivocal result is when a valid result falls within the range between a negative or positive result. Refer to the last instructions for the indicated range of equivocality for this specific test. Equivocal does not refer to an UI test result, e.g., optical density test due to presence of factors that cause non-specific background reactions.

7. In the case of a first-equivalent test result, the test can be repeated based on the type of IgM assay used. Cost considerations should be taken into account and direct referral of specimen with equivocal results (without retesting) to a RRL is advisable. If an equivocal second test result is obtained a second sample may be requested and tested. If the second sample repeats as equivocal both samples should be sent to the RRL.

8. A positive IgM result alone is not confirmatory but considered presumptive evidence of infection. Additional clinical and epidemiologic criteria, such as history of vaccination, must be used for the final interpretation of the results and classification of the suspected YF case. It is not essential to perform serology testing to differentiate yellow fever and other flaviviruses on specimens where local transmission of YF has already been confirmed.

9. Once an outbreak of YF has been confirmed in a specific area, the decision might be made to not refer outpatient samples from patients residing in the same area to a RRL for confirmatory testing but to report as presumptive infection. However, cases from a new geographic area, particularly if adjacent or linked to outbreak areas, or with atypical or unusual presentation of illness, should be referred to RRL for further confirmatory testing.

10. A second sample taken 10 days post onset of illness should only be requested and tested if the case is from a distinct area where there is still no sign of an outbreak.

11. Fresh and fixed tissue samples (≥1 cm³) should be collected (liver and kidney tissue should always be collected; other organs may also be collected) and submitted to a RRL for confirmatory testing. Fresh tissue samples collected ≤7 days post symptom onset and/or collected from jaundice or hemorrhagic fever case should be tested with available assay. If fixed tissue samples collected >7 days post symptom onset or from non-fatal cases should only be requested and tested if the case is from a new geographic area, particularly if adjacent or linked to outbreak areas, or with atypical or unusual presentation of illness, it is possible to perform the test up to 14 days after symptom onset.

12. A negative RT-qPCR in tissue from fatal cases can be followed up with serology if serum or plasma was not available. A second sample from unvaccinated cases of the same area to a RRL for confirmatory testing but to report as Presumptive infection. To confirm the infection, particularly in areas where no YF virus circulation has been recently described, differential neutralization testing with flavivirus standard to the areas of exposure or neutralization testing of appropriately paired samples to demonstrate re-convalescence should be performed in an RRL.

13. A second sample taken 10 days post symptom onset may be requested and tested if the case is from a distinct area where there is still no sign of an outbreak.

14. A suspected YF case AND/OR clinical suspicion of YF1

FIGURE 3.

Yellow fever testing algorithm during outbreak.

Yellow Fever Testing Algorithm for Outbreak

<table>
<thead>
<tr>
<th>Serology available</th>
<th>RT-qPCR available</th>
<th>IHC available</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>Negative</td>
<td>Equivocal</td>
</tr>
<tr>
<td>Fatal</td>
<td>Non-fatal</td>
<td></td>
</tr>
<tr>
<td>Serum or plasma (≥ 2ml), or whole blood (≥ 4ml)</td>
<td>Fresh Tissue</td>
<td>Fixed Tissue</td>
</tr>
<tr>
<td>YF RT-qPCR³</td>
<td>YF RT-qPCR³</td>
<td>YF IHC</td>
</tr>
<tr>
<td>Not available</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>YF IgM testing⁵</td>
<td>Re-test⁶</td>
<td></td>
</tr>
<tr>
<td>Documented history of YF vaccination?</td>
<td>YES</td>
<td></td>
</tr>
<tr>
<td>NO, Vaccination history unknown, or case known to be unvaccinated</td>
<td>Report as: CONFIRMED YF INFECTION⁴ (with mandatory blood investigation form which includes: vaccination history; AND the specimen referral form; mentioning the testing history)</td>
<td></td>
</tr>
<tr>
<td>Refer at least 0.5ml (ideally ≥ 1ml) of serum or plasma to RRL for confirmation⁸, ⁹, ¹³</td>
<td>Report as: PRESENCE OF YF IgM IN VACCINATED INDIVIDUAL. Interpret with care, considering clinical presentation &amp; epidemiological context¹⁴</td>
<td></td>
</tr>
<tr>
<td>Collected ≤7 days post symptom onset</td>
<td>Report as: EXCLUDE YF INFECTION⁴</td>
<td>Report as: EXCLUDE YF INFECTION⁴</td>
</tr>
<tr>
<td>Collected &gt;7 days post symptom onset and/or collected from jaundice or hemorrhagic fever case</td>
<td>Report as: CONFIRMED YF INFECTION⁴</td>
<td>Report as: CONFIRMED YF INFECTION⁴</td>
</tr>
</tbody>
</table>

