Laboratory manual for yellow fever

World Health Organization

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This document was developed in consultation with:

Individual experts also members of the Eliminate Yellow Fever Epidemics (EYE) Strategy's advisory Laboratory Technical Working Group (LTWG): Alison Jane Basile, Amy Lambert, Ana Bispo, Christin Goodman, Cristina Domingo-Carrasco, Eric Mossel, Felipe Nevaca, Gamou Fall, Huguette Simo, John Kayiwa, Julius Lutwama, Mary Dooshima, Matthias Niedrig, Naazneen Moolla, Ngu Abanda, and Zsofia Igloi.

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Abbreviations and acronyms

ACIP	Advisory Committee on Immunization Practices
AVR	Analysis, visualization, and reporting
BF	Booking form
BSC	Biosafety cabinet
CDC	Centers of Disease Control and Prevention
CLIA	Clinical Laboratory Improvement Amendments
CLSI	Clinical and Laboratory Standards Institute
CPE	Cytopathic effect
CSV	Comma-separated values
ELISA	Enzyme-linked immunosorbent assay
EPI	Expanded Programme on Immunization
EQA	External quality assessment
EYE	Eliminate Yellow Fever Epidemics
LFA	Lateral flow assay
GLC	Global Laboratory Coordinator
GMRLN	Global Measles and Rubella Laboratory Network
GPLN	Global Polio Laboratory Network
GSL	Global specialized laboratory
GYFLaN	Global Yellow Fever Laboratory Network
IATA	International Air Transport Association
Ig	Immunoglobulin
IgM	immunoglobulin M antibody
IHC	Immunohistochemistry
IHR	International Health Regulations
IQA	Internal quality assessments
ISO	International Standards Organization
LAC	Latin America and the Caribbean
LFA	Lateral flow assay
LoD	Limit of detection

Abbreviations and acronyms

LQSI	Laboratory quality stepwise implementation
LTWG	Laboratory technical working group
LQMS	Laboratory quality management system
MAC-ELISA	IgM antibody capture enzyme-linked immunosorbent assay
MOH	Ministry of Health
NATA	National Association of Testing Authorities
NC	Negative control
NL	National laboratories
NPHL	National Public Health Laboratories
NTC	No-template control
OD	Optical density
PAHO	Pan American Health Organization
PBS	Phosphate-buffered saline
PC	Positive control
PDF	Portable document format
PFU	Plaque forming unit
PPE	Personal protective equipment
PRNT	Plaque reduction neutralization test
QA	Quality assurance
PDF	Portable document format
PFU	Plaque forming unit
PPE	Personal protective equipment
PRNT	Plaque reduction neutralization test
QA	Quality assurance
QC	Quality control
QMS	Quality management system
QSE	Quality system essentials

Abbreviations and acronyms

RELDA	Arbovirus Diagnosis Laboratory Network of the Americas
RLC	Regional Laboratory Coordinator
RRL	Regional reference laboratory
RT-qPCR	quantitative reverse transcriptase-polymerase chain reaction
SAGE	Strategic Advisory Group of Experts on Immunization
SLIPTA	Stepwise Laboratory Quality Improvement Process Towards Accreditation
SLMTA	Strengthening Laboratory Management Toward Accreditation
SNL	Subnational laboratories
SOP	Standard Operating Protocols
UNICEF	United Nations Children's Fund
UVRI	Uganda Virus Research Institute
VPD	Vaccine preventable diseases
WHO	World Health Organization
YF	Yellow fever

Executive summary

Across tropical and subtropical regions of Africa and South America, a total of 47 countries are home to the vaccine-preventable mosquito-borne yellow fever (YF) disease. The virus causing it cannot be eradicated as non-human primates are part of the transmission cycle; therefore, mass vaccination of susceptible human populations and of travelers to YF-endemic areas is the primary control method. However, insufficient vaccination coverage has led to major outbreaks in Angola (2015-16), Democratic Republic of the Congo (2016), and Brazil (2017-18), underscoring the urgency to address this insufficiency.

In response, the World Health Organization (WHO) together with an international coalition of partners established the Eliminate Yellow Fever Epidemics Strategy (EYE Strategy) in 2016, which was subsequently adopted by Member States at risk of YF, with the goal of eliminating epidemics and major outbreaks of YF by 2026.

A prerequisite for effective prevention and control of YF is accurate and timely identification of the disease. While the EYE Strategy helps to address various YF vaccine supply-chain issues, complete vaccination coverage takes time and strategic use of resources. Laboratory identification of the virus is complex but crucial to rapidly respond to outbreaks. In consultation with individual experts ranging from members of EYE Strategy's Laboratory Technical Working Group, as well as laboratory staff working within routine YF national surveillance programmes, WHO has undertaken a comprehensive update of YF laboratory practices in the Global YF Laboratory Network (GYFLaN) to align with the EYE Strategy's needs.

A comprehensive on-site laboratory assessment in 2018 of all the YF laboratories on the African continent was used to guide this undertaking. The aims were to document the current laboratory capacities and identify areas for improvement. Analyses revealed major inconsistencies in testing methodologies; training, equipment, and maintenance gaps; shortage of reagents; and frequent delays in shipping of diagnostic specimens especially for confirmatory testing. It revealed the need to expand molecular testing and to improve consistency by shifting from in-house assays to quality-assured standardized commercial assays. The EYE-LTWG sought to tackle the identified weak areas and thereby enhance the overall laboratory capacity of the GYFLaN. Significant improvements to the timeliness and quality of testing have already been documented.

The manual for the monitoring of yellow fever virus infection was published in 2004 (first edition) and acted as the first laboratory manual for yellow fever surveillance. Since then, technological advances and the influx of financial and logistical support for YF laboratory activities necessitated an update. The current document entitled Laboratory Manual for Yellow Fever (Second edition, 2023) builds on the 2004 version, covering recent approaches to all aspects of YF laboratory operations. New chapters describe network-wide testing and interpretation algorithms, serological and molecular testing methodologies, laboratory practices for Regional Reference Laboratories, and improvements in logistics, including new international shipping procedures. Quality is emphasized throughout, including the newly introduced network-wide external quality assurance activities. The manual is intended as a comprehensive reference for laboratories members of the GYFLaN to enhance the quality, consistency, timeliness, and availability of laboratory identification of YF, with the overarching goal of aiding in the elimination of YF epidemics.

Chapter 1

Yellow fever: An overview

Yellow fever (YF) is a mosquito-borne viral disease endemic in sub-Saharan Africa and Central/South America. Although immunization with the 17D YF vaccine is possible and highly recommended in these regions, due to having a non-human primate reservoir, YF is not a disease that can be targeted for eradication. The vaccine, which is safe, inexpensive, and reliable, can confer sustained immunity and lifelong protection against YF disease; to dramatically reduce case-fatality rate (1). However, low vaccine coverage in some regions, will inevitably carry the risk of outbreak among unimmunized persons. Therefore, strong YF surveillance programmes are paramount in these regions to assess and plan accordingly if an outbreak is likely. A YF surveillance system should be able to detect, investigate, collect samples, and have a reporting system in place for the suspected cases.

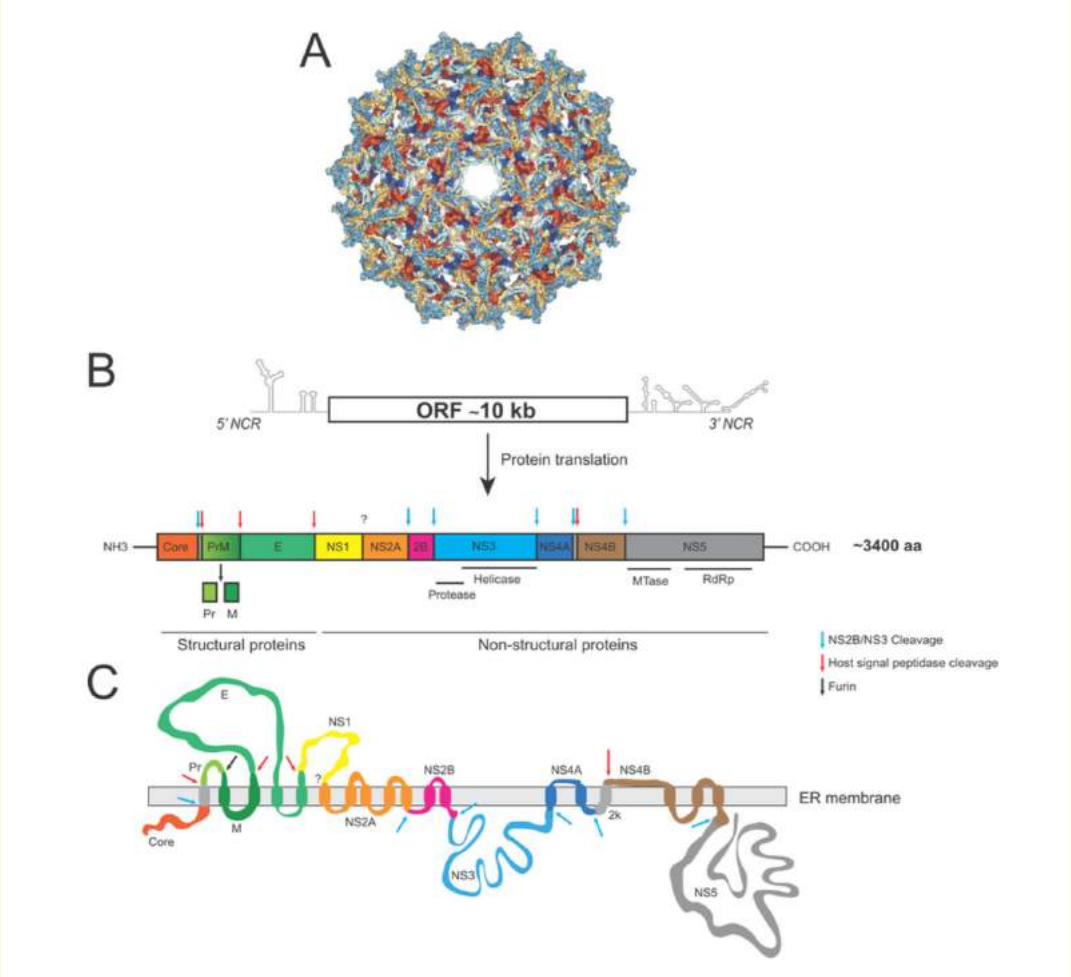
Chapter content:

- The structure and biology of YF virus
- Transmission of YF virus
- YF virus geographical distribution
- The epidemiology of human disease
- YF virus infection and immune response
- YF surveillance
- The clinical description and case definition of YF
- Laboratory diagnosis of YF Case information

1.1 THE STRUCTURE AND BIOLOGY OF YF VIRUS

YF virus belongs to the genus Orthoflavivirus, which consists of 53 species, many of which can cause a variety of human and animal diseases (2, 3). YF virus is a small, spherical virion with icosahedral symmetry, approximately 50 nm in diameter, and is comprised of a viral envelope and membrane proteins arranged in head-to-tail heterodimers, and embedded in a host cell-derived lipid bilayer, which surrounds a nucleocapsid core (Figure 1.1) (2). The positive-sense, single-stranded ribonucleic acid (RNA) genome, approximately 11 kb in length, is packaged in the nucleocapsid. The genome functions as an mRNA, encoding three structural proteins and seven nonstructural proteins in one open reading frame, which is flanked at both the 5' and 3' ends by untranslated regions and capped at the 5' end. The virion outer envelope aids the entry of the virus into the cell. The major immunogenic epitopes are also located on the envelope proteins, which are targeted by serological diagnostic assays.

FIGURE 1.1 **Yellow fever virus genome organization and translation and processing of the polyprotein.** See Box 1.1 for detailed legend.



Source: taken from (4) Douam F, Ploss A. Yellow Fever Virus: Knowledge Gaps Impeding the Fight Against an Old Foe. Trends Microbiol. 2018 Nov;26(11):913-928.

Box 1.1 Details of legend from Figure 1.1

A. Cryo-EM representation of an immature FYF virus particle (PDB 1NA4).

B. Schematic representation of YF virus viral RNA and polyprotein, each viral protein is shown by a distinct colour, arrows indicate cleavage sites in the polyprotein processed by proteases of cellular (red or black arrow) or viral (blue arrow) origin.

C. Schematic representation of the YF virus polyprotein anchored into the endoplasmic reticulum membrane following translation. Abbreviations: E, envelope; prM/M, premembrane/membrane; NCR, noncoding region; NS, nonstructural; ER endoplasmic reticulum.

YF virus is heat-labile and can be heat inactivated after 30 minutes at 56°C. The virus is sensitive to UV and gamma irradiation (30 kGry); inactivated by organic solvents and detergents; and susceptible to a wide range of disinfectants such as 70% ethanol, 2% glutaraldehyde, 3-8% formaldehyde, 1% sodium hypochlorite, iodine, and phenol iodophors. However, the virus appears to be relatively stable after freeze-drying and can be stored at –70°C or –170°C for decades (5). The virus loses infectivity outside the host in ambient temperatures on surfaces or objects (fomites). However, in mosquito eggs, which are resistant to desiccation, the virus can survive for long periods (5).

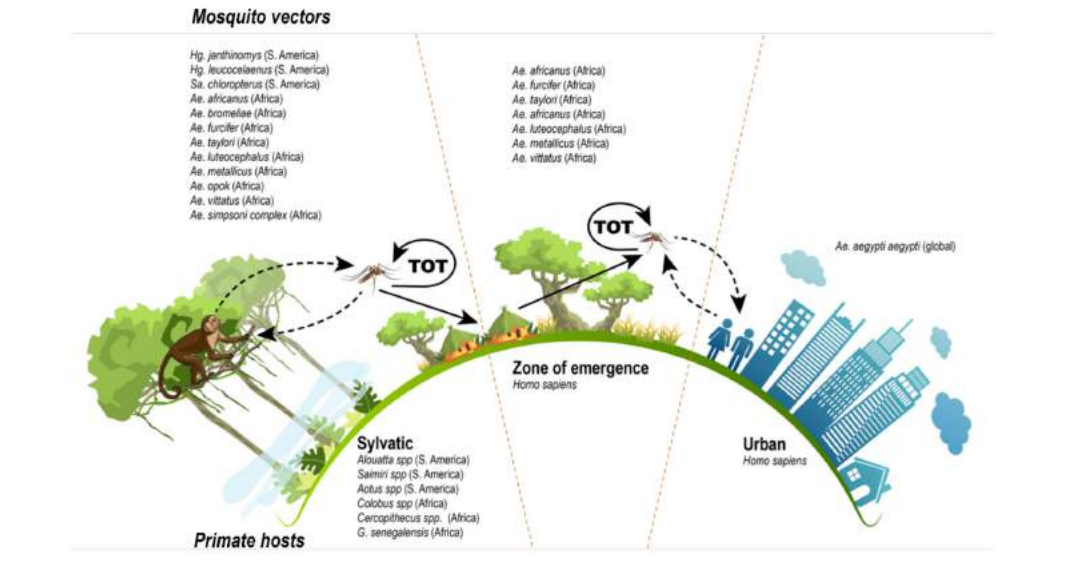
The YF virus has the potential for generation of variants during replication due to the lack of proofreading capacity of RNA-dependent polymerases (6). However, the stringency of the need to infect and replicate in both nonvertebrate and vertebrate hosts exerts a selection pressure resulting in lower variation than that which may occur with other RNA viruses (5).

1.2 TRANSMISSION OF YF VIRUS

YF virus is a mosquito-borne virus (arbovirus) and is transmitted between infected mosquitoes and primates (human and non-human) through a bite (Figure 1.2) (4, 7). Upon biting, the virus is inoculated into the host via the mosquito's saliva. The incubation period is three to seven days. Viremic hosts subsequently transmit the virus to naive mosquitoes that feed on them, usually during the first three to four days of illness (8, 9). Mosquitoes are infective for life and pass on the virus to their eggs through transovarial transmission, which hatch in suitable breeding places, usually in stagnant water.

The virus is transmitted in both forest (sylvatic) and urban cycles. In the forest cycle, the sylvatic vectors are in high density and virus-infected mosquitoes (*Aedes africanus, Haemagogus* species and others) breed in abundance in trees. When humans enter these regions, they are at-risk of becoming infected if virus-infected mosquitoes bite them. The risk is amplified when these infected humans then travel, becoming the principal host of epidemic transmission (10). In Africa, there is also an intermediate zone between forest and savannah that is an important transitional zone for transmission to humans (Figure 1.2) (7, 11). In the urban cycle, infected *Aedes aegypti*, can transmit the virus to humans. If the virus goes undetected, it then has the potential to trigger an epidemic (11).

FIGURE 1.2 **Transmission cycle of yellow fever virus. Abbreviation: TOT, transovarial transmission.**



Source: adapted from (12) Sacchetto L, Drumond B P, Han B A, Nogueira M L, Vasilakis N, Re-emergence of yellow fever in the neotropics – quo vadis? Emerging Topics in Life Science 2020, 4:411-422.

- **Sylvatic YF virus transmission.** In tropical rainforests, YF virus circulates between non-human primates and tree-top canopy mosquitoes (i.e. *Haemagogus* and *Sabethes* spp. in South America and *Aedes africanus* in Africa). Humans become infected when they are present in the forest and are bitten by mosquitoes carrying the YF virus;
- **Urban YF virus transmission.** Large outbreaks can occur when infected humans travel to densely populated areas where a large portion of the population are not immune against the virus. For example, if the *Aedes aegypti* mosquitoes, which often breed around homes in containers and readily bite humans, are exposed to infected humans, the YF virus can be easily transmitted in this high-density urban setting. Thus, there is a high-risk for a YF outbreak when a YF case emerges undetected in an urban area with a population with low YF vaccine coverage (11). The recent detection of the YF virus in *Aedes albopictus* mosquitoes captured in urban and rural areas in various parts of the world is of concern and is still being monitored (8, 13–17);
- **Intermediate YF virus transmission.** Small-scale epidemics, which occur in savannah areas located on the edge of forest areas in humid or semi-humid parts of Africa are believed to be the most common form of transmission in Africa. Semi-domestic and peri-domestic *Aedes* spp. mosquitoes, which breed in the wild and around households, infect both non-human and human primates. Interaction between people and the infected mosquitoes leads to transmission.

In addition to mosquito-borne transmission of the virus, YF virus has also been found to be transmitted to humans by exposure to infected blood and aerosols in the laboratory (18) and to newborns perinatally (i.e., around the time of birth) (19, 20). YF vaccine containing a live attenuated virus has also been shown to be transmitted via blood transfusion (21) and, although rare, to infants through breastfeeding (22).

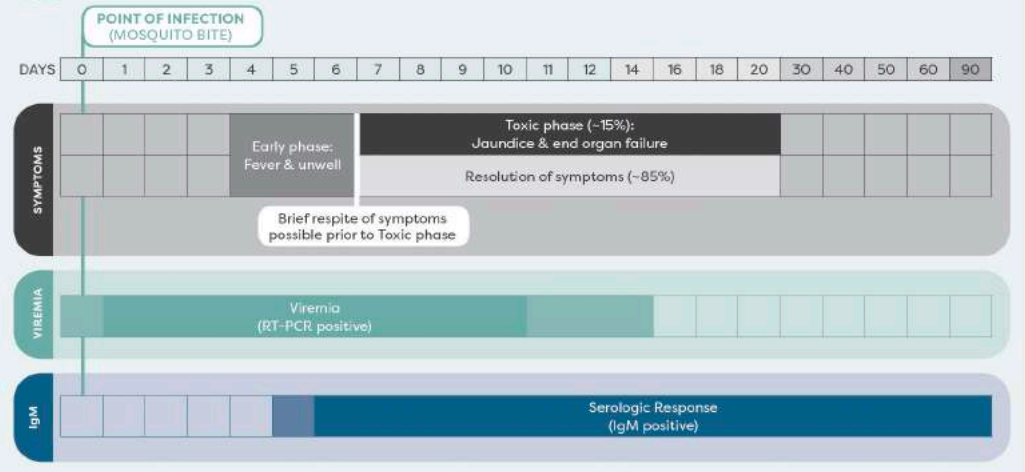
1.3 YF VIRUS INFECTION AND IMMUNE RESPONSE

In natural infections, an infected mosquito inoculates YF virus into the skin during a bite. Initial virus replication occurs in lymphoid tissues (spleen and lymph nodes). The YF virus is viscerotropic, with the liver being the most affected organ, although the kidneys, spleen, lymph nodes, and heart can also be damaged by the virus (7, 23). The lethality and severity of hepatic disease is associated with the viral load detected in the blood. Pathogenesis is primarily caused by the host immune response to the infection. The innate immune system is initially down-regulated in favour of the virus, whereas end-stage immunopathological mechanisms contribute to death. Apoptosis in the liver, induced by transforming growth factor beta and anti-inflammatory cytokines, is the main mechanism of cellular death in severe YF disease (7). The clinical outcome of YF correlates with the degree of liver damage, which can be measured by hepatic aminotransferases. Severe liver damage is accompanied by reduced synthesis of clotting factors, resulting in haemorrhagic diathesis, a sign of fatal YF. Renal failure is also a hallmark of severe and fatal YF. The final stage of disease is characterized by rapid deterioration, multiorgan failure, and circulatory shock. Enhanced expression of pro-inflammatory cytokines in the late stage of disease results in severe systemic inflammatory syndrome, which also contributes to lethality. Most survivors of a YF virus infection are protected for life and the liver recovers without sequelae.

The virus can be cultured from the blood of symptomatic patients during the first 5 days of illness (24). However, the highly sensitive real-time reverse transcriptase-polymerase chain reaction (RT-PCR) test can increase the ability and time to detect viral RNA up to 10-15 days following onset of symptoms, but there is not enough data to recommend molecular testing after 15 days (Figure 1.3). There is some evidence that YF viral RNA may be detected in the urine of patients up to 24 days following onset of symptoms. Hence, routine testing of urine as a specimen type has been evaluated (25).

Both immunoglobulin M and G (IgM and IgG) antibodies are produced during the primary immune response and YF virus-specific IgM can be detected in the serum at ≥5 days post illness onset in 90% of YF cases using sensitive immunoassays. IgM antibody levels peak about 7–10 days after the onset of illness and then decline rapidly to undetectable levels after 6–8 weeks in most cases (26). However, IgM has been detected in a small proportion of YF vaccinated individuals up to a year after vaccination (24, 27). It is sometimes possible to detect virus-specific IgM in post-exposure serum specimens among individuals that remain asymptomatic.

FIGURE 1.3 **Timeline of clinical disease and laboratory diagnostic testing relative to point of infection. See Box 1.2 for detailed legend.**



Source: taken from (28) World Health Organisation (WHO), "Yellow Fever Vaccine-Preventable Diseases Surveillance Standards" (2020).

Box 1.2 Details of legend from Figure 1.3

Dark colours indicate where the majority of persons are viremic or IgM positive.

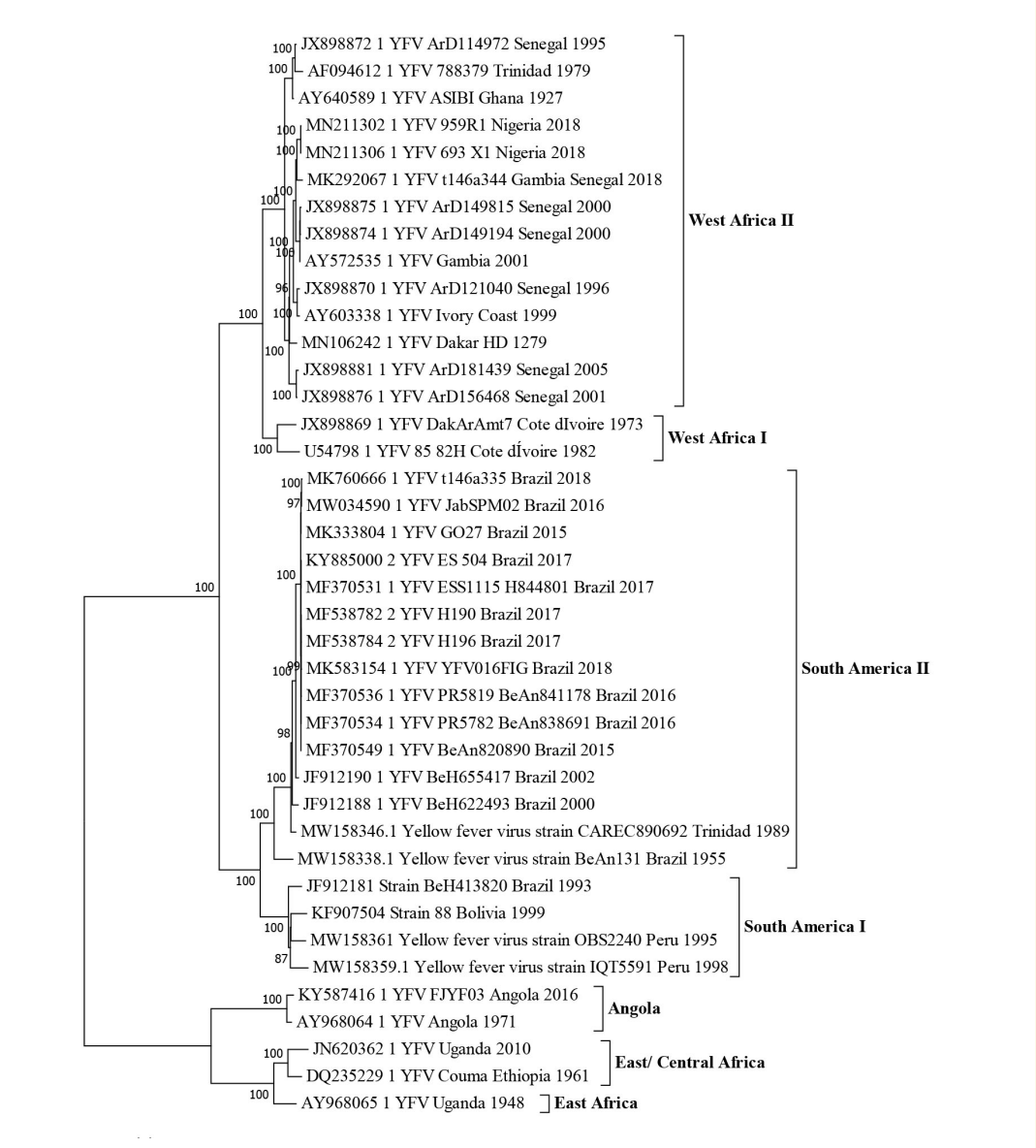
Lighter colours signify periods where some persons can be positive, but the diagnostic measure is less reliable.

1.4 YF VIRUS ORIGIN AND GEOGRAPHICAL DISTRIBUTION

YF virus is thought to have originated in Central Africa between 700-1200 years ago (7, 23). All strains of YF virus are comprised of one serotype, with five major lineages in Africa occurring in overlapping geographical regions [West Africa I (West/Central), West Africa II (West), East Africa, East/Central Africa, and Angola] and two lineages in South America [South America I (widely dispersed) and South America II (western South America)] (Figure 1.4) (7, 24, 29). There is considerable heterogeneity between isolates from Africa and South America. Among the genotypes, the East African genotype is the most divergent. The two South America genotypes are believed to have diverged from West African strains during the transport of enslaved Africans to the Americas in the 1500s. Nucleic acid sequencing and phylogenetic analysis of isolates indicate that YF virus strains circulate in discrete foci, maintained in both horizontal and vertical transmission cycles, with periodic emergences limited to accessible corridors with appropriate vectors and hosts (7).

FIGURE 1.4

Yellow fever phylogenetic analysis showing major yellow fever virus genotypes, based on alignment of the complete genomes of 40 representative African and American yellow fever virus strains, using the Maximum Likelihood method based on the general time reversible model. Individual strains are defined by name and country/year of isolation.



Source: figure developed and graciously provided by Cristina Domingo-Carrasco, personal communication.

In South America, non-human primates are susceptible to YF virus infection, and primate epizootics and die-offs are used as sentinels in YF surveillance. In Africa, the majority of non-human primates have inapparent, although viremic, YF virus infections and do not die from YF virus infection (24). This difference is most likely due to the more recent introduction of YF virus in the Americas than to differences in virulence between strains. There is little evidence for differences in virulence between wild-type strains of YF virus, although until recently there has been little genetic analyses of YF strains. With more whole genome sequencing of isolates and better characterization of outbreaks, a clearer understanding of the differences in virulence between strains, particularly between African and South American isolates, may provide a deeper understanding. Most importantly, the genetic variation does not appear to be biologically significant, as all genotypes are neutralized by YF vaccine-induced antibodies (30).

1.5 THE EPIDEMIOLOGY OF HUMAN DISEASE

YF is endemic and intermittently epidemic in tropical and subtropical regions of South America and Africa (23, 24, 31) (Chapter 2). Forty-seven countries, 34 in Africa and 13 in Central and South America, are either endemic or have regions that are endemic for YF as of 2023 (32). A modelling study based on African data sources estimated the burden of YF in 2013 to be 84 000–170 000 severe cases and 29 000–60 000 deaths (33). In 2014, using data from 11 YF studies, the estimated proportion of YF virus infections in humans that are likely to be asymptomatic was approximately 55%, 33% resulted in mild disease, and 12% resulted in severe illness characterized by fever with jaundice or haemorrhagic symptoms (33). The case-fatality rate among those with severe disease was estimated to be 47%, similar to the 40%-50% case-fatality rates of other studies (33). In 2018, using demographic and vaccination data, the estimates were 109 000 (95% credible interval [CrI] [67 000-173 000]) severe infections and 51 000 (95% CrI [31 000-82 000]) deaths due to YF in Africa and South America (34). However, disease incidence is difficult to assess (35), as only 1%–2% of cases are probably reported, and the WHO indicates that underreporting is a concern, and the real number of cases could be 10 to 250 times of what is currently reported (32).

The epidemiology of the disease differs between the continents. In South America, transmission is mainly sylvatic, with humans infected when they enter forest areas where the virus occurs in an enzootic cycle. The epidemiology of YF in Africa is often mixed, involving both sylvatic and domestic vector species in intermediate YF virus transmission.

1.6 YF VACCINATION

The most important measure for the prevention of YF is vaccination. The live, attenuated YF vaccine is safe and effective; a single dose has been shown to provide lifelong protection against YF disease (36). In clinical trials, 80%–100% of vaccine recipients develop protective immunity within 10 days and 99% do so within 30 days. Although adverse events are uncommon, contraindications and safe immunization practices must be followed (37). Several recommended immunization strategies are in place for high-risk countries based on the Eliminate Yellow Fever Epidemics (EYE) Strategy goals (Figure 1.5).

Vaccination programmes comprise of:

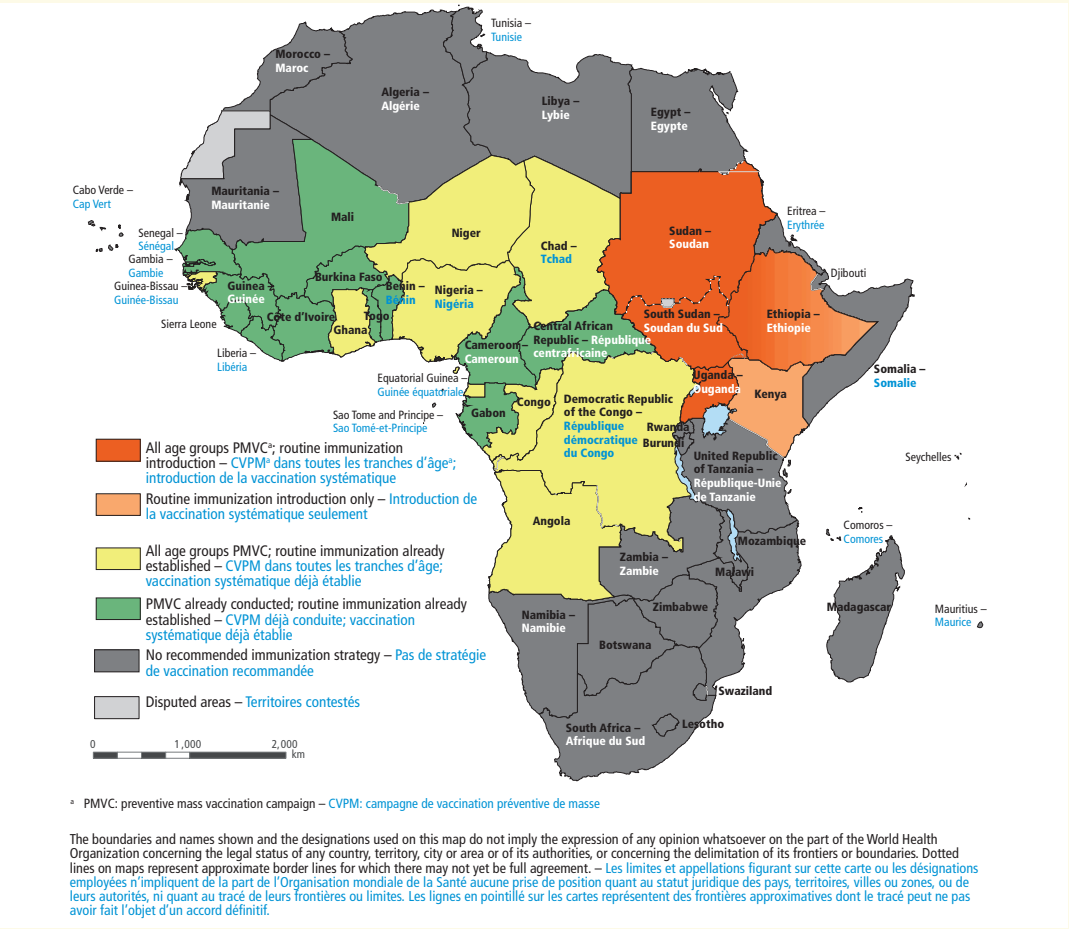
- routine infant immunization for children aged 9 months or above;
- preventive and reactive mass vaccination campaigns aimed at wider age ranges;
- vaccination of travellers going to YF endemic areas;
- vaccination of at-risk laboratory workers.

Early outbreak detection and investigation followed by reactive mass vaccination campaigns are also important strategies (37). Other strategies for YF prevention are personal protection against mosquito bites and community vector control.

Following the urban YF epidemic in Angola in 2016 that depleted vaccine supplies, WHO and partners recognized the need for better prevention, detection, and response to YF. In response, a comprehensive global strategy named EYE was implemented in 2017. The EYE Strategy emphasizes on protecting populations at-risk for YF, preventing international spread, and containing outbreaks rapidly (28, 32, 38, 39). The essential role of the diagnostic laboratory in the EYE Strategy is discussed in Chapter 2 and Chapter 7.

FIGURE 1.5A

Recommended immunizations activities for yellow fever in high-risk countries in Africa 2017-2026.

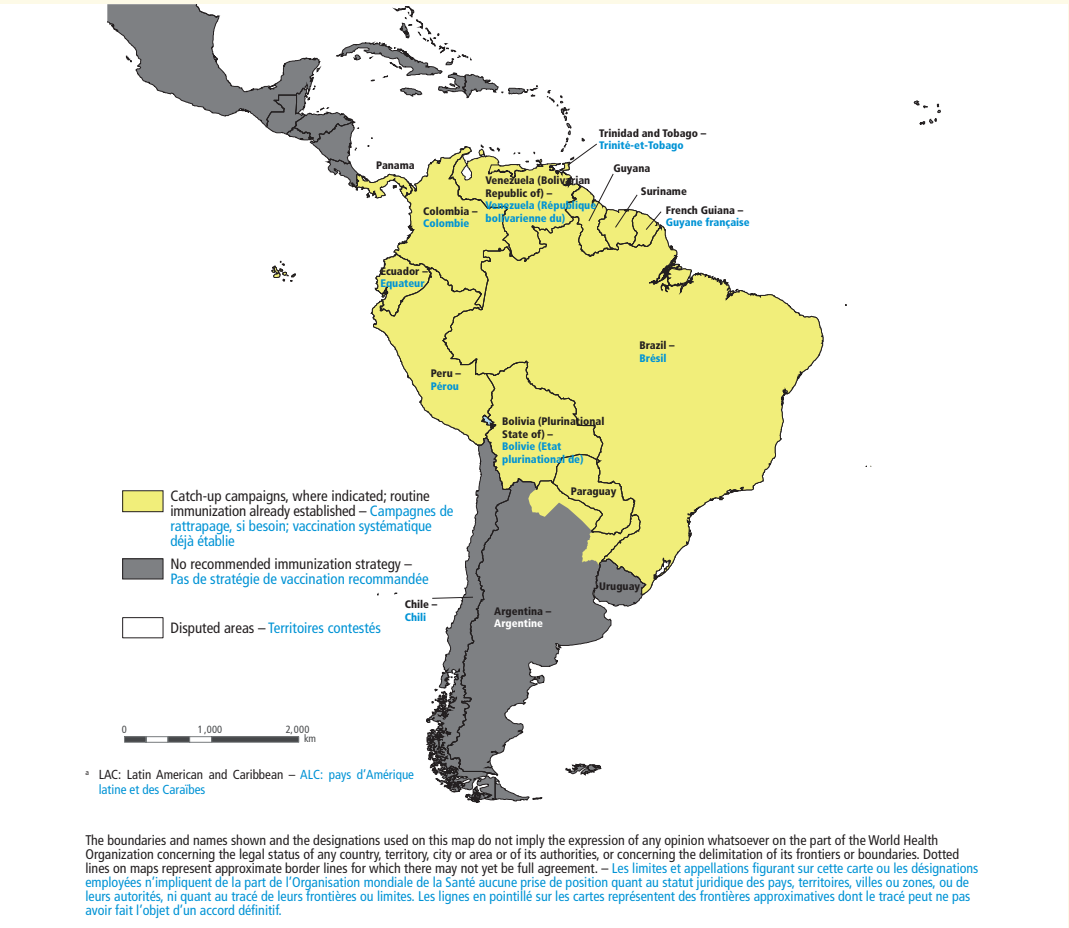


[a] PMVC: preventive mass vaccination campaign – CVPM: campagne de vaccination préventive de masse

The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement. – Les limites et appellations figurant sur cette carte ou les désignations employées n'impliquent de la part de l'Organisation mondiale de la Santé aucune prise de position quant au statut juridique des pays, territoires, villes ou zones, ou de leurs autorités, ni quant au tracé de leurs frontières ou limites. Les lignes en pointillé sur les cartes représentent des frontières approximatives dont le tracé peut ne pas avoir fait l'objet d'un accord définitif.

Source: taken from (38) World Health Organisation (WHO), Eliminate Yellow fever Epidemics (EYE): a global strategy, 2017–2026. Relev. Epidemiol. Hebd. 92, 193–204 (2017).

FIGURE 1.5B **Recommended immunizations activities for yellow fever in high-risk countries in Latin America and Caribbean 2017-2026.**



Source: taken from (38) World Health Organisation (WHO), Eliminate Yellow fever Epidemics (EYE): a global strategy, 2017–2026. Relev. Epidemiol. Hebd. 92, 193–204 (2017).

1.7 YF SURVEILLANCE

High vaccination coverage reduces human incidence but does not disrupt enzootic transmission between mosquitoes and non-human primates. Because elimination or eradication of YF virus and continuous surveillance of YF infected mosquitoes in the environment is not possible, continual vaccination of naive populations is required to protect humans living in areas endemic for YF (28, 40). YF surveillance is critical for monitoring the incidence of the disease and allowing the prediction and early detection of outbreaks to guide control measures. Through continuous surveillance, populations at-risk can be identified, and appropriate preventive strategies planned and implemented. Over time, good surveillance helps to identify factors that may put populations at increased risk and permit assessment of the impact of public health interventions designed to reduce the burden of disease.

Human surveillance: Case-based surveillance with laboratory confirmation for YF has resulted in earlier detection of outbreaks and allowed for a timely response. However, experience has also shown that only 1%–3% of suspected YF cases test positive for YF IgM; of these, approximately half might be confirmed by additional investigation (28). Introducing real-time RT-PCR to the testing algorithm in the National Laboratory (NL) level in the African Region is expected to change the percentage of confirmed cases. However, mild cases may remain undetected because the patients are treated at home or presumed to have another etiology (e.g., malaria, COVID-19) and do not seek care in a health facility.

For every confirmed case of YF with jaundice, there are an estimated nine cases with mild or asymptomatic infections (33). Therefore, one confirmed case is sufficient to consider a potential outbreak and justify planning for early investigation and intervention. The type and magnitude of response should be guided by high-quality field investigation and risk assessment. Information on vaccination coverage among different age groups is essential to determine an intervention strategy. Case investigations and outbreak responses should be adapted to the local context; a confirmed case in an unvaccinated urban population requires rapid intervention; if there is a cluster of probable cases, every effort must be made to rapidly identify the cause of the outbreak (39).

Surveillance of non-human primates: The role of non-human primates in YF epidemiology in Africa and the Americas is very different. In the Americas, sylvatic YF often occurs in unvaccinated people working in jungles or sylvatic environment, and in the perisylvatic areas. In sylvatic enzootic areas, monkey deaths due to YF virus often precede human cases. For this reason, surveillance systems to detect deaths of non-human primates are often put in place. When there is a cluster of non-human primate deaths, tissue samples are taken and tested for YF virus infection. Results are then reported to the health system authorities and preventive vaccination activities in people may be undertaken. In Africa, non-human primate species usually do not die from YF virus infection and instead acquire immunity against YF when they are infected. Therefore, surveillance systems to detect deaths of non-human primates are generally not effective (10).

1.8 THE CLINICAL DESCRIPTION AND CASE DEFINITION OF YF

Most people infected with the YF virus are asymptomatic; the ratio of inapparent to symptomatic infection is estimated from studies in Africa to be 7-12:1 (7). The clinical course of disease is comprised of three stages: infection, remission, and intoxication, often without clear demarcation (24). The incubation period between infection and onset of illness is approximately 3 to 7 days. Viremia peaks in the first 3 to 4 days of illness (Figure 1.3). Symptomatic illness is characterized by sudden onset of fever; chills; head, back, and muscle pain; and nausea and vomiting (28). Symptoms usually subside 3-4 days post-onset of illness; the patient will then go into remission or deteriorate into the intoxication phase. An estimated 5%–25% of clinical cases progress to more severe disease, including jaundice, renal insufficiency, cardiovascular instability, or haemorrhage (e.g., epistaxis, hematemesis, melena, haematuria, petechiae, or ecchymoses). The case-fatality rate among severe YF cases is estimated at 47% (33). Factors contributing to the heterogeneity in infection rates and potentially disease rates include the local environment; characteristics of the human, vector and virus populations, including pre-existing YF virus immunity due to previous exposure or vaccination; interventions in response to outbreaks; and the prevalence of other health conditions that may influence the severity of disease (such as malnutrition or HIV infection) (33).

Suspicion of YF is based on the patient's clinical features, travel history (if the patient is from a non-endemic country or area), activities, and epidemiologic background of the location where the presumed infection occurred. It is difficult to make a definitive diagnosis based on clinical symptoms alone because the signs and symptoms of the disease are similar to those of other infections, such as viral hepatitis, malaria, leptospirosis, typhoid fever, and other viral haemorrhagic fevers (e.g., Ebola virus disease). Laboratory confirmation is therefore essential for the differential diagnosis of YF.

The WHO case definition of YF for public health surveillance is as follows (28):

Suspected case: A suspected case is any person with acute onset of fever, with jaundice appearing within 14 days of onset of the first symptoms. The syndromic definition of suspected cases is broad and has many possible differential diagnoses, making it a sensitive but not very specific definition. It is expected that approximately 1%-3% of suspected cases are actually infected with YF virus. All cases of acute fever and jaundice in high-risk areas should include laboratory confirmation to confirm the diagnosis. However, this sensitive definition provides an alert within an early warning system that there might be an outbreak, and it should trigger the activation of an appropriate outbreak response.

FINAL CASE CLASSIFICATION

Probable case:

A suspected case AND at least one of the following:

- Presence of YF IgM antibody in the absence of YF immunization within 30 days of illness onset;

Epidemiological link to a confirmed case or an outbreak (e.g., household members or persons in close proximity to a case through work, residence in past month)

Confirmed case:

A probable case AND at least one of the following:

- Negative results of differential neutralization testing with flaviviruses (e.g., dengue, West Nile, Zika) endemic in the area of exposure;
- Seroconversion in appropriately paired samples tested by YF neutralization testing

AND

- Absence of YF immunization within 30 days before onset of illness

OR

A suspected case AND at least one of the following:

- Detection of YF virus genome in blood or other organs by real-time reverse RT-PCR;
- Detection of YF antigen in liver or other organs by immunohistochemistry;
- Isolation of YF virus

AND

- Absence of YF immunization within 14 days before onset of illness

In the event of complex test results, results must be carefully interpreted by surveillance and laboratory experts with review of relevant clinical and epidemiological details (e.g., if both YF and differential neutralization testing yields positive results).

Discarded case:

> A person who tests negative for YF antibody testing (with specimen collected >7 days post-onset of illness) or negative immunohistochemistry on organ tissue samples.

Note: A negative RT-PCR result does not rule out a case.

Note: The International Health Regulations require all YF cases to be reported to WHO within 24 hours of detection (103).

1.9 LABORATORY DIAGNOSIS OF YF

The YF laboratory testing algorithms in outbreak and non-outbreak settings and interpretation of results are discussed in Chapter 4. Detection of YF virus-specific IgM antibody from serum specimens (Chapter 5) and the detection of YF viral RNA by real-time or quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR) from tissue and serum specimens (Chapter 6) are standard methods that are used among laboratories in the Global YF Laboratory Network (GYFLaN) to diagnose suspected acute cases of YF. Histopathologic examination of tissue is also an important method of diagnosing YF, particularly in South America, where autopsy and testing are more readily performed (Chapter 6).

YF vaccine also elicits IgM production and recent vaccine history must be considered when evaluating suspected cases by serology for case confirmation. Current laboratory tests cannot differentiate between YF virus IgM developed after vaccination or in response to infection with YF wild-type virus. Therefore, laboratory results in people who have received a YF vaccine within 30 days must be interpreted with care and assessed on a case-by-case basis, considering the clinical presentation and epidemiological context along with the laboratory results. Interpretations of laboratory test results in vaccinated individuals are discussed in Chapters 4, 5, and 6.

A positive serological test for YF IgM alone is not sufficient to confirm a YF virus infection, as other flaviviruses co-circulating in the area, such as dengue, West Nile, and Zika, can give false positive results in YF serological assays (41). Differential diagnostic testing for other flaviviruses, as determined by local epidemiology, must be performed. Confirmatory differential diagnostic testing in the Regional Reference Laboratory (RRL) is part of the YF testing algorithm in Africa and discussed in Chapter 4.

Development of molecular diagnostic tools has significantly advanced the definitive diagnosis of YF. In South America in recent outbreaks, RT-qPCR has become the first-line testing method (42). The high sensitivity (approximately 1-10 virus particles) and specificity of RT-qPCR, including differentiation between wild-type and vaccine YF strains, underlies current approaches to YF diagnostics (Chapter 6).

Specimen collection and transport are discussed in Chapter 3. All specimens should be transported to laboratories with appropriate patient and clinical information (e.g., age, sex, place of residence, onset of symptoms, YF vaccination history and travel history) in the case investigation and laboratory request forms. Laboratory test results cannot be interpreted correctly without this information. Serum specimens should be tested as soon as possible, preferably within 24 hours of arrival at the laboratory, and stored properly. Serum should be separated from whole blood as soon as possible after clotting to avoid haemolysis and resulting test interference. The time when blood is collected – that is the length of time after onset of symptoms – affects the interpretation of the results of both molecular and serological tests. Therefore, it is important to remember that the time estimate is based on the history given by the patient and may not always be accurate.

In localities where local transmission has not yet been confirmed, serum specimens should be obtained from all people with suspected YF. If the laboratory has reached maximum 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 between YF and other *flaviviruses* on specimens from areas where local transmission has already been confirmed, unless more than one flavivirus has been documented to be currently circulating in that area. However, all specimens should be properly stored for future analysis if needed. In at-risk countries that do not have confirmed YF outbreaks, laboratory testing should be used to detect the first (index) case (Chapter 4).

All biological samples obtained for YF testing, including whole blood, serum, or fresh tissue, are potentially infectious (43). Procedures for handling infected tissue cultures, animals, or arthropods should be carefully assessed according to national regulations and the biosafety regulations of each laboratory, and the use of class II biosafety cabinets should be considered (43). All laboratory personnel working with YF virus or suspected YF cases should be vaccinated. In endemic areas with competent vectors, to prevent intra-hospital transmission, YF patients should be placed under mosquito nets.

Chapter 2

The Global Yellow Fever Laboratory Network and the role of the yellow fever diagnostic laboratory network in the EYE Strategy

To support Member States, in 2005 the WHO established the GYFLaN in order to improve laboratory-based diagnosis and surveillance for YF in countries at-risk of YF outbreaks, and provide essential, rapid, and reliable data on YF cases (44). Within the GYFLaN framework, the collective experience of all laboratories provides essential input for the development of improved methodologies, testing strategies, and to establish quality assurance programmes.

In 2016, two linked YF outbreaks in Angola and the Democratic Republic of the Congo resulted in unprecedented rapid rural and urban spread in the capitals, Luanda and Kinshasa, respectively, as well as many of the provinces. This resulted in the exhaustion of global stockpiles of YF vaccine (45, 46). Cases associated with this YF outbreak were reported in countries as far as China, due to spread via international travellers. This demonstrates how quickly the virus can reach new areas, where transmission in urban settings can be established, and how far YF can spread in urban settings if left unchecked (47). In response to these outbreaks, the EYE Global Strategy was developed (48). The YF diagnostic laboratory component of the EYE Strategy is an essential contribution to the overall success of the EYE Strategy objectives. Strong diagnostic laboratories and the structure of the GYFLaN should allow all EYE strategic objectives to be met. Funds from Global Alliance for Vaccine and Immunization (Gavi) support multiple components of the laboratory system and facilitate rapid detection and containment of outbreaks, which allows evidence-based decisions to be made on how a finite supply of vaccine can best be allocated between routine immunization, preventive campaigns, and reactive vaccination campaigns.

Chapter content:

- Establishment of the GYFLaN
- Objectives and organization of the global laboratory network
- Coordination of the GYFLaN
- Role and major activities of the network laboratories
- Overview of the EYE Strategy
- The importance of high-quality diagnostic laboratories in the EYE Strategy
- Laboratory strengthening in the EYE Strategy

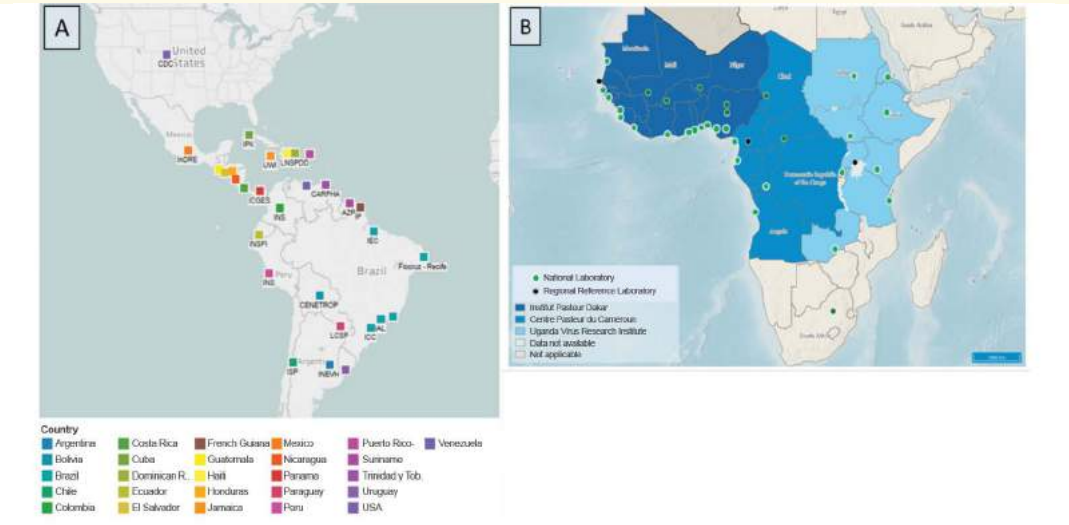
2.1 ESTABLISHMENT OF THE GYFLaN

Since 1988, WHO has developed global networks of laboratories with complementary capacities to ensure the availability of high-quality surveillance of vaccine preventable diseases. The laboratory network approach has a strong capacity-strengthening element, with RRLs providing training and technical support to NLs. The GYFLaN was established by WHO in 2005 and currently consists of more than 40 laboratories in the Region of the Americas and the African Regions; however, the laboratories in the two regions may use different testing protocols. The GYFLaN has focused on developing YF-specific testing skills, building upon existing cross-cutting laboratory capacities that are in place for the Global Polio Laboratory Network (GPLN) and the Global Measles and Rubella Laboratory Network (GMRLN), using a translational approach to set up similar technical and managing capacities as used for different diseases (44).

The NLs for YF within the GYFLaN were developed and expanded in conjunction with regional surveillance and control programmes. Regional YF laboratory networks have been established in the Americas and in Africa (Figure 2.1) and may also be linked to the surveillance of other vaccine preventable diseases such as measles and rubella, be fully integrated with arbovirus surveillance, or include syndromic surveillance of high threat pathogens such as Ebola (49).

The Pan American Health Organization (PAHO) Dengue Laboratory Network of the Americas was created in 2008 with the objective of strengthening scientific and technical capabilities and establishing a standardized laboratory protocol for dengue diagnosis. In 2016, expanding its range of action against the many arboviruses circulating in the Latin American region (including dengue, Zika, chikungunya, YF, West Nile, Mayaro, and Oropouche) and integrated with arbovirus surveillance, it became the Arbovirus Diagnostic Laboratory Network (RELDA). RELDA is made up of 30 laboratories from 26 countries and territories in the Region of the Americas (50). In Africa, a network for the detection and laboratory confirmation of YF cases in the WHO African Region was established in 2001, when international efforts were aimed to reduce YF mortality rates (38, 48).

FIGURE 2.1 **The yellow fever laboratory networks.** See Box 2.1 for detailed legend.



Source: Courtesy of the WHO/EYE Secretariat.

Box 2.1 Details of legend from Figure 2.1

(A) In the Americas, there are 30 National Public Health Laboratories (NPHLs) in 26 countries, five of which are WHO/PAHO Collaborating Centres (Argentina, Brazil, Cuba, Mexico, and the United States). All NPHLs have molecular detection capacity for at least four arboviral diseases (dengue, chikungunya, Zika, yellow fever viruses) and enzyme-linked immunosorbent assay (ELISA) platforms to diagnose at least three arboviral diseases (dengue, chikungunya, Zika). Sixteen NPHLs have biosafety level 3 (BSL-3) laboratories. Source: Courtesy of Jairo Mendez from PAHO.

(B) In Africa, there are 38 National Laboratories in 33 countries and three Regional Reference Laboratories at the Institute Pasteur de Dakar, Senegal for the West African Region; Centre Pasteur du Cameroun, for the Central African Region; and Uganda Virus Research Institute, Uganda for the Eastern Southern African Region.

2.2 OBJECTIVES AND ORGANIZATION OF THE GLOBAL LABORATORY NETWORK

There are several key objectives for maintaining a unified network of laboratories to support YF programme goals:

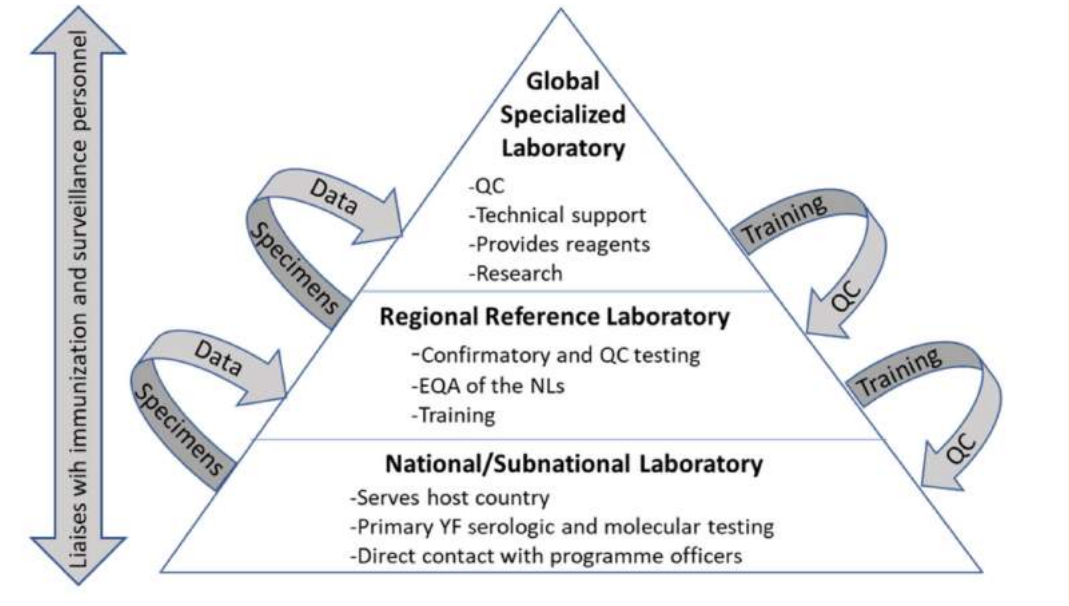
- Develop, improve, validate, and subsequently introduce standardized protocols for the laboratory confirmation of YF;
- Establish standards for quality assurance (QA) and quality control (QC) to achieve standardized surveillance;
- Administer and implement standardized external QA through global proficiency testing;
- Monitor the performance of network laboratories through annual accreditation;
- Provide training resources and facilities for staff of network laboratories;
- Facilitate inter-laboratory cooperation for expanding national and regional capacity;
- Maximize the efficient use of limited reagents and validated reference materials;
- Share expertise and data to track transmission of YF virus;
- Provide support including WHO funding to enhance and maintain laboratories in areas with limited resources.

The GYFLaN was modelled based on the GPLN and GMRLN (Figure 2.2) (44, 51). These networks are organized into four tiers: Global Specialized Laboratories (GSLs), RRLs, NLs, and subnational laboratories (SNLs). The GSL and RRLs have been established as centres of excellence and act as reference facilities for NLs and SNLs, which are nominated by the Ministry of Health (MOH) of each Member State. The SNLs may be organized according to governing administrative levels and/or geographical areas (e.g., province/state, district/prefecture) (44).

A tiered approach makes efficient use of resources by networking laboratories with complementary capacities and roles. Each progressive tier provides access to more advanced diagnostic services and offers cascading technical support, external QA, and QC to the tiers below.

NLs identify presumptive YF cases and rule out YF as the diagnosis for other suspected YF cases. RRLs provide referral testing to confirm presumptive YF virus infections and can also provide training and technical support. RRLs also assist WHO in coordinating QA/QC testing. GSLs improve existing testing methods or develop new diagnostic tools, provide more sophisticated tests (e.g., genetic sequencing), assist WHO in coordinating laboratory networks, and facilitate training.

FIGURE 2.2 **Tiered structure and functions of the Global Yellow Fever Laboratory Network.** See Box 2.2 for detailed legend.



Source: modified from (51) Diop et al. The Global Polio Laboratory Network as a platform for the viral vaccine preventable and emerging diseases laboratory networks. J Infect Disease 2017:216(suppl_1):S299-S307.

Box 2.2 Details of legend from Figure 2.2

The network provides access to services and technical support so that every country need not have the same capacity. Abbreviations: EQA, external quality assessment; NL, National Laboratory; QC, quality control.

Some of the laboratories may have more than one designation. For example, the RRLs also act as NLs and are therefore also responsible for first-line testing of specimens by YF virus-specific IgM and/or RNA detection. A description of general areas of responsibilities for the four levels of network laboratories is given below.

2.2.1 GLOBAL SPECIALIZED LABORATORY

The responsibilities of the GSL extend to YF laboratories in all regions and countries.

- **Technical support/training:** Provides technical advice, consultation, and specialized training to RRLs and NLs. Assists in preparing global reports and summaries of network activities. Develops periodic external quality assessment (EQA) proficiency testing panels for regional laboratories;
- **Research and development:** Contributes to the development and validation of novel methods and the standardization of procedures and protocols. Evaluates diagnostic kits and develops and improves methods. Conducts molecular epidemiologic research;
- **QA:** Prepares standards, proficiency panels, and training materials;

- **Biobanking:** Provides storage of reference materials, specimens, reference virus strains, and virus isolates;
- **Reference testing:** Develops and evaluates diagnostic assays and protocols; investigates use of alternative specimen types (e.g., urine);
- **Investigation of special cases:** Provides comprehensive testing of specimens from cases that may be difficult to classify.

2.2.2 REGIONAL REFERENCE LABORATORY

Laboratories designated as RRLs have demonstrated the capacity to undertake international responsibilities and collaborate closely with the GSLs. The general roles and responsibilities of the RRL are listed below; specific activities of the RRL to support the GYFLaN are provided in detail in Chapter 7.

- **Reference testing:** Samples tested in the NLs and SNLs are referred to an RRL for further testing to confirm results and provide case classification using standard and specialized methods, including differential diagnostic testing, plaque reduction neutralization, and RT-qPCR. The results from the RRL should rule over the one from the NL, except, if possible, specimen degradation is suspected. This is particularly true for specimens that tested positive by RT-qPCR at the NL level, which is likely to be undetectable by the RRL due to a chance of RNA degradation during transportation errors. Where needed, the RRL can also provide support for genetic characterization of samples referred for virologic surveillance;
- **Technical support/training:** Provides technical support and training to NL staff to improve their capacity to test for YF and to introduce newly developed laboratory diagnostic procedures and methods;
- **Data management and reporting:** Submits final test results through national database. Reports results of confirmatory and reference testing to country MOH, NLs and SNLs, country WHO programme manager, and WHO Headquarters;
- **QA:** Performs validation of NL test results using a validated assay and internal controls. Coordinates EQA proficiency testing programmes and provides QC retesting for NLs;
- **Research:** Collaborates with the GSL in the development and evaluation of new or improved methods.

2.2.3 NATIONAL LABORATORY

The NL communicates directly with immunization programme managers.

- **Testing:** Provides presumptive YF virus infection classification for clinically suspected YF by using IgM detection assays or YF virus infection confirmation by RT-qPCR if the capacity exists. If the facility does not have the capacity to support serologic testing, the NL forwards clinical specimens to the RRL for testing. Performs epidemiologically essential serological surveys;
- **Data management and reporting:** Submits results into MOH and WHO databases. Reports results to country MOH, country WHO programme manager, RRL, and SNLs, if applicable;
- **QA:** Participates in EQA proficiency testing programmes. A proportion of specimens are sent to the RRL for QC retesting. Monitors the quality of SNLs under its responsibility.

2.2.4 SUBNATIONAL LABORATORY

In many countries, the responsibility of first-line testing of specimens for case classification is shared by laboratories at the subnational level. This may be necessary because of population size, geographical distribution, and/or logistical challenges.

- **Testing:** Provides presumptive YF virus infection classification for clinically suspected YF by using IgM detection assays or YF virus infection confirmation by RT-qPCR if the capacity exists. If the facility does not have the capacity to support serologic testing, the SNL forwards clinical specimens to the NL for testing;
- **Data management and reporting:** Submits test results to national database. Reports the results to the MOH, WHO country programme manager, and NL;
- **QA:** Participates in EQA proficiency testing programmes. A proportion of specimens are sent to the NL for QC retesting.

2.3 COORDINATION OF THE GYFLaN

Coordination of the GYFLaN is carried out by WHO Headquarters, based on the experience gained in establishing the Global Laboratory Networks (42, 44). Each of the WHO regions has a WHO Regional Laboratory Coordinator (RLC) responsible for the laboratories within their region. The RLC works closely with the GSLs, RRLs, and WHO Headquarters to coordinate training activities. Each of the regions work in partnership with the Global Laboratory Coordinator (GLC) who is based at WHO Headquarters in Geneva, Switzerland. The GLC and RLCs share comments, requests, and queries, and provide ongoing communication through summaries and regional meetings. Procurement and distribution of essential standardized laboratory equipment and reagents are also coordinated through WHO.

An established system of indicators purposively designed for the monitoring of laboratory performance, including EQA for IgM detection, molecular detection and characterization, and laboratory accreditation has proven beneficial for the participating laboratories. The analysis of quality assessment activities helps to guide training needs and the data is valuable for tailoring recommendations intended to improve testing methods as well as to respond to changes in technology. YF serology and molecular EQA proficiency testing programmes have been established. Comprehensive training programmes will follow with the goal of improving laboratory capacity and expanding the number of laboratories routinely performing molecular techniques for YF surveillance.

Laboratory virologists and epidemiologists at all levels must establish mechanisms to exchange information on a regular basis to monitor and evaluate performance indicators of the surveillance system and to link laboratory and epidemiology data. Therefore, while responsibilities for YF testing (along with documentation and reporting requirements) increase the workload for laboratories in the GYFLaN, the technical collaborations and collegial relationships are important benefits for member laboratories. The network laboratories interact closely with national immunization programmes, and timely information exchange is critical for integrated surveillance and to provide necessary data to inform strategic interventions. The reliability of the data reporting system must also be examined during the laboratory quality management system (QMS) assessment (see Chapter 9). Better diagnosis leads to better immunization programmes, not only because a response can be activated sooner through improved diagnosis and surveillance, but also because unnecessary intervention can be avoided.

Thedventages of providing updates and reviewing aggregate data in a scheduled meeting format are now well established. Representatives of the GSLs and RRLs should hold meetings on an annual basis, and many actually participate more frequently through memberships in one or more research working groups. Representatives from the NLs should hold meetings with their immunization programme counterparts at least once a month and participate in regional meetings every year.

An effective and efficient laboratory network depends on collaboration and information exchange within the network, and with the disease control field staff, and colleagues in the immunization programme. Standard referral and reporting forms have been developed to ensure that all essential patient information is transmitted in a harmonized manner (see Chapter 8). Collection and dissemination of relevant information that guides both disease control activities and testing strategies is the cornerstone of effective surveillance.

2.4 ROLE AND MAJOR ACTIVITIES OF THE NETWORK LABORATORIES

Laboratory confirmation of suspected YF virus infections is critical for effective case management and surveillance. The primary role of the laboratories in the GYFLaN is to provide reliable laboratory testing to support the goals of the national, regional, and global YF surveillance and immunization programmes. It is essential that the laboratory meets the indicators for high-quality, laboratory-based surveillance. To be effective and efficient members of the GYFLaN, NLs and SNLs should have:

- Established links with the immunization and surveillance units at the MOH;
- Proven capability to perform testing, adhere to the GYFLaN testing algorithm, and interpret multi-testing results based on clinical and epidemiological context;
- Appropriately trained scientists and technicians;
- Adequate laboratory facilities and resources to cover running costs;
- Suitable equipment to conduct routine serological assays;
- Capabilities for data management and rapid communication of results, with both feed forward and feedback of data.

There are three broad areas of activities that network laboratories carry out in support of these surveillance programmes. These are discussed in detail below.

1. Laboratory testing for surveillance and early detection of YF cases
2. Communication and documentation of laboratory results and data
3. Participation in WHO quality assessment and accreditation programmes

2.4.1 LABORATORY TESTING FOR SURVEILLANCE AND EARLY DETECTION OF YF CASES

Sustained YF control strategies rely on strong surveillance and diagnostic capacities for early detection of outbreaks and rapid implementation of control measures that can help mitigate the risk of spread and the use of extensive resources. Therefore, laboratory testing of clinical specimens and results reported for clinically suspected cases must be timely and accurate. Antibody-based detection using an immunoassay with confirmation through differential testing and neutralization assays and/or direct virological detection by molecular testing (such as RT-qPCR), antigen-detection assays, and immunohistochemistry (IHC) have been validated for use in the NLs. Proper documentation of patient vaccination and clinical history, as well as epidemiological context data are critical for case classification.

The SNLs and NLs that do not perform all testing required for the confirmation of a YF virus infection should promptly notify the appropriate NL or RRL regarding referral of specimens for such testing. It is essential that specimens are properly processed and stored to preserve the integrity of the specimens.

2.4.2 COMMUNICATION AND DOCUMENTATION OF LABORATORY RESULTS AND DATA

The format and timing of results reporting at the local, national, and regional levels will be based on global standards, but operational procedures should be drafted in consultation with appropriate national surveillance and immunization programme staff. These operational procedures for conducting surveillance should be clearly assigned, understood, and standardized throughout the network to enable consistent and comparable monitoring from a supranational perspective. All pertinent information must be transmitted rapidly and reliably from the local units to higher levels of the surveillance or health centre offices, nationally, regionally, and globally. Zero reporting (reporting even if no cases are confirmed) may be required on either a weekly or monthly basis, depending on the recent history of YF activity. This is an especially important activity in low incidence settings.

RLCs and NLs should ensure that the activities completed by the laboratory in support of programme goals are documented. Calculation of the laboratory indicators requires careful management of data. All NLs and RRLs in the GYFLaN are requested to provide reports of results to WHO on a regular, standardized schedule (e.g., monthly, weekly). For more information on data management and reporting in the GYFLaN, refer to Chapter 8.

2.4.3 PARTICIPATION IN WHO QA, QC, AND ACCREDITATION PROGRAMMES

Laboratories in the network should participate in annual proficiency testing in selected techniques and be evaluated on an annual basis through the WHO accreditation programme. In addition, all laboratories should refer specimens for retesting for QC in a standardized approach across the network. Records of specimens referred to the RRL for QC retesting, and other necessary elements that demonstrate QC and QA should be maintained. Chapter 9 covers the requirements for all aspects of QA and QC, including the WHO-sponsored proficiency programmes and accreditation.

2.5 OVERVIEW OF THE EYE STRATEGY

The EYE Strategy is a comprehensive and long-term strategy that aims at ending YF epidemics by 2026, and consists of three strategic objectives:

1. protect at-risk populations;
2. prevent international spread;
3. contain outbreaks rapidly.

The EYE Strategy calls for a multitargeted approach to prevent YF and contain any potential outbreaks, so that epidemics do not develop. The components within the strategic objectives include (48):

- preventive and reactive vaccination campaigns;
- urban readiness;
- continuous surveillance;
- timely and reliable diagnostic laboratory testing;
- improving the application of International Health Regulations (IHR);
- strengthening capacities to rapidly respond to outbreaks to avoid major epidemics.

To achieve these objectives, there are five cross-cutting competencies for success:

1. affordable vaccines and sustained vaccine market;
2. strong political commitment at all levels;
3. high level governance;
4. synergies with relevant programmes and sectors;
5. research and development for better tools and practices.

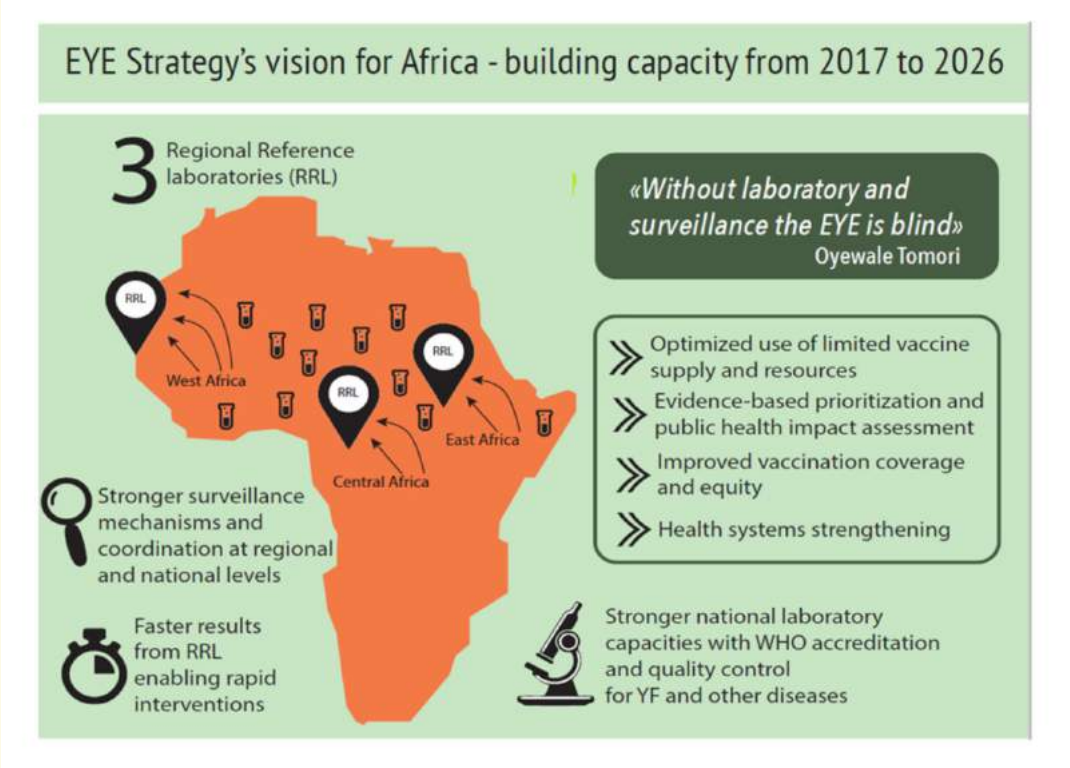
The principles outlined in the EYE Strategy were validated by the Strategic Advisory Group of Experts on Immunization (SAGE) in October 2016 and approved by the Gavi Board in December 2016. In 2017, the EYE Strategy launched an unprecedented initiative. With more than 50 partners involved, the EYE partnership supports 40 at-risk countries in Africa and the Americas to prevent, detect, and respond to YF suspected cases and outbreaks. By 2026, it is expected that more than 1 billion people will be protected with the YF vaccine.

Early detection of YF cases is critical to achieve rapid response and containment at the source of YF outbreaks. Rapidly controlling outbreaks ensures that resources can be controlled with the aim of protecting at-risk populations to reduce the likelihood of international spread.

YF incidence during inter-epidemic periods is low and clinical symptoms are nonspecific, and largely similar to those of other diseases occurring in risk areas, such as viral hepatitis, malaria and leptospirosis; other arboviruses (dengue and Zika); and other haemorrhagic fevers such as Ebola, Lassa, or Crimean Congo. Diagnosis based on clinical symptoms alone is often not accurate. Therefore, serum specimens collected from patients must be tested in the diagnostic laboratory.

By introducing improved tests and rapidly ruling "in" or "out" the diagnosis of YF, appropriate public health interventions can be tailored accordingly, and available resources optimally used to identify index cases in new areas, control spread, and minimize impact (e.g., mass vaccination for a confirmed YF case versus interventions aimed specifically at susceptible populations). The activities outlined in the YF diagnostics investment programme support strong disease surveillance and health systems in high-risk countries in Africa and help optimize the YF vaccine distribution with emphasis on strong routine immunization. This effort aligns with the EYE Strategy's vision on laboratory capacity-building by 2026 (Figure 2.3) and supports the direction set by the EYE laboratory technical working group created in December 2017.