¹ A suspected YF case is any person with acute onset of fever, with jaundice appearing within 14 days of onset of the first symptoms. Clinical suspicion of YF may be made prior to the appearance of jaundice and is based on other clinically compatible symptoms such as fever, headache, myalgia, arthralgia, vomiting, and urticaria and on the epidemiologic context.

² Applicable to RT-qPCR testing and IgM RDT only. EDTA blood tubes should ideally be used. Avoid using sodium heparin tubes as interfering with PCR.

³ Whenever available, RT-qPCR should be the first-line test during an outbreak, irrespective of the number of days since symptom onset. Therefore, RT-qPCR might be reported in all samples collected as a primary test. A positive result in these samples will confirm YF infection. Whereas a negative result would not exclude the possibility of a YF infection. Samples with negative RT-qPCR results should be referred for IgM testing regardless of the day post-onset of illness that they were collected as a negative molecular result does not rule out YF and serology should be done. In case of non-specificity of the typical blood-based specimens, RT-qPCR on alternative specimens such as saliva, urine, and sputum could be useful to support case confirmation. For fatal cases, RT-qPCR should be performed on all available samples, independent of the collection date. If the laboratory has capacity to only test by RT-qPCR or IgM serology, all samples at any number of days post-onset of illness should be tested with the available assay.

⁴ For cases with no history of vaccination, vaccination, history unknown, or vaccinated >14 days before onset of illness.

⁵ If IgM results are uninterpretable (UI) due to high background and/or potential inhibitory factors, consider repeating the test, and request a 2nd specimen if repeat result is also UI. If testing is not continuous to be UI, must be negative. If a rapid IgM LEISA test is used, serial testing including a confirmation round of a MAC-ELISA based assay and communications of preliminary results might be required; please refer to the Operational guidance on the use of YF test for more details.

⁶ In the case where a MAC-ELISA method is used, an equivocal result is when a valid result falls within the range between a negative or positive result. Refer to the last instructions for the indicated range of equivocality for this specific test. Equivocal does not refer to an UI test result, e.g., optical density test due to presence of factors that cause non-specific background reactions.

⁷ In the case of a first-equivalent test result, the test can be repeated based on the type of IgM assay used. Cost considerations should be taken into account and direct referral of specimen with equivocal results (without retesting) to a RRL is advisable. If an equivocal second test result is obtained a second sample may be requested and tested. If the second sample repeats as equivocal both samples should be sent to the RRL.

⁸ A positive IgM result alone is not confirmatory but considered presumptive evidence of infection. Additional clinical and epidemiologic criteria, such as history of vaccination, must be used for the final interpretation of the results and classification of the suspected YF case. It is not essential to perform serology testing to differentiate yellow fever and other flaviviruses on specimens where local transmission of YF has already been confirmed.

⁹ Once an outbreak of YF has been confirmed in a specific area, the decision might be made to not refer outpatient samples from patients residing in the same area to a RRL for confirmatory testing but to report as presumptive infection. However, cases from a new geographic area, particularly if adjacent or linked to outbreak areas, or with atypical or unusual presentation of illness, should be referred to RRL for further confirmatory testing.

¹⁰ A second sample taken 10 days post symptom onset of illness should only be requested and tested if the case is from a distinct area where there is still no sign of an outbreak.

¹¹ Fresh and fixed tissue samples (≥1 cm³) should be collected (liver and kidney tissue should always be collected; other organs may also be collected) and submitted to a RRL for confirmatory testing. Fresh tissue samples collected ≤7 days post symptom onset and/or collected from jaundice or hemorrhagic fever case should be tested with available assay. If fixed tissue samples collected >7 days post symptom onset or from non-fatal cases should only be requested and tested if the case is from a new geographic area, particularly if adjacent or linked to outbreak areas, or with atypical or unusual presentation of illness, it is possible to perform the test up to 14 days after symptom onset.