FIGURE 2.3 **EYE vision for strengthened yellow fever laboratory and network capacity in Africa.**



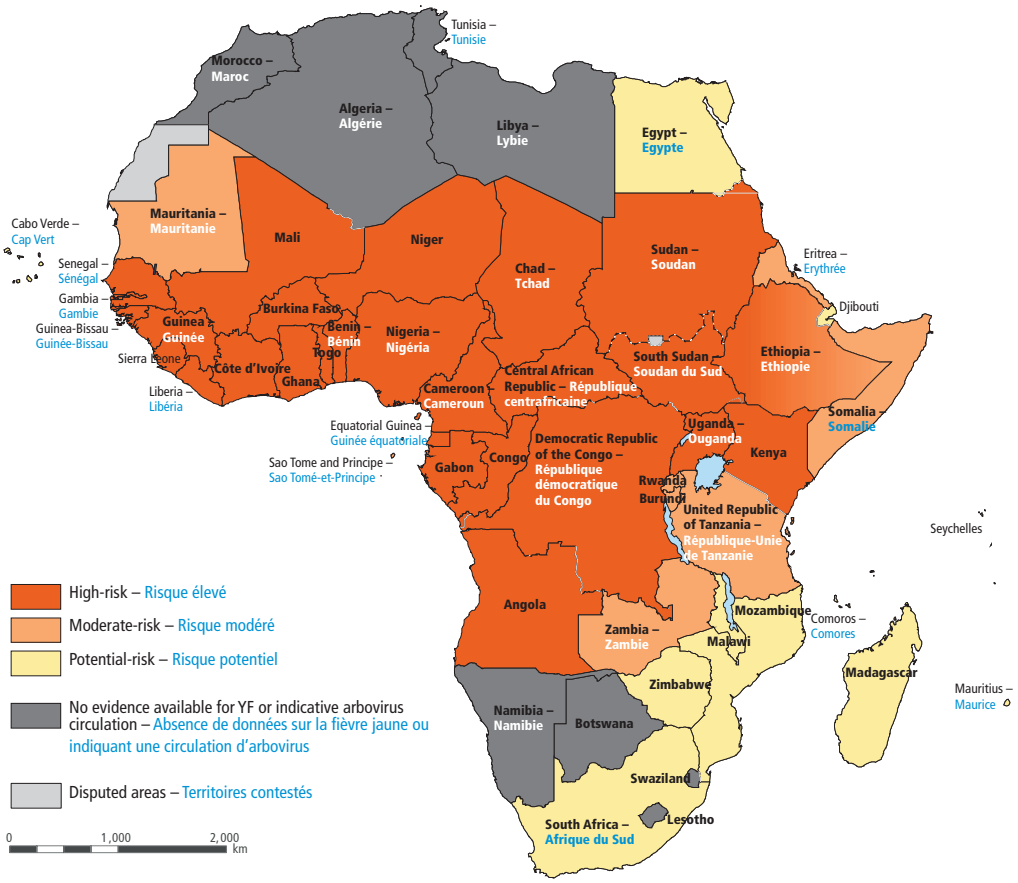
Source: modified from (52) World Health Organization. Eliminate Yellow Fever Epidemics (EYE). Strategy Partners meeting report. 2018:2.

The EYE Strategy targets countries and regions that are considered most vulnerable to YF outbreaks. The classification of countries at-risk for YF epidemics has been updated to consider changing epidemiology such as environmental factors, population density, and vector prevalence. Forty-seven countries (34 in Africa, and 13 in Central and South America) are either endemic for or have regions that are endemic for YF (32).

2.5.1 AFRICA

For Africa, a three-step approach was used to reclassify the 34 countries into high, moderate, and potential risk categories and to propose preventive strategies accordingly. The first step is using an estimation of crude risk for YF transmission based on timing and intensity of YF virus circulation in the country, estimates of the transmission potential in terms of the basic reproduction number, and the assessment of urban outbreak risk based on reports of recent or current outbreaks of Aedes aegypti-transmitted viral diseases. This analysis enabled identification of 27 "high-risk" and eight "moderate-risk" countries in Africa (Figure 2.4) (48).

FIGURE 2.4 **Yellow fever risk classification by country: Africa, 2016.**



The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement. – Les limites et appellations figurant sur cette carte ou les désignations employées n'impliquent de la part de l'Organisation mondiale de la Santé aucune prise de position quant au statut juridique des pays, territoires, villes ou zones, ou de leurs autorités, ni quant au tracé de leurs frontières ou limites. Les lignes en pointillé sur les cartes représentent des frontières approximatives dont le tracé peut ne pas avoir fait l'objet d'un accord définitif.

Source: taken from (38) World Health Organization (WHO). Eliminate Yellow Fever Epidemics (EYE): a global strategy, 2017–2026. Wkly Epidemiol Rec 2017;92:193–204.

2.5.2 LATIN AMERICA AND THE CARIBBEAN (LAC)

For LAC, YF is a significant public health problem for the 13 countries with endemic areas, and all are considered to be at high-risk (Figure 2.5). Over the last 30 years and throughout 2016, YF virus activity has been restricted to the enzootic area shared by the Pluri-national State of Bolivia, Brazil, Colombia, Ecuador, French Guyana, Guyana, Panama, Peru, Suriname, Trinidad and Tobago, and the Bolivarian Republic of Venezuela (48).

FIGURE 2.5 **Yellow fever risk classification by country as per the EYE Strategy: Latin America and Caribbean, 2016.**

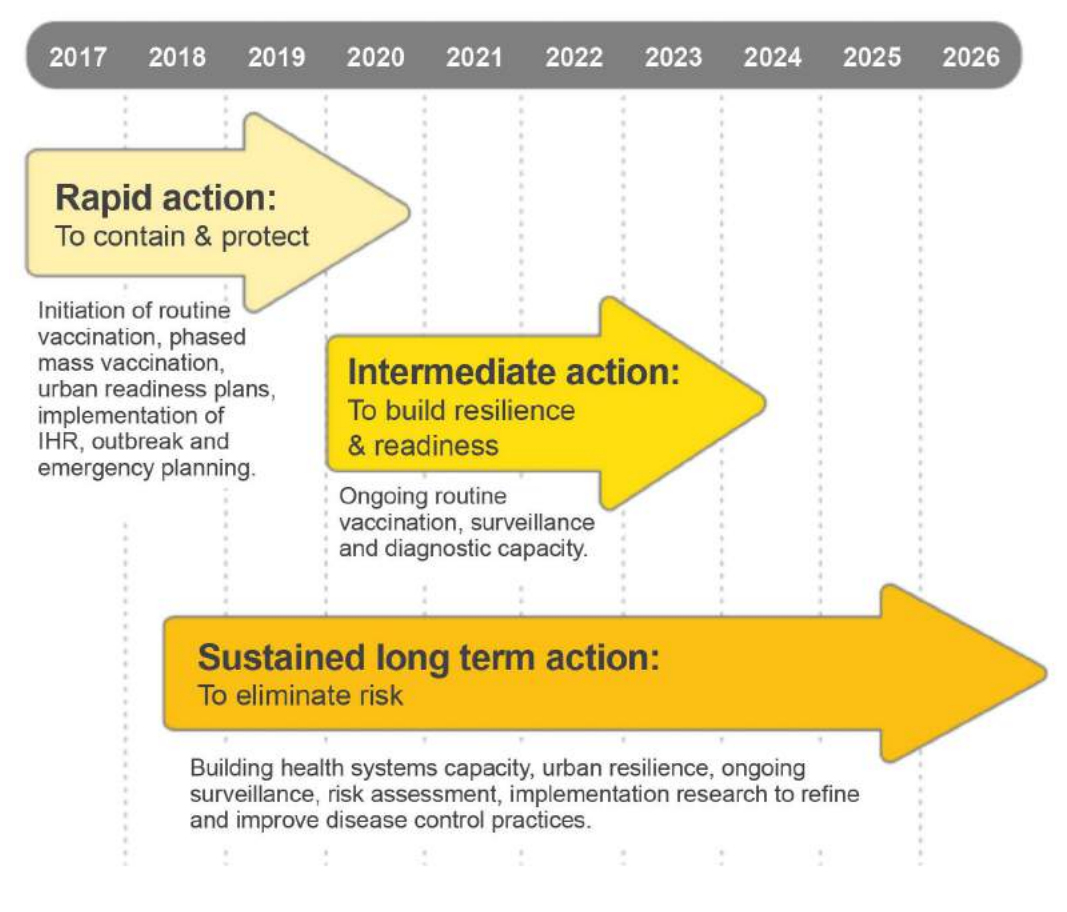
Source: taken from (38) World Health Organization (WHO). Eliminate Yellow Fever Epidemics (EYE): a global strategy, 2017–2026. Wkly Epidemiol Rec 2017;92:193–204.

Intense YF virus circulation with extensive epizootics (animal outbreaks) and outbreaks of human cases have been reported in LAC since late 2007. Prior to this, the last confirmed urban outbreak of YF in LAC occurred in 1942 in Brazil (48). As of 2016, national immunization schedules include YF vaccine in every country with enzootic areas in LAC. In some countries, such as Argentina and Panama, the vaccine is only administered in areas of potential risk and with enzootic activity. Following the outbreaks reported in the south-eastern urban regions between 2016 and 2018, Brazil has expanded the scope of vaccination nationwide. With the exception of the island of Trinidad, there is no YF circulation reported in the Caribbean countries and territories, which are not endemic for the disease. Nonetheless, large densely populated regions of South America, Central America, and the Caribbean outside the enzootic zone are now described as countries with "potential for YF transmission" on the same basis as the risk categorization proposed for Africa, due to the intense and rapid spread of both the Chikungunya and Zika viruses and recurrent dengue epidemics (48). More recently, in 2022, there have been 10 confirmed cases of YF in three countries: Bolivia (5 confirmed cases), Brazil (3 confirmed cases), and Peru (2 confirmed cases) (53). The PAHO/WHO encourage Member States to continue with YF surveillance and immunizing the at-risk population, as well as taking actions to inform and vaccinate travellers to endemic regions (53).

2.5.3 GOALS AND MILESTONES

A summary of the goals and key milestones outlined by the EYE Strategy is highlighted in Figure 2.6. The development of the EYE Strategy has helped assure greatly improved vaccine supply outlook due to the work of Gavi Alliance partners and vaccine manufacturers, which has provided the opportunity to significantly scale up YF control efforts. Despite the improved supply of vaccine, prioritization is still required to meet a base demand scenario and current supply would not be able to cover an ambitious scenario meeting all potential country requests simultaneously. The introduction of YF into Asia would have an unprecedented impact on the current vaccine situation and would be very difficult to manage. Because epidemic risk might exist through imported cases, strict implementation of the IHR for travellers in and out of countries at-risk for YF, as well as increased surveillance and preparedness, is paramount to prevent, detect, and respond to potential epidemic threats. Port and border control authorities need to be engaged to identify gaps and ensure that the vaccination status of all travellers entering and leaving endemic areas is known and appropriately managed. This is particularly important at points of entry into the at-risk countries. The EYE Strategy has made progress, allowing for coordinated efforts to increase protection of at-risk populations. However, there are areas that could benefit from acceleration and all activities are being implemented on a 'continued learning' basis.

FIGURE 2.6 **EYE timelines for implementation.**

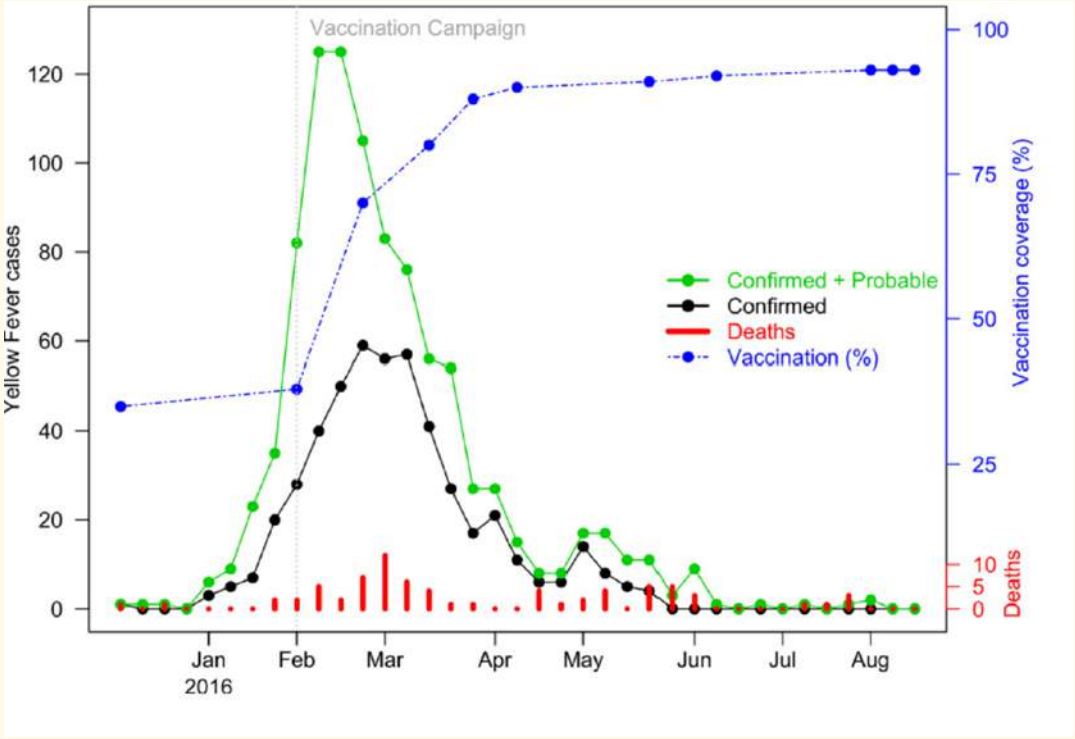


Source: taken from (52) World Health Organisation (WHO), "A WHO report of the Eliminate Yellow Fever Epidemics (EYE) strategy regional kick-off meeting for Africa" (2018).

The success of the EYE Strategy will be facilitated by strong surveillance and high-quality diagnostic laboratories to allow data-driven prioritization of YF immunization efforts as well as early outbreak alert, detection, and response. The EYE Strategy aims to minimize the risk of the potentially devastating consequences of YF spread (e.g., export of cases to YF I populations). It relies on strong surveillance backed by reliable, rapid laboratory diagnosis, so that sporadic cases and outbreaks are detected and contained before they escalate to epidemics.

The course of the 2015-2016 YF outbreak in Luanda, Angola illustrates the importance of rapid outbreak detection and vaccination response (Figure 2.7) (54).

FIGURE 2.7 **The observed yellow fever outbreak in Luanda, Angola from December 5, 2015 to August 18, 2016.**



Source: taken from (54) Zhao S, Stone L, Gao D, et al., Modelling the large-scale yellow fever outbreak in Luanda, Angola, and the impact of vaccination. PLoS Negl. Trop. Dis. 12 (2018), doi:10.1371/journal.pntd.0006158.

Once the YF reactive vaccination campaign began, the number of cases began to decline rapidly, preventing an estimated 6242 cases and 444 deaths in Luanda alone (54). If the reactive campaign had been initiated earlier, significant numbers of YF cases and deaths could have been prevented, potentially preventing the spread of the outbreak to the Democratic Republic of Congo. Without the spread of the outbreak to the Democratic Republic of Congo, the quantities of vaccine that were required to respond to the outbreak would have been much less.

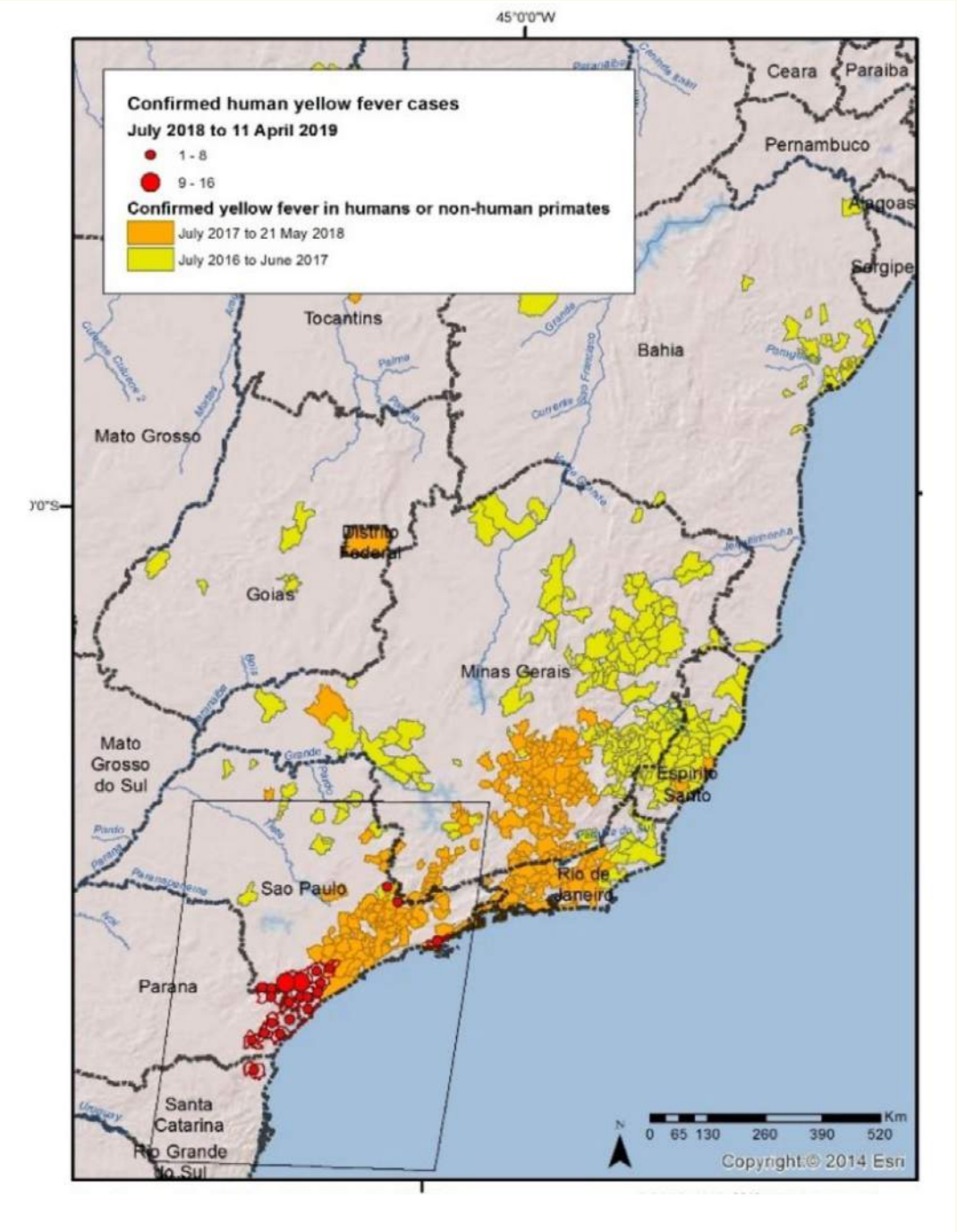
The 47 days needed to confirm that the first known case of the outbreak in Angola was, in fact YF, was the most time-consuming step prior to the launch of the Angolan reactive vaccination campaign (54, 55). However, confirmation is crucial for ensuring that unnecessary YF reactive vaccination campaigns are not launched. Faster confirmation of future outbreaks is essential to allow YF vaccine to be used as efficiently and effectively as possible.

Strong surveillance incorporating timely, reliable laboratory confirmation is needed throughout Africa to guide prioritization of YF vaccination activities, i.e., determine the sequence of introducing YF vaccine into routine immunization in additional countries and preventing mass vaccination campaigns in different areas. Risk analysis models are only as good as their underlying data on YF distribution and risk factors.

The recent experience in Brazil illustrates how the adjustment of risk assessments based on surveillance data can help drive immunization programme decisions (Figure 2.8). Until 2017, Brazil did not use routine YF immunization in coastal areas due to a very low risk of transmission following decades of mosquito control (56). However, in 2017, given the presence of other flaviviruses such as Zika in Brazil, the detection of YF cases heading towards urban areas, and surveillance data showing an expansion in areas at-risk, YF risk in coastal areas changed from a low level, in which conserving resources by not vaccinating made sense, to a higher level in which vaccination was justified (57). Because Brazil had confidence in the laboratory – with strong confirmation that the cases really were YF – they were able to build a convincing case and decide to implement large-scale vaccination in coastal areas.

FIGURE 2.8

Distribution of epizootics and confirmed human cases in Brazil from July 2018 to March 2019.



Source: taken from (13) World Health Organization (WHO), Yellow fever: Disease Outbreak –ews - Brazil (2019), (available at https://www.who.int/emergencies/disease-outbreak-news/item/18-april-2019-yellow-fever-brazil-en).

2.6 LABORATORY STRENGTHENING IN THE EYE STRATEGY

Given the importance of a laboratory's capacity in the success of the EYE Strategy, a laboratory technical working group (LTWG) has been established within the EYE governance structure to provide expert technical advice and guidance on YF diagnostic laboratory technical questions, including the updating of laboratory diagnostic algorithms (Chapter 4). This working group has a central role in guiding efforts to strengthen YF laboratory capacity.

The LTWG convened for the first time in December 2017 to identify the priorities for laboratory strengthening within the EYE Strategy and met again in September 2018 to provide further input to the proposal and address the limitations in African YF laboratory capacity. The following LTWG priorities are addressed by the proposal:

1. Strengthened laboratory networks, including strong QA/QC processes;
2. Improved specimen storage, processing, and transportation times;
3. Improved procurement systems;
4. New and/or improved laboratory tests and updated testing algorithms;
5. Strengthened and streamlined data and information sharing.

The transport of diagnostic specimens is being supported through the establishment of the EYE.ops operational and transport mechanism (eye.ops@who.int). Supported by an innovative investment from Gavi to strengthen YF diagnostics and faster confirmation of cases, which has been instrumental to this programme, EYE.ops aims to facilitate international transportation to the RRL from the African NLs (see Chapter 3). This support includes the financial coverage of all the operations, thus leading to substantial savings for laboratories requesting the EYE.ops assistance. The EYE.ops started roll out at the end of 2019, at first with four pilot countries. Currently, EYE.ops supports all African YF NLs in shipping their samples internationally, but also has the capacity to extend its support to the rest of the GYFLaN.

In addition to the international transport of samples, the EYE.ops aims to further support the laboratories in the African Yellow Fever Laboratory Network by prepositioning dedicated triple packaging on an annual basis. Furthermore, a contingency stockpile of triple packaging is being managed by the EYE Secretariat and upon request can be deployed immediately to any country facing a shortage. Information can be obtained through the EYE Secretariat.

YF cases are rare during non-epidemic periods and, due to the challenges of distinguishing YF from other diseases, multiple diagnostic tests are needed to confirm cases. Strengthening YF laboratory capacity in high-risk African countries will need reliable diagnostic assays, cascading technical support, and access to specialized analysis in a tiered approach to deal with these challenges.

Although basic issues of test availability and performance as well as sample transportation are the most immediate challenges currently affecting YF diagnostic laboratory capacity in some countries of the African Yellow Fever Laboratory Network, rapid and accurate sharing of data is also important, not only for identifying and assessing possible YF cases but also for triggering appropriate action when YF cases have been confirmed. As a result, integrating laboratory and surveillance data systems are essential. Further work by the LTWG will complement such efforts by advancing criteria and norms for sharing information within countries between surveillance and laboratory systems and between tiers within the laboratory network.

Further to immunization, the EYE Strategy aims to prevent outbreaks through early detection of YF cases. All countries are recommended to implement case-based surveillance for YF, regardless of underlying risk status (49). For YF confirmation, one must consider the laboratory diagnostics results in conjunction with available details about the clinical, vaccination history, and epidemiological context to inform final interpretation and classification. In Africa, this can be carried out within a functional Integrated Disease Surveillance and Response Programme, including case-based surveillance that applies a standard case definition and follows up each suspected case. This also requires a strengthening of laboratory testing capacities, with functional NLs, well-resourced regional and subregional reference laboratories, and a rapid sample referral and information exchange system between all levels. Innovative laboratory methods and alternative specimen types are needed to extend the time window for virus detection (e.g., YF genome has been successfully detected by RT-qPCR from urine or saliva for a longer period than in serum). This will help to exclude false positive results often seen in YF serological assays due to cross-reactivity with other flaviviruses, and better distinguish post-infection from post-vaccination seropositivity in the absence of reliable vaccination information.

Given the EYE Strategy's goal of protecting populations from YF and preventing epidemics, improved and well-coordinated laboratory and surveillance data are essential for tracking disease and informing progress on those goals. By augmenting efforts to prevent, detect, and contain YF outbreaks as efficiently and effectively as possible, a strengthened laboratory system should improve the overall value for money of the EYE Strategy.

Useful links:

EYE Strategy website: Eliminate yellow fever epidemics (EYE) strategy 2017-2026 (who.int)

YF vaccines stockpiles: Yellow fever vaccines stockpiles (who.int)

YF outbreak toolbox: Yellow fever Outbreak Toolbox (who.int)

Chapter 3

Clinical specimens for the laboratory diagnosis of yellow fever

This chapter describes the best practices for collecting and processing appropriate specimens for the laboratory diagnosis of YF. Serum is the most used patient specimen collected for both molecular (reverse transcriptase-quantitative polymerase chain reaction, RT-qPCR) and serological (ELISA; immunofluorescence assay, LFA; lateral flow assay) testing. A positive RT-qPCR result is considered a confirmed diagnosis of YF virus infection, whereas an IgM positive result provides a presumptive diagnosis, requiring the sample to be sent to the RRL for confirmation or additional testing (Chapter 4).

A second serum sample obtained from a suspected YF case may be needed to confirm an unclear diagnosis. For example, paired serum samples collected at appropriate intervals tested for IgM (or IgG) with a negative or equivocal result in the earlier collected sample and positive result in the second sample indicates a YF seroconversion of the patient.

Positive tissue samples from fatal cases are important specimen types used to test the efficiency of RT-qPCR and IHC testing. The various sample types used in the specific YF testing methods employed at the NLs and RRLs are discussed in Chapter 4 and Chapter 7, respectively.

Accurate test results depend on high-quality samples. Instructions for collecting and processing specimens for YF testing should be available in regional surveillance guidelines or field guides (49). These protocols should be updated and reviewed by laboratory and epidemiological staff at appropriate intervals. Communication between the laboratory and field staff is essential to ensure that samples are collected with adequate volume for testing at the NL and RRL, processed on-site, and transported properly to the laboratory to assure sample quality for testing.

Case-based surveillance should be implemented in countries identified by WHO as being at-risk for YF (11, 58). Ideally, blood samples should be obtained from every suspected case according to the YF case definition (Chapter 1). During outbreaks, a link between the laboratory and the epidemiology counterparts should be used to facilitate case confirmation by prioritizing laboratory testing for suspected cases. Ongoing coordination between the laboratory and the field staff can reduce unnecessary blood collection and testing.

Chapter content:

- Best practices for collection, processing, and storage of serum specimens
- Timing of blood collection for YF testing
- Collection and processing of clinical specimens for YF testing in fatal cases
- Alternative specimens for YF testing
- Collection of specimens from non-human primates
- Transport of clinical specimens
- Safety procedures for incoming clinical specimens

3.1 BEST PRACTICES FOR COLLECTION, PROCESSING, AND STORAGE OF SERUM SPECIMENS

3.1.1 BLOOD COLLECTION

Collecting a blood sample is performed by venepuncture using a sterile, plain collection tube (red-top tube) or serum separator tube without additives. If plasma is to be obtained, using sodium citrate (light blue top) tubes should be considered. Staff involved with the collection of patients' blood specimens should be fully trained in the process, including the appropriate use and disposal of needles and syringes and the best practices to avoid haemolysis (59, 60).

> **Note:** Refer to WHO guidelines on drawing blood for details, Section 2.3 onwards illustrates the best practices in phlebotomy (61).

At least 2-5 ml of blood should be collected from adults to yield 1-2 ml of serum; collection of 1-2 ml of blood is acceptable from infants. Collection of an adequate sample volume is critical to complete all the testing that may be needed for diagnosis. In addition to the molecular and/serological testing conducted at the NL, an absolute minimum volume of 0.5 ml serum is required by the RRL to conduct confirmatory or differential diagnostic testing, and testing for other diseases with similar clinical manifestations, such as fever and jaundice (e.g., malaria, leptospirosis, dengue, etc.). However, whenever possible NL should send up to 1.0 ml to the RRL to allow additional future analyses if required. Inadequate sample volume is a recurrent issue and limits the type of testing that can be done by the RRL to confirm or rule out a YF virus infection.

3.1.2 PROCESSING BLOOD SPECIMENS

Procedure for processing blood when 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. If it is not possible to process immediately, whole blood can be stored at (+)4–8°C (never freeze whole blood) for up to 24 hours or for 6 hours at (+)20-25°C before the serum is separated by centrifugation.

After the blood has clotted, the stoppered tube is centrifuged at 1000 x g for 10 minutes to separate the serum from the clot. If a serum separator tube was used, the serum should be carefully removed from above the separator, being sure not to touch the gel with the pipette tip. If a plain tube was used, the serum should be removed carefully without disturbing the red cell layer. In both cases, the serum should be transferred to a sterile, labelled vial. The screw-capped vial should have a durable label attached with the patient's identifier, date of collection, and specimen type.

Procedure for processing blood without a centrifuge: Blood should be placed in a refrigerator immediately after it is drawn in a standing rack or position. Transport should be organized promptly as specimens can be stored up to 6 hours at (+)20-25°C before the serum is separated by centrifugation. Serum can be pipetted out aseptically in a serum separator tube after the red blood cells have separated (about 6 hours at (+)4°C). If the blood was collected in a plain tube, the blood may be refrigerated until there is complete retraction of the clot from the serum (no longer than 24 hour). The serum should be carefully removed with a fine-bore pipette, to avoid extracting red cells, and transferred aseptically to a sterile, labelled vial.

Note: Haemolysis in samples can interfere with accurate test results by affecting the performance of the RT-qPCR test and causing a reduction in IgM-mediated signal in serological assays. Improper blood collection (e.g., incorrect puncture site, needle size, tube mixing, or filling of tubes) or sample handling (e.g., moving the tube after clotting without centrifugation or transporting the blood off-site to process) may lead to haemolysis. Therefore, to avoid causing haemolysis, phlebotomy personnel should be well trained in the proper procedures for collecting blood and specimens should be processed on-site using best practices, as described above (11).

Note: Collection tubes containing sodium heparin may have detrimental effects on molecular downstream applications and should be avoided. For such applications, EDTA-treated blood tubes should ideally be used.

3.1.3 SERUM STORAGE AND TRANSPORT TO THE LABORATORY

Serum should be stored at refrigeration temperature (+4-8°C) until shipment, but ideally should not be stored at (+) 4-8°C for longer than 7 days. When a delay is anticipated in shipping or in testing, the serum specimens should be frozen at -20°C, or colder (e.g., -80°C) if available. Aliquots of serum specimens should be prepared prior to freezing.

YF virus and serum antibodies are sensitive to heat and temperature fluctuations (freeze-thawing). Repeated freeze-thaw cycles of serum samples should be avoided, as it can have negative effects on the integrity of the IgM antibodies and viral RNA and diminish or destroy virus viability. A frost-free freezer, which has a periodic warming cycle to prevent ice build-up, should not be used.

Serum specimens should be shipped to the laboratory as soon as possible, ideally within 48 hours of collection, and should not be delayed by collecting additional specimens. The samples that have been refrigerated at (+) 4-8°C should be shipped with cold packs. However, samples transported within the first 24 hours for short distances can be transported at room temperature but should never exceed (+) 20°C during storage and/or transport. If transport is delayed, serum should be kept frozen at -20°C and shipped to a testing laboratory packed with frozen cold/ice packs in a sufficiently insulated container (see details below in Section 3.6). If the transit interval from the sender to arrival at the receiving laboratory is greater than 3-5 days, it is best to preserve the sample at -80°C and transport on dry ice, if it is available.

3.2 TIMING OF BLOOD COLLECTION FOR YF TESTING

Blood should be collected from a suspected case of YF at first contact (Chapter 4). In most instances, a single serum specimen will be sufficient for investigating a suspected YF case by molecular and/or serological analysis.

Prompt collection of a sample from a patient with suspected YF is the best approach to maximize the likelihood of obtaining a positive (confirmed) signal using RT-qPCR, which is more efficient when samples are collected closer to illness onset when the patient is viremic, before jaundice presentation. Serum collected up to 14 days after illness onset can be tested by RT-qPCR, although RNA detection rates decrease at the end of the viraemic phase, approximately after 10 days post illness onset if the patient recovers (31).

Samples tested by serology should be collected within the first 21 days after the onset of illness, as anti-YF virus IgM antibodies develop within a few days and wane after 30-60 days (27, 50). However, note that a sample obtained during the first week of clinical symptoms (≤7 days) from a true YF case could yield negative results in both IgM and RT-qPCR assays, as viremia may decline below the threshold of RNA detection and IgM may not be fully elicited to a detectable level.

A second specimen collected at least 1 week after the first specimen may be helpful for laboratory diagnosis if the indicated YF symptoms persist and the first sample was collected ≤7 days post-onset of illness and produced a negative (IgM and RT-qPCR) result. The collection of paired sera allows the demonstration of seroconversion (negative acute and positive convalescent) to YF virus (Chapter 4).

3.3 COLLECTION AND PROCESSING OF CLINICAL SPECIMENS FOR YF TESTING IN FATAL CASES

Histopathological analysis by IHC performed on liver sections and other tissues can be used to confirm diagnosis of fatal YF cases (biopsy in living patients is not indicated and should not be attempted). In the Americas, routine YF surveillance has been improved by histopathological review of liver specimens collected at postmortem from patients who died following an acute febrile illness (40, 49).

Fatal cases should have fresh and fixed tissue collected as well as blood, if possible. Blood can be collected by cardiac puncture and processed as discussed above in Section 3.1. Liver and kidney are the tissues of choice for histopathological and IHC analyses. Spleen, brain, lung, heart, and lymph node samples may also be useful. Molecular testing of serum and fresh or paraffin-embedded tissue samples can also be performed for case confirmation.

Specific instructions for collecting a liver sample from a fatal case are available from WHO regional offices and WHO Headquarters, together with instructions for sending the sample to an appropriate RRL (Chapter 7) (11). In general, fresh tissue samples of approximately 1 cm3 can be frozen at -80°C and sent to a RRL on dry ice. If this is not possible, fresh tissue should be stored in sterile saline or refrigerated phosphate-buffered saline (PBS) and shipped with refrigerant gels (do not freeze the material).

> **Note:** Frozen tissue may lose the structural integrity observed in paraffin-embedded tissue, which will affect histology, but epitopes are easily retrieved for IHC testing. To preserve the integrity for histopathological and IHC analyses, tissue samples need to be fixed in 10% buffered formalin and transported to the pathology laboratory 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. However, RNA-preserving agents affect the immunogenicity of tissues in IHC testing.

3.4 ALTERNATIVE SPECIMENS FOR YF TESTING

RT-qPCR testing may be done on plasma (minimum 1 ml) or whole blood (minimum 2 ml, so that there is sufficient volume to send to the RRL, if necessary). Use of the proper RNA extraction kits for these sample types is essential.

Clinical specimens (serum and dried blood spots) collected for molecular characterization may be processed and shipped on commercially produced paper cards (FTA® Cards) that inactivate virus but preserve the nucleic acids. Shipment of the FTA cards does not require a cold chain. Protocols for the preparation, shipment, and extraction of dried blood and serum spots are currently under evaluation (62). The use of dried blood spots in serology is currently being evaluated for use. Information will be updated as it becomes available.

Other patient specimen types, including whole blood, plasma and urine have been successfully used for RT-qPCR testing but have not been validated by WHO for use by the GYFLaN (25, 63, 64).

3.5 COLLECTION OF SPECIMENS FROM NON-HUMAN PRIMATES

In sylvatic YF enzootic areas of the Americas, surveillance systems are designed to detect epizootic deaths of non-human primates due to YF. When there is a cluster of non-human primate deaths, tissue samples (e.g., liver, kidney) from dead primates are obtained for YF histological and virological testing (e.g., RT-qPCR, IHC, virus isolation). Sample collection, processing, and transport should be coordinated with the PAHO YF RLC (10). All samples should be considered as potentially infectious not only with YF virus, but other highly pathogenic organisms (e.g., simian herpesviruses) as well. Appropriate biosafety practices should be strictly followed in collecting, processing, and testing specimens from non-human primates.

3.6 TRANSPORT OF CLINICAL SPECIMENS

Successful shipments of materials require advanced planning and communication between the sender, carrier, and receiver. The requirements for shipping diagnostic blood or tissue specimens may vary according to national conditions and international regulations regarding the human or animal origin of the specimens (65, 66). All specimens included in the shipment must be properly labelled and should be accompanied with paperwork that includes the identification number that matches the label on the vial. The laboratory request form and any other paperwork for each specimen should be placed in a separate plastic bag. In addition, all necessary details about each specimen should be provided to the receiving laboratory, including all relevant clinical and epidemiologic information regarding the suspected case. The onset and collection dates, as well as the date of the patients most recent vaccination are critical data to include for appropriate testing and interpretation of results. The documentation required and the patient information that should accompany specimens for YF testing is discussed in Chapter 8. A standardized laboratory request form is recommended to ensure that all the pertinent information is provided. An example of an appropriate laboratory request form is provided in Annex 8.2 (also available at *https://www.who.int/publications/i/item/9789240084476)*.

3.6.1 NATIONAL SHIPMENTS

Shipment of specimens within the country either by air or land must meet all national requirements and laboratories should work closely with domestic carriers to ensure the packaging (triple packaging system, Figure 3.1), documentation and labelling are appropriate. The complexity of the process requires an ongoing effort to keep participating laboratories informed of any updates or modifications made to shipping or packaging requirements.

FIGURE 3.1

Examples of basic triple packaging system.

As the name suggested, any package used to contain an infectious substance must be comprised of three layers:

1. The primary receptacle, containing the infectious substance, must be watertight, and impermeable to the substance held within (i.e. leakproof- for liquid, or sift-proof- for solids). The primary receptacle should be appropriately labelled as to content.

The primary receptacle must not become punctured, broken, weakened or affected by contact with the infectious substance. For example, the primary receptacle should not be corroded by preservation media used to hold a patient specimen.

If the infectious substance contains a liquid, or semi-liquid substance, the primary receptacle must be wrapped in enough absorbent material to absorb all the fluid in the rare event of a breakage or leakage.

2. A second watertight, leakproof or sift- proof container should then be used to enclose and protect the primary receptacle, and its absorbent material.

Several primary receptacles may be placed in a single secondary container, provided they are all infectious substances of the same class. If the primary receptacle is fragile, each must be wrapped and placed in the secondary container individually, or in a way that prevents contact between them. Cushioning material may be required to secure the primary receptacles within the secondary container.

3. A third, outer layer of packaging is used to protect the secondary container from physical damage while in transit. It must therefore be of an appropriate strength for the weight, size and composition of the inner packages to be protected. At least one surface of the outer packaging must have a minimum dimension of 100 mm x 100 mm.

Source: taken from (67) World Health Organization (WHO) Guidance on regulations for the transport of infectious substances, 2021-2022.

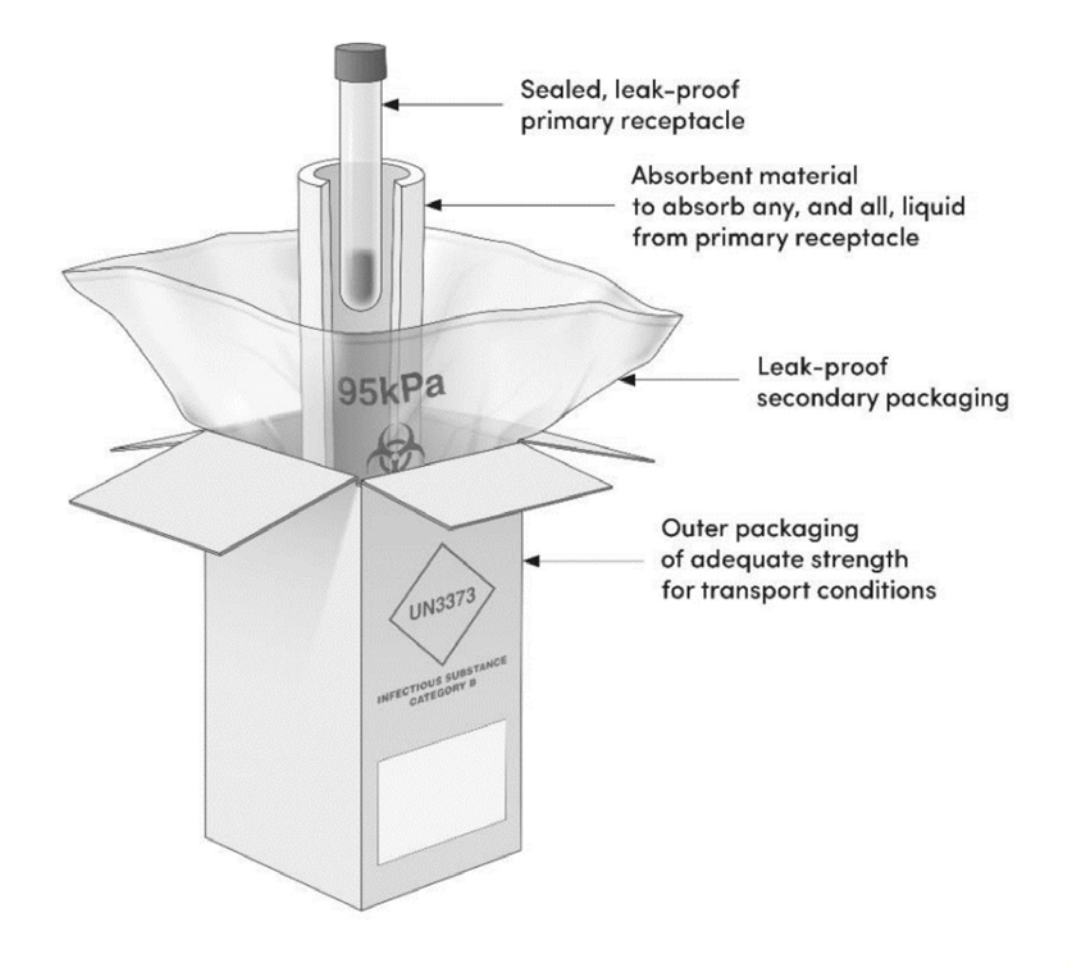
3.6.2 INTERNATIONAL SHIPMENTS

EYE.ops mechanism for international shipping of specimens: The EYE Secretariat set up an operational mechanism (EYE.ops) in 2019 to strengthen and support sample transport between NLs and their respective RRL or other designated laboratories within the network. EYE.ops facilitates the international shipments of samples through a courier specialized in international transport of category B substances, using a standardized procedure and booking form (BF) (Annex 3.1). EYE.ops coordinates with the international courier company on all aspects associated with shipping out and delivery of specimens. The EYE.ops mechanism covers all transport costs and sending laboratories are released from any administrative follow-up and financial commitment. The EYE.ops shipping procedure is as follows:

1. **Contact with EYE.ops:** The NL initiates the request for shipment by filling in a dedicated BF, providing all the details of the shipment (Annex 3.1). The completed BF needs to be sent to the EYE.ops Secretariat via email (EYE.ops@who.int). It is important that all the mandatory fields in the BF are filled out, especially the type of request, the names of the shipping and receiving laboratories, the required temperature for the sample types to be shipped, the volume of sample being shipped (note that a minimum of 0.5 ml of serum is required for comprehensive testing at the RRL), etc.
2. **Acknowledgement and clearance of request by EYE.ops:** Upon reception and within 24 hours, EYE.ops will acknowledge receipt and begin processing the shipment or request further information, if needed. The shipment will not be processed if there is missing information in the BF document.
3. **EYE.ops arranges international courier from the NL:** EYE.ops liaises with the specialized courier in international transport to arrange for the shipment of specimens. Subsequently, the courier company contacts the NL to arrange the package pick up.

The chosen carrier(s) used in EYE.ops have been selected to match the international requirements and are all International Air Transport Association (IATA) certified. The carrier may require specific documents and may provide advice on packaging or specific instructions necessary to ensure safe arrival of the shipment, including how to store the package in the event of a delay during transit (Figure 3.2). Triple packaging material is provided by the courier company if not available at the laboratory. Most of the YF laboratories are provided with adequate triple packaging every year. In addition, the courier company has specific instructions for either delivery or storage at the required temperature in case of weekend arrivals.

FIGURE 3.2 **Example of triple packaging materials that may be used to comply with P650 for Category B infectious substances.**



Source: taken from (67) World Health Organization (WHO) Guidance on regulations for the transport of infectious substances, 2021-2022.

Please refer to the Information Video on the EYE Strategy International Samples Transportation (EYE.ops), available at https://youtu.be/iBEAmwcbxn0, for more information.

Other international shipping procedures: For laboratories that do not use the EYE.ops mechanism to ship samples, it is the responsibility of the sender to ensure that all requirements are met for shipment of materials sent from the laboratory. It is essential that the sending laboratory adheres to requirements for documentation, standardized packaging, and labelling. The methods and details of the shipping (courier, date of shipping) should be arranged or communicated between the sender and the receiving laboratory prior to shipment. Before initiating the shipment, the recipient laboratory must be informed of the quantity and type of specimens that will be sent, the diagnostic testing requested, transport conditions, and any biosafety issues. Once the receiving laboratory agrees to accept the samples, the shipment can be scheduled. The sender should inquire about any import permits required by the receiving laboratory's national government. The receiving laboratory must obtain the current permit or other required paperwork and provide them to the shipping laboratory. The sender and receiver should then make advance arrangements for a mutually convenient time for the shipment to ensure that the appropriate staff are available to receive the package.

The sender should inquire whether the receiver prefers a specific carrier or can provide helpful information regarding previous experiences or problems encountered with incoming shipments. The international courier must be familiar with the country regulations regarding shipping and receiving infectious substances and diagnostic specimens. For example, in shipping highly pathogenic agents, it may be mandatory for the recipient to certify that the receiving institution has adequate facilities for handling the specimens. The carrier may require specific documents and may provide advice on packaging or specific instructions necessary to ensure safe arrival of the shipment, including how to store the package in the event of a delay during transit. It is useful to know the itinerary to anticipate delays and recommended to avoid weekend or holiday delivery of the packages.

Proper packaging (triple packaging system) and labelling of the clinical specimens or materials being shipped is vital to maintain the integrity of the specimens, prevent accidents, and ensure there are no delays due to failure to adhere to shipping regulations. The packaging requirements for some types of laboratory materials are subject to international and national regulations. The IATA Dangerous Goods Regulations provide guidance on the classification and transportation of biological specimens (66).

Category B agents are considered to present a reduced risk because they are not easily transmissible and basic precautions and hygienic practices will serve to prevent exposure and infection in the event of an incident. Patient diagnostic specimens and other materials are classified as category B agents and are not subject to dangerous goods requirements and regulations if there is minimal likelihood that viable pathogens are present (formalin fixed tissue samples, for instance) and only if they are transported in packaging which will prevent any leakage and are correctly labelled. The exterior packaging must be marked as "Exempt human specimen". The WHO Guidance on regulations for the Transport of Infectious Substances 2021-2022 covers all aspects of shipping including packaging and documentation (67). However, category B samples are considered dangerous goods by some carriers (e.g., FEDEX) and they may not accept them.

The vials should be packaged with absorbent material and protected against crushing of the contents. The laboratory request form and any other paperwork for each specimen should be placed in a separate plastic bag inside the package. Adequate refrigerants should also be included in the package such as ice or ice packs that have been frozen at -20°C; ice packs can maintain temperatures of 4-8°C in a well-insulated shipping container for up to 3 days. Ice should be placed in a leak-proof container outside the secondary receptacle; the outer packaging should also be leak-proof.

To maintain cold chain conditions for longer than 3 days, the use of dry ice is recommended if it can be readily obtained and the required packaging for dry ice shipments is available (inside specially designed insulated packaging which permits the release of carbon dioxide gas as the dry ice undergoes sublimation). However, note that dry ice by itself is regulated as a hazardous material in air transport and needs to be declared separately; ICAO/IATA Packing Instruction PI954 applies (66, 68). The outermost packaging must carry the appropriate hazard label for dry ice.

The documentation required for shipping materials is determined by the nature of the materials being sent. In general, each shipment should be accompanied with the airway bill (if shipped by air), any required export/import documentation, and the outer packaging should inform the receiving laboratory of the proper contents and proper storage conditions.

For example:

URGENT: DO NOT DELAY: Biological specimens – highly perishable – store at (+) 4°C to (+) 8°C

Once the package has been sent, the receiver should be immediately notified of the following:

- Number of cartons and weight;
- Flight and arrival date/time;
- Airway bill number.

A copy of the airway bill should be provided from the sender and the receiving laboratory should confirm that the airway bill was received. Upon receipt of the package and inspection of the contents, the receiver should provide confirmation of delivery and inform the sender of the condition of the materials. This can be facilitated by the sender including a "fax back" form in the shipment. If the quantity or types of specimens received does not match the accompanying documentation or does not agree with the information that was provided prior to shipment, the receiver should immediately contact the sender and resolve the apparent discrepancy.

3.7 SAFETY PROCEDURES FOR INCOMING CLINICAL SPECIMENS

On arrival into the laboratory, shipping cartons or packages must be promptly unpacked in the designated area. It is recommended that each laboratory develop specific standard operating procedures for opening packages and logging in specimens. All specimens should always be considered as potentially infectious, and packages should be opened and the contents removed for inspection and specimen accessioning within a biosafety cabinet (BSC). Personnel who receive and unpack specimens should be aware of the potential health hazards involved and trained to adopt biosafety precautions, including wearing appropriate personal protective equipment (e.g., laboratory coat and latex or nitrile gloves), particularly when dealing with broken or leaking containers. Unpacking and recording of specimens should preferably be carried out by two persons: one records data while the other is gloved and is responsible for opening the package and checking for broken or damaged sample containers and evidence of leakage. Any potentially contaminated paperwork should be contained within the BSC while the information is manually recorded on a fresh sheet of paper outside the BSC. Contaminated documents should be handled in the same manner as infectious waste. Information that should be recorded when accessioning samples is provided in Chapter 8.

A BSC should be used for opening primary specimen containers and transferring specimens to another container, such as when preparing aliquots of clinical specimens (43). Disinfectants such as 70% ethanol are used to wipe down surfaces and a 1:10 solution of sodium hypochlorite (bleach) solution 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.

Samples that are received frozen at the laboratory should be maintained frozen and stored at -20°C or the lowest available temperature until tested. If a sample is delivered to the laboratory at room temperature or (+)4-8°C but will be tested within a few days, it can be stored in the refrigerator. However, if testing will occur more than a week after the sample is received or if the sample is to be stored for an extended period of time for follow-up testing or biobanking, small aliquots of serum (0.5–1.0 ml) should be prepared and frozen at -20°C or the lowest available temperature (e.g., -80°C) (49). A frost-free freezer, which has a periodic warming cycle to prevent ice build-up, should not be used.

Chapter 4

Laboratory testing algorithms for the diagnosis of yellow fever virus infections

The clinical spectrum of YF ranges from asymptomatic or mild infection to potentially fatal severe conditions with haemorrhage and jaundice (32, 58, 69). Suspicion of YF is based on the patient's clinical features, travel dates and locations (if the patient is from a non-endemic country or area and thus, likely lacking natural immunity), and activities and epidemiological history of the location where the presumed infection occurred. Laboratory diagnosis is essential to identify YF virus infections in samples collected from YF suspected cases meeting the YF clinical case definition in both surveillance and outbreak settings.

Unified testing algorithms were developed by the WHO in consultation with individual experts from the EYE Strategy LTWG and personnel from the WHO GYFLaN after reviewing regional laboratory networks and region-specific algorithms for the diagnosis of YF in the African and LAC regions (42, 70). The algorithms were harmonized between the two regions and the sequence and types of tests used to diagnose YF virus infections were standardized. The main challenges have included aligning testing protocols among laboratories in the light of the strengths and weaknesses of different YF tests, the availability of testing supplies, and limited financial and human resources in the laboratories.

Chapter content:

- Timeline of clinical symptoms and immune response following YF virus infection
- Sample types and timing of sample collection for YF testing
- Laboratory assays used for diagnosis of YF
- The GYFLaN testing algorithm
- Interpretation of test results
- Limitations of YF laboratory assays
- Referral of samples to the Regional Reference Laboratory
- YF case classification

4.1 TIMELINE OF CLINICAL SYMPTOMS AND IMMUNE RESPONSE FOLLOWING YF VIRUS INFECTION

The incubation period for YF infection is usually 4–6 days after transmission through a bite by an infected mosquito (see timeline of infection and immune response in Chapter 1, Figure 1.5). In most infected persons, the course of infection is asymptomatic or mild. However, in severe cases, infected persons enter a second, more toxic phase of illness within 24 hours of recovering from initial symptoms. Approximately 15% of infected persons develop this severe form of YF disease, and it is during this toxic phase that the severe signs and symptoms classically associated with YF present, including severe abdominal pain, jaundice and liver failure, renal insufficiency, and haemorrhagic signs such as bleeding from the mouth, nose, eyes, or stomach. Death occurs in 20%-50% of people who develop hepatorenal failure, which is marked by a coagulative necrosis of hepatocytes in the mid-zone of the liver lobule (49).

Infected persons are viremic for approximately a week from the onset of symptoms. RNA can be detected in blood and infected organs during the viremic phase of the infection. After 10 days, detection of RNA decreases as viremia is cleared by the immune system. In very severe cases, positive results have been observed in samples collected at much later time periods. Rapidly after disease onset and by 7 days, the serological immune response is elicited to YF virus infection, which involves production of anti-YF virus IgM antibodies. The IgM titre decreases after 30-60 days in most persons, although in some individuals IgM has been shown to persist for months or years after infection. The immune response likely provides lifelong protection against repeat infection by antibody and cellular-mediated immunity (49).

4.2 SAMPLE TYPES AND TIMING OF SAMPLE COLLECTION FOR YF TESTING

Serum is the principal diagnostic sample type tested; molecular testing can also be done with whole blood and plasma. In fatal cases, tissue samples (in particular, liver and kidney) are collected during autopsy for molecular testing and IHC and, if possible, blood is collected for serology and molecular testing (Chapter 5 and chapter 6).

A blood sample should be collected on first contact with every suspected YF case when there is not an outbreak, not waiting for the ideal window, and within 14 days of symptom onset. If results of molecular and serologic testing are negative in a serum collected ≤7 days after symptom onset, a second sample should be collected >7 days after onset and tested by serology (IgM), if possible. Guidelines for specimen collection, transport, and storage are provided in Chapter 3.

> **Note:** All biological samples (whole blood, serum, tissue) should be considered as potentially infectious. Samples should be processed and tested in a class II BSC (in endemic areas) and laboratory personnel handling these samples must use appropriate personal protective equipment to avoid percutaneous exposure and be vaccinated against YF (Chapter 3) (43). Procedures for handling non-human samples should be carefully assessed according to national regulations and the biosafety manual of each laboratory.

4.3 LABORATORY ASSAYS USED FOR DIAGNOSING YF

YF virus infection is diagnosed through virological (detection of the viral genome, detection of viral antigens, IHC, or virus isolation) and/or serological methods (detection of IgM by ELISA, rapid test, or immunofluorescent assay) (Figure 4.1) (24, 71). The main assay categories conducted by the NLs are reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) and YF IgM detection. The testing algorithm is based on the sensitivities and specificities of the two assay categories.

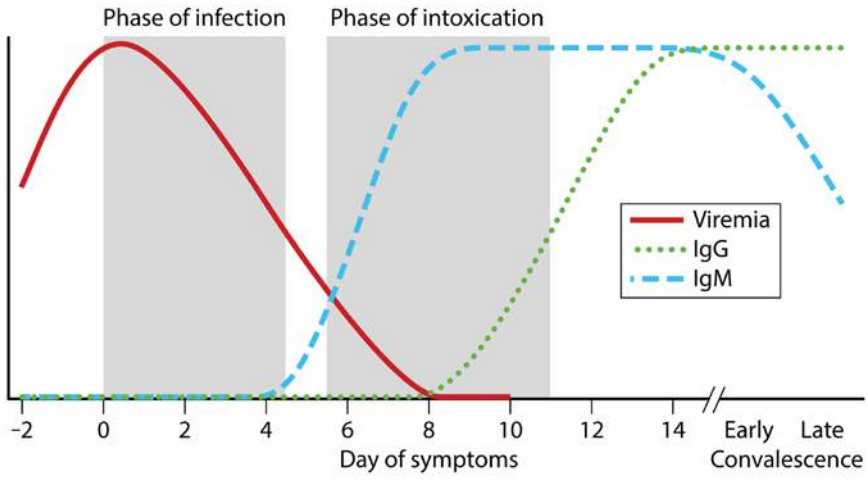
Note for health workers: Sensitivity varies depending on the timing of blood collection, it is important to document:

- Symptom onset date;
- Specimen collection date;
- YF vaccination history.

See details in Chapter 8.2

Sensitivity varies depending on the timing of blood collection, with RT-qPCR decreasing with days post-onset of symptoms as viremia is cleared through the immune response, and IgM detection increasing in sensitivity (Figure 4.1). The testing algorithm, presented in the next section, has been developed to optimize diagnosis of YF virus infection based on the characteristics of these assays.

FIGURE 4.1 **Model time course for YF.** See Box 4.1 for detailed legend.



Source: Modified from (31) Waggoner JJ, Rojas A, Pinsky BA. 2018. Yellow fever virus: diagnostics for a persistent arboviral threat. J Clin Microbiol 56:e00827-18.

Box 4.1 Details of legend from Figure 4.1

Shaded areas represent periods of infection and intoxication. For many patients, YF will resolve during the phase of remission (unshaded region between the aforementioned phases), and it is estimated that 12% of patients will enter the period of intoxication. YFV RNA may be detectable in urine into early convalescence, although sensitivity of detection is unknown. Duration of viremia has been variable and may extend past the identified time period in severe cases.

Molecular diagnostics: RT-qPCR detects YF viral RNA, both wild-type and vaccine strains, during the viremic phase of disease (Chapter 6). RT-qPCR assay sensitivity is highest in the first few days from symptom onset and decreases with time, as viremia is cleared by the immune response. RT-qPCR has high specificity for YF genome; thus, a positive result confirms the diagnosis of YF virus infection.

IgM detection: IgM detection assays have high sensitivities for anti-YF virial IgM antibodies, elicited both from the wild-type and live attenuated vaccine YF viruses, by 7 days (>90% of cases) after onset of symptoms and decreases as IgM response wanes after 30-60 days (Chapter 5). IgM detection assays for flaviviruses have low specificity, as there is significant cross-reactivity of other flavivirus IgM antibodies with YF antigens used in the assay. Thus, in areas where other flaviviruses co-circulate (especially dengue, West Nile, and Zika viruses), the possibility of a false positive result is high. Therefore, a positive result in a single sample indicates a presumptive acute YF virus infection, and confirmatory testing in the RRL by plaque reduction neutralization test (PRNT) is required (see Chapter 7).

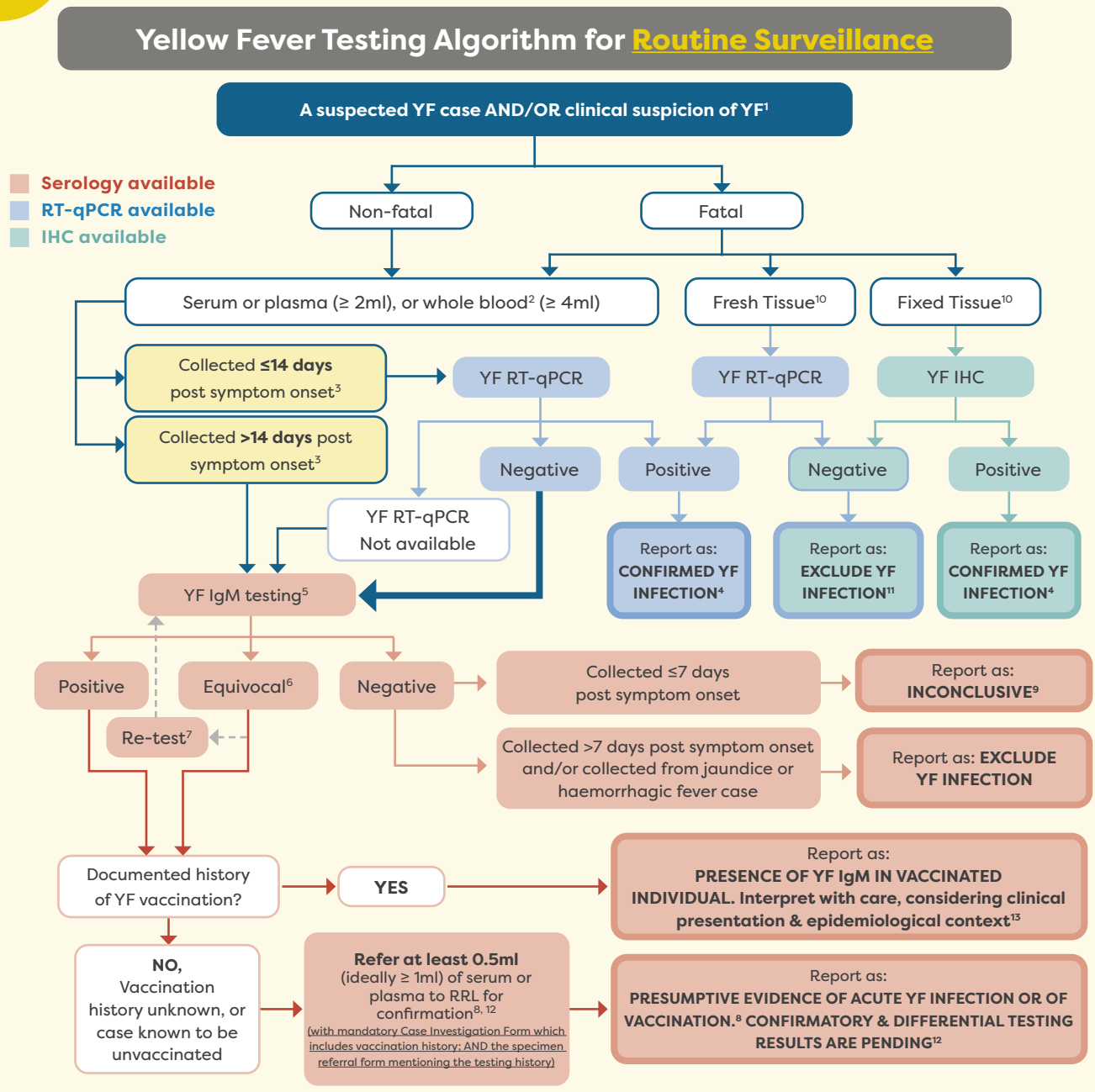
Immunohistochemistry: Viral antigens present in tissue collected during autopsy can be detected by IHC. IHC testing of fresh and fixed tissue is performed in reference laboratories by trained pathologists and laboratory personnel (24). The simplified procedure to perform YF IHC using the MACH 4 Universal AP Polymer Kit is available in Annex 4.1.

4.4 THE GYFLaN TESTING ALGORITHM

The YF testing algorithms are shown for routine surveillance in Figure 4.2 and during outbreaks in Figure 4.3. The use of RT-qPCR should be prioritized, since a positive result obtained from RT-qPCR is classified as a confirmed YF virus infection, even if antibodies against YF virus were not detected or not wet tested, thus reducing diagnostic turnaround time. The YF RT-qPCR has also demonstrated high sensitivity for detecting YF viral RNA up to 14 days after the onset of symptoms, particularly in severe (and fatal) cases (31, 72). Therefore, it is recommended that serum collected up to 14 days should be routinely tested by RT-qPCR, although the probability of detecting RNA decreases after 10 days. A sample with a RT-qPCR negative result does not rule out the infection because the sample may have been collected too late for RNA detection, as the clinical case definition includes jaundice, which occurs several days after onset of symptoms (49). Samples with negative RT-qPCR results should subsequently be tested by serology.

FIGURE 4.2

Yellow fever testing algorithm for routine YF surveillance in the GYFLaN. See Box 4.2 for detailed legend.



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, 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 RT-qPCR sensitivity is higher in the first 10 days from symptom onset, decreasing as viremia is cleared. However, detection up to 14 days has been reported, particularly in severe cases. In immunosuppressed cases, viremia may last even longer. Therefore, RT-qPCR might be attempted in samples collected ≤14 days from onset. A positive result in those samples will confirm a 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. 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.

4 For cases with no history of vaccination, vaccination history unknown or vaccinated >14 days before onset 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 it to RRL when referring specimen).

5 If the 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 still UI. If testing is or continues to be UI, treat as negative. If a rapid IgM LFA test is used, serial testing including a combined use of a MAC-ELISA based assay and communication of preliminary results might be required; please refer to the Operational guidance(73) on the use of YF tests 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 test instructions for the indicated range of equivocality for this specific test. Equivocal does 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. Cost considerations should be taken into account and direct referral of specimen with equivocal results (without 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 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 epidemiological criteria, such as history of vaccination, must be used for the final interpretation of the results and classification of the suspected YF case.

To confirm the infection, particularly in areas where no YF virus circulation has been recently described, differential neutralization testing with flaviviruses endemic to the area of exposure or neutralization testing of appropriately paired sample set to demonstrate seroconversion should be performed in an RRL.

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; additionally, spleen, lung, brain and heart tissue can be collected) and tested in fatal cases regardless of sampling date after onset of symptoms. If no other specimen is available, paraffin-embedded tissue could also be used for RT-qPCR testing.

11 A negative RT-qPCR in tissue from fatal cases can be followed up with serology if serum or plasma was collected before death.

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 In recent vaccinees (<30 days) who develop classical symptoms of YF infection, targeted sequencing or use of discriminatory RT-qPCR should aim to differentiate between infections with wild-type YF and the vaccine virus strain. Note: YF IgM antibodies can persist for months to years post-vaccination.

Box 4.2 Details of legend from Figure 4.2

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, 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 interfering with PCR.

3 RT-qPCR sensitivity is higher in the first 10 days from symptom onset, decreasing as viremia is cleared. However, detection up to 14 days has been reported, particularly in severe cases. In immunosuppressed cases, viremia may even last longer. Therefore, RT-qPCR might be attempted in samples collected ≤14 days from onset. A positive result in those samples will confirm a 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. 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.

4 For cases with no history of vaccination, vaccination history unknown or vaccinated >14 days before onset 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 it to RRL when referring specimen).

5 If the IgM results are uninterpretable (UI) due to high background and/or potential inhibitory factors, consider repeating the test, and request a second specimen if repeat result is still UI. If testing is or continues to be UI, treat as negative. If a rapid IgM LFA test is used, serial testing including a combined used of a MAC-ELISA based assay and communication of preliminary results might be required; please refer to the Operational guidance(73) on the use of YF tests 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 test instructions for the indicated range of equivocality for this specific test. Equivocal does not refer to an UI test result, e.g., equivocal is not due to presence of factors that cause nonspecific 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. Cost considerations should be taken into account and direct referral of specimen with equivocal results (without retesting) to an RRL is advisable. If a repeated equivocal test result remains equivocal upon retesting, 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 epidemiological criteria, such as history of vaccination, must be used for the final interpretation of the results and classification of the suspected YF case.

To confirm the infection, particularly in areas where no YF virus circulation has been recently described, differential neutralization testing with flaviviruses endemic to the area of exposure or neutralization testing of appropriately paired sample set to demonstrate seroconversion should be performed in an RRL.

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 (≥ 1 cm3) should be collected (liver and kidney tissue should always be collected; additionally, spleen, lung, brain and heart tissue can be collected) and tested in fatal cases regardless of sampling date after onset of symptoms. If no other specimen is available, paraffin-embedded tissue could also be used for RT-qPCR testing.

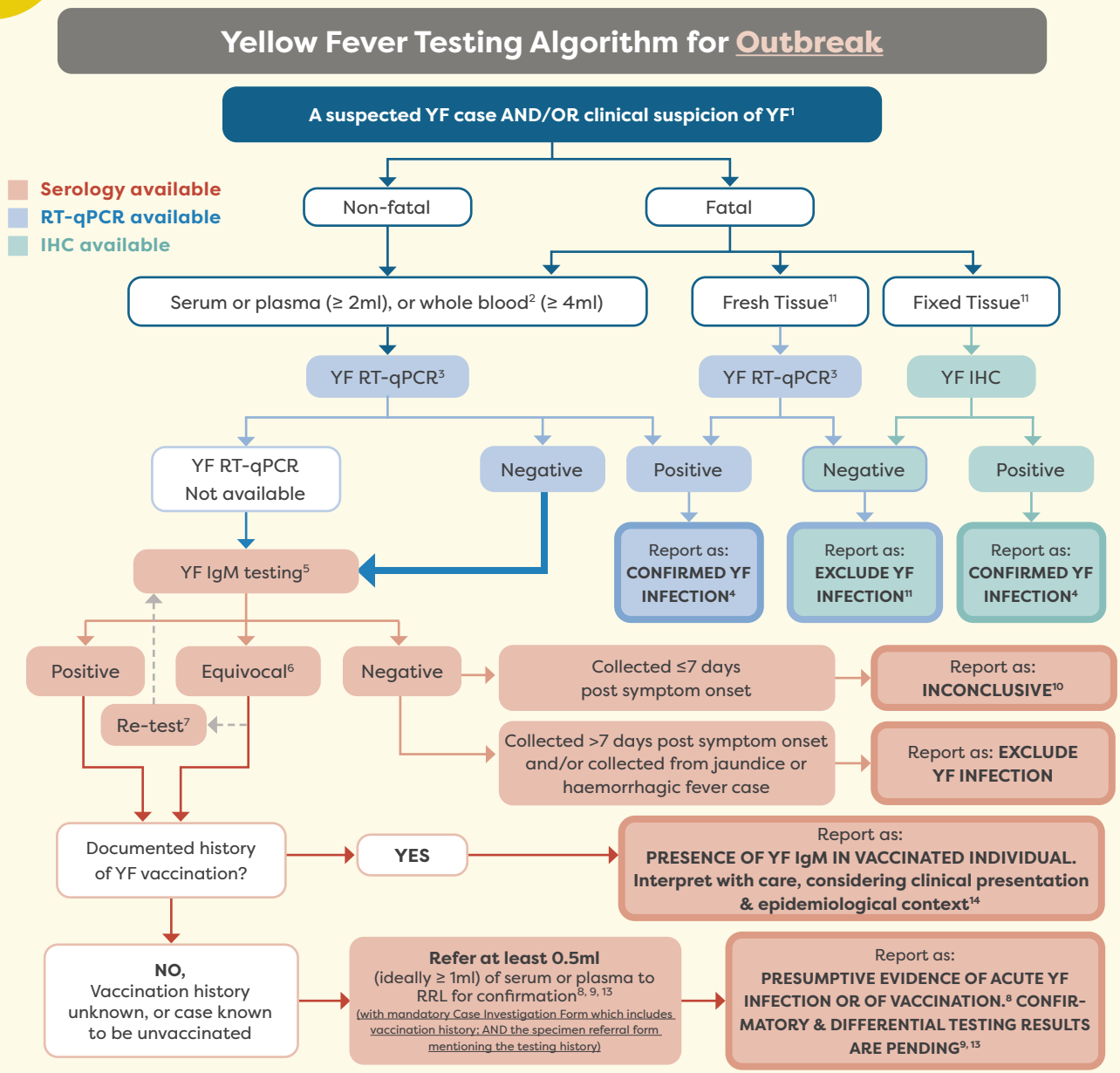
11 A negative RT-qPCR in tissue from fatal cases can be followed up with serology if serum or plasma was collected before death.

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 In recent vaccinees (<30 days) who develop classical symptoms of YF infection, targeted sequencing or use of discriminatory RT-qPCR should aim to differentiate between infections with wild-type YF and the vaccine virus strain. Note: YF IgM antibodies can persist for months to years post-vaccination.

FIGURE 4.3

The yellow fever testing algorithm for National Laboratories during an outbreak. See Box 4.3 for detailed legend.



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, 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 interfering with PCR.

3 Whenever available, RT-qPCR should be the first-line test during an outbreak, irrespective of the number of days since symptoms onset. Therefore, RT-qPCR might be attempted in all samples collected as a primary test. A positive result in those samples will confirm a 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 unavailability of the typical blood-based specimens, RT-qPCR on alternative specimen 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 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 still UI. If testing is or continues to be UI, treat as negative. If a rapid IgM LFA test is used, serial testing including a combined used of a MAC-ELISA based assay and communication of preliminary results might be required; please refer to the Operational guidance *(73)* on the use of YF tests for more details.

6 In the case where a MAC-ELISA method is used, an equivocal results is when a valid result falls within the range between a negative or positive result. Refer to the test instructions for the indicated range of equivocality for this specific test. Equivocal does 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. Cost considerations should be taken into account and direct referral of specimen with equivocal results (without 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 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 epidemiological 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.

To confirm the infection, particularly in areas where no YF virus circulation has been recently described, differential neutralization testing with flaviviruses endemic to the area of exposure or neutralization testing of appropriately paired sample set to demonstrate seroconversion should be performed in an RRL.

9 Once an outbreak of YF has been confirmed in a specific area, the decision might be made to not refer subsequent samples from unvaccinated cases of the same area to a RRL for confirmatory testing but to report as Presumptive infection based on a YF IgM positive result. However, cases from a new geographic area, particularly if adjacent or linked to outbreak areas, or with atypical or unusual clinical presentation, 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 district where there is still no sign of an outbreak.

11 Fresh and fixed tissue samples (≥1cm3) should be collected (liver and kidney tissue should always be collected; additionally, spleen, lung, brain and heart tissue can be collected) and tested in fatal cases regardless of sampling date after onset of symptoms. If no other specimen is available, paraffin-embedded tissue could also be used for RT-qPCR testing.

12 A negative RT-qPCR in tissue from fatal cases can be followed up with serology if serum or plasma was collected before death.

13 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).

14 In recent vaccinees (<30 days) who develop classical symptoms of YF infection, targeted sequencing or use of discriminatory RT-qPCR should aim to differentiate between infections with wild-type YF and the vaccine virus strain. Note: YF IgM antibodies can persist for months to years post-vaccination.

Box 4.3 Details of legend from Figure 4.3

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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.

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4 For cases with no history of vaccination, vaccination history unknown or vaccinated >14 days before onset 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 it to RRL when referring specimen).

5 If the IgM results are uninterpretable (UI) due to high background and/or potential inhibitory factors, consider repeating the test, and request a second specimen if repeat result is still UI. If testing is or continues to be UI, treat as negative. If a rapid IgM LFA test is used, serial testing including a combined used of a MAC-ELISA based assay and communication of preliminary results might be required; please refer to the Operational guidance(73) on the use of YF tests 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 test instructions for the indicated range of equivocality for this specific test. Equivocal does not refer to an UI test result, e.g., equivocal is not due to presence of factors that cause nonspecific 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. Cost considerations should be taken into account and direct referral of specimen with equivocal results (without retesting) to an RRL is advisable. If a repeated equivocal test result remains equivocal upon retesting, 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 epidemiological criteria, such as history of vaccination, must be used for the final interpretation of the results and classification of the suspected YF case.

To confirm the infection, particularly in areas where no YF virus circulation has been recently described, differential neutralization testing with flaviviruses endemic to the area of exposure or neutralization testing of appropriately paired sample set to demonstrate seroconversion should be performed in an RRL.

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 (≥ 1 cm3) should be collected (liver and kidney tissue should always be collected; additionally, spleen, lung, brain and heart tissue can be collected) and tested in fatal cases regardless of sampling date after onset of symptoms. If no other specimen is available, paraffin-embedded tissue could also be used for RT-qPCR testing.

11 A negative RT-qPCR in tissue from fatal cases can be followed up with serology if serum or plasma was collected before death.

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 In recent vaccinees (<30 days) who develop classical symptoms of YF infection, targeted sequencing or use of discriminatory RT-qPCR should aim to differentiate between infections with wild-type YF and the vaccine virus strain. Note: YF IgM antibodies can persist for months to years post-vaccination.

14 In recent vaccinees (<30 days) who develop classical symptoms of YF infection, targeted sequencing or use of discriminatory RT-qPCR should aim to differentiate between infections with wild-type YF and the vaccine virus strain. Note: YF IgM antibodies can persist for months to years post-vaccination.