¹² A negative RT-qPCR in tissue from fatal cases can be followed up with serology if serum or plasma was not available. A second sample from unvaccinated cases of the same area to a RRL for confirmatory testing but to report as presumptive infection. To confirm the infection, particularly in areas where no YF virus circulation has been recently described, differential neutralization testing with flavivirus standard to the areas of exposure or neutralization testing of appropriately paired samples to demonstrate re-convalescence should be performed in an RRL.

¹³ A second sample taken 10 days post symptom onset may be requested and tested if the case is from a distinct area where there is still no sign of an outbreak.

¹⁴ A suspected YF case AND/OR clinical suspicion of YF.
TESTING WHERE OTHER PATHOGENS ARE CIRCULATING

Testing of YF may be incorporated into local algorithms together with potentially co-circulating pathogens e.g., chikungunya virus, Ebola viruses, malaria, DEN virus, ZIK virus, WN virus, coronaviruses, either sequentially or in parallel. Though generally uncommon, coinfection with more than one arbovirus has been documented (9-11).

TESTING WHERE METHODS ARE LIMITED

A simplified YF testing algorithm for countries with no access to YF RT-qPCR, YF IgM ELISA and PRNT is provided in Figure 4. Interpretations of results must take into account clinical presentation and epidemiological context.
Yellow Fever SIMPLIFIED Testing Algorithm Using Arbo Panel kit for Countries with no access to YF RT-qPCR, YF ELISA and PRNT testing

**FIGURE 4.** Yellow fever simplified testing algorithm using SD Biosensor Standard Q Arbo Panel 1 kit for countries with no access to YF RT-qPCR, YF ELISA and PRNT testing.

### Interpretation guide of results of the Arbo Panel I (Z/D/C/Y) Test kit

<table>
<thead>
<tr>
<th>YFV IgM result</th>
<th>Dengue IgM result</th>
<th>Zika IgM result</th>
<th>CHIKV IgM result</th>
<th>Dengue NS1 Ag result</th>
<th>Differential Interpretation based on all test results of the Arbo Panel I (Z/D/C/Y) Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Evidence of recent YF virus infection OR of YF vaccination³</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Evidence of recent Flavivirus infection⁴</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Evidence of concurrent acute Dengue virus infection and recent Chikungunya virus infection⁵,⁶</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>Evidence of recent Dengue virus infection⁴</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Evidence of recent Dengue virus infection⁴</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Evidence of recent Zika virus infection⁴</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Evidence of recent Dengue virus infection⁴</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Evidence of recent Dengue virus infection⁴</td>
</tr>
<tr>
<td>-</td>
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<td>-</td>
<td>+</td>
<td>-</td>
<td>Evidence of recent Dengue virus infection⁴</td>
</tr>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Evidence of recent Dengue virus infection⁴</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Evidence of recent Zika virus infection⁴</td>
</tr>
</tbody>
</table>

**Evidence of recent YF virus infection OR of YF vaccination³**

**Evidence of recent Flavivirus infection⁴**

**Evidence of concurrent acute Dengue virus infection and recent Chikungunya virus infection⁵,⁶**

**Evidence of recent Dengue virus infection⁴**

**Evidence of recent Dengue virus infection⁴**

**Evidence of recent Zika virus infection⁴**

**Evidence of recent Dengue virus infection⁴**

**Evidence of recent Zika virus infection⁴**

**Evidence of recent Chikungunya virus infection⁴,⁷**

**Negative YF IgM result.** YF infection can be excluded if the specimen was collected 7-14 days post symptom-onset symptoms and/or collected from a jaundice or haemorrhagic fever case³.

---

1. A suspected YF case is any patient with acute onset of fever, with jaundice appearing within 14 days of onset of the first symptoms. Clinical suspicion of YF may be made prior to the appearance of jaundice and is based on other clinically compatible symptoms such as fever, headache, myalgia, nausea, vomiting, and fatigue and on epidemiologic factors. Assessment of YF vaccination history, medical testing history, travel history, and history of contact with known YF cases should be recorded and taken into consideration when interpreting test results.