Serological testing based on YF IgM detection should be conducted on all samples with RT-qPCR negative results, or if RT-qPCR is not available at the NL, on all samples each day post-onset of illness. If the laboratory has the capacity to only test for IgM or by RT-qPCR, all samples should be tested by the available assay, regardless of days post-onset of illness.

With the increased availability of WHO-listed commercial assays, it is important to ensure efficient use of resources, and as such a combined strategy of IgM testing can be used. For example, the use of a YF IgM LFA as an initial "rule-in" test can help expedite preliminary IgM results, where samples with positive IgM results can then be immediately referred for confirmatory testing to the RRL. Samples with negative results using the LFA can be batched and further tested by the MAC-ELISA method using a complete plate for efficient use of resources. Please refer to the operational guidance for YF testing (73).

A NL may not have the capacity to test all specimens collected during an outbreak. If the laboratory has reached maximum capacity, priority should be given to testing specimens from those areas where local transmission has not yet been confirmed. Samples that have not been tested at the NL should be referred to the RRL for initial testing.

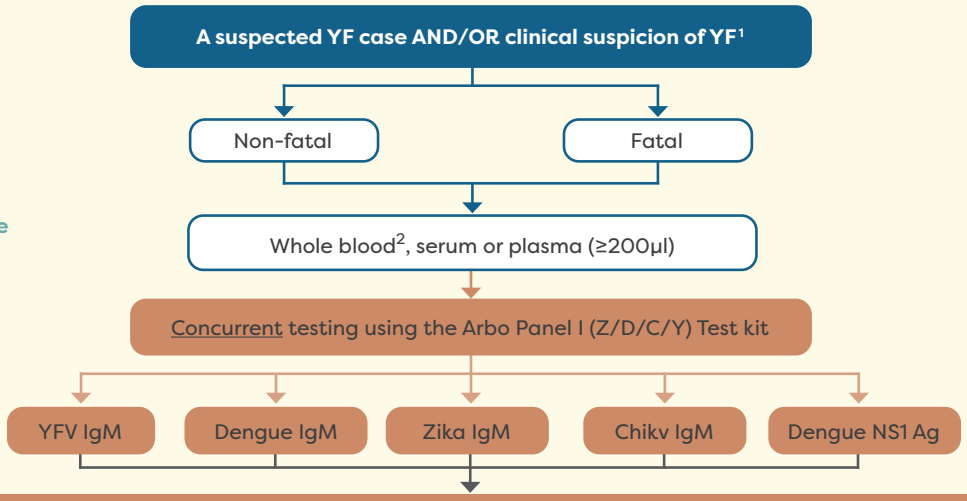
In countries without a WHO-accredited laboratory for performing YF serology IgM testing and who do not have access to international referral testing (including confirmatory PRNT testing), the STANDARD Q Arbo Panel I (Z/D/CY) Test Kit (SD Biosensor) can be used in accordance with the YF simplified testing algorithm using the Arbo Panel kit and strictly adhering to its interpretation guide (Figure 4.4). Final STANDARD Q Arbo Panel I testing outcomes of suspected YF cases should be reported immediately to the national surveillance programme.

FIGURE 4.4

YF simplified testing algorithm and interpretation guide using the STANDARD Q Arbo Panel I (Z/D/CY) test kit for countries without access to YF IgM, RT-qPCR, and PRNT.
See Box 4.4 for more detailed information on the legend.

Yellow Fever **SIMPLIFIED** Testing Algorithm Using Arbo Panel kit for Countries with no access to YF RT-qPCR, YF ELISA and PRNT testing

- SD Biosensor Arbo Panel I (Z/D/C/Y) Test Kit available
- RT-qPCR NOT available
- IHC NOT available
- PRNT NOT available
- IgM ELISA NOT available



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

YFV IgM result	Dengue IgM result	Zika IgM result	CHIKV IgM result	Dengue NS1 Ag result[6]	Differential Interpretation based on all test results of the Arbo Panel I (Z/D/C/Y) Test
+	-	-	-	-	Evidence of recent YF virus infection OR of YF vaccination[3]
+	-	-	-	+	Evidence of recent Flavivirus infection[4]
+	+	-	-	+ or -	
+	+	+	-	+ or -	
+	-	+	-	+ or -	
-	+	+	-	+ or -	
-	-	+	-	+	
+	+	+	+	+ or -	Evidence of Chikungunya[7] co-infection with at least one Flavivirus[4]
+	-	+	+	+ or -	
+	-	-	+	+ or -	
-	+	+	+	+ or -	
-	-	+	+	+ or -	
-	-	-	+	+	Evidence of concurrent acute Dengue virus infection and recent Chikungunya virus infection[4,7]
-	+	-	-	+ or -	Evidence of recent Dengue virus infection[4]
-	-	-	-	+	Evidence of recent Dengue virus infection[4]
-	-	+	-	-	Evidence of recent Zika virus infection[4]
-	-	-	+	-	Evidence of recent Chikungunya virus infection[4, 7]
-	-	-	-	-	**Negative YF IgM result.** YF infection can be excluded if the specimen was collected >7 days post symptom-onset symptoms and/or collected from a jaundice or haemorrhagic fever case[5]

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, nausea, vomiting, and fatigue and on epidemiologic factors. Assessment of YF vaccination history, malaria 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 tube 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 test 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.

Box 4.4 Details of legend from Figure 4.4

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, nausea, vomiting, and fatigue and on epidemiologic factors. Assessment of YF vaccination history, malaria 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 anticoagulant tube such as heparin, EDTA or sodium citrate by venipuncture. If venous whole blood in an anticoagulant 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 haemolysed blood samples.

3 In the event of an individual with a documented history of YF vaccination, 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.

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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.

4.5 INTERPRETATION OF TEST RESULTS

Interpretations of test results based on specimen type, timing of sample collection, and vaccination history, with guidance on further testing needed in the RRL, are listed below and shown in Figures 4.2 and 4.3. Results should be reported to the surveillance programme after all testing is complete.

Positive by RT-qPCR: A positive RT-qPCR result is interpreted as a confirmed YF virus infection in the absence of vaccination within 14 days.

Negative by YF RT-qPCR: A negative RT-qPCR result does not exclude the possibility of a YF virus infection. Samples with negative RT-qPCR results should be tested for IgM regardless of the day post-onset of illness that they were collected.

YF IgM positive: A positive YF IgM result is considered presumptive evidence of acute YF virus infection but is not sufficient to confirm infection due to the possible cross-reactivity with other flaviviruses. The sample should be immediately referred to the RRL for differential neutralization testing with flaviviruses endemic to the area of exposure to confirm the infection. Confirmatory testing should be prioritized for samples coming from areas where no YF virus circulation has been recently described (Chapter 7). During an outbreak, confirmatory testing may be completed later.

YF IgM equivocal: Test results are equivocal when a valid result falls within the range between a negative or positive result, as defined in the assay instructions for use (not to an uninterpretable test result). In the case of a first equivocal test result, the test should be repeated on the sample. If the repeated equivocal test remains equivocal upon retesting, the result should be reported as equivocal. A second sample should be requested and tested, but if a second sample is not available, the initial sample should be sent to the RRL. If the second sample repeats as equivocal, results of the first and second samples should be reported as equivocal and both the first and second samples should be sent to the RRL for further testing.

YF IgM negative: YF can be ruled out if the IgM test is negative in a sample collected from day 8 post-symptom onset or from a jaundice or haemorrhagic fever case. YF is excluded and no further YF testing is done. A negative YF IgM test is inconclusive for samples collected within 7 days post-symptom onset.

Uninterpretable: A sample with a YF IgM test result that is uninterpretable due to high background and potential inhibitory factors should be retested. If the result continues to be uninterpretable after retesting, it should be interpreted as negative.

Positive by IHC: A positive IHC result is interpreted as a confirmed YF virus infection in the absence of vaccination within 14 days.

Negative by IHC: A negative IHC result excludes the diagnosis of YF virus infection if no other testing (e.g., RT-qPCR) was done on the sample or if results of the other testing were also negative. The clinical interpretation of any staining or its absence needs to be complemented by morphological studies and proper controls and evaluated within the context of the patient's clinical history and other diagnostic tests.

4.6 LIMITATIONS OF YF LABORATORY ASSAYS

YF vaccine viremia: Vaccination with the live attenuated YF virus normally induces a low viremia that decreases after 4 to 7 days. The YF RT-qPCR assay is highly sensitive and may detect viremia due to vaccination in samples collected shortly after vaccination. Samples with YF RT-qPCR results collected from a patient with documented history of YF vaccine should be referred to the RRL for differential molecular testing.

Immune response to YF vaccine: YF vaccine also elicits IgM and neutralizing antibody production. IgM assays cannot differentiate between YF IgM stimulated by vaccination or by infection with YF wild-type virus. In addition, in a significant proportion of individuals, the IgM response can be detected for one month and in some cases up to 3-4 years post-vaccination (30). Thus, knowledge of a patient's YF vaccination status is critical to inform the testing algorithm but also for case classification as in the absence of direct epidemiologic link; a documented YF vaccinated case is likely going to be discarded, even when PRNT is positive.

Adverse reaction to YF vaccination: Serious adverse effects caused by a YF vaccination are very rare (~4.7 cases per 100 000 vaccine doses) (74). However, if epidemiological and clinical criteria for a vaccine-related fever are met, it is possible that a suspected case with a recent YF vaccination may be attributed to a vaccine reaction and discarded without identification of a vaccine strain by molecular testing. Since serology cannot discern a wild-type infection from vaccination, the case must be investigated appropriately. Further evaluation of the case may provide evidence for a vaccine-related reaction. However, it should be noted that when jaundice occurs 7-14 days post-vaccination it may be due to an infection with another pathogen rather than a reaction to the recent vaccination, but if the case meets the criteria for a vaccine-related fever, the fever is generally attributed to the vaccine unless laboratory confirmation of another cause of jaundice and fever is identified.

Flavivirus cross-reactivity: Members of the Flaviviridae family (YF, dengue, West Nile, Zika) have similar epitopes and IgM in serum from a patient with a flavivirus infection other than YF may give a false positive result in YF IgM testing. Epidemiological evidence of other flaviviruses circulating in a region should be considered when interpreting IgM test results. Concurrent differential neutralization testing is required for YF confirmation (75).

4.7 REFERRAL OF SAMPLES TO THE REGIONAL REFERENCE LABORATORY

While the NL perform IgM testing and, when available, RT-qPCR, the RRL conducts confirmatory, reference, and QC testing of samples referred from the NLs (Chapter 7). Guidelines for sending the sample for further testing to the RRL based on the NL test results are as follows:

> **Note:** NL test results should be reported to the surveillance system immediately upon completion of testing and prior to sending the sample to the RRL.

- **The YF IgM result is positive** and the sample was either negative or not tested by YF RT-qPCR. The sample should be immediately referred to the RRL to confirm the infection;
- **The YF RT-qPCR result is positive** and the sample is from an area where YF virus infections have not been reported recently. Immediate confirmatory testing at the RRL is required;
- **The YF RT-qPCR result is positive** and the sample was collected from a recent vaccinee (<14 days) who develops classical symptoms of YF virus infection. The sample should be sent to the RRL for sequencing or use of discriminatory RT-qPCR, to differentiate between infections with wild-type and vaccine YF virus strains;
- **The YF RT-qPCR result is negative** and the NL does not have the capacity to test by a YF IgM assay. The sample should be sent to the RRL for IgM testing, regardless of the day post-onset of illness that it was collected;
- **The YF IgM result is negative** and the sample was collected ≤7 days after onset of illness, a second sample taken 10 days post-onset of illness is not available, and the NL does not have the capacity to test by YF RT-qPCR. The sample should be sent to the RRL for molecular testing;
- **The YF IgM results are equivocal** after testing is repeated. The sample should be sent to the RRL for further testing.

4.8 YF CASE CLASSIFICATION

The reporting of YF cases is a requirement of the IHR. NL and RRL test results are used to determine case classification by the surveillance team; thus, having a direct effect on the YF control and prevention programmes. All YF RT-qPCR and/or IgM positive test results at the NL should be reported immediately to the surveillance programme and classified as confirmed and presumptive YF virus infections, respectively. Samples with IgM or equivocal results referred to the RRL for confirmatory testing with positive results should be reported immediately to the country surveillance programme and WHO (Chapter 7).

Additional clinical and epidemiological data, such as history of vaccination, must be used for the final interpretation of the results and classification of the suspected YF case by surveillance and laboratory experts. Therefore, sample tracing and data management are essential in the GYFLaN (Chapter 8). Although some WHO regions may have different reporting criteria for negative samples, all IgM results should be reported by the NL within the time frame designated by the surveillance programme. Samples tested at the RRL with no evidence of YF virus infection should be reported in a timely and accurate manner back to the national surveillance programme and WHO.

Chapter 5

Antibody detection methods for yellow fever diagnosis

The most common serological method used by the GYFLaN to diagnose suspected acute cases of YF is the ELISA, which can detect virus-specific immunoglobulin (IgM) in a single serum specimen (Chapter 4). Other serological assays that detect the presence of YF IgM antibodies are also commercially available and have recently been recommended by WHO for routine use in YF surveillance following an in-depth kit performance evaluation (76, 77). A positive YF IgM result is interpreted as a presumptive acute YF virus infection in a suspected case with no YF vaccination history (see YF testing algorithm in Chapter 4). However, the interpretation of a YF IgM positive result should consider the epidemiologic context, detailed vaccination history of the individual, timing of the specimen collection, and possible co-circulation of other flaviviruses in the region. All IgM positive results in individuals with no known history of YF vaccination should be confirmed by concurrent YF and differential PRNT as per the confirmatory testing algorithm (Chapter 7). There are important issues regarding the interpretation of results using serological assays for YF diagnosis, as antibodies directed against other flaviviruses can react in YF serological assays, causing false positive results. Therefore, the detection of YF viral RNA by real-time, or RT-qPCR is the preferred first-line test at the National Laboratory (NL), as it gives a definitive confirmation of a YF virus infection (Chapter 4). IgM testing is conducted if the result of the RT-qPCR test is negative or if molecular testing is not available in the NL. PRNT and other differential diagnostic flavivirus testing is done in the RRL following a positive YF IgM result, but PRNT is not a primary test for YF diagnosis.

Chapter content:

- Kinetics of the immune response against YF virus infection and the IgM immunoassay test principle
- Selection and comparison of IgM detection assays
- Setting up the YF IgM assays in the laboratory
- Calculation and interpretation of YF IgM test results
- Alternative specimens for IgM detection
- Other serological techniques and methodologies

5.1 KINETICS OF THE IMMUNE RESPONSE AGAINST YF VIRUS INFECTION AND THE IgM IMMUNOASSAY TEST PRINCIPLE

In natural infections, an infected mosquito inoculates YF virus into the skin during a bite (see Chapter 1 and Figure 1.3). The incubation period between infection and onset of illness is approximately 3 to 7 days (23). Viremia peaks in the first 3 to 4 days of illness and is detectable by highly sensitive real-time RT-PCR methods up to 10-15 days in some cases (23, 31).

The IgM diagnostic assay is intended for the qualitative detection of YF virus IgM antibodies in human sera collected from individuals meeting WHO YF clinical criteria and case definition (e.g., clinical signs and symptoms associated with YF virus infection) and/or YF virus epidemiological criteria (e.g., history of residence in or travel to a geographical region with YF virus transmission at the time of travel, or other epidemiologic criteria for which YF virus testing may be indicated) (see Chapter 1). IgM detection assays are based on the principle of IgM antibody capture and are advantageous because they detect antibodies produced during the first few days after onset of clinical symptoms in the acute phase of a primary infection, obviating the need for convalescent-phase specimens in many cases (41). Anti-YF IgM antibodies can be detected by ELISA (mainly IgM antibody capture, MAC-ELISA), indirect immunofluorescence, or IgM lateral flow immunochromatographic assays, also known as rapid tests (24).

5.2 SELECTION AND COMPARISON OF IgM DETECTION ASSAYS

Recently, commercial YF IgM detection assays in kit format and rapid tests became available following the finalization of guidelines for the external validation of serologic kits by the WHO Product Review Panel and external evaluations of kit-based IgM detection assays (71). Gavi, the Vaccine Alliance is funding procurement for such new commercial assays through a centralized supply channel system via United Nations Children's Fund (UNICEF). Guidance and recommendations by WHO on the use of these assays following the outcome of the respective Kit performance evaluations (76, 77), is available in the 'Operational guidance on the use of YF assays in the context of surveillance' (73).

ELISA assays for YF IgM: Until such commercial assays (e.g. the ATCC MAC-HD) became more widely available, the in-house CDC YF MAC-ELISA method, developed using a combination of approximately 10 commercially available and in-house produced reagents, has been used and continues to be used by some NLs for YF testing as part of the routine surveillance programme. The complete list of bundled reagents required to perform such in-house method is available in Annex 5.2, whereas Annex 5.1A describes the concept of the MAC-ELISA procedure. The CDC YF MAC-ELISA protocol is shown in Annex 5.1B; an example of the YF MAC-ELISA in a practicum format is given in Annex 5.1C, and a results calculation worksheet is shown in Annex 5.1D (78)(also available at *https://www.who.int/publications/i/item/9789240084476*).

Rapid tests for YF IgM: Rapid (<30 minutes) tests based on lateral flow technology are widely used for human immunodeficiency and hepatitis virus screening. Recently, rapid tests for detecting YF IgM with good sensitivity and specificity have been developed. These assays are designed to be read visually either in a clinic or by surveillance teams and as such give rapid results. A serological rapid test for YF has been evaluated by WHO (76, 77). Chapter 4 provides guidance on the use of YF IgM assays in the YF testing algorithm.

5.3 SETTING UP THE YF IgM ASSAYS IN THE LABORATORY

5.3.1 LABORATORY SAFETY

Proper biosafety precautions, including personal protective equipment, must be used when handling specimen materials. Specimens should be processed in a BSC, if available. Laboratory technicians should aim to work according to the good laboratory practice guidelines (79), including wearing clean gowns and new, powder-free gloves during assay reagent setup and handling. Gloves should be changed whenever they become contaminated.

General considerations

- Store reagents at appropriate temperatures (see product inserts);
- Do not use reagents or kits beyond their expiration dates;
- Keep reagent tubes capped while not in use;
- Use calibrated pipettes only.

5.3.2 VALIDATING YF IgM ASSAYS IN THE LABORATORY

The YF IgM detection assays should be standardized and validated prior to use in the laboratory and re-standardized when equipment is calibrated, repaired, or replaced, and at least once a year. This is particularly important when using in-house methods, such as the CDC MAC-ELISA method. Re-standardization of reagents should occur when new lot numbers of reagents are introduced. Reagent and assay standardization may be confirmed by testing verification panels. An example of the protocol used for verification of a new serological assay in the laboratory is provided in Annex 5.3. A worksheet to standardize reagents by titration when using the in-house CDC YF MAC-ELISA method is shown in Annex 5.4 (also available at *https://www.who.int/publications/i/item/9789240084476)*.

As with all laboratory assays, quality management of the IgM assay is essential. Personnel must be trained in the specific protocol, the use of all instruments, and use the appropriate controls. Adequate records must be kept of every round of analysis to help traceability of results. Protocols must be in place for quality management systems, including quality assessment and QC, as well as calibration and maintenance of pipettors, plate readers and plate washers. The use of an in-house positive control and tracking of controls using Levey-Jennings charts/Westgard rules are provided in Chapter 9.

5.3.3 SPECIMEN HANDLING AND PROCESSING

An adequate serum specimen for IgM testing is collected within 21 days from onset of jaundice. As discussed above, although YF MAC-ELISAs are highly sensitive, a negative IgM result may be obtained from a proportion of YF cases if serum was collected ≤7 days after illness onset, normally before jaundice. Because a single confirmed positive YF case requires initiation of an investigation, a second serum specimen is recommended if a negative IgM result is obtained from serum collected during the time frame that is considered early for IgM detection by serological assays. Despite the possibility of a false negative IgM result, a blood sample should be collected immediately from a suspected YF case, particularly as an acute patient specimen is recommended for RT-qPCR testing. The interpretation of YF laboratory test results is affected by the timing of sample drawn following the onset of symptoms. Therefore, it is important that the time estimate is based on the accurate travel, vaccination, and disease history given by the patient; Chapter 4 and Chapter 3 refers to best practices for the timing of collecting serum specimens.

All specimens should be transported to laboratories with appropriate patient information (e.g., age, sex, place of residence, onset of symptoms, vaccination history, travel history). Laboratory test results cannot be interpreted correctly without this information. Proper collection, storage, and transport of specimens are essential for sample integrity. Serum specimens should be tested as soon as possible, preferably within 24 hours of arrival at the laboratory. If the NL conducts YF testing once per week or when they receive sufficient samples to complete a full ELISA plate, an aliquot of the serum can be made and stored at 4°C until tested, with the rest stored at –20°C or –80°C. Serum samples should not be kept at 4°C for extended periods of time. Multiple freeze-thaw cycles of samples should be avoided. Further guidance on specimen processing and storage is available in Chapter 3.

5.4 CALCULATION AND INTERPRETATION OF YF IgM TEST RESULTS

5.4.1 VALIDATION OF TEST RESULTS

Check expiry dates of individual reagents or the kit before running the assay. Before the results can be calculated for the clinical specimens, the test must be confirmed valid for use. Test validity must be determined for each plate or rapid test strip, according to the criteria in the protocol or manufacturer's instructions. Results for clinical specimens may only be determined if the test is valid. If the test run is not valid, then the test must be repeated.

When using a plate-based IgM antibody capture technique, such as the MAC-HD kit or the CDC MAC-ELISA in-house method, the proper controls should be included in each test run (Annex 5.1A). Positive and negative assay controls should be run concurrently with all test samples.

- **Positive control (PC) serum:** flavivirus IgM PC or human clinical specimen with well-characterized YF IgM antibody levels. For assay validity, the PC must have reactivity above the required threshold, as defined in the protocol. If the PC fails to produce a valid result, the test must be repeated. If after a repeat, the PC still falls below the thresholds, then one or more of the reagents, the PC itself, or test parameters was likely the error and troubleshooting should be performed. Validity criteria for commercial assays are given in the manufacturer's instructions and vary between assays. An example of the validity criteria of the in-house CDC YF MAC-ELISA method is shown in Annex 5.1C;
- **Negative control (NC):** The NC can be serum which is nonreactive in the test; with the optical density (OD) or P/N falling below the required threshold. Rapid tests do not contain NCs. Therefore, it is good practice to test an in-house NC serum periodically to monitor nonspecific reactivity of the assay.

If the requirements are met, the test is valid and the test specimen results can be read and interpreted, according to the manufacturer's instruction. As for the in-house CDC method, Annexes 5.5A and B provide MAC-ELISA technical and troubleshooting guides, respectively, to aid in identifying problems for test runs that are not valid.

5.4.2 CALCULATING TEST RESULTS FOR MAC-ELISA ASSAYS

To determine whether a clinical specimen contains IgM directed against YF virus, which would indicate a recent infection, the results are calculated according to the protocol or manufacturer's instructions. It is important to follow the standard operating protocols (SOPs) provided by any manufacturer's protocols, especially in terms of threshold, which will vary depending on the products provided. For example, cut off criteria used for the ATCC MAC-HD kit and those used in the CDC YF MAC-ELISA method are different. Although both assays use criteria based on the P/N ratio calculated for the test specimen, defined as the mean OD (if running multiple replicates) of the test specimen reacted with YF viral antigen (P) divided by the mean OD of the negative human serum reacted with YF viral antigen (N), the positivity cut off value of such P/N ratio differs between those methods. Nonetheless, it should be stressed that the OD or P/N value for a specimen is not an indication of absolute antibody concentration, i.e., the OD or P/N value is not quantitative.

Additionally, in some assays it must be determined whether nonspecific background reactivity is being generated for each specimen. If this requirement is not met, nonspecific background is being generated (e.g., due to haemolysed sera) and the result MUST be reported as inconclusive. Inconclusive specimens should be retested or alternatively, confirmatory PRNTs can be performed. If repeat testing yields inconclusive results, the specimen should be forwarded to the RRL for further analysis. Although rarely available, the analysis of consecutive serum samples from the patient could resolve equivocal or inconclusive results, providing a clear indication of seroconversion or an increasing IgM response.

IgM positive result: A test specimen with a calculated value greater than or equal to the designated threshold should be reported as IgM positive (presumptive YF virus infection) if it meets the background requirements (Chapter 4).

IgM equivocal result: Most YF MAC-ELISA protocols have an equivocal range between the negative and positive thresholds. If the IgM result for the serum specimen is equivocal, the standard procedure is to repeat the test on the specimen. If after retesting the result remains equivocal, it should be reported as such. Rapid tests generally have positive or negative interpretations only.

IgM negative result: All specimens for which the specimen OD or index values are below the threshold designated in the protocol are considered negative. Timing of the specimen collection determines if further analysis is required for final interpretation according to the testing algorithm in Chapter 4.

5.4.3 REPORTING RESULTS

Laboratory test results should be reported promptly with appropriate accompanying information as described in Chapter 8. Laboratories are required to immediately report positive results to the appropriate public health authorities. Samples requiring further testing in the RRL to confirm an IgM positive result or to clarify an IgM equivocal result, should be prepared and sent to the RRL or other reference laboratory within 7 days so that final diagnosis can be determined. This is especially important if the result is doubtful or unexpected due to a non-classic clinical presentation and/or the epidemiological investigation fails to identify a source of infection.

5.4.4 INTERPRETATION OF RESULTS IN THE DIAGNOSIS OF YF

The flow chart for YF testing, interpretation, and requirements for further testing is shown in the testing algorithms in Chapter 4.

Interpretation of an IgM positive result: A sample with a positive IgM result, in the absence of vaccination history, is considered as presumptive positive for YF virus infection (probable YF case), requiring confirmatory testing. A positive IgM result in a single sample is not definitive for diagnosis of YF virus infection and results of this test cannot be used as the sole basis for an outbreak investigation and response. Further testing combined with clinical observations, patient history, epidemiological information, and other laboratory evidence must be used for the final interpretation, particularly in areas where no YF virus circulation has been described recently.

In the WHO African Region, confirmation of the presence of anti-YF IgM antibodies in equivocal or presumptive positive specimens requires detection of YF virus-specific neutralizing antibodies by the PRNT (Chapter 7) in the RRL, according to the GYFLaN testing algorithm (Chapter 4).

False positive IgM results: Interpretation of YF IgM results must consider the possibility of false positive results.

- **Cross-reactivity with other flavivirus IgM:** The most common cause of false positive results is cross-reactivity with IgM elicited against other flavivirus infections due to the common antigenic epitopes among the flaviviruses. In many regions where YF virus is circulating, other arthropod-borne flaviviruses such as dengue, Zika, and West Nile viruses may be co-circulating and differential diagnostic IgM testing should be considered for these viruses (Chapter 7). The possible causes for cross-reactivity and nonspecific reactions in the IgM assay are discussed in Chapter 4, Section 4.2.2;
- **IgM response to recent YF vaccine:** The IgM response to the attenuated YF vaccine virus is identical to the IgM response to wild-type YF virus infection, and serologic techniques cannot distinguish between an immune response to natural infection and antibodies elicited by a recent immunization. Low levels of YF IgM have been detected for months or years following infection or vaccination in some individuals (27). Therefore, serological laboratory results in people who have received a YF vaccine must be interpreted with care and assessed on a case-by-case basis, and must consider the clinical presentation and epidemiological context along with the laboratory results (see Chapter 4 and (28));
- **IgM response to other pathogens and nonspecific reactivity to endogenous factors:** Another type of false positive IgM result, a nonspecific positive result, may occur due to components within the serum specimen. Medical conditions such as malaria, rheumatic diseases, or autoimmune diseases can cause interference and generate false positive IgM results in some immunoassays. When serum is tested for YF IgM from patients with fever illnesses or jaundice due to other causes, nonspecific reactivity can cause a positive result. False positivity caused by other agents with similar clinical symptoms to YF, such as malaria, have been observed and can confound YF diagnosis (76, 77). This is especially true in settings with low prevalence of disease because no test is 100% specific. Guidance for additional testing to address the lower predictive value of positive IgM results in control settings is discussed in Chapter 4.

Interpretation of an IgM negative result: YF IgM levels are variable, but generally are detected at 3-4 days post-onset of symptoms and continue for three or more months following initial infection.

False negative IgM results: A negative IgM result in an acute case of YF can occur due to early collection of specimens, before IgM rises to detectable levels, prior to the appearance of jaundice. The guidelines for the interpretation of results obtained for IgM detection includes a caveat that a false negative may be obtained when serum is collected too early (Chapter 4) (80). This refers to the possibility that the negative IgM result may not diagnose a true YF infection (a proportion of early serum specimens from true cases will have a negative IgM result). A negative IgM result under such circumstances is not technically a false negative result but represents a valid result as measured by the assays since the patient has either not yet produced an IgM response or the level of virus-specific IgM is below the detection threshold for the assay. Therefore, a negative YF IgM test result is inconclusive for samples collected up to and including day 7 post-symptom onset. The analysis of a convalescent serum specimen might help to evaluate the unclear finding to clarify if the patient is reported as negative for a recent YF infection.

Another cause of false negative results is low sensitivity of the assay. Generally, rapid tests have been shown to have lower sensitivity compared to plate-based MAC-ELISAs. Therefore, in the case of an appropriately timed specimen (>7 days post-onset of illness) collected from a patient meeting the clinical definition of YF with a negative result in the rapid test, the specimen should be retested by an assay with higher sensitivity (e.g., ATCC YF MAC-HD Kit) before ruling out YF (see testing algorithm in Chapter 4).

Failure to follow the assay procedures and poor specimen conditions, which degrades the IgM or haemolysis specimens (Chapter 3), may also contribute to a false negative result. Negative results may also occur when specimens are collected after IgM levels have decreased below detectable levels (typically months post-onset of symptoms).

Interpretation of an IgM equivocal result: Equivocal results may be due to IgM levels at the limit of detection, low levels of cross-reactivity, or detectable nonspecific reactivity. If the IgM result for the serum specimen after retesting remains equivocal, the result should be reported as equivocal and further tests should be performed in the RRL. Collection of a second sample timed from a few days to 1 week is recommended, but in practice is rarely possible (refer to 'Timing of the serum specimen' in Section 5.2, above).

5.5 ALTERNATIVE SPECIMENS FOR IgM DETECTION

Dried blood spots prepared from capillary or venous blood might be an alternative option for sampling in areas where a cold chain and/or collection of venous blood is not feasible. This alternative type of sample requires that proper techniques for collection and processing are followed. The best practices for the collection, optimal timing, and preparation of dried blood spots are presented in Chapter 3. Whole blood samples are being evaluated by specialized and reference laboratories in the GYFLaN for detection of YF-specific IgM as an alternative to testing serum.

5.6 OTHER SEROLOGICAL TECHNIQUES AND METHODOLOGIES

As validated improvements or alternative methodologies become available for YF diagnostics, updates will be posted to the GYFLaN website. Types of assays or techniques that show promise for expanding throughput or have specific utility in the field are described below.

IgG detection immunoassays: IgG testing is not part of the GYFLaN testing algorithm but is in limited use in reference laboratories. After a YF virus infection, IgG persists in serum for the lifetime of the individual, and therefore is not useful for diagnosis of acute YF virus infections using a single serum specimen (81). In addition, there is extensive cross-reactivity of IgG among the flaviviruses. However, IgG testing of paired specimens can be useful. This method relies on the availability of two specimens, usually collected 10-21 days apart, that are tested together in the same assay. For example, a seroconversion, in which the acute phase sample has a negative result for IgG and the second or convalescent specimen has a positive IgG result, confirms a positive IgM result. In addition, IgG testing may be utilized when risk assessments are conducted to determine the need for introducing YF vaccination.

NS1 IgM detection assays: Because the immune response against the NS1 antigen is less prominent compared to the YF envelope protein, the NS1 antigen-detection assays have lower sensitivity compared to IgM assays targeting the YF envelope protein. However, as shown with dengue and Zika testing, NS1 IgM detection assays show less cross-reactivity with other flavivirus NS1 antigens and may be useful in differential diagnosis to confirm a YF virus infection (82).

Chapter 6

Detection of viral RNA by real-time RT-PCR for the confirmation of yellow fever virus infection

Real-time or RT-qPCR detection of YF viral genomic RNA directly from clinical specimens is an important assay for laboratory diagnosis in the viraemic phase of the infection (see Chapter 4) (41). The sensitivity of molecular diagnostics has improved in recent years and viral RNA can be efficiently detected for longer periods in the blood of severe cases and in autopsy tissues. Successful amplification of YF virus-specific RNA by RT-qPCR from serum specimens collected as late as 14-20 days after illness onset has been reported (7). However, YF diagnosis by RNA detection can be challenging, as the level and duration of viremia varies between individuals and, even when collected early, the concentration of virus particles in serum may be below the level of detection. In addition, the date of illness onset reported is not always accurate (24). Therefore, interpretation of results must be carefully considered. Although a positive RT-qPCR result is considered evidence of an acute YF virus infection, a negative result does not rule out YF (see the YF testing algorithm in Chapter 4).

Protocols for performing YF RT-qPCR have been developed and evaluated, including commercial assays, and many of the laboratories in the GYFLaN are equipped and trained to conduct RT-qPCR (24, 84, 85). Each laboratory in the GYFLaN should work with their national surveillance programme and the WHO RLC before incorporating RT-qPCR as a tool for routine testing and should follow the YF testing algorithm guidelines for RT-qPCR and IgM testing in Chapter 4 (41).

Chapter content:

- Best practices for collecting and processing clinical specimens and extracting YF RNA
- Considerations for the use of molecular diagnostic methods
- YF RNA detection by RT-qPCR
- Quality assurance and quality control of RT-qPCR testing
- Test validity, data interpretation, and assay limitations

6.1 BEST PRACTICES FOR COLLECTING AND PROCESSING CLINICAL SPECIMENS AND EXTRACTING YF RNA

Serum and tissue specimens (see Chapter 3) are the primary specimen types tested in routine surveillance by the GYFLaN. In fatal cases, RNA extracted from blood and fresh tissue from the liver, spleen or kidney and paraffin-embedded tissue samples can be tested by RT-qPCR. Other patient specimen types, including whole blood, plasma and urine have been successfully used for RT-qPCR testing but have not been validated by WHO for use by the GYFLaN (25, 63, 64). Dried blood or serum spots on FTA™ cards are being evaluated as a source of RNA when routine virologic specimens cannot be obtained or properly transported, particularly in remote areas.

The most important factor for successful amplification and identification of YF RNA by any detection method is the timing of sampling. The recommended timing for collection of various types of clinical specimens is provided in Chapter 1 and Chapter 3. Specimens should be collected shortly after the onset of disease in the acute viraemic phase of the infection, generally within 14 days, and often before jaundice appears (Chapter 4). The sensitivity of RNA detection methods from most types of specimens declines considerably by 10 days if the patient recovers, which is reflected in the testing algorithm discussed in Chapter 4 (24). However, although the chances to detect RNA decreases after 10 days, it is suggested that serum collected up to 14 days can be routinely tested by RT-qPCR (Chapter 4).

The timely and proper sampling of specimens from suspected YF cases for virus nucleic acid testing is crucial, as the integrity of the viral RNA can be compromised with improper handling during specimen collection, processing, and transport. To minimize degradation and loss of quality of the samples, the procedures and recommendations described in Chapter 3 should be followed.

Upon arrival at the laboratory, the contents of the package containing the clinical specimen is inspected and the condition is recorded (see Chapter 8). If not accessioned and processed immediately, the specimens should be transferred to a refrigerator (4°-8°C) or frozen at –20°C or preferably at –70°C, according to the SOP for the type of specimen. Repeated cycles of freezing and thawing should be avoided to prevent degradation of the RNA.

The extraction of RNA from a clinical specimen must be performed accurately to maximize the yield of RNA. The best approach for RNA extraction is to use a suitable commercial RNA extraction kit or commercially available reagents. RNA extraction protocols for clinical specimens that are in use by GYFLaN laboratories are available in Annex 6.1.

6.2 CONSIDERATIONS FOR THE USE OF MOLECULAR DIAGNOSTIC METHODS

RT-qPCR is the recommended method for RNA testing, as it offers very high specificity and analytical sensitivity and the risk for cross-contamination is reduced compared to conventional and nested RT-PCR testing. In addition, there is improved capacity for higher throughput without the need for post-amplification processing. Another advantage of RT-qPCR is that the assay can be configured for detecting multiple targets in the same reaction, such as the YF viral RNA and an internal control (e.g., cellular reference gene) to monitor sample quality.

Multiplex RT-qPCR assays also have been developed to simultaneously detect YF- and dengue-specific RNA as well as a cellular gene or different strains or variants of YF virus (86).

Most RT-qPCR assays are designed as one-step assays, which combine the reverse transcription and PCR amplification steps in a single reaction. This reduces the repeated pipetting steps that increase the likelihood of cross-contamination. It is important to use a validated assay and to include positive and negative controls in all test runs (see Section 6.4.2 below). Some assays include an in-process control, which targets a housekeeping (cellular) gene and monitors the quality of sample extraction and presence of inhibitory factors in the reaction. Laboratories should develop SOPs using validated RT-qPCR protocols with established performance characteristics including:

- Defined lower limit of detection (LoD): the analytical sensitivity has been measured using samples with a known RNA copy number (e.g., RNA copies/reaction);
- Diagnostical sensitivity: the demonstrated ability to detect YF RNA in clinical samples from truly infected individuals;
- Specificity: the absence of a signal when tested against other pathogens causing haemorrhagic and/or jaundice and febrile illness. Specificity can be evaluated in silico by submitting the primer sequences to a BLAST search on GenBank® and/or analytically by testing with RNA from a selection of viruses and bacteria;
- Repeatability and reproducibility: results show limited intra-assay and inter-assay variations;
- PC RNAs of known sequence, preferably with genetic markers that clearly identify control reactions;
- Optimized and defined reaction conditions;
- Demonstrated ability to detect all known circulating genotypes of wild-type YF virus;
- Flexible platform/chemistry;
- Flexible analytical software.

The laboratory must ensure that validation and appropriate QC/QA plans are in place. The quality indicators to be monitored for each assay should be indicated in the laboratory SOP

6.3 YF RNA DETECTION BY RT-QPCR

A variety of RT-qPCR assays have been developed to detect different regions of the YF genome. However, some of these were developed for research purposes and are not suitable for diagnosis. In molecular testing specifically for laboratory diagnosis (virus-specific RNA detection), the specific target amplified by the primers must detect all wild-type YF genotypes and have sensitivity within the LoD relevant to the concentration of RNA in a clinical specimen (23, 76).

Regional laboratory workshops sponsored by WHO to support molecular detection of YF may offer different protocols for RT-qPCR. As with immunoassays for antibody detection, various validated in-house and commercial kits may demonstrate acceptable performance, but all RT-qPCR protocols must meet WHO validation criteria (e.g., standardized self-contained kit, stable at room temperature, high sensitivity and specificity) (87). Regional workshops may use different kits, but all workshops emphasize the technical aspects of RT-qPCR as well as QA and QC components.

An example of an in-house RT-qPCR method that amplifies an 88-nucleotide region of the YF 5' noncoding region with the acceptable ranges of threshold cycles (Ct) values for both controls is provided in Annex 6.2. Annex 6.3 gives an example commercial kits for the RNA extraction through the RT-qPCR procedure and published primers and probes (24, 84).

The WHO has evaluated and recommends the use of the RealStar® Yellow Fever Virus RT-PCR Kit 1.0 manufactured by Altona (Hamburg, Germany) (88) as an in vitro diagnostic assay used for the qualitative detection of YF virus-specific RNA; however other commercially available kits can be used according to their guidelines of use. The kit was developed for the detection of all known YF virus strains including the vaccine strain 17D. Manufacturer's instructions for use are shown in Annex 6.4.

6.4 QA AND QC OF RT-QPCR TESTING

RT-qPCR is a sensitive testing method and should be performed following strict QC and QA procedures. A description of the QA programme administered through WHO for molecular internal and EQA is provided in Chapter 9. All the laboratories that use RT-qPCR for YF diagnosis must participate in the annual molecular EQA programme and include appropriate QC procedures in the laboratory SOP. In addition, the WHO accreditation process for the GYFLaN includes evaluations of the facilities for molecular testing internal QA performance, which includes documentation of internal and external QA/QC processes.

6.4.1 WORKFLOW IN THE MOLECULAR LABORATORY

A clearly structured, unidirectional workflow proceeding from the area for RNA extraction/reagent preparation (pre-amplification area) to the PCR amplification room (post-amplification area) must be implemented and maintained to avoid cross-contamination of samples tested by RT-qPCR as follows (see also Annex 6.5).

- RNA extraction in a class II BSC (initial steps until inactivation);
- Preparation of the RT-qPCR pre-mix;
- Addition of RNA template and controls;
- RT-qPCR amplification and post-amplification analysis;
- Manipulation and disposal of the products of amplification.

It is important that separate work areas are maintained for each activity. Each work area should have dedicated equipment (e.g., pipettes, microcentrifuges), supplies (e.g., microcentrifuge tubes, pipette tips) and personal protective equipment (PPE) (e.g., gowns, gloves) which should not be moved from one work area to another.

The initial steps of RNA extraction should be performed in a class II Type A2 BSC because clinical samples are potentially infectious until inactivated by the addition of ethanol to the sample/RNA lysis buffer mix. Subsequent steps in RNA extraction and RT-qPCR can be performed in a BSC or a dedicated PCR cabinet. Following these guidelines will help minimize the chance of false positive results. Important practices that should be included in RT-qPCR SOPs are listed below and in the guidelines "Establishment of PCR laboratory in developing countries" provided by WHO (89).

- Wear clean, disposable gowns and new, powder-free gloves during pre-mix setup and handling of extracted nucleic acids;
- Store primer/probes and enzyme master mix at appropriate temperatures according to the manufacturer's recommendations (see package inserts);
- Do not use reagents beyond their expiration dates;
- Wear gloves when touching materials; keep reagent and reaction tubes capped as much as possible; avoid working or passing hands over tubes;

- Clean work surfaces and equipment (pipettors, tube racks, etc.) using a commercial product that destroys DNA and RNA or a freshly prepared chlorine bleach solution (10%). Attention should be made to not mix these products with the PCR products to prevent inactivation of PCR products;
- Use aerosol barrier (filter) pipette tips only.

All personnel must be familiar with the protocols and instruments used. Equipment should be properly maintained, with documentation that verifies that routine preventive maintenance and calibration are regularly performed (see guidelines in Chapter 9).

6.4.2 ASSAY CONTROLS

The use of appropriate controls is essential for RT-qPCR assays. Virus-specific and in-process DNA controls are often included in the test protocol or kit. However, if positive and negative controls are not provided with the molecular YF test kit the use of laboratory "in-house" positive and negative controls are recommended.

Positive control RNA: Viral RNA from infected culture or synthetic viral RNA are commonly used as PCs. Serum from an acute YF RT-qPCR positive case is rarely used, as the volume is limited.

- It is important to use an adequate and calibrated control throughout all test runs;
- The PC concentration should be 1-2 logs over the LoD of the assay. However, high concentration PCs (PC with low Cts) increase the risk of contamination and mask small, but important, fluctuations in the performance of the assay, and may only be visible when there are large performance deviations;
- Two different concentrations of PCs are recommended, one with a medium Ct (approximately 28 Ct) as primary control for validation together with a second one (approximately 32 Ct) very near the LoD of the assay, which will provide information on small fluctuations between the test runs (90);
- The European Virus Archive – Global (EVAg) provides quantified, full virus genome PC RNA and can be sourced from the Robert Koch Institute (https://www.european-virus-archive.com/node/2495). Laboratories can have access to the controls via EVAg free of charge.

Negative extraction control: A mock RNA sample, to detect any nonspecific or interfering substances and to detect cross-contamination which may occur in the RNA extraction process.

- Ideally, the same specimen type (e.g., serum, tissue) from non-infected patients should be used;
- Sterile water can also be substituted but should go through the same extraction process as the samples.

No-template control (NTC): Sterile, nuclease-free water is added to the reaction mix instead of extracted RNA.

- NTC detects extraneous nucleic acid contamination which may be present in the reaction mix or introduced during assay set up;
- NTCs have been useful to identify (for instance) pipetting errors and "sweeping" from one well to the next one, particularly when they are in different position in the plate.

Reference gene: To evaluate the adequacy of the clinical sample, quality of the RNA extraction, and efficiency of amplification.

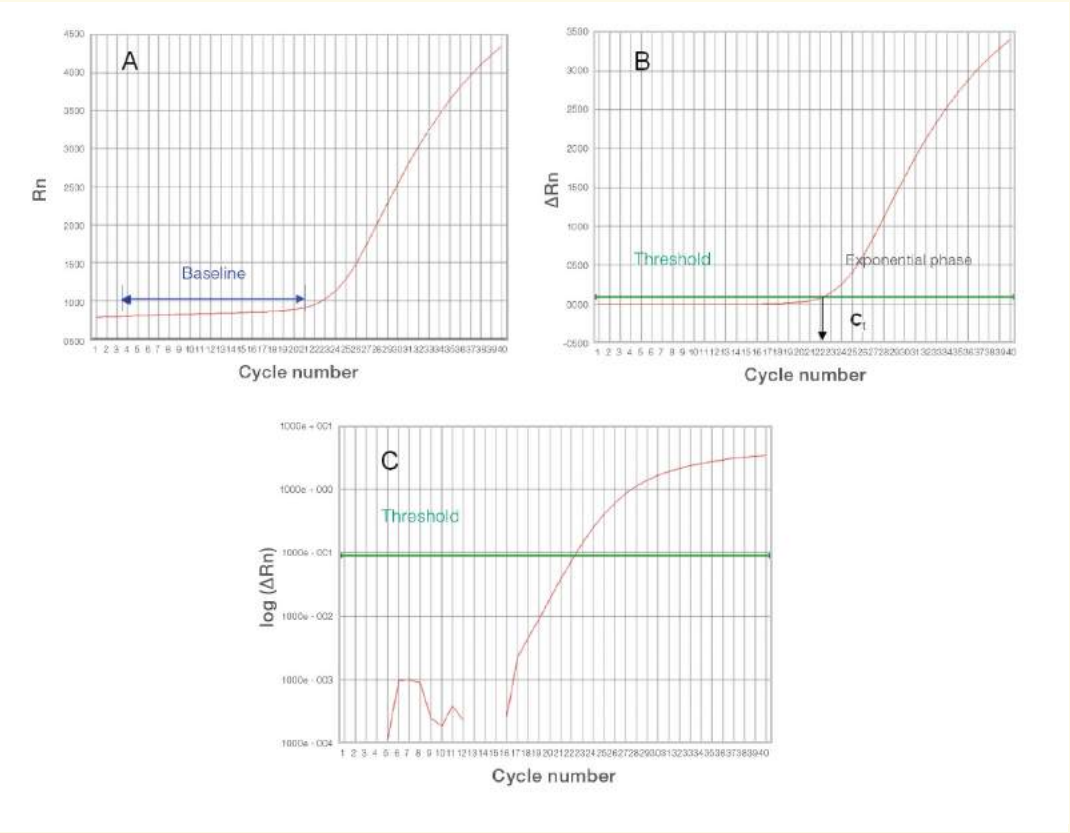
- Reference cellular or housekeeping genes are expressed in a wide variety of tissues and cell types and show no or only minimal changes in expression levels between the individual samples and experimental conditions;
- Failure to detect the reference gene indicates that insufficient cellular material was collected, inhibitory substances are present in the sample, or the sample was transported or processed improperly;
- The use of a reference gene for RT-qPCR assays used for case confirmation is strongly recommended by the WHO;
- Reference gene controls and the primer/probes to detect them are included in most commercial kits;
- Kit-based RT-qPCR assays are usually designed to detect cellular RNA from a reference gene, or "housekeeping gene". The most frequently targeted reference genes, human ribonuclease P (RNase P) and glyceraldehyde 3-phosphate dehydrogenase (GADPH), are present in all human cells and are used to evaluate the adequacy of the clinical sample and the quality of the RNA extraction;
- Detection of a reference gene is performed either in a multiplex format or as a separate reaction.

Documentation of the use of appropriate controls is necessary to ensure accuracy. The RT-qPCR SOP should identify quality indicators that will be monitored for each assay to help identify trends that may affect assay performance. For example, many laboratories plot the Ct value of one of the standard PC reactions.

6.5 TEST VALIDITY, DATA INTERPRETATION, AND ASSAY LIMITATIONS

After completing the run, the data should be validated and then analysed. A valid test run requires that all positive and negative controls yield the expected results. The test is not valid if any PC is negative or any of the negative controls are positive. It is critical to carefully evaluate the data for each sample. The threshold and baseline are set according to the protocol or manufacturer's instructions prior to analysis, as it will affect the Ct value and thus, the interpretation. The amplification curves must cross the set threshold for the virus-specific RT-qPCR to be considered positive and visual inspection should be performed to verify that the amplification curves cross the threshold at the linear phase (Figure 6.1). The exponential phase in Figure 6.1B corresponds to the linear phase in Figure 6.1C. Some manufacturers and distributors of RT-qPCR reagents created and made publicly available a number of educational resources of RT-qPCR methods and principles. Examples include the Real-Time PCR (qPCR) Learning Center from ThermoFischer Scientific (https://www.thermofisher.com/ch/en/home/life-science/pcr/real-time-pcr/real-time-pcr-learning-center.html), and the Altona Diagnostics training videos describing validation and analysis procedures for the RealStar RT-PCR Kit evaluated by WHO for use in the context of surveillance (https://www.youtube.com/playlist?list=PLcDmavw-Uq6sA1w_4w-cilNfZKPdSZxbB).

FIGURE 6.1 **Graphical representation of real-time PCR data.** See Box 6.1 for detailed legend.



Source: taken from Applied Biosystems™'s Application Note entitled "Real-time PCR: Understanding C t", available at: https://www.thermofisher.com/content/dam/LifeTech/Documents/PDFs/PG1503-PJ9169-CO019879-Re-brand-Real-Time-PCR-Understanding-Ct-Value-Americas-FHR.pdf

Box 6.1 Details of legend from Figure 6.1

Rn is the fluorescence of the reporter dye divided by the fluorescence of a passive reference dye; i.e., Rn is the reporter signal normalized to the fluorescence signal of ROX dye.

(A) Rn is plotted against PCR cycle number.

(B) ΔRn is Rn minus the baseline; ΔRn is plotted against PCR cycle number.

(C) An amplification plot shows the variation of log (ΔRn) with PCR cycle number.

Test validation: Validation of the test should be determined with the criteria in the instructions for use and include:

- PC and reference gene (if included) should be positive (reaction generates a curve that crosses the threshold at <38 Ct). If negative, repeat the plate;
- NC and NTC (if included) should be negative (reactions generate curves that cross ≥38 Ct OR that fails to cross the threshold). If any NC has a positive result, the test is considered invalid. The following steps should be taken:
  - Immediately discard all working stocks of reagent dilutions and remake from fresh stock;
  - Bench surfaces, pipettes, and other equipment should be cleaned properly to destroy nucleic acids in the reagent setup and template addition work areas.

Data analysis: If all control results are acceptable, proceed to analyse each sample result.

- A result is interpreted as negative if the reaction generates a curve that crosses the threshold ≥38 Ct OR that fails to cross the threshold;
- A result is interpreted as positive if the reaction generates a curve that crosses the threshold at <38 Ct.

In RT-qPCR assays that contain a reference gene:

- Positive results for both YF virus- and reference- gene specific RT-qPCR is considered valid and positive for YF;
- A negative result for the YF virus-specific RT-qPCR but a positive result for the reference gene specific RT-qPCR is considered a valid test and negative result for YF.

If all controls are as expected some further issues which cause a 'false negative' result should be evaluated as described below.

- Negative results for both the reference gene specific RT-qPCR and the YF virus-specific RT-qPCR is reported as undetermined (inconclusive), as the quality of the RNA in the sample may have been compromised. Re-extract the RNA and repeat the test;
- Extremely weak positive results (high Ct values close to the cut off Ct) in test samples should be interpreted with caution. Ideally, the test samples should be tested in duplicate. If the test is valid (the PC and reference gene are positive and the NTC is negative) but there are inconsistent results between duplicate samples (positive/negative, positive/undetermined), the samples should be retested

Interpretation of RT-qPCR results: The YF RT-qPCR is a very sensitive assay and the presence of only a few RNA molecules can be detected in a YF virus-infected patient. A positive result indicates that YF RNA was present in the clinical sample. The positive predictive value that the patient with a positive RT-qPCR result is infected with YF virus is very high. The result is of public health importance and the findings need to be reported to the national public health institution to start public health measures. Confirmatory testing should still be done at the RRL, particularly with a single suspected case in an area where YF has not been previously reported.

If the suspected case comes from a person who had been vaccinated within 7-14 days, the positive result may be due to a vaccine-associated infection. In those instances where the patient received a YF vaccination following potential exposure to a confirmed case or was vaccinated as part an outbreak response, it is necessary to identify and differentiate a vaccine strain (17D) by use of a validated vaccine-specific RT-qPCR assay or sequencing to discard the case or confirm that the infection is caused by natural infection of a wild-type YF virus strain. These tests are normally conducted at the RRL or other reference laboratory. For more information on distinguishing vaccine strains from wild-type infections see Section 6.4 and Chapter 7.

A negative test result may be also obtained from a true case of YF. The abundance of virus-specific RNA is generally much lower in infected cells compared to the cellular RNA of the reference gene. Therefore, a positive signal from the reference gene may be obtained from specimens collected from true cases while the virus-specific RT-qPCR result is negative. A 'false negative' result may be obtained due to:

- Specimen collection outside the viremia phase;
- Poor sample collection, transportation, or processing;
- Partial degradation of RNA, reducing YF viral RNA below detection levels;
- Failure to follow the assay protocol or equipment failure;
- Failure to use specified extraction kit and platform.

Therefore, a negative RT-qPCR result should be interpreted carefully. A negative RT-qPCR result can be helpful to corroborate other negative laboratory results but should not be the sole basis for ruling out a suspected case. The final classification of suspected cases that are negative by RT-qPCR with inconclusive IgM results (i.e., negative IgM from serum collected ≤7 days after onset of illness or before appearance of jaundice) must be based on clinical and epidemiological assessment or discarded based on laboratory confirmation of other jaundice-causing illness. Additional testing may also be necessary. The testing algorithm in Chapter 4 provides guidance for the interpretation of RT-qPCR results and recommendations for additional testing.

Chapter 7

Yellow fever confirmatory and reference testing at the Regional Reference Laboratory

Within the tiered structure of the GYFLaN, RRLs provide support to the NLs by conducting confirmatory and reference YF testing (Chapter 2). Whereas detection of YF viral RNA by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) confirms a YF virus infection, detection of IgM by serology is interpreted as a presumptive YF virus infection due to the extensive cross-reactivity of flavivirus IgM antibodies to YF antigens used in the IgM assays (41, 91). A differential flavivirus neutralization test conducted in the RRL is required for confirmation or exclusion of a presumptive YF virus infection (Chapter 4).

RRLs also provide reference and primary testing to laboratories within the GYFLaN and conduct further investigations to characterize YF transmission for surveillance programmes and during outbreaks.

Chapter content:

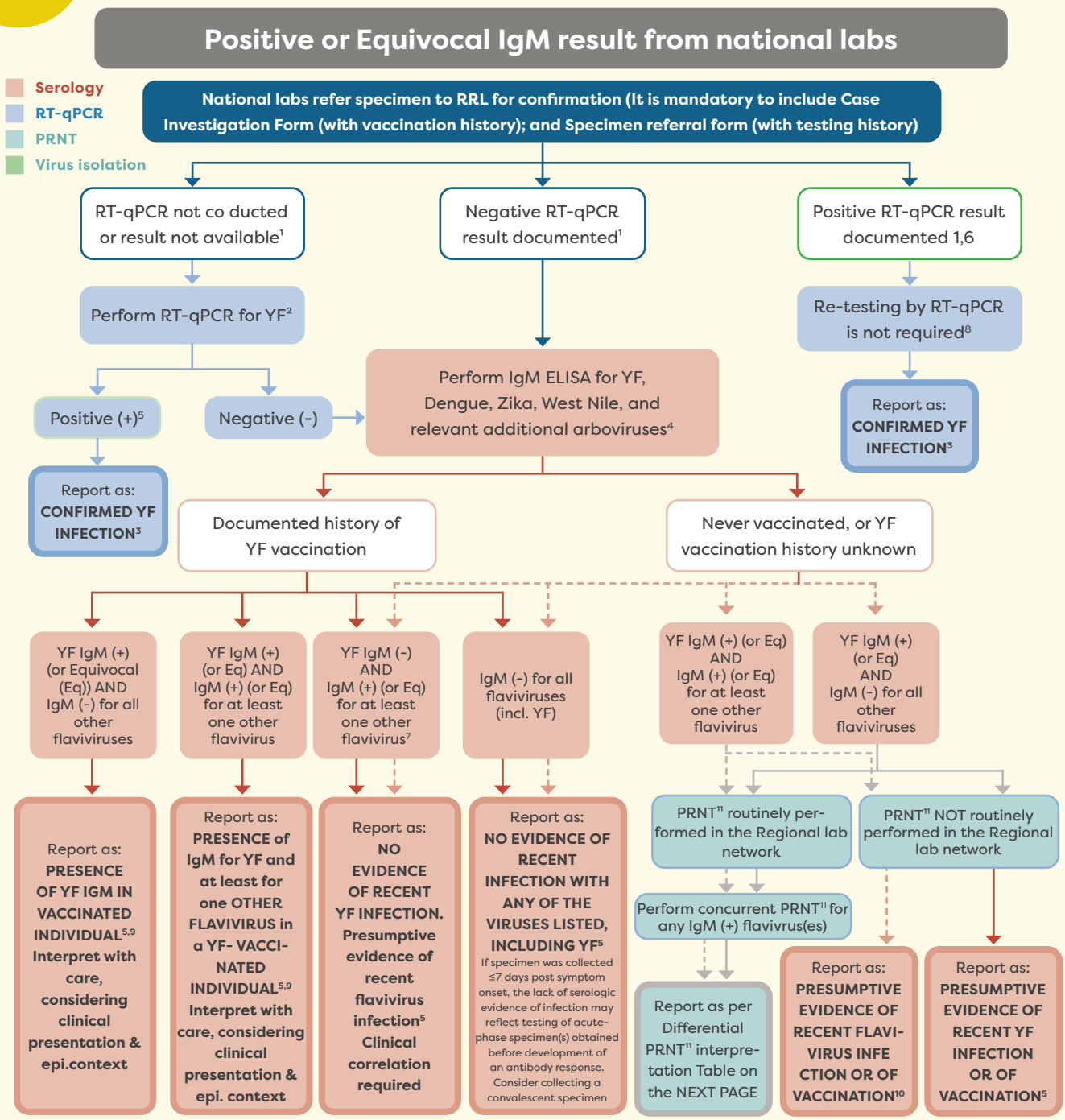
- The RRL YF confirmatory testing algorithm
- The neutralizing antibody assay
- Reporting results of RRL testing
- Differential diagnostic flavivirus IgM testing
- Virus isolation
- Genomic sequencing

7.1 THE REGIONAL REFERENCE LABORATORY YF CONFIRMATORY TESTING ALGORITHM

The RRL testing algorithm for referred specimens is shown in Figure 7.1A. Serum specimens tested for IgM in the NLs with YF IgM positive and equivocal results are submitted to the RRL to confirm or rule out a YF virus infection by a neutralizing antibody test, such as the PRNT used in the GYFLaN. Note that neutralization assays are not primary diagnostic tests and not useful for characterizing samples with IgM negative results. The RRL may also attempt YF RT-qPCR if there is sufficient volume to conduct all the tests needed.

FIGURE 7.1A

Yellow Fever CONFIRMATORY Testing Algorithm for Regional Reference Laboratorie. See Box 7.1 for detailed legend.



1 Laboratories must be part of the GYFLaN and accredited by WHO to perform RT-qPCR. If patient specimen was collected ≤14 days from symptom onset and tested for YF RT-qPCR by the national lab, there is no need for the RRL to repeat the RT-qPCR, unless specifically requested by national lab. However, if there are no documented RT-qPCR results from national lab, the RRL should perform the RT-qPCR for YF. In recent vaccinees (<30 days) who develop classical symptoms of YF infection, and for which a positive RT-qPCR result from the national lab is documented, the RRL should perform targeted sequencing or use of discriminatory RT-qPCR in order to differentiate between infections with wild-type YF virus and the vaccine virus strain.

2 Whenever available, RT-qPCR should be the first-line test, irrespective of the number of days since symptoms onset. A positive result in those samples will confirm a YF infection, whereas a negative result would not exclude the possibility of a YF virus 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. For fatal cases, RT-qPCR should be performed on all available samples, independent of the collection date.

3 Clinical correlation required. For cases with no history of vaccination, vaccination history unknown, or vaccinated >14 days before symptom onset, this YF RT-qPCR positive results supports the evidence of active YF virus circulation.

4 IgM testing for YF, Dengue, Zika, and West Nile are part of the minimum package for YF surveillance purposes. Testing for other arboviruses with similar clinical presentation or in the same genus can be added, if these arboviruses infections are common in this region and therefore epidemiologically relevant and specific tests are available (ex: Rift Valley fever, Crimean-Congo Haemorrhagic Fever, etc.)

5 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, etc.)

6 Virus isolation can be attempted following RT-qPCR positive result if the Ct value is <30 to support forward-thinking efforts requiring strain characterization or virus isolate for banking purposes. The outcome of the viral isolation should not delay or affect YF case surveillance reporting.

7 Further PRNT testing for other flaviviruses with IgM positive or equivocal result can be attempted but is not mandatory as part of YF surveillance. Reporting on the absence of evidence of recent YF virus infection should not be delayed.

8 Routine RT-qPCR testing is only conducted by accredited national laboratories. Additionally, considering the risk of specimen degradation during transportation, re-testing by RT-qPCR by RRL is not required

To confirm the infection, particularly in areas where no YF virus circulation has been recently described, differential neutralization testing with flaviviruses endemic to the area of exposure or neutralization testing of appropriately paired sample set to demonstrate seroconversion should be performed in an RRL.

9 In recent vaccinees (<30 days) who develop classical symptoms of YF infection, targeted sequencing or use of discriminatory RT-qPCR should aim to differentiate between infections with wild-type YF and the vaccine virus strain. Note: YF IgM antibodies can persist for months to years post-vaccination. Consider documented cross-reactivity of IgM detection among flaviviruses.

10 Consider documented cross-reactivity of IgM detection among flaviviruses. However, in areas where no YF circulation has been described recently, this result does not rule out yellow fever. Consider performing PRNT in a Regional Reference Laboratory. This should also prompt further clinical and epidemiological investigation.

11 PRNT= Plaque Reduction Neutralization Test

Note: According to the GYFLaN testing algorithm in Chapter 4, the sample would not have been tested by RT-qPCR or would have been tested and had a negative result in the NL. Abbreviations: RRL, Regional Reference Laboratories; YF, yellow fever; PRNT, plaque reduction neutralization test; GYFLaN, Global Yellow Fever Laboratory Network.

Box 7.1 Details of legend from Figure 7.1A

1 Laboratories must be part of the GYFLaN and accredited by WHO to perform RT-qPCR. If patient specimen was collected ≤14 days from symptom onset and tested for YF RT-qPCR by the national lab, there is no need for the RRL to repeat the RT-qPCR, unless specifically requested by national lab. However, if there are no documented RT-qPCR results from national lab, the RRL should perform the RT-qPCR for YF. In recent vaccinees (<30 days) who develop classical symptoms of YF infection, and for which a positive RT-qPCR result from the national lab is documented, the RRL should perform targeted sequencing or use of discriminatory RT-qPCR in order to differentiate between infections with wild-type YF virus and the vaccine virus strain.

2 Whenever available, RT-qPCR should be the first-line test, irrespective of the number of days since symptoms onset. A positive result in those samples will confirm a YF infection, whereas a negative result would not exclude the possibility of a YF virus 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. For fatal cases, RT-qPCR should be performed on all available samples, independent of the collection date.

3 Clinical correlation required. For cases with no history of vaccination, vaccination history unknown, or vaccinated >14 days before symptom onset, this YF RT-qPCR positive results supports the evidence of active YF virus circulation.

4 IgM testing for YF, Dengue, Zika, and West Nile are part of the minimum package for YF surveillance purposes. Testing for other arboviruses with similar clinical presentation or in the same genus can be added, if these arboviruses infections are common in this region and therefore epidemiological relevant and specific tests are available (ex: Rift Valley fever, Crimean-Congo Haemorrhagic Fever, etc.)

5 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, etc.)

6 Virus isolation can be attempted following RT-qPCR positive result if the Ct value is <30 to support forward-thinking efforts requiring strain characterization or virus isolate for banking purpose. The outcome of the viral isolation should not delay or affect YF case surveillance reporting.

7 Further PRNT testing for other flaviviruses with IgM positive or equivocal result can be attempted but is not mandatory as part of YF surveillance. Reporting on the absence of evidence of recent YF virus infection should not be delayed.

8 Routine RT-qPCR testing is only conducted by accredited national laboratories. Additionally, considering the risk of specimen degradation during transportation, re-testing by RT-qPCR by RRL is not required

9 In recent vaccinees (<30 days) who develop classical symptoms of YF infection, targeted sequencing or use of discriminatory RT-qPCR should aim to differentiate between infections with wild-type YF and the vaccine virus strain. Note: YF IgM antibodies can persist for months to years post-vaccination. Consider documented cross-reactivity of IgM detection among flaviviruses.

10 Consider documented cross-reactivity of IgM detection among flaviviruses. However, in areas where no YF circulation has been described recently, this result does not rule out yellow fever. Consider performing PRNT in a Regional Reference Laboratory. This should also prompt further clinical and epidemiological investigation.

11 PRNT= Plaque Reduction Neutralization Test

FIGURE 7.1B

Yellow fever confirmatory testing algorithm for Regional Reference Laboratories table for the interpretation of PRNT results for IgM positive specimen. See Box 7.2 for more detailed information on the legend.

Yellow Fever **CONFIRMATORY** Testing Algorithm for **Regional Reference Laboratories**

Table for the interpretation of PRNT[6] results for IgM positive specimen[4]

Yellow Fever[4] (YF) PRNT result	Dengue[4] (D) PRNT result	Zika[4] (Z) PRNT result	West Nile[4] (WN) PRNT result (if tested)	Differential Interpretation[1]
+	-	-	-	**Evidence of recent YF virus infection[2,3]**
+	+	-	-	**Differential diagnosis considering YF IgM positive result and differences in PRNT titres among IgM- positive viruses tested[5]** - If the YF titre is positive, and at least 4-fold higher than any of the D/Z/WN positive PRNT titres, the interpretation is **Evidence of recent YELLOW FEVER virus infection[2].** - If there is a <4-fold difference between any of the 2 highest positive PRNT titres, the interpretation is **Evidence of recent FLAVIVIRUS infection.** - If the YF PRNT titre is positive but one of D, Zor WN has a positive PRNT titre at least 4-fold higher than all the other PRNT titres, then the interpretation is **Evidence of recent infection of that SPECIFIC FLAVIVIRUS (D, Z or WN).** - If the YF titre is positive, but at least 4-fold lower than the closest other positive PRNT titre, AND where two or more of the non-YF viruses have a difference in titres less than 4-fold from one another, then the interpretation is **Evidence of recent FLAVIVIRUS infection.**
+	+	-	+	
+	+	+	-	
+	+	+	+	
+	-	+	+	
+	-	-	+	
+	-	+	-	
-	+	-	-	**Evidence of recent Dengue virus infection**
-	+	-	+	**Evidence of recent Flavivirus infection** (unless D or WN has a titre ≥4 greater than the other one)
-	+	+	-	**Evidence of recent Flavivirus infection** (unless D or Z has a titre ≥ 4 greater than the other one)
-	+	+	+	**Evidence of recent Flavivirus infection** (unless D or Z or WN has a titre ≥4 greater than the other one)
-	-	+	+	**Evidence of recent Flavivirus infection** (unless Z or WN has a titre ≥4 greater than the other one)
-	-	-	+	**Evidence of recent West Nile virus infection**
-	-	+	-	**Evidence of recent Zika virus infection**
-	-	-	-	**Positive YF IgM result not confirmed by neutralization testing, suggesting a non-specific IgM result.**

1 Final interpretation to be reported and advice on conclusion should occur after all testing is complete.

2 Case classification to 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 PRNT result.

3 Interpretation also valid for YF-only PRNT (i.e., non-differential PRNT) if all IgM test results for other flaviviruses were all negative. Note that performing concurrent PRNT for any IgM-positive flavivirus(es) remains mandatory for correct differential interpretation.

4 PRNT testing for YF must always be accompanied by concurrent PRNT testing for any other IgM-Positive flaviviruses tested as part of the differential IgM scheme. The interpretation of a YF PRNT result can ONLY be done as part of a differential PRNT interpretation due to known cross-reactivity among flaviviruses.

5 Interpretation provided are considering a YF or differential PRNT SOP where the titre difference criteria between viruses tested is set to a ≥4-fold difference.

6 PRNT= Plaque Reduction Neutralization Test

Note: According to the GYFLaN testing algorithm in Chapter 4, the sample would not have been tested by RT-qPCR or would have been tested and had a negative result in the NL. Abbreviations: RRL, Regional Reference Laboratories; YF, yellow fever; PRNT, plaque reduction neutralization test; GYFLaN, Global Yellow Fever Laboratory Network.

Box 7.2 Details of legend from Figure 7.1B

1 Final interpretation to be reported and advice on conclusion should occur after all testing is complete

2 Case classification to 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 PRNT result.

3 Interpretation also valid for YF-only PRNT (i.e., non-differential PRNT) if all IgM test results for other flaviviruses were all negative. Note that performing concurrent PRNT for any IgM-positive flavivirus(es) remains mandatory for correct differential interpretation.

4 PRNT testing for YF must always be accompanying with concurrent PRNT testing for any other IgM-Positive flaviviruses tested as part of the differential IgM scheme. The interpretation of a YF PRNT result can ONLY be done as part of a differential PRNT interpretation due to known cross-reactivity among flaviviruses.

5 Interpretation provided are considering a YF or differential PRNT SOP where the titre difference criteria between viruses tested is set to a ≥4-fold difference.

6 PRNT= Plaque Reduction Neutralization Test.

7.2 THE NEUTRALIZING ANTIBODY ASSAY

7.2.1 KINETICS OF THE IMMUNE RESPONSE AGAINST YF VIRUS INFECTION AND THE NEUTRALIZING ANTIBODY TEST PRINCIPL

Neutralizing antibodies are produced during virus infection as part of the immune response and block virus attachment to cells, inhibiting the virus infection. Neutralizing antibodies (mostly IgG) against YF can be detected in the serum at approximately 7-10 days following wild-type infection or YF vaccination. Immunity to YF is lifelong and neutralizing antibodies are detectable throughout a person's life, although the antibody titer may decline over time.

In the neutralization test such as the PRNT, a known concentration of reference YF virus is mixed with serial dilutions of the test serum, alongside controls using antisera of known reactivity. If YF virus-specific neutralizing antibodies are present, they bind to surface proteins of the YF virus, inhibiting the incorporation of the infectious virus into the cells. The antibody-virus complex mixture is then inoculated onto a monolayer of susceptible cells.

Depending on the concentration of neutralizing antibodies bound to the virus, infection of the cells is measurably inhibited or reduced, i.e., the virus is neutralized. A single virus particle infects a single cell in a fixed cell monolayer, then continues to infect neighbouring cells forming a plaque, and is defined as one plaque forming unit (PFU). Plaques can be visualized by staining the cell monolayer; viable cells take up the dye, whereas the plaques are colourless. Reduction in the number of PFU in a sample compared to the control, which contains virus but no neutralizing antibody, indicates neutralization. The end-point of this assay is calculated as the test serum titer (expressed as a dilution) at which point a designated proportion of the virus, usually 50% or 90%, is neutralized compared to non-neutralized virus controls on the cell monolayer (92).

The neutralization test offers higher specificity than most serological assays for the detection of IgM and IgG antibodies in primary flavivirus infections and is used in the GYFLaN as a quantitative confirmatory assay. However, cross-reactivity among flaviviruses also occurs in neutralization assays in secondary flavivirus infections. Thus, it is recommended that this technique should be performed with a differential panel of flaviviruses endemic to the particular region.

A lack of differentiation is diagnostically informative of a secondary infection even though the current infecting virus cannot be identified.

In the GYFLaN, the neutralization assay is normally performed in RRLs because the test requires a cell culture laboratory, appropriate biosafety conditions for propagating live virus, and laboratory personnel experienced in virus cell culture and titration techniques. An example of the PRNT protocol used at the RRL in the Uganda Virus Research Institute (UVRI) is shown in Annex 7.1. The following describes the outline for the detection of neutralizing antibodies to YF virus in human sera using the UVRI PRNT protocol.

1. Aliquots of serum samples are heat inactivated at 56°C for 30 min to destroy complement and to inactivate adventitious viruses.
2. Serial dilutions (twofold) of patient serum are made, usually starting at 1:5.
3. Reference virus is diluted in media to correct concentration.
4. Reference virus is added to the serum dilutions and to the positive and NC serum.
5. The virus-serum mixtures are incubated at 37°C for one hour or overnight at 4°C.
6. Inoculation into a cell culture well (e.g., in a 12-well plate) containing a confluent monolayer of cells:
   - The virus-serum mixtures, including virus-PC and virus-NC serum;
   - Reference virus back titration;
   - Media is added to cell culture control wells.
7. The cell culture is overlayed with nutrient medium containing agarose to immobilize the virus-serum particles.
8. The cell culture plates are incubated at 37°C until plaques are formed.
9. Plaques are visualized by a second overlay of nutrient medium/agarose containing neutral red as a vital stain after 2-6 days incubation according to the virus used.
10. The plates are inspected after one or two days for evidence of plaques, which appear as clear dots on the red field of cells.
11. Plaques are marked and counted to calculate the neutralization titer compared to the back titration.

> **Note:** Assay controls must be run concurrently with serum samples, and when a specimen is tested by PRNT using multiple flaviviruses (to test for cross-neutralization), the assays should all be started on the same day.

1. **Positive control:** Homologous viral antiserum with standardized known neutralizing antibody titer.
2. **Negative control:** Serum without virus-specific neutralizing antibody.
3. **Virus back titration control:** Three dilutions of the reference virus (102, 101, 100 dilutions compared to the virus used in the assay) are added to cells. This documents that an appropriate plaque count has been used and determines the maximum number of plaques below which the chosen per cent neutralization is achieved.
4. **Cell control and nutrient medium control:** The cell layer should remain confluent throughout the duration of the assay without plaques or extensive cytopathic effect (CPE), or any other structural changes in a host cell resulting from viral infection. CPE occurs when the infecting virus causes lysis (dissolution) of the host cell or when the cell dies without lysis because of its inability to reproduce.

7.2.2 LABORATORY SAFETY

The neutralization assay requires cell culture and processing of live virus and must be performed in a certified BSC. All personnel conducting these procedures should wear appropriate PPE, including a laboratory coat, gloves, safety glasses, and closed-toe shoes. Procedures shall be conducted using universal best laboratory precautions and according to the biological safety regulations of the laboratory. Personnel need to be thoroughly trained in aseptic techniques, cell culture, and working with infectious agents. Hazardous infectious material waste should be disposed of according to the regulations of the institution. All personnel conducting the neutralization tests should be vaccinated against YF virus at least 15 days before starting laboratory work (43).

7.2.3 BIOLOGICAL COMPONENTS OF THE NEUTRALIZATION ASSAY

1. Monolayers of cells that will support growth of YF virus e.g. Vero cells.
2. Reference YF virus (preferably attenuated YF strain 17D or ChimeriVax-YF vaccine virus).
3. Patient's serum: An aliquot with sufficient volume should be heat inactivated.
4. Homologous viral antiserum for PC.
5. NC serum.
6. Cell culture media.