2. Including the possibility of using either capillary or venous whole blood. Collect the venous whole blood into the commercially available anti-coagulant tubes such as heparin, EDTA or sodium citrate by venipuncture. If venous whole blood in an anti-coagulant tube is stored in a refrigerator at 2-8°C, the specimen can be used for testing within 1-2 days after collection. Do not use hemolyzed blood samples.

3. In the event the individual was never vaccinated against YF or has an unknown YF vaccination history, report as “PRESENCE OF YF IGm IN VACCINATED INDIVIDUAL. Interpret with care, considering clinical presentation & epidemiological context”. Whereas in the event of an individual never vaccinated against YF or with an unknown YF vaccination history, report as “PRESUMPTIVE Evidence of ACUTE YF Infection”. Final case confirmation requires plaque-reduction neutralization testing (PRNT). If PRNT is not available in the region, such case could be classified as a Probable YF case in the light of the clinical presentation & epidemiological context.

4. Case classification should consider the epidemiologic context of co-circulation of other flaviviruses and previous vaccination of the individual. Also, malaria and rheumatic diseases should also be considered as there is documented cross-reactivity affecting the specificity of the IgM result.

5. If the specimen was collected within 7 days post onset of illness, a second sample taken ≥10 days post onset of illness should be requested and tested again whenever possible to account for possible seroconversion.

6. The SD Biosensor Arbo Panel I (Z/D/C/Y) Test kit also includes testing for Dengue NS1 antigen, which is optional in the context of YF surveillance. A positive Dengue NS1 result is an indication of an acute Dengue infection.

7. The result suggests a recent Chikungunya virus infection. However, the documented risk of cross-reactivity with Mayaro and O’nyong nyong virus IgM cannot be excluded and should be considered if epidemiologically relevant.
BACKGROUND

The WHO and EYE LTWG have worked together to enhance molecular and serological testing in the GYFLaN while taking into account the dynamics of YF infection and the most suited markers of infection to be tested for at various times of specimen collection.

Their aim is to help shape the market for quality-assured YF assays, by ensuring availability of reliable options. The goal is to provide fully evaluated assays, reagents, and equipment and coordinate their distribution to Gavi funding-eligible countries, making them accessible to all GYFLaN laboratories.

Through the WHO kit performance evaluation programme for YF, assays intended for use in the context of surveillance can be evaluated independently. If successful, the assays are recommended for use and are included in the list of products available through the UNICEF procurement services. The evaluations include assessment of manufacturer’s claim and quality management system documentation through a dossier review, along with an independent laboratory evaluation of the assay performance.

WHO routinely publishes Expression of Interest calls to invite manufacturers to submit their applications via the United Nations Global Marketplace. Various YF assays have already been evaluated and recommended for use in the context of surveillance, and are listed in UNICEF’s Supply Catalogue (https://supply.unicef.org/). Use of these validated and approved commercial YF assays is recommended throughout the GYFLaN to improve the quality, consistency, and timeliness of YF testing.

INVENTORY OF TEST KITS, REAGENTS, AND CONSUMABLES

An adequate supply of test kits, reagents and supplies must always be available to avoid disruptions in testing capacity. This requires forecasting of future needs, ideally informed by historical consumption to which a buffer is added to account for sudden surge. A monthly physical count is advisable to accurately anticipate when to place the next order while limiting risks of wastage of expired reagents. Considering the relatively short shelf life of laboratory reagents (often 12 months from date of manufacture), National Programmes might strategically opt for an ordering scheme of two orders per year made six months apart, where each order accounts for six months’ worth of forecasted consumption (at the estimated time of arrival of the products in country) plus a three-month rolling buffer. Laboratories can estimate the amounts required for the rolling buffer by multiplying by three the estimated monthly forecasted consumption of a given product and then subtracting the quantity of the current stock of the product expected to be remaining in their storage at the time of delivery of the new order.
Example:

Forecasted monthly consumption = 3 kits/month
Six-month supply (6 x 3) = 18 kits
Three-month buffer (3 x 3) = 9 kits
Remaining stock at product arrival = 6 kits
Order 18 + 9 - 6 = 21 kits

This methodology offers the advantage to constantly have a surge buffer for sudden outbreaks and to rapidly re-adjust ordering quantities in the context of varying seasonal trends in testing.