Preparation of virus stock for the neutralization assay: The stock YF virus needs to be prepared ahead of time and accurately titrated. Single-use aliquots should be made and stored at -80°C until used. A sufficient volume of virus needs to be diluted to a standard concentration before starting the neutralization protocol. See Annex 7.2 for an example on making dilutions of stock viruses and calculating the volume of diluted virus needed for the number of samples to be tested.

Preparation of serum samples: An aliquot of the serum sample is made with enough volume to test each sample in duplicate and make serial dilutions for all viruses used in differential testing. The serum aliquot is heated for 30 min at 56°C to inactivate adventitious viruses and destroy complement without adversely affecting sample quality. The serum is then diluted twofold from 1:5 to the end-point. An example of serum dilutions to 1:160 is shown in Table 7.1. However, it should be noted that 1:160 is often not the end-point neutralization titer in a sample containing a high concentration of neutralizing antibody.

TABLE 7.1 **Calculations of serum and medium needed to make serial dilutions for the neutralization assay.**

Dilution	*Serum [µl]*	*Medium [µl]*
1:5	24	96
1:10	60 (from 1:5)	60
1:20	60 (from 1:10)	60
1:40	60 (from 1:20)	60
1:80	60 (from 1:40)	60
1:160	60 (from 1:80)	60

7.2.4 VALIDATION AND CALCULATION OF NEUTRALIZING ANTIBODY TEST RESULTS

Before the results can be calculated for the clinical specimens, the test must be determined to be valid. This includes the following:

- Neutralizing antibody titer in the wells containing PC serum similar to that previously determined;
- No neutralization in the wells containing NC serum; i.e., plaques too numerous to count or extensive CPE;
- Reference virus back titrations display the correct number of plaques (e.g., 100 [too many to count, TMTC], 10, 1);
- The cell layers in the cell control wells are confluent without plaques or CPE

Results for clinical specimens may only be determined if the test is valid. If the test is not valid, then the test must be repeated.

Calculation of neutralizing antibody titer: Virus-specific neutralizing antibody titers are determined by a per cent end-point. For example, if neutralization is defined as a ≥90% reduction of the plaques count in the viral inoculum (10-1 back titration) and the back titration indicates that the test inoculum had 100 plaques, then the highest serum dilution with 10 or fewer plaques is the 90% end-point. The serum neutralization titer is calculated as the reciprocal of the highest dilution of serum that neutralizes the virus inoculum at least 90% (e.g., ≤10). A neutralization titer of ≥10 is generally considered positive for neutralizing antibodies. An example of a worksheet for recording plaque counts and calculating neutralizing antibody titer is given in Annex 7.3.

7.2.5 DIFFERENTIAL DIAGNOSTIC NEUTRALIZATION TESTING

Differential diagnostic neutralization testing with YF and another flavivirus is recommended in areas where YF virus co-circulates with other flaviviruses, such as the dengue, Zika, or West Nile viruses. A neutralizing antibody titer to YF virus only, or a titer ≥fourfold over the heterologous flavivirus tested, is interpreted as positive for YF-specific antibody (see the table for the interpretation of PRNT results for IgM positive specimen in Figure 7.1B). If the difference in titer is less than fourfold, a differential diagnostic interpretation cannot be made. Secondary flavivirus infections are common in individuals who live in areas where multiple flaviviruses co-circulate. The neutralization assay test might not be useful as a virus-specific diagnostic assay in sera from patients with secondary flavivirus infections, as antibodies from a previous flavivirus infection (e.g., YF virus) and the current flavivirus infection (e.g., dengue virus) may both react with the reference YF virus, whether it is the current infecting virus or not. Each RRL will determine the applicability of the neutralization assay and interpret the test results based on conditions (YF vaccine coverage, other flavivirus transmission, etc.) specific for the region and patient information (vaccination status) to classify cases that are PRNT positive.

7.2.6 INTERPRETATION OF NEUTRALIZATION ASSAY RESULTS

Interpretation of neutralization assay results in YF vaccinated individuals: YF vaccine virus elicits neutralizing antibodies that are present throughout the vaccinee's life, although the titer may decline over time. The neutralization assay cannot differentiate between neutralizing antibodies elicited against YF wild-type or live attenuated vaccine virus infection. In fact, neutralization tests are used to measure the level of protective antibody following vaccinations. It is important to obtain the vaccine history from individuals with suspected YF upon sample collection.

Interpretation of YF MAC-ELISA and PRNT results for confirmation of YF virus infection: Neutralization results by themselves are not diagnostic and should be interpreted together with IgM testing results. Neutralizing antibodies are comprised of IgM, IgG, and other antibodies. Whereas IgM is usually present in an acute YF infection or recent vaccination, IgG may be present from a past YF virus infection or vaccination, or other flavivirus infection. A positive neutralizing titer to YF virus in a single specimen can only be interpreted as the presence of neutralizing antibody to YF virus infection at some time in the patient's life, either naturally or through vaccination. Therefore, the neutralization assay is not used as a primary test for YF diagnosis in a single serum sample but in combination with IgM testing to confirm or clarify positive or equivocal IgM results, i.e., presumptive acute YF virus infections. Final interpretations of serological testing (IgM and neutralization) in a single serum specimen are shown in Figure 7.1B.

As with IgM testing, interpretation of YF laboratory test results is affected by the timing of sample draw following onset of symptoms (Chapter 3). Note that neutralizing antibodies (shown as IgG) rise to detectable levels after IgM in primary YF virus infections. There is a window where the IgM results are positive and PRNT results are still negative in an acute primary YF virus infection.

7.3 REPORTING RESULTS OF RRL TESTING

At the completion of testing, the RRL reports results to the country MOH as well as to WHO. The surveillance programme will consider the results from all the tests, the clinical information (e.g., vaccination status of the individual, timing of the specimen collection), and the epidemiologic context (e.g., possible co-circulation of other flaviviruses) to classify the case (Chapter 8). If tests conducted in the NL were repeated by the RRL (e.g., YF MAC-ELISA) and the results were discrepant, the RRL results would supersede those of the NL, while ensuring adequate reporting of the final result back to the surveillance programme. The RRL should provide technical assistance to investigate the cause of the discrepancy and measures to improve the NL accuracy.

The RRLs also collect, analyse, and report information originating from other network laboratories to WHO, including:

- The number of samples received from each NL that were positive for YF at those NLs and, for each specimen, the date the sample was received, which types of YF test were used, and the results from and dates of completion of each of those tests;
- The number of samples received from each NL that were negative for YF at those NLs and, for each specimen, the date the sample was received, which types of YF test were used, and the results from and dates of completion of each of those tests;
- Any challenges in receiving specimens from NLs, testing those specimens, and reporting on results to NLs, should describe in sufficient detail the specific specimens and which episodes of each challenge was associated with that specimen, which will help to guide future corrective action;
- Observations on how to improve the functioning of YF laboratory network referral and testing system.

7.4 DIFFERENTIAL DIAGNOSTIC FLAVIVIRUS IgM TESTING

In many regions where the YF virus is circulating, other arthropod-borne flaviviruses such as dengue, Zika, and West Nile viruses may also be actively transmitted. Because of common immunogenic epitopes mostly on the envelope protein among the flaviviruses, IgM elicited in response to another flavivirus infection may cross-react with YF antigen in the YF IgM assay, yielding a false positive result (91). IgM testing for other expected flaviviruses can be informative and differential IgM diagnostic testing against one or more flaviviruses, most commonly dengue, is part of the testing algorithm in the LAC Region, where neutralization testing is not routinely used to confirm YF virus infections. Reference laboratories may use analyte-specific reagents for laboratory-developed IgM assays, commercial assays, or use a combination of laboratory-developed and commercial assays. Because these tests are not calibrated with one another, comparison of IgM titers is not valid. Therefore, a sample tested for YF and other flaviviruses with more than one IgM positive result is interpreted as an acute flavivirus infection. A positive YF IgM test with a negative differential test should be interpreted as a probable YF case; however, differential diagnostic MAC-ELISAs are not confirmatory tests.

7.5 VIRUS ISOLATION

Viral isolation is a tool to complement or to better characterize a YF virus strain, which might be particularly important where it is emerging/re-emerging or where any atypical epidemiology or clinical profile is detected, but virus isolation should not be used as a diagnostic methodology. Generally, virus isolation is used in a context of arbovirus surveillance or virus discovery, not for YF surveillance. Virus isolation can be attempted following RT-qPCR positive result if the Ct value is <30 to support forward thinking efforts requiring strain characterization or virus isolate for banking purposes. The outcome of the viral isolation should not delay or affect YF case surveillance reporting.

7.6 GENOMIC SEQUENCING

Genetic analysis of YF virus strains can be useful in the context of temporal and geographical details of cases and outbreaks and for molecular epidemiologic studies. In recent vaccinees (<30 days) who develop classical symptoms of YF infection, targeted sequencing or use of discriminatory RT-qPCR should aim to differentiate between infections with wild-type YF and the vaccine virus strain (93). However, genomic sequencing is not standard practice in the YF surveillance programme or GYFLaN testing algorithm, as the sequencing results do not impact case classification, or outbreak identification and response. There is no network wide YF nucleotide surveillance database, but any YF genomic data should be shared with the country surveillance and GYFLaN coordinators.

Direct sequencing of clinical samples is often unsuccessful, as most samples with detectable YF RNA by RT-qPCR have very high Ct values (Ct >30) or very low virus concentration. Virus isolation and amplification through cell culture may be needed to get a better yield for the sequencing process.

Chapter 8

Data management and reporting of laboratory results

An essential part of the work in every laboratory is to record and manage test results, QA data, and other related information that establishes the output and performance of the laboratory. Laboratory protocols should be in place to clearly outline the processes and methods for generating and maintaining records. NL test results should be promptly communicated without delays to the relevant partners. Data sharing for reporting, which would include data aggregation, is reported regularly and in standardized formats, although the frequency and way laboratory results are reported will vary according to the needs of the recipient, i.e. the submitter, local and national public health authorities, or WHO Country and Regional Offices.

The term "data management" encompasses several activities and processes and is an essential component of any disease surveillance system (28). WHO regional offices will provide details of specific data reporting requirements appropriate to the level and activities of WHO YF laboratories within each regional laboratory network (11).

Specific guidance in data management and in the preparation and distribution of reports are covered in this chapter. An overview of laboratory QA and QC, accreditation requirements, and related quality systems such as document control are described in Chapter 9. In addition to recording relevant data and results, the laboratory is responsible for the analysis and interpreting results, identifying epidemiologic patterns or trends, and preparing regular reports.

Chapter content:

- Data management goals
- Data recorded for incoming specimens (accessioning)
- Recording laboratory results
- Information and data to maintain in laboratory database or records
- Reporting to Expanded Programme on Immunization (EPI) teams and to WHO
- Data management and results reporting for specimens sent to the Regional Reference Laboratory for confirmatory testing

8.1 DATA MANAGEMENT GOALS

Maintenance of laboratory records that are accurate and relevant requires good management practices and a clear designation of responsibilities. The success or failure of any public health or disease control initiative depends on establishing and maintaining a good information exchange system, with accurate and timely data being assigned an appropriate action. The importance of good laboratory data management cannot be overstated.

To create an efficient data management system, the following operational considerations should guide decision-making:

- The meaning of the information generated;
- What information needs to be provided outside of the laboratory?
- Who needs the information?
- How frequently does the information need to be transmitted?
- How frequently is the data analysed, summarized, and distributed?
- What is the data quality? Are some data missing, dates entered incorrectly, transcribing errors? Do patient identifiers match the specimen vials?

Every laboratory will need to determine the processes and documentation required to transform data, records, and related laboratory activities into template formats standardized per Regional Yellow Fever Laboratory Network, and supported through WHO Regional Laboratory Network Coordinators. Such network-standardized templates should be in place for:

- Test results, provided in a report form, to the Expanded Programme on Immunization (EPI) and to the submitter or the organization that requested the testing;
- Annual reports or progress reports for the director or head of the institute;
- Summary reports consisting of activities and data that help justify continued support and funding

Data quality is a key responsibility of the laboratory. The documentation that is required to meet the goals above should be concise and complete, avoiding unnecessary information and excessive data entry to reduce omissions and errors. The concept definitions of the six dimensions of data quality (accuracy, reliability, precision, completeness, timeliness, integrity) are listed in Table 8.1 below.

TABLE 8.1

Data quality concept definitions. *Source: Adapted from Annex 1B in (94).*

Dimension of Data Quality	***Operational Definition***
Accuracy	Data accuracy is the degree to which data represent real-world things, events, or an agreed-upon source. Also known as validity. Accurate data are considered correct: the data measure what they are intended to measure? Accurate data minimizes errors (e.g., recording or interviewer bias, transcription error, sampling error) to a point of being negligible.
Reliability	Data are considered reliable when they are generated by a programme's information system based on protocols and procedures that do not change according to who is using them and when or how often they are used. Data should yield the same results on repeated collection, processing, storing, and display of information. Thus, data should be consistent. The data are reliable because they are measured and collected consistently.
Precision	Precision means that the data have sufficient detail. For example, an indicator may require the number or proportion of individuals who were presumptive YF cases that got tested on a certain assay, and that received their results within X number of days. An information system lacks precision in this context if it is not designed to record the total number of presumptive YF cases identified or the date of result receipt.
Completeness	Completeness means that an information system from which the results are derived is appropriately inclusive: It represents the complete list of eligible persons or units and not just a fraction of the list.
Timeliness	Data are timely when they are up to date (current) and when the information is available on time. Timeliness is affected by: (1) the rate at which the programme's information system is updated, (2) the rate of change of actual programme activities, and (3) when the information is actually used or required.
Integrity	Data have integrity when the system that is used to generate them is protected from undue pressure, deliberate bias, or manipulation for political or personal reasons.

The next step in data management is to decide how the information should be physically recorded and stored. Laboratories have traditionally maintained laboratory results in books or ledgers. These are often in the form of paper records, recorded line by line, with all the information relating to the specimen or case entered fields in specific columns, or line-listings.

Simple spreadsheet systems (using software such as Excel), reflecting the line-listing of paper records, have also been widely used to meet regional network requirements. However, while Excel is preferable to paper records, it should be noted that it requires a lot of discipline to keep the data quality at a satisfactory level over time. The file needs to be backed up frequently as it is fairly easy to accidently delete or edit multiple records unless the sheet is protected. In addition, although useful for some types of analysis, computer spreadsheets are not very easy to manipulate when using large amounts of information.

Efforts should be made to promote the use and standardization of computer record systems to meet all the reporting requirements. These systems should be widely available throughout the laboratory network and easy to use. The software and hardware selected to computerize laboratory record keeping is beyond the scope of this manual. However, the manager should seek out available options that are low cost and can be maintained through local expertise. At a minimum, the system should be user-friendly, allow rapid and accurate access to selected records, have the capability to perform simple calculations such as frequencies and time intervals, and have functions available to create tables and graphs. The format should be machine-readable, i.e., a structured format that can automatically be read and processed by a computer such as comma-separated values (CSV), JavaScript Object Notation (JSON) or Extensible Markup Language (XML). "Machine-readable format" does not include portable document format (PDF).

Data entry should include drop-down menus where feasible. Ideally, laboratory records systems should include:

- The ability to detect errors in the entered information;
- Routine backup of data;
- Routine analysis and reporting;
- Version tracking (show when a specific entry and or line-listing was edited and by whom, this will help with trouble shooting in case of a data entry error and identify the focal point in case of further need for clarification);
- An efficient way to extract the information without personalized information, ideally the ability to link it to other systems or analysis/visualization software via an API.

There are a variety of tools (e.g., Go.Data, ODK, SORMAS, EPI-Info) that can be used for outbreak investigations; for developing small to mid-sized disease surveillance systems; as analysis, visualization, and reporting components of larger systems; and in the continuing education in the science of epidemiology and public health analytical methods at schools of public health around the world. Go.Data is one such important tool that can be readily implemented by countries, supported through WHO (https://www.who.int/tools/godata).

Most recently, global efforts to develop standardized tools has generated tools for surveillance using the DHIS2 platform. Examples currently used in various countries include the DHIS2 module for Integrated Disease Surveillance Aggregate System (can be found at the following link: https://docs.dhis2.org/en/topics/metadata/disease-surveillance/ids-aggregate/version-120/design.html), and the even more recent Case Surveillance Tracker Design for vaccine preventable diseases, including YF (can be found at the following link: https://docs.dhis2.org/en/topics/metadata/disease-surveillance/vpd-case-surveillance/design.html).

In designing any recording and reporting system for laboratory results, it is essential to request input from colleagues in all areas related to disease surveillance and control so that the system is understood and functions well across departments and to higher levels of the organization. The information flow should be clearly established from one level to the next so that none of the intended recipients are missed. Information can also be "broadcast" or sent to several recipients at different levels at the same time.

Once a pattern of information flow is established, it is very important that it is followed without exception. The system should undergo review periodically to make sure that it is functioning well and determine whether improvements can be made. If any changes are made to the system, it is essential that all parties involved are informed of the changes and agree to them

8.2 DATA RECORDED FOR INCOMING SPECIMENS (ACCESSIONING)

A case investigation/laboratory request form is completed for each suspected YF case investigated at the time of specimen collection and should accompany all specimens sent to the laboratory (there are two separate forms in some YF surveillance programmes). Examples of case investigation/laboratory request forms are shown in Annexes 8.1A-C in English, Spanish, and French. Information on specimen labels must be carefully checked to ensure that it matches information on the laboratory request form. Information on the form must be complete in order to process the specimen and include:

- Epidemiology (EPID) or case number;
- Symptom onset date;
- Specimen collection date;
- YF vaccination history

The complete information that should be included can be viewed on the laboratory request form templates in Annexes 8.1A-C. The receiving laboratory should actively contact the sender if critical information is missing and inform them that without the information the specimen cannot be processed.

Upon receipt of a specimen the laboratory should record the following information in the laboratory ledger or database:

- Date and type of specimen received in laboratory;
- Specimen adequacy (e.g., volume);
- Specimen condition (e.g., container integrity, temperature, haemolysis);
- Processing steps if appropriate (e.g., elution, separation, centrifugation);
- Storage location.

Any problems or issues with the timeliness of samples or the condition or adequacy of the specimen should be communicated immediately to the EPI manager. The specimen rejection rate should be documented, for example, in "accept or reject for processing" the input should be documented after the testing has been attempted, and in case there is not sufficient amount of sample to perform additional assays.

Each specimen should be assigned a laboratory processing number, which is entered in the laboratory ledger or database, as well as on the accompanying request form and specimen vial or container. The laboratory processing number is distinct from the patient/case number EPI identification (ID) number on the case identification/laboratory request form. This may be an abbreviated version of the patient/case number or a sequential unique laboratory number. The laboratory processing number must be used on all containers, centrifuge tubes, test tubes, and vials throughout subsequent laboratory procedures. Different aliquots should also have separate numbers (may be derived from the laboratory number with records kept as to where the aliquots are stored). If possible, samples and aliquots should be labelled with a bar-code to reduce transcription errors. Each sample must have a link to the case identification number used by the country's surveillance programme. The patient/case number and laboratory processing number should both be captured in the laboratory ledger for future reference.

8.3 RECORDING LABORATORY RESULTS

As soon as test results become available and have been validated, a record should be entered into the laboratory database. Most laboratories maintain some form of digital database, often on dedicated computer servers, which are backed up frequently to avoid loss of data and to facilitate interaction with requestors and national and international databases (e.g., WHO). The results and additional information that are collected on each specimen should be entered into the record and include the following data, at a minimum:

- Case identification number;
- Laboratory processing number;
- Date of symptom/jaundice onset;
- Date of sample collection;
- Vaccination history of case;
- Travel history of case;
- Course of disease and clinical presentation (ideally, drop-down for standardization and ease of entry);
- Arrival date of the specimen into the laboratory;
- Date of test result;
- Type of assay (e.g., MAC-ELISA, rapid test, RT-qPCR, neutralization);
- Raw data (e.g., OD for MAC-ELISA, threshold cycles [Cts] for RT-qPCR, titre for neutralization assay);
- Interpretation of test results (e.g., detected/not detected, positive/negative/equivocal);
- Date result reported and to whom (requesting physician, EPI manager, WHO);
- Information (dates, volumes, tests requested, etc.) about sample referral to higher tiers such as for the case confirmation by the RRL;
- The time it took the specimen to arrive from the positive test result in the NL to the RRL;
- The time it took from the arrival of the specimen to the final test result in the RRL.

If an aliquot was forwarded to the RRL (see Section 8.6), the following information should be included in the laboratory record:

- Name of RRL;
- Purpose of referral;
- Laboratory identifier of specimen;
- Date specimen forwarded to RRL;
- Date of reception of results from RRL;
- RRL result;
- Date of reporting and to whom.

8.4 INFORMATION AND DATA TO RETAIN IN LABORATORY DATABASE OR RECORDS

Worksheets can document the details of all samples being tested and the composition of the test during any given procedure. Recording details of all variables in a particular test or assay will allow analysis and development of trends, which can aid in troubleshooting and in verifying the quality and performance of test procedures. A review of a laboratory's raw data is required during an on-site review for the WHO accreditation process (Chapter 9).

Worksheets can be a paper-based system, where variables are recorded by hand on a printed worksheet. For an electronic system, worksheets may be computer generated and may be partially generated from the programme associated with the test/assay. Patient information, such as date of symptoms onset, date of sample collection, and vaccination history should be included in the worksheet, as it will directly impact the type of test to be performed as well as the differential diagnosis. Information recorded on a worksheet should also include:

- Sample identification number;
- Sample information (sample condition, dates of symptom onset and collection);
- Type of test/assay;
- Date of test performed;
- Name of operator, name of person validating test;
- Lot numbers and expiry date of reagents/kits;
- Reagent concentration and identity of diluent(s);
- Control sample details: ID, date, lot number, reference value;
- Validity of test/assay;
- Results: raw data, calculated values/units;
- Interpretation of final result to be reported;
- Date of reporting results;
- Comments: record of any issues with assay and how they were resolved;
- Attachments: Raw data print outs, digital images if generated.

There are certain points in data handling when it is easy for transcription errors to occur, such as during manual transfer of data from a patient history form to worksheets or databases, keyboard data entry into a computerized information system, or data entry from worksheets to reports. The laboratory should put processes in place to safeguard against errors at these points. Formal checking processes are necessary to ensure the accuracy of data recording and transmission of handwritten or keyed information. One example of a simple checking process is to always have two people review data transcription to verify its accuracy.

A common error occurs when the date format varies from a referring laboratory. Always request countries to specify the format their dates are recorded in, for example DD/MM/YYYY. Although some commonly used databases can use drop-down menus or be programmed to allow automatic conversion of date formats, error checking is still advisable.

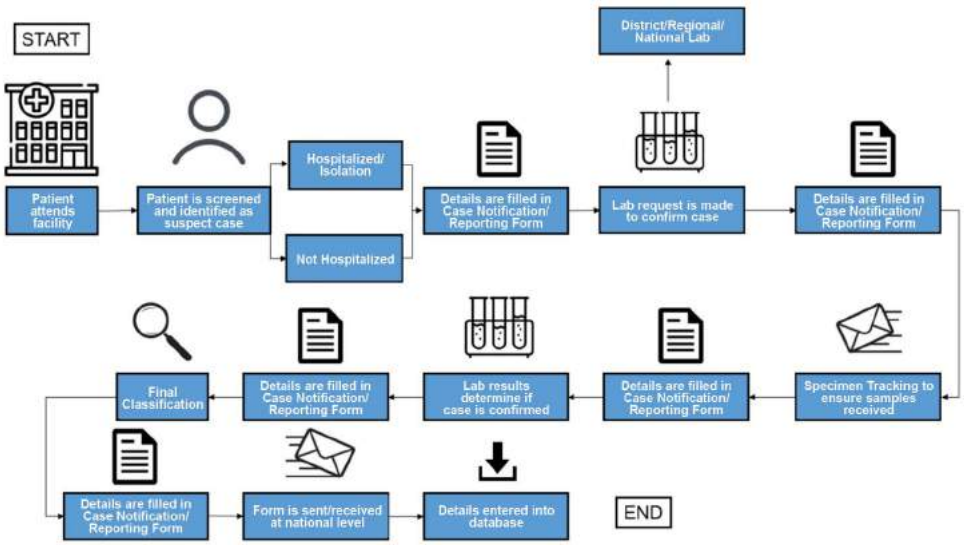
8.5 REPORTING TO EPI TEAMS AND TO WHO

Rapid diagnosis and reporting of YF cases are critical for triggering effective responses to contain outbreaks; one of the EYE strategic goals. Although the details of how and when NLs report results to EPI managers should be arranged locally, the EYE Strategy monitoring and evaluation indicators are as follows (28, 40, 95):

- In YF high-risk countries, samples are sent to the NL within 3 days following sample collection;
- All results from suspected cases should be reported within a short turnaround time (3-7 days) as established by the national or regional guidelines;
- In the absence of recent cases, a positive result should be reported within 24 hours.

Whereas results necessary for appropriate case follow-up and outbreak response will be of an urgent nature, data generated to guide preventive immunization activities should be compiled, analysed, and transmitted according to the time frame established for the programme (Figure 8.1). Monthly exchange of information may be helpful to the programme and to the laboratory for the purpose of comparing and reviewing the completeness of data for suspected cases. In addition, feedback related to the adequacy of samples and any problems encountered can help identify issues and challenges to timeliness of specimen arrival and reporting. All reports should be available to the EPI managers on request.

FIGURE 8.1 **Workflow for all diseases, example illustrating the processes resulting in all relevant case forms being entered into a centralized level case-based surveillance data management system, based on multiple sources of data (e.g., clinical information captured at the facility, laboratory diagnosis, additional details that may be completed by surveillance officers during case investigation).**



This workflow example is closely aligned to the existing centralized reporting system using Epi-Info. Source: modified from (96) VPD Case Surveillance Tracker Design (2022) https://docs.dhis2.org/en/topics/metadata/disease-surveillance/vpd-case-surveillance/design.html

All NLs are requested to provide a report of results to WHO. This information is used to update country summaries, monitor laboratory performance, and coordinate international agency activity. Data provided in the reports is essential to coordinate the surveillance programme as a whole. It should be a priority activity of all laboratories in the network to send reports in a timely and accurate manner.

Because of the amount of data involved and the time required to analyse the information it is essential that laboratories processing more than 100 specimens a year provide their reports of data in machine-readable format. WHO Headquarters can provide a set of laboratory data management programmes suitable for most of the GYFLaN laboratories.

8.6 DATA MANAGEMENT AND RESULTS REPORTING FOR SPECIMENS SENT TO THE RRL FOR CONFIRMATORY TESTING

The RRLs conduct further testing of specimens submitted from NLs to confirm or rule out YF virus infections (Chapter 4 and Chapter 7). Final RRL test results are reported to the surveillance programme to guide final case classification, as shown in Figure 8.1.

The NL is responsible for collecting and documenting all critical patient and specimen information on the case identification form prior to referral to the RRL, as well as properly documenting it in their national database. Before shipping samples to the RRL for further investigation, the RRL should be contacted to provide relevant patient clinical information, the tests conducted at the NL and the diagnostic tests requested (Chapter 3). An example of a specimen referral form is shown in Annex 8.2 (also available at *https://www.who.int/publications/i/item/9789240084476*).

The RRL also collates and analyses information originating from the NLs such as:

- The number of samples received at the RRL from each national YF NL;
- The types of testing done at the NL and test results for each specimen received at the RRL;
- The date the sample was received at the RRL;
- The types of testing done at the RRL and the results;
- Dates of completion of each of the tests on the specimen;
- Challenges and problems the RRL had in receiving specimens from NLs and testing those specimens (e.g., inadequate sample volume, poor sample condition, missing or incomplete sample referral forms) with sufficient detail to guide future corrective action.

The RRL is responsible for timely reporting of final test results to the country surveillance programmes and WHO Country Offices and Headquarters to guide case classification and for further investigation, if needed.

Chapter 9

Quality assurance, quality control, and assessment of laboratory performance

The success of YF immunization activities can only be determined through qualified laboratory-based surveillance activities. Decisions for outbreak control and public health measures rely on accurate laboratory testing. The laboratories in the GYFLaN must meet accreditation or equivalent standards and must perform all the tests according to SOPs following QA criteria.

Periodic review of procedures and processes drive continual improvement. By following procedures that have been established to minimize errors and generate the appropriate documentation for subsequent review and critical evaluation of laboratory processes, the laboratory can achieve the highest possible level of accuracy.

It is becoming more common for risk management principles to be applied to clinical laboratory environments (43, 97). In risk analysis, the testing process is mapped as individual steps and any possible weaknesses or hazards are identified. Then processes are formulated to avoid errors or inconsistencies at each step. This analysis of the process, from pre-analytical to post-analytical phase, is customized for a specific laboratory test. The potential source of error or hazard at each step is identified and a plan is formulated to mitigate the risks of error.

By adopting a QMS model, activities along the path of workflow across departments are systematically analysed and quality policies can be built into all processes in a stepwise, and eventually, as an integrated whole. Coordination of policies across departments with the aim of quality improvement can only be achieved with a dedicated QMS.

Chapter content:

- The establishment and benefits of a quality management system (QMS)
- Technical elements of QMS
- Laboratory management structure
- Quality assurance
- Laboratory facilities, equipment, and supplies
- Document control
- WHO assessment and accreditation
- External laboratory quality management and accreditation programs

9.1 THE ESTABLISHMENT AND BENEFITS OF A QUALITY MANAGEMENT SYSTEM

Implementing a quality plan or a QMS is essential to integrate all aspects of laboratory processes and management. A well-functioning QMS not only oversees QA activities but also encompasses all operations within an organization that affect laboratory performance. Setting up a QMS in a laboratory requires an analysis of the organizational structure, personnel responsibilities, procedures, processes, and resources to achieve the highest accuracy and reliability possible. By adopting a QMS model, activities along the path of workflow across departments are systematically analysed. Quality policies can be integrated into all processes in a stepwise manner and quality improvement can be built into the system as a whole. Coordination of policies across departments with the aim of quality improvement can only be achieved with a dedicated QMS.

It is the responsibility of the head of the laboratory or designated quality manager to establish, implement, and ensure compliance of the QMS requirements. However, the success of a QMS is the responsibility of all personnel contributing to the laboratory work. A strong supporting organizational structure with the committed management is crucial. It is recommended that a dedicated quality manager coordinate all the activities of implementation and documentation.

When all the laboratory procedures and processes are organized into a workable structure, the opportunity to ensure that all components are appropriately managed is increased. A well-functioning QMS enables standardization to 'best practices' in all areas of an organization that influence quality. This comprehensive approach directs and substantially controls the quality of the organization.

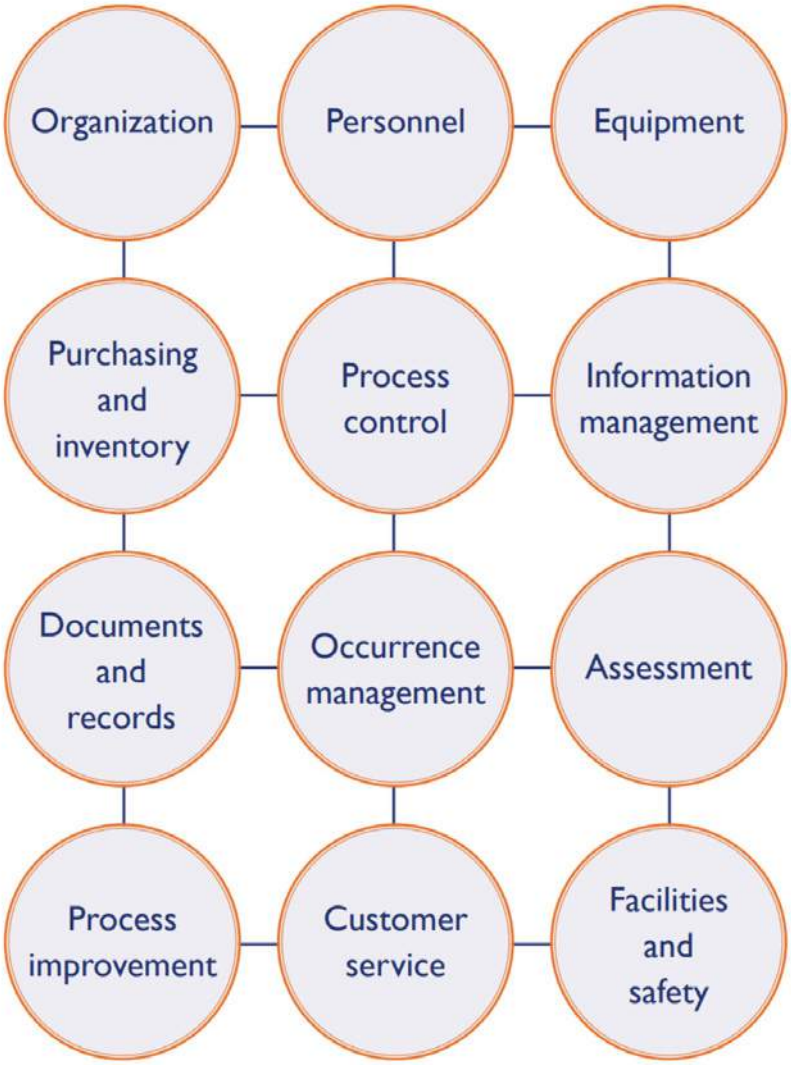
Implementing a QMS can be initiated at different starting places, adding on building blocks which are continually refined and expanded. There are various online resources to guide the laboratory in developing and implementing a QMS:

9.1.1 THE WHO LABORATORY QUALITY MANAGEMENT SYSTEM (LQMS) HANDBOOK

This handbook (https://www.who.int/publications/i/item/9789241548274) (90) was developed through a collaboration between the WHO Lyon Office for National Epidemic Preparedness and Response, the US Centers for Disease Control and Prevention (CDC) Division of Laboratory Systems, and the Clinical and Laboratory Standards Institute (CLSI). It is based on training sessions and modules provided by the CDC and WHO in more than 25 countries, and on guidelines for implementation of International Standards Organization (ISO) 15189 in diagnostic laboratories, developed by CLSI.

The LQMS handbook describes 12 building blocks for planning and implementing a QMS. These coordinated activities, or quality system essentials (QSE) are often identified as the cornerstone for establishing a QMS (Figure 9.1).

FIGURE 9.1 **The 12 quality system essentials following the framework developed by Clinical and Laboratory Standards Institute.**



Source: taken from (90) World Health Organization (WHO), Laboratory quality management system: handbook (2011), (available at https://www.who.int/publications/i/item/9789241548274).

9.1.2 THE LABORATORY QUALITY STEPWISE IMPLEMENTATION (LQSI) TOOL

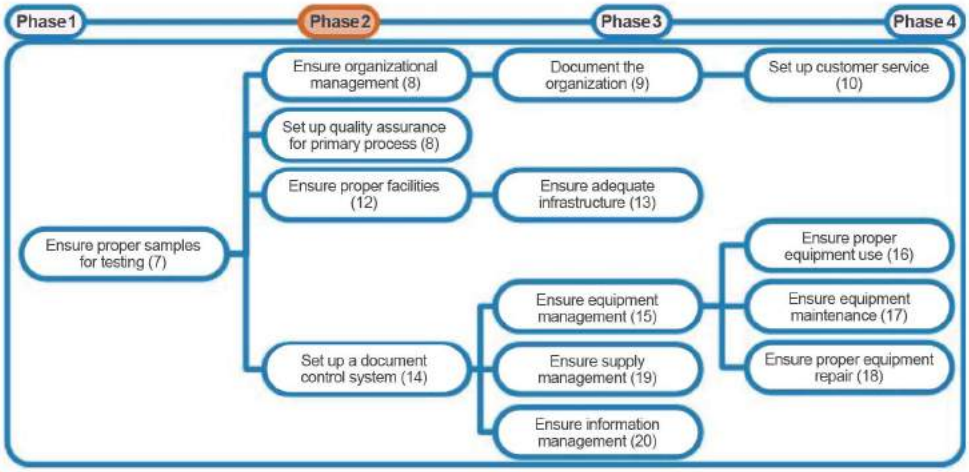
The LQSI tool website (https://extranet.who.int/lqsi/content/homepage) (98) provides a practical guide for laboratories to implement a QMS by following a stepwise plan in compliance with ISO 15189. It contains the same activities as the QSE framework, but, whereas the QSE framework shows an overview of activities to be completed per QSE, the activities in the LQSI are structured in such a way that they form a sequence of events for day-to-day implementation. Note that it is not a requirement to adhere strictly to the provided roadmap; it is just an indication of a potential proper sequence of activities for day-to-day implementation. When another sequence better suits the local situation in the laboratory, the optimal sequence can be modified according to the needs of the laboratory. A tutorial on how to use the WHO: LQSI tool can be found on YouTube at: https://youtu.be/V_7ofaDtMBQ.

To implement the QMS in a logical way, the activities are divided over four phases of implementation, with each phase having a specific focus (Figure 9.2).

- **Phase 1**: Ensuring that the primary process of the laboratory operates correctly and safely;
- **Phase 2:** Controlling and assuring quality and creating traceability;
- **Phase 3:** Ensuring proper management, leadership, and organization;
- **Phase 4:** Creating continuous improvement and preparing for accreditation.

The tool is constructed such that, even when a laboratory does not reach full implementation of the QMS, it has already improved its quality service provision from the moment it completes phase 1, and as such has benefited already.

FIGURE 9.2 **Stepwise plan to implement a QMS, that will contain the same activities as the quality system essentials (QSE) framework, but whereas the QSE framework shows an overview of activities to be completed per QSE, here the activities are structured in such a way that they form a sequence of events for day-to-day implementation.**



Source: taken from (98) World Health Organization (WHO), Laboratory Quality Stepwise Implementation tool, (available at https://extranet.who.int/lqsi/content/homepage).