UNICEF procurement services are available for all member countries of the GYFLaN irrespective of funding stream. However, countries not eligible for Gavi funding for diagnostics are requested to make advance payments when placing an order. Countries are advised to plan for sufficient procurement lead time (minimum of three months) in order to avoid shortages.

STORAGE AND DISPOSAL

All assay and kit components should be stored and disposed of following expiry according to the manufacturer’s instructions and laboratory SOPs.

MOLECULAR ASSAYS

RT-qPCR assay sensitivity is highest around symptom onset, decreasing with time up to 14 days as the immune response clears the viremia. RT-qPCR has high specificity for the YF genome; thus, in a WHO accredited laboratory, a positive result confirms the diagnosis of YF virus infection, allowing for early specific identification of YF. Molecular methods are highly susceptible to contamination; thus, contamination mitigation procedures must be carefully adhered to, as must quality control measures. For laboratories not yet accredited by WHO, specimens resulting in a RT-qPCR positive result by the NL should be referred to the RRL or nearest WHO Collaborative Centre performing routine RT-qPCR testing for YF. For further information, refer to Chapter 6 of WHO Laboratory manual for yellow fever, 2023 edition (12).

BIOSAFETY FOR MOLECULAR ASSAYS

Specimens must be presumed to be infectious prior to inactivation for RNA extraction, and must be manipulated in a Class II BSC, and proper decontamination of surfaces and waste disposal procedures must be observed.
The extraction of RNA from a clinical specimen must be performed accurately to maximize the quality and quantity of RNA available for RT-qPCR testing. The best approach for RNA extraction is to use a suitable commercial RNA extraction kit which are available through the UNICEF Supply Division.

### YF MOLECULAR ASSAY KITS

The commercial assay, RealStar® Yellow Fever Virus RT-PCR kit 1.0 (Altona Diagnostics, Hamburg, Germany) was independently evaluated by WHO and was found to be appropriate for use in the GYFLaN (9, 10). The RealStar® kit detects YF viral RNA from both wild-type and vaccine strains and was found to be of comparable performance to the reference method. The limit of detection was 690 copies/ml for prototype wild-type YF virus with clinical sensitivities and specificities of 100%. Buffer, reverse transcriptase, DNA polymerase, magnesium salt, primers, probes and controls are included in the kit, and the assay has been validated for use with a range of real-time PCR instruments. The assay has a shelf life of 12 months from manufacture. The manufacturer has produced the [RealStar® workflow video](https://www.youtube.com/playlist?list=PLcDmavw-Uq6sA1w_4w-cj1NfZKP4SZxbB) to aid in the performance of the assay and the instructions for use (IFU) are available on the manufacturer’s website under the product sub-section “Manuals” at: [https://altona-diagnostics.com/en/products/reagents-140/reagents/realstar-real-time-pcr-reagents/realstar-yellow-fever-virus-rt-pcr-kit-ce.html](https://altona-diagnostics.com/en/products/reagents-140/reagents/realstar-real-time-pcr-reagents/realstar-yellow-fever-virus-rt-pcr-kit-ce.html)

The WHO kit performance evaluation programme continues to evaluate additional commercial molecular products through Expression of Interest calls. The outcomes of other successful products also will be made publicly available on the WHO website.

### SEROLOGICAL ASSAYS

IgM antibody levels for YF virus rise in the first few days after symptoms appear and peak in one to three weeks after symptom onset. After this time, the response typically begins to wane but some level of IgM may be detectable for months or years (11). Most YF IgM antibodies target the envelope (E)-protein on which epitopes common to related flaviviruses and their vaccines exist, causing some false positivity in E-based assays. Positive IgM results are therefore presumptive, and confirmation at RRLs by more specific methods, such as PRNT, is necessary.

Serological assays remain crucial for YF identification, as patients often seek medical attention after the window period for molecular testing has passed/elapsed, and a history of YF vaccination is important to inform correct/appropriate assay interpretation. More information, including quality control, can be found in Chapter 5 of the WHO Laboratory manual for yellow fever, 2023 edition (12).
BIOSAFETY FOR SEROLOGICAL ASSAYS

Specimens must be presumed to be infectious due to YF or other infectious agents. Proper biosafety precautions, including use of PPE, must be used when handling specimens. Specimen manipulation and serological assays should be performed in a BSC, especially during and after specimen addition and washing steps. Plate washers should be placed in the BSC and a freshly-prepared sodium hypochlorite solution containing 5000 ppm Cl (corresponding to a freshly prepared 1:10 dilution of typical household bleach containing 5% sodium hypochlorite) should be added to waste containers. Proper decontamination of surfaces and waste disposal procedures must be observed.

YF IgM KITS

Two YF IgM assays have been independently evaluated by WHO and recommended for use in the context of surveillance within the GYFLaN (16, 17).

The YF MAC-HD 1.0 (ATCC®, Manassas, Virginia, USA) is a commercially available ELISA kit adapted from the 3-day MAC-ELISA method developed by the US Centers for Disease Control and Prevention (CDC) but features a shorter time to results of approximately 4 hours. The YF MAC-HD kit detects YF IgM from both wild-type and vaccine strains. The kit has a clinical sensitivity of 93% and a clinical specificity of 98% determined by independent evaluation compared with a reference method. The analytical specificity of the kit is 60% with DEN specimens and 90-100% with other regionally-important flaviviruses. Some reactivity may be seen with other organisms and substances, including malaria and rheumatoid factor. The assay is single-use. It includes a pre-coated plate, and all required buffers, antigens, conjugate, substrate, stop solution, controls and IFU are included in the kit. It can be used with automated or manual washing and a plate reader is used. Results calculations are automated using a provided workbook. The IFU and calculations workbook are available at ATCC-YF-MAC-HD-IFU-and-calcualtions-sheet. To note that the use of the optional “overnight protocol” provided in the instruction for use by the manufacturer for further evaluation of equivocal results through triplicate testing is only recommended to be used by RRLs in order to ensure efficient use of resources. As such, NLs can directly refer all specimens with an initial equivocal IgM result to RRLs for confirmation in order to avoid delays. The shelf life of the YF MAC-HD is 12 months from manufacture and a video has been produced to aid in the use of the assay, available at Use-of-YF-MAC-HD-video.

The STANDARD™ Q Yellow Fever IgM Test (SD Biosensor, Gyeonggi-do, Republic of Korea) is an LFA that uses lateral flow technology. Time to results is 15 minutes and the manufacturer validates the assay for use with 10 microliters of whole blood, plasma and serum. The assay detects YF IgM from both wild-type and vaccine strains, with a clinical sensitivity of 87% and a clinical specificity of 98% confirmed by independent evaluation when comparing to a reference method, which used serum as the sample type. Analytical specificity is 90-100% with regionally-important flaviviruses. Some reactivity was seen with other organisms and substances including malaria and rheumatoid factor. Assays are analysed manually and the evaluated inter-reader variability was shown to be 0%. A test device, buffer, sample collector, and IFU are included in the kit. The shelf life of the STANDARD™ Q Yellow Fever IgM Test is 24 months from manufacture.

Additional commercial serological assays and an antigen detection assay may be evaluated in the near future.
RECOMMENDED USE OF IgM ASSAYS

Testing algorithms described in Figures 2 and 3 should be attentively followed when testing for YF. The algorithms were designed to account for various types of assays and know overall characteristics, performance and limitations, and in line with the disease progression. Whereas these algorithms are product agnostic, the IgM testing workflow (Figure 5) proposes a more specific sequenced testing strategy using jointly the STANDARD Q Yellow Fever IgM Test and the YF MAC-HD 1.0 assay in order to ascertain the presence of IgM directed against YF in suspected individuals. As such, it provides the an example of what the “YF IgM Testing” box of the algorithms could entail when using such two specific commercial assays, based on their specific respective performance and accounting for efficient use of resources.

Both evaluated IgM assays have good performance profiles in the limited Kit performance evaluations performed, with the YF MAC-HD being somewhat more sensitive than the STANDARD™ Q Yellow Fever IgM Test. However, the YF MAC-HD assay may also be less specific (against a defined analytical specificity panel), is a single-use kit, and is more costly than the STANDARD™ Q Yellow Fever IgM Test (12,13).

To gather more data on the use of these assays in YF endemic countries, and to simultaneously maximize their cost-efficiency, these two commercial IgM assays should be used sequentially in NLs until further notice as follows* and as shown in Figure 5:

- All laboratory registers should be updated to accommodate the recording of both the STANDARD™ Q Yellow Fever IgM Test and the YF MAC-HD 1.0 assay results for each suspected specimen eligible for IgM testing;
- At sub-NLs, NLs (including RRL acting in the capacity of NL), all specimens eligible for IgM testing as per the algorithm in Figure 2 or Figure 3 should be first tested using the STANDARD™ Q Yellow Fever IgM Test;
- Positive results by the STANDARD™ Q Yellow Fever IgM Test for specimens taken from individuals known to be vaccinated against YF should be recorded in the laboratory register and reported as: “Presence of YF IgM in vaccinated individual. Interpret with care, consider clinical presentation and epidemiological context”. No further testing is necessary for these specimens;
- Positive results by the STANDARD™ Q Yellow Fever IgM Test for specimens from either unvaccinated individuals or those with unknown vaccination history should be recorded in the laboratory register, and can be immediately reported as: “Presumptive evidence of acute YF infection or of vaccination. Confirmatory and differential testing are pending”. These specimens should be referred directly (no additional testing by NLs is required) to the RRL for confirmation and differential testing;
- Negative results from the STANDARD™ Q Yellow Fever IgM Test should be recorded in the laboratory register and can be immediately reported as: “No YF IgM was detected in preliminary testing. Further IgM testing pending, prior to final case classification”;
- While Negative IgM results on the STANDARD™ Q Yellow Fever IgM Test should be reported immediately as preliminary results as described in the above point, the specimens giving such result should be retained for subsequent testing by the NLs using the YF MAC-HD 1.0 assay in order to inform the final case classification. Specimens should be stored together at 4°C until a total of 24 negative specimens (or 23 if an in-house positive control is routinely used) are available, at which time, they should be tested using a full plate of the YF MAC-HD 1.0 assay;
- Negative results from the YF MAC-HD 1.0 assay should be recorded in the laboratory register, and can be immediately reported as: “No YF IgM was detected in final IgM testing”. If the specimen was collected >7 days post symptom onset, YF is excluded. If the specimen was collected within a week following symptom onset, the IgM result is inconclusive, and a new specimen should be collected;
- Positive and equivocal results from the YF MAC-HD 1.0 assay should be recorded in the laboratory register, and can be immediately reported as: “Additional IgM testing resulted in presumptive evidence of acute YF infection or of vaccination. Confirmatory and differential testing are pending” until further confirmation by RRL becomes available;
- Any specimens yielding positive or equivocal results by YF MAC-HD 1.0 assay should be directly referred to the RRL (Singlicate retesting of equivocal by NLs is advisable. However NLs may opt out of such retesting in order to avoid delays and refer directly for confirmatory testing by the RRL).
This workflow shows a sequential use of two distinct assays and is to be used by national laboratories of the GYFLaN when using rapid lateral flow assays (e.g., STANDARD™ Q Yellow fever IgM Test) and in full alignment with the testing algorithms.
RRLs receiving samples referred from NLs will use the YF MAC-HD 1.0 assay along with differential testing for other viruses prior to performing YF PRNT testing (and differential PRNT if relevant).

All laboratories should maintain adequate stock of both the STANDARD™ Q Yellow Fever IgM Test and the YF MAC-HD 1.0 assay in order to ensure initial testing of all specimens eligible for IgM testing and serial ELISA testing of those yielding a negative preliminary result using the LFA.

*Laboratories that cannot ship specimens across international borders for result confirmation and those that are not accredited by WHO to perform MAC-ELISA and PRNT, should consider using the STANDARD™ Q Arbo Panel I kit from SD Biosensor. This assay includes distinct IgM tests for ZIK, Chikungunya, YF and DEN viruses, and an antigen test for DEN NS1. All five LFAs included in the kit should be used at the same time for each specimen. A simplified interpretation guide for results from the STANDARD™ Q Arbo Panel I kit is provided in Figure 4.

**VERIFICATION OF ASSAYS**

To ensure assays are performing adequately under individual laboratory conditions, all new assays, including test kits, should be verified prior to routine use in each NL. This should occur regardless of whether the assay has been externally validated. Generally, panels consisting of 10 known positive and 10 negative samples should be compiled and tested for accuracy, precision and reportable range. After the assay is verified for use, all staff involved in YF testing should test a blinded panel to ensure proficiency.
Logistics and funding for YF test kits are in place according to a standardized procurement mechanism through UNICEF Supply Division. NLs in Gavi funding-eligible countries that receive more than 50 YF specimens per year and that are WHO accredited, may obtain the following currently-available items:

- **YF RT-qPCR test kits such as:**
  - Altona Diagnostics RealStar® Yellow Fever Virus RT-PCR kit 1.0 and related consumable supplies including RNA extraction kits;
  - Other commercially available RT-qPCR might be added to the list in the future (contingent to successful evaluation and recommendation for use by WHO, and agreed upon by Unicef and Gavi).

- **YF IgM serology test kits such as:**
  - ATCC® YF MAC-HD 1.0 assay and related consumables supplies required to perform the assay;
  - SD Biosensor STANDARD™ Q Yellow Fever IgM Test kits;
  - Other commercially available YF IgM serology products might be added to the list in the future (contingent to successful evaluation and recommendation for use by WHO, and agreed upon by Unicef and Gavi).

- **Personal protective supplies;**

- **Equipment (ELISA washer, ELISA reader, real-time PCR machine, biosafety cabinet) if a need has been confirmed through a WHO-associated laboratory capacity assessment visit**

An application form must be submitted to obtain YF laboratory support. The form includes a request for recent specimen submission numbers to estimate expected testing volumes and materials requirements. Materials for higher-than-expected submission volumes are kept in reserve. The amounts and types of consumable supplies needed for testing a given number of specimens is determined based on input from the WHO and UNICEF. Additional supplies and consumables are available for quality assurance testing purposes. Commercial serology kits will be made available soon using this mechanism.

The application form and further information can be found on pages 83-85 of the Gavi Vaccine Funding Guidelines available at Gavi-funding-guidelines. NLs in countries that are not eligible for Gavi support should contact their Regional YF Laboratory Coordinator for help in obtaining test materials and supplies or refer to the UNICEF Supply Catalogue at https://supply.unicef.org/.

Gavi diagnostic procurement funding does not currently support the procurement of the STANDARD™ Q Arbo Panel I kit from SD Biosensor. However the kit is available for a cost through direct purchase from the manufacturer as well as through the UNICEF Supply Catalogue. Countries wishing to place an order through UNICEF Procurement Services can have access to UNICEF’s pre-negotiated terms with the manufacturer. Countries should also be aware that in both instances of direct purchase of such product from the manufacturer or of purchase through UNICEF Procurement Services, pre-payment will be required before the order can be processed.
Laboratories should follow national reporting requirements. All positive, equivocal, or negative test results should be immediately reported to national authorities, including additional results from differential and confirmatory testing performed at the RRL, in order to contribute to evidence used for case classification. States Parties to the International Health Regulations (IHR) are reminded of their obligations to share with WHO relevant public health information for events for which they notified WHO, using the decision instrument in Annex 2 of the IHR (2005). Member States are requested to immediately notify WHO of all positive laboratory results, including any YF laboratory result that is awaiting confirmation. WHO can assist Member States to access testing through referral via their WHO RC.

Reporting may be required on either a weekly or monthly basis, even in the event of absence of confirmed cases. Frequency of reporting may be influenced and dependent on recent history of YF activity, especially in low incidence settings and serves to document laboratory activities in support of YF surveillance. All NLs and RRLs in the GYFLaN are requested to provide reports of results to WHO monthly or weekly. For more information, please contact your WHO country office focal point, WHO Regional YF Laboratory coordinator, or WHO Global YF Laboratory Network Coordinator using the following address: GYFLaN@who.int
This guidance provides recommendations for identifying cases suspected to be infected with YF for surveillance purposes and is based on current knowledge, available technologies, and needs.

The WHO is closely monitoring developments related to YF outbreaks and the availability of new YF assays and will revise and release updated guidance when necessary.

For more detailed information on YF laboratory practices, please refer to the WHO Laboratory manual for yellow fever, 2023 edition (12).
References

17. WHO public report for the kit performance evaluation of STANDARD™ Q Yellow fever IgM Test to detect IgM to Yellow fever virus in the context of surveillance. 2023 (available at https://www.who.int/publications/i/item/9789240068957).