9.1.3 THE WHO LABORATORY BIOSAFETY MANUAL, FOURTH EDITION (LBM4)

The LBM4 (https://www.who.int/publications/i/item/9789240011311) (43) provides guidance in good laboratory practice; biosafety, including a national oversight system; training; best working practices; and a risk assessment framework to promote a responsible safety culture that builds country capacity and complies with the IHR (43). With particular emphasis on evidence, local biological risk assessment, and personnel competence, the LBM4 presents a novel approach that allows each facility to take a feasible and most effective combination of risk control measures. This technology-neutral approach will help attain much desired equitable, adequate, and sustainable access to necessary laboratory services and life science research across all countries, without compromising safety. Subject-specific monographs include:

- Risk assessment;
- Laboratory design and maintenance;
- Biological safety cabinets and other primary containment devices;
- PPE;
- Decontamination and waste management;
- Biosafety programme management;
- Outbreak preparedness and resilience.

9.2 TECHNICAL ELEMENTS OF QMS

There are a range of activities within QMS that relate directly to the processes that ensure reliable laboratory results and prevention of errors. As shown in Phase 2 in the LQSI framework (Figure 9.2), the four technical elements of QMS are organizational management (Section 9.3); QA and QC processes (Section 9.4); facilities, equipment, and supplies management (Section 9.5); and document control systems (Section 9.6) (98).

9.3 LABORATORY MANAGEMENT STRUCTURE

Good quality laboratory outputs depend on qualified, motivated staff. All personnel in the laboratory must have a full understanding of QMS and the importance of teamwork in successfully implementing the QMS. An organizational chart should be prepared and distributed that clearly shows the appropriate lines of authority and the functions and responsibilities of each person. A YF laboratory should have staff with qualifications and experience enabling them to carry out all the required functions and responsibilities safely and accurately. It is essential that every person working in the laboratory has undergone appropriate orientation and training in the functioning and operation of the laboratory.

Human resource staff and the laboratory manager should regularly review training needs and ensure that technical and scientific staff receive training according to those identified needs (see in LQSI tool (98): https://extranet.who.int/lqsi/content/perform-staff-competency-assessments). The training offered to staff should directly contribute to the success of the objectives of the QMS. A continuing education programme must be developed which includes on-site as well as external training. The staff training programme should be formalized into records that are maintained as part of the required documentation.

Opportunities should be provided for staff to acquire skills outside their own discipline or cross training to allow for flexibility in shifting or reassigning personnel if needed.
Training can be in several formats:

- one-on-one or group;
- hands-on or theoretical;
- on-site or online;
- institutional tutors or external tutors.

The general topics of training should include development of protocols and procedures, QA/QC, and biosafety. WHO periodically holds hands-on training workshops for the performance of specific tests and every opportunity should be taken for the persons routinely performing these tests to attend the training workshops. RRLs and GSLs are also instrumental in providing on-site training and learning opportunities to the NLs in their constituencies. Records of staff education and training achievements should be maintained.

All new staff should be acquainted with the risks involved in working in a YF laboratory before commencing with any activities and adequate safety training should be provided. The director is responsible for implementation of and compliance with the provisions of the LBM4. Vaccination against Hepatitis B and YF is mandatory for laboratory staff working with patient specimens. Safe handling of blood products and precautions for opening packages of clinical specimens is provided in Chapter 3.

Mechanisms should be in place for regular meetings for information exchange and on-site training, which should include periodic updating of staff on technical issues. Arrangements for staff absences, either scheduled leave or due to illness, are required to ensure services are maintained. Contingency plans for increased workload, such as during outbreaks, should be developed with anticipated staff and resource needs determined and possible solutions identified.

Assessment of an employee's performance in the laboratory should occur periodically. Refresher courses should be required to ensure technical skills and knowledge of and adherence to safety rules and policies are maintained. Positive feedback, as well as suggestions for improvement, should be provided. All identified personnel problems should be addressed with the employee when they occur, so that they can correct any issue before it can have a major impact.

9.4 QUALITY ASSURANCE

Laboratory QA is the overall programme that encompasses all procedures and processes in the laboratory that affect the output of the laboratory processes (SOPs), QC, the interpretation of results, and timely reporting. The goal of QA is to assure that procedures are performed consistently and that errors are prevented. QA is a systematic approach to define the best practices for all processes associated with the functioning of the laboratory that can affect the reported results. QA establishes procedures to review performance and workflow procedures and directs both proactive and corrective proactive measures to improve laboratory quality. A combination of internal quality assessments (IQAs), performed within the laboratory using its own staff, and EQAs, conducted by a group or agency outside the laboratory, is the recommended approach to ensuring quality. Laboratory quality standards are an important part of the assessment process, serving as benchmarks for the laboratory. Performance monitoring should be a continuous process in each laboratory. Important indicators of performance include the timeliness of reporting results and summary reports of laboratory data and the concordance rates for QC testing and EQA testing. Improvement in the quality can be achieved by documentation, adherence to the SOPs, and tools to assess laboratory output and performance.

9.4.1 STANDARD OPERATING PROCEDURES

SOPs are documents that contain written step-by-step instructions that laboratory staff should follow when performing a procedure. SOPs should be prepared by experienced, technical staff in the laboratory, revised by their immediate supervisor as needed, and approved by the head of the laboratory. A laboratory will have an SOP for each process (such as accessioning samples) and all laboratory tests that are conducted in the laboratory.

SOPs should be prepared for general procedures as indicated:

- **General:** preparation of SOPs, correction of notes and documentation, and preparation of protocols, and reports;
- **Test systems:** preparation and maintenance of work areas;
- **Laboratory operations:** receipt, recording, and labelling of specimens; sterilization of material; storage of samples; labelling of materials and reagents; and preparation of media and solutions;
- **Relating to staff:** training, handling of hazardous materials, laboratory safety, staffing of each laboratory subunit;
- **Reference materials:** identification, characterization, handling, reception, storage, and use;
- **Archives:** maintenance, distribution, and updating;
- **Equipment:** regular calibration, cleaning, and preventive maintenance;
- **Test methods:** methods for processing and testing samples sent to a laboratory

Guidance on developing specific SOPs for sample collection, handling, and reception; instrument maintenance (including one for biosafety cabinets); use of disinfectants; and inventory and stock management can be found in the WHO LQSI tool by typing "SOP" in the "search-this-site" box (https://extranet.who.int/lqsi/).

See for example:

- How to Write a Master SOP (https://extranet.who.int/lqsi/content/write-master-sop)
- Template Master SOP (https://extranet.who.int/lqsi/content/template-master-sop)
- Example of Procedure SOP (https://extranet.who.int/lqsi/content/example-procedure-sop)
- Example of Analysis SOP (https://extranet.who.int/lqsi/content/example-analysis-sop)
- Example of Equipment SOP (https://extranet.who.int/lqsi/content/example-equipment-sop)
- How to Write an SOP for Recording, Reporting and Archiving of Results (https://extranet.who.int/lqsi/content/write-sop-recording-reporting-and-archiving-results)
- How to Write an SOP for EQA procedures (https://extranet.who.int/lqsi/content/write-sop-external-quality-assessment-procedures)

The goal of an SOP is to provide standardized, detailed instructions of a procedure to ensure that all staff members understand and perform the activity or test in a uniform manner to ensure uniform outputs. An experienced technician should first train staff who do not normally perform the procedure, before that staff member performs the test according to the SOP. It should be emphasized that all laboratory staff must follow the SOPs.

It is important that SOPs are written in a clear and concise manner that eliminates ambiguity. All steps in the procedure should be clearly described to avoid potential "error points". These methods should closely follow the WHO-recommended procedures.

Each SOP should have a standard format and the following information on each page:

- logo and name of organization;
- department or unit issuing the procedure;
- title and version number;

- signature of person who drew up the procedure, with date (day, month, and year);
- signature of person who reviewed it, with date (day, month, and year);
- signature of person who authorized it, with date (day, month, and year);
- duration of validity;
- date of review;
- code;
- page number and total number of pages in document.

Any method or steps that undergo changes from the previous SOP, no matter how minor or major, must be validated before being put into practice. Changes in SOPs should be implemented by specialized technical staff in the laboratory, revised by their immediate supervisors, and approved by the head of the laboratory. There should be a version numbering system with registration of changes made and date of validation. The following characteristics should be compared with those of the previous version:

- **Accuracy:** the degree of correlation with the value achieved by the previous method;
- **Precision:** the variation in the results as represented by the standard deviation or the coefficient of variation;
- **Sensitivity:** the capacity of the test procedure to record small variations between concentrations;
- **Reproducibility:** the precision of the procedure when it is performed under different conditions;
- **Specificity:** the degree of uniformity of the response to the substance in question;
- **Robustness:** the ability to provide accurate and precise results under a variety of conditions

The SOPs for laboratory tests and assays must be sensitive enough for detecting errors, and direct the steps and documentation required for timely correction of detected errors or non-conformance to SOPs. Reviews of non-conforming events, corrective action taken, and the effectiveness of that corrective action to address the problem are important for ongoing quality improvement. IQAs or on-site audits should be conducted to identify non-compliance with SOPs or deficiencies in the SOPs that need attention.

9.4.2 QUALITY CONTROL

Whereas QA includes activities or measures taken to prevent errors, a QC programme is a systematic process that monitors the validity of an assay by incorporating a method to measure accuracy and precision and to detect errors. QC measures must be included in each assay to verify that the complete analytical workflow is conducted properly and are completed prior to the release of the test result. The QC protocols include guidelines for addressing any detected errors (troubleshooting) and specifies the corrective action and any remedial action that may be required. Remedial action, or remediation, addresses any consequences that may result from an error.

The use of appropriate positive or negative controls is a fundamental component for conducting the analytical phase of testing. If possible, internal and external controls should be used for validation and to monitor test performance. QC materials such as kit controls and in-house controls are included in all runs to quantify the normal variability of an assay by establishing a normal range. The aim is to include controls that are sufficient to differentiate between normal variation and technical errors. QC materials should be available in sufficient quantities to minimize the number of times that control ranges must be prepared. For more information, refer to the WHO QMS Handbook, Section 7-3, Establishing the value range for the control material from (90).

For IgM testing, statistical analysis can be used for the daily monitoring of values obtained from in-house controls samples. Use of a Levey-Jennings chart is recommended to visualize the performance range of an assay and to monitor trends or variation in assay performance (Annex 9.1). If controls are out-of-range, corrective action and troubleshooting should be undertaken and the problem should be resolved, and the test repeated before reporting assay results. Westgard rules can be applied to determine whether the results from the in-house controls sample are valid and sample results can be reported, or if they need to be rerun (Annex 9.1). Westgard rules are helpful to avoid rejecting runs that may be acceptable for a particular assay and can be used to detect both random and systematic errors. When control sample values are outside the acceptable range, troubleshooting must be undertaken to identify the problem. All variables should be checked systematically and preferably only one remedial action at a time should be undertaken before repeating the assay. All tests with control values out-of-range, inconsistent results, or any form of equipment failure should be documented, with the original problem, the problem resolution, and the remedial action clearly noted and brought to the attention of the QA manager and the laboratory director.

9.4.3 QUALITY ASSESSMENTS

Internal quality assessments

(1) QC testing: QC testing or retesting of a subset of specimens submitted to the laboratory for YF testing serves as an internal measure of a laboratory's performance over a period of time. Specimens selected for validation should be representative of all results determined by the NL (positive, negative, and equivocal) and be chronologically and geographically representative of the country and selected from multiple outbreaks, if applicable.

(2) Monitoring laboratory output and performance: The assessment process is a tool for examining laboratory performance and comparing it to standards, benchmarks, or the performance of other laboratories. All laboratories should have regular and thorough processes for monitoring the accuracy and performance of their procedures and the persons performing them.

In performing an assay, one of the most common sources of error, and usually among the easiest to address, is the operator. Poor performance may not be due to the operator's skill level or failure to follow the SOP correctly, but rather due to other factors. Among the potential factors that can affect operator performance:

- Time pressure to complete a heavy workload;
- Distractions from phone calls or colleagues interrupting workflow;
- Incorrectly labelled samples or selection of incorrect samples.

It is critical that a supervisor or other highly experienced staff member review the performance of every assay and confirm that all the validity criteria are met and that no transcription errors have occurred before signing the worksheet. Test results should not be reported without supervisory sign-off confirming that the assay has been performed according to accepted criteria. Errors may also be due to poorly or incorrectly written procedures or SOPs and failed or poorly calibrated equipment.

External quality assessment

QA/assessment is a continual process and demonstrating the quality of individual laboratories in the GYFLaN is critical, specifically to maintain confidence by stakeholders and colleagues in the surveillance programme that the laboratories are providing results with high accuracy. EQA refers to a system that checks the laboratory's performance through an external, usually reference, facility. The GYFLaN YF EQA programme, coordinated by WHO, is comprised of the following activities (44):

1. Proficiency testing.
2. QC retesting.
3. On-site evaluation.

(1) Proficiency testing: In the GYFLaN YF EQA programme, the RRL or any another reference laboratory sends a panel of samples that were pretested at the YF NLs. The samples should be tested according to the routine diagnostic laboratory procedure. The raw data and test results are reported back to the EQA coordinator who then compares the results with the values from the pretesting. The EQA coordinator analyses the results and scores the proficiency of the NL.

Incorrect results indicate errors or weaknesses in the NL QA system, which must be further investigated, addressed and eliminated. Although proficiency panels provide only a snapshot for QA, the results provide an assessment tool that has proven to be valuable for the individual laboratory performance and for aggregate laboratory network evaluation. In addition, the assays and methods that may vary across laboratories can be compared through the distribution and testing of a global proficiency panel. In this way, inconsistencies with assays used for YF surveillance in individual YF laboratories can be detected.

The performance of each laboratory is assessed according to the concordance of expected results, completeness of data provided, and timeliness of the results transmitted to the EQA coordinator. Points are deducted if incorrect sample results are reported, which may be due to an error in interpretation or in the transcription of results. In addition, deductions to the final score will occur if kit or assay information, validation data, and details of any in-house control sample used are not provided or not complete. A score of <90% is considered a red flag and requires an immediate plan for improving assay performance. Continued poor test performance will have a negative impact on the laboratory's accreditation status. Scores below 80% require urgent action to resolve any deficiencies in the laboratory or in the performance of their assays. The details of the proficiency programmes for YF IgM and for molecular testing available for laboratories in GYFLaN laboratories are provided below.

YF serological proficiency panels:

Proficiency programmes for YF IgM and neutralization testing within the GYFLaN have been established by WHO. The specimen panels consist of serum samples that have been thoroughly evaluated prior to being included in the panel. All laboratories in the GYFLaN that receive reagents for the IgM test are required to participate in the IgM proficiency testing programme. Reference laboratories with capacity to conduct neutralization assays must participate in the WHO plaque reduction neutralization EQA programme as part of their responsibilities. Laboratories test the panels as routine samples and report detailed results of the samples as well as assay validation data within the prescribed timelines.

YF nucleic acid detection proficiency panel:

The WHO programme for molecular external quality assurance (mEQA) for the YF laboratories in the GYFLaN was initiated in 2011 in conjunction with the Robert Koch Institute, Berlin, Germany (99). The mEQA panels consist of serum specimens spiked with various concentrations of YF virus from cell cultures and lyophilized for ease of transport. Laboratories must reconstitute the samples and extract and elute the RNA correctly prior to conducting the RT-qPCR test. To achieve the maximum points, the reported results should demonstrate:

- Correct identification of YF RNA from all positive samples;
- No false positive results from the negative samples;
- Adequate positive and negative controls included in RT-qPCR reactions;
- Ct values that fall within an expected range, based on reference testing at the laboratory preparing the panels.

All network laboratories performing molecular techniques for YF surveillance must achieve a passing score in the annual mEQA so that molecular results reported to the surveillance programme can be accepted. Evidence of performance issues in individual laboratories will require the development of an action plan to address these issues and successful completion of a subsequent mEQA panel before routine molecular testing can proceed.

(2) QC retesting: Retesting and validating a subset of samples tested in the NL by a designated reference laboratory provides an appropriate external measure of a laboratory's performance over a much longer period of time. Specimens sent from the NL for QC testing should be representative of all results determined by the NL (positive, negative, and equivocal), chronologically and geographically representative of the country, and selected from multiple outbreaks, if applicable. The proportion of specimens sent to the RRL for QC testing is dependent on the quality of the laboratory and may range from 10%-100%, with the lower range for a fully accredited laboratory and 100% for a laboratory which has failed accreditation. The proposed list of selected samples (number and distribution) should be shared with the RLC and RRL director for endorsement before being sent to the RRL.

As with any shipment of samples, the sending laboratory should communicate with the receiving laboratory to determine the optimum time to ship the specimens, and by which means. A minimum volume of 0.5 ml should be sent to ensure that the RRL has an adequate sample to undertake and complete all the tests required (Chapter 3).

Concordance of the final results should be high, with the exception of results in the equivocal range or for positive and negative samples with test values close to the cut-offs. Although a score of 90% is a passing score for confirmatory testing, laboratories should carefully review every discordant result and try to resolve any issues. Any discordant result should be retested by the NL and if the discordance remains, the reference laboratory should consult with the RLC and NL director to identify a course of action and identify possible causes. Careful documentation of the discordant result and the process to resolve it should be retained, as this will be reviewed at the time of the on-site accreditation review.

(3) On-site review: On-site evaluations have proven to be useful in NLs lacking competency or proficiency. Real-time observation and analysis of the laboratory activities allows for troubleshooting, technical assistance, and training to improve laboratory performance. On-site reviews can be combined with on-site reviews of other WHO programmes (e.g., measles/rubella).

9.5 LABORATORY FACILITIES, EQUIPMENT, AND SUPPLIES

9.5.1 LABORATORY SPACE

The workspace in the laboratory should be allocated to provide adequate space and separation as required for activities performed. In addition, attention must be given to elements of the laboratory environment that can be adjusted to allow for safe performance of laboratory work and for cleaning, disinfecting, and maintenance. Walls, ceilings, and floors should be smooth, easy to clean, impermeable to liquids, and resistant to the chemicals and disinfectants normally used in the laboratory. Bench tops should be impervious to water and resistant to disinfectants, acids, alkalis, organic solvents, and moderate heat. Illumination should be adequate for all activities.

Laboratory furniture should be sturdy and under bench storage cabinets and equipment should be accessible for cleaning. Ideally, storage space for supplies in use should be available and adequate to prevent clutter on bench tops and in aisles. Additional long-term storage space, located outside the laboratory working areas, should also be provided. There should be a clear separation of infectious and clean areas with designated, separate areas for office work and eating or drinking.

Microbiological containment should be appropriate for the levels of risk of infection of the potential pathogens in the material being worked with by the laboratory technician. Areas where the risk of contamination of microorganisms or PCR products is high should be isolated from areas that could be adversely impacted. Use of colour-coded gowns, pipettes, and other mobile equipment or supplies for each specialized area enhances the chances that these are not used inappropriately in clean areas. Infectious material should be stored appropriately in separate containments.

Additional important aspects of laboratory design are provided in the LQSI tool (https://extranet.who.int/lqsi/node/107) and the LBM4 (https://www.who.int/publications/i/item/9789240011311) (43).

9.5.2 LABORATORY EQUIPMENT

A variety of equipment is utilized in the laboratory, and all must function properly. Choosing the right equipment, installing it correctly, ensuring that it works properly, and having a system for maintenance are all necessary for QA. Guidance on equipment maintenance can be found in the WHO Maintenance manual for laboratory equipment, second edition: (https://apps.who.int/iris/bitstream/handle/10665/43835/9789241596350_eng_low.pdf) (100). Setting up equipment maintenance and calibration programmes can be accessed on the LQSI tool at https://extranet.who.int/lqsi/activities/2/34. The laboratory should have a list of equipment and instruments indicating for each item:

- Name and location of equipment;
- Brand;
- Donor or supplier;
- Maintenance company;
- Maintenance schedule;
- Inventory number;
- Serial number;
- Model and year;
- Location;
- Date of purchase or receipt from donor;
- Date of first use;
- Copy of manufacturer's handbook.

Equipment such as pipettes, incubators, refrigerators, freezers, thermometers, and thermocyclers are essential components for generating accurate test results. Failure or non-compliance of the equipment can be the cause of assay failure resulting in invalidated runs. It is essential that regular calibration and preventive maintenance be undertaken. Often the equipment manufacturer will provide a user manual that includes instructions and a suggested schedule for equipment maintenance and/or calibration. Following the manufacturer's recommendations will reduce failure or non-compliance.

All equipment should have clear and accessible documentation of the processes required for calibration, maintenance, and performance recording. Refrigerators, freezers, and incubators should be monitored continually either through once or twice daily manual temperature recording or a continuous electronic monitoring system with out-of-range alarms established. SOPs should be written for each instrument that cover basic operation and maintenance procedures. Detailed logbooks should be maintained with documentation of preventive maintenance, non-routine maintenance, and repairs. An example of an equipment maintenance log sheet can be accessed in the LQSI tool (98) (https://extranet.who.int/lqsi/content/develop-maintenance-and-usage-log-sheetslogbooks-each-piece-equipment).

9.5.3 REAGENTS AND SUPPLIES

All reagents should be inspected to ensure that the seals are intact upon receipt into the stockroom or when distributed to the laboratory. These inspections should be recorded with the initials of the person responsible for the inspection and the date on the label. Expiration dates should be recorded and monitored. Reagents that are mixed or diluted in the laboratory should be prepared in conformity with written SOPs.

At least a six months' reserve stock of reagents and supplies should be held in the laboratory at all times. Reagents should be ordered 6 to 12 months ahead of estimated needs, given the long delivery times and difficulty of transporting reagents and supplies to some countries, although predicting the utilization of reagents can be challenging due to the seasonality and epidemiology of YF disease. An outbreak requiring the immediate testing of hundreds of samples can rapidly consume current stocks of reagents and require rapid revision of forecasted needs.

Managing stocks of reagents requires regular inventory checks at scheduled intervals to record stock utilization and balances. This is especially important when supplies are ordered from international suppliers with the need to factor in long and unpredictable lead times for shipping and delivery. Some WHO regional offices however have the capacity to hold reserve stocks of reagents should a large outbreak occur. However, the need for regular communication of reagent stock levels to the regional coordinators is vital.

It is recommended that an inventory system is maintained for supplies. A central inventory or logbook of reagents should be kept containing the following:

- Name of reagent;
- Supplier (origin);
- Lot number;
- Date of receipt;
- Expiration date, where applicable;
- Place and conditions of storage (with corresponding SOP);
- Date of analysis to determine whether it complies with stipulated requirements;
- Quantity of material, with dynamic and trigger points for replenishing.

The inventory should contain all the information relating to the properties of the items. All staff should be aware of the need to complete an inventory update as and when they use or replenish supplies. However, overall responsibility for managing the inventory should be in the hands of the laboratory manager or a designated staff member.

9.6 DOCUMENT CONTROL

The product of the laboratory is information, primarily in the form of test reporting. Information (data) needs to be carefully managed to ensure accuracy and confidentiality, as well as accessibility to the laboratory staff and to the health care providers. Records must be meticulously entered and maintained so that the data is accurate and accessible. Information may be managed and conveyed with either paper systems or with computers; both are discussed, along with the use of worksheets, in Chapter 8.

It is important to address both the use and maintenance of documents and records. Documents, by definition, require updating. A system must be established for managing them so that current versions are always available. Documents such as SOPs are easier to manage and update by using standardized formats and a document numbering system. Even without a QMS in place, a document management system can be developed using the LQSI tool on setting up a document control system (https://extranet.who.int/lqsi/activities/2/33) as well as in Section 16.5 of the WHO Handbook, Laboratory QMS (90).

Documenting QA activities not only leads to improved accuracy but also demonstrates the quality of the results generated by the laboratory. The performance of any assay can be influenced by a multitude of factors, including the lot-to-lot variability of microplates and reagents, and the performance of the components or reagents in the assay. External factors, such as the operator, incubation time and temperature, and the accuracy of delivery devices must also be considered. For this reason, it is critical to maintain equipment maintenance records, pipette calibration certifications, and logbooks for daily temperature measurements of incubators/refrigerators/freezers to avoid variation due to in-process problems. In addition, these records provide evidence of adherence to QA procedures that are assessed during WHO evaluations as well as internal audits.

9.7 WHO ASSESSMENT AND ACCREDITATION

Accreditation provides documentation of the laboratory's qualifications and capacity to detect, identify, and promptly report YF positive samples to the national surveillance programme and the regional and global WHO programmes. The accreditation process also provides a learning opportunity and serves as a mechanism to identify resource and training needs. The WHO YF accreditation programme was established based on the polio and measles laboratory networks and consists of an assessment or external audit of laboratories within the network (44). The YF accreditation document, aligned with the other vaccine preventable disease surveillance checklists, is comprised of eight programmatic criteria that need to be met for accreditation in addition to the checklist minimal criteria (>80% in scoring points + all checklist mandatory criteria fully met). The checklist contains additional operational criteria to include current and expanded practices as the laboratory capacity increases. The WHO YF laboratory accreditation and annual review checklists are shown in Annex 9.2.

9.7.1 PROGRAMMATIC CRITERIA

The eight programmatic criteria are the following:

1. **The accuracy of YF and YF IgM detection is at least 90%.** Accuracy is determined by the agreement in test results on sera submitted by the NL or SNL to the supervisory laboratory (RRL or NL, respectively) for QC testing during the 12-month review period.
2. **IgM tests should be performed on at least 50 specimens annually.** To maintain skills in performing serological assays, virus laboratories should maintain appropriate reagents and assay kits to have capacity to test continually throughout the year. To maintain expertise, it is required that laboratories test a minimum of 50 specimens for IgM detection annually, spread across the year. Where surveillance specimens are insufficient to meet this indicator then the lab may use PC specimens for completing the minimum requirement.
3. **Internal QC procedures are implemented.** Appropriate QC procedures are in place and followed, including appropriate serological and molecular controls (such as in-house positive and negative controls and assay controls), micro-pipettor calibration, and temperature recording of incubators and refrigerators/freezers. QC data sheets and summaries of corrective action are retained and available for review.
4. **The score on the most recent WHO approved serological proficiency test is at least 90%.** Proficiency test results to be reported within 14 days of panel receipt to receive full credit. In the event a "conditional pass" score is obtained (between 80% and 90%), the lab must have implemented the recommended corrective actions.
5. **The score on the most recent WHO YF molecular proficiency test panel is at least 90%.** Molecular EQA panel results are reported within 14 days of panel receipt to receive full credit. In the event a "conditional pass" score is obtained (between 80% and 90%), the laboratory has implemented the recommended corrective actions. Note: This applies only to laboratories routinely performing molecular testing.

6. **Results from virus isolation/detection and genotyping (if performed) are completed within 2 months of receipt of specimen AND data reported to WHO monthly, for ≥80% of the specimens appropriate for genetic analysis.** Genotype information can assist national control programmes to determine transmission pathways and needs to be provided in a timely manner. Genetic data on appropriate specimens collected from separate chains of infection should be supplied to the national programme as soon as they become available. Laboratories are also encouraged to submit sequence data to GenBank once sequencing is completed.
7. **The scoring points obtained in each of the relevant Checklist Assessments is at least 80%, along with ALL checklist mandatory criteria being fully met,** as evidenced in the General Laboratory sections and the serology, molecular, and/or virus isolation sections. For laboratories with consistently high-performance indicators, the RLC may waive the on-site review upon satisfactory completion of the annual checklist by the laboratory.
8. **80% of YF IgM results are communicated within 7 days of receipt by the laboratory.** Subsequent YF IgM testing, when needed, are reported to the EPI programme promptly following efficient use of resources.

9.7.2 THE CHECKLISTS

The assessment contains four checklists, one general and three assay specific (IgM testing, molecular testing, and virus isolation). The general (G) checklist describes general laboratory practices not linked to one specific assay. Information in the general checklist must be completed by the laboratory to inform the assessor before assessment. The general laboratory checklist includes:

Part G-I: Laboratory profile: Provides a profile of the laboratory and serves to identify resource needs, including:

1. Description of the YF diagnostic laboratory facility.
2. Description of the YF diagnostic laboratory equipment.
3. Personnel resources.

Part G-II: YF laboratory testing: Summarizes performance for the last calendar year.

1. List of diagnostic tests performed in the laboratory
2. SOPs

Part G-III: National surveillance data: Summarizes national surveillance data for the last calendar year.

1. Country-specific data.
2. Laboratory reporting.

The YF serology (S), molecular (M), and virus isolation (V) checklists contain sections as follows:

3. Description of the YF diagnostic laboratory equipment.
4. Performance of YF diagnostic testing in the previous 12 months.
5. Result of most recent YF proficiency tests (if available).

9.7.3 LABORATORY ASSESSMENT

The assay specific checklists also contain sections to be filled out by the laboratory before the assessment (see Annex 9.2). During the site visit the assessor evaluates and scores the laboratory performance and provides a summary of the review and a recommendation of accredited, provisionally accredited, or not accredited.

Accredited: The laboratory meets necessary requirements. The eight programmatic criteria are met in addition to the checklist minimal criteria (>80% in scoring points + all checklist mandatory criteria fully met). The distinct sections allow for some laboratories to be accredited for various methods based on the variety of testing they are performing.

Provisionally accredited: The laboratory fulfilled most of the necessary requirements but must improve on one or more relevant conditions to maintain good and reliable diagnostic testing. A partial accreditation, or a non-accreditation result, will set in motion a series of activities to build the capacity of the laboratory needed to reach full accreditation status as soon as possible.

Not accredited: The laboratory did not fulfil the necessary requirements. Analysis of laboratory activities and programme planning will be initiated for NLs lacking competency or proficiency, with technical assistance, troubleshooting, and training provided to improve performance of the laboratory.

Any accreditation reviews of network laboratories carried out should follow the checklist in Annex 9.2. The outcomes and recommendations should be reported within the prescribed time frame. All conclusions and recommendations from the review should be presented to the relevant laboratory staff, institute director, and the MOH at the conclusion of the review in the form of a draft document which will be finalized after consultation with the RLC. Due to the tiered structure of the GYFLaN, some laboratories in the global network will have responsibility for monitoring the quality of other network laboratories (see Chapter 2). All interactions with other laboratories in the network, such as confirmatory testing, technical advice, or training and share a summary of the interaction with the relevant RLC or GLC must be documented.

It is important to communicate issues that arise to the RLC. Often, a quick resolution may be possible by an exchange of information since similar issues may have been previously encountered by other NLs in the region. In addition, some issues may have consequences for the entire region or even the global network. Notifications regarding important laboratory issues by email with other laboratories should include a carbon copy (cc) to the appropriate RLC. The RLC will ensure that issues are brought to the attention of those RRLs or NLs that are responsible for subnational or other non-network laboratories.

9.8 EXTERNAL LABORATORY QUALITY MANAGEMENT AND ACCREDITATION PROGRAMMES

There are several international organizations involved in the promotion of laboratory quality (Table 9.1). The largest of these is the ISO, which has developed and published international standards applicable to various kinds of organizations, including clinical and public health laboratories. Many laboratories in the GYFLaN have undergone ISO accreditation to the ISO standard 15189:2014 (Medical laboratories — Requirements for quality and competence). This standard specifies the requirements for quality and competence in medical laboratories and can be used by network laboratories in developing their QMS and assessing their own competence.

Although there are many parallels between the WHO accreditation programme and ISO 15189, the ISO standard is considerably more comprehensive, especially in relation to the documentation of quality systems and management. Other international organizations include CLSI, a standards-developing organization that promotes the development and use of voluntary consensus standards and guidelines within the health care community.

The Arbovirus Diagnosis Laboratory Network of the Americas (RELDA) established a QA system in YF NLs and SNLs in the Americas and standardized laboratory testing algorithms for the diagnosis and surveillance of arboviral diseases, including evaluation and standardization of reagents and protocols as well as the validation of commercial YF test kits.

The Stepwise Laboratory Quality Improvement Process Towards Accreditation (SLIPTA) programme is a framework for improving the quality of public health laboratories in the African Region to achieve ISO 15189 standards (101). Based on the principles of affordability, scalability, measurability, and accessibility, SLIPTA promotes country ownership of the process and sustainability of improved quality laboratories. Under SLIPTA, laboratories are audited using a checklist based on the QSE from CLSI and scored according to the WHO African Region five-stage accreditation-preparedness point system. SLIPTA provides the roadmap and motivation for laboratories to make steady improvement in service delivery and patient care. The WHO Guide for SLIPTA in the African Region can be found at: https://www.afro.who.int/publications/who-guide-stepwise-laboratory-improvement-process-towards-accreditation-slipta-african.

The Strengthening Laboratory Management Toward Accreditation (SLMTA) programme was created in 2009 in response to the observed need for structured laboratory management training and quality improvement (102). The SLMTA curriculum includes training towards development of management competencies and guides laboratories in the development of QMS, using a practical approach to address everyday challenges with available resources. Each laboratory participating in SLMTA conducts an internal audit at the beginning (baseline) and the end (exit) of the programme using the SLIPTA checklist, which provides the SLMTA programme with a means to identify gaps and benchmark progress. The difference between baseline and exit scores, as well as their respective star ratings, is calculated to identify weaknesses and areas that require improvement, measure success of the programme, and indicate future goals for the laboratory. SLMTA equips laboratory management with the ability to implement QMS to improve their performance on the SLIPTA scale and eventually achieve formal accreditation status. To support this link, individual SLIPTA checklist items are mapped to each of the instructional activities in the SLMTA curriculum so that participants know exactly which management action will fulfil the requirements of any given checklist item. In addition to the SLIPTA scores, laboratories demonstrate their progress through improvement project data, such as turnaround time, sample rejection rate, stock out rate, customer satisfaction survey results, and before-and-after photographs of physical changes. Implementation of improvement projects requires teamwork involving the entire laboratory staff, thus ensuring that the projects become part of the laboratory's continuous improvement processes.

Some countries have also established a certification process for medical laboratories to regulate testing and reporting, such as Clinical Laboratory Improvement Amendments (CLIA) in the USA and National Association of Testing Authorities (NATA) in Australia. All laboratories in the GYFLaN should consider becoming accredited to ISO and/or any national accreditation authority, as an adjunct to the WHO YF accreditation programme.

TABLE 9.1

Laboratory quality and standards organizations.

Organization	*Abbreviation*	*Country or region*	*Website*
International Standards Organization	ISO	International	https://www.iso.org/home.html
Clinical and Laboratory Standards Institute	CLSI	International	https://clsi.org
Arbovirus Diagnosis Laboratory Network of the Americas	RELDA	Pan American Region	https://www.paho.org/en/topics/dengue/arbovirus-diagnosis-laboratory-network-americas-relda
Stepwise Laboratory Quality Improvement Process Towards Accreditation	SLIPTA	African Region	https://www.afro.who.int/publications/who-guide-stepwise-laboratory-improvement-process-towards-accreditation-slipta-african
Strengthening Laboratory Management Toward Accreditation	SLMTA	African Region	http://dx.doi.org/10.4102/ajlm.v3i2.194
Clinical Laboratory Improvement Amendments	CLIA	USA	https://www.cdc.gov/clia/about.html
National Association of Testing Authorities	NATA	Australia	https://www.nata.com.au/

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Annexes

PLEASE NOTE:

The numbering of the Annexes is aligned with the corresponding chapter from the main document. There are no annex references for chapters one and two. The first annex relates to chapter three, therefore numbering begins with Annex 3.

Annex 3.

Booking Form and EYE Strategy Arboviruses

Also available at *https://tinyurl.com/eyeopsbookingform*

Organisation mondiale de la Santé

BOOKING FORM / EYE STRATEGY

YELLOW FEVER

Use one form per shipment

Please send this booking to the World Health Organization (EYE.ops@who.int).

The courier country representative will arrange pick up of the materials described below.

Information of booking form	
DATE: (dd, mm, yyyy)	**:**
PAGE(S):	**:**
From: Name of the laboratory	**:**

Type of request: Emergency ☐ **EQA** ☐ **pls cross one box only**

Sent by email ☐ **Sent by fax:** ☐ **Please (cross the box(es) you chose)**

TO: EYE OPS eye.ops@who.int , WHE/ EYE Secretariat

CC Jimmy Odongo Odongoj@who.int, Maurice DEMANOU demanoum@who.int and Laurence Cibrelus cibrelusl@who.int

Laboratory contact person for the pick up	:
Mobile phone	:

Laboratory to collect the samples:	**Laboratory to deliver the samples**
Laboratory Name:	**WHO Yellow Fever Collaborating Centres.**
Address:	
Address:	☐ Institute Pasteur Senegal- Dakar
City:	
Zip Code:	☐ Institute Pasteur - Cameroun
Country:	
Contact:	☐ UVRI Entebbe -Uganda

WHO ACCOUNT: to be filled in by the EYE Secretariat

THE LOCAL OFFICE OF THE SHIPPING COMPANY WILL PROVIDE DRY ICE, ADEQUATE PACKAGING MATERIALS and REQUIRED PAPERWORK (DGD; AWB, other, FOR THIS SHIPMENT.

Please cross the box(es) below and the specify the type of temperature control required

☐ INFECTIOUS SUBSTANCES AFFECTING HUMANS' CATEGORY A - UN 2814

☐ Ambient / ☐ Refrigerated (Gel packs +2/+8°C) ☐ Frozen (-20°C) ☐ Dry Ice (-80°C)

NUMBER OF VIALS AND ML: _____

☐ BIOLOGICAL SUBSTANCES CATEGORY B - UN3373

☐ Ambient / ☐ Refrigerated (Gel packs +2/+8°C) ☐ Frozen (-20°C) ☐ Dry Ice (-80°C)

NUMBER OF VIALS AND ML: _____

☐ OTHER

☐ Ambient / ☐ Refrigerated (Gel packs +2/+8°C) ☐ Frozen (-20°C) ☐ Dry Ice (-80°C)

NUMBER OF VIALS AND ML: _____

Number of inner packaging and size (if available):

☐ Other information: Import Permit / Export permit /detailed packing list

Date, Name and Signature of requestor

Annex 4.

Yellow Fever Immunohistochemistry MACH Technique 4 (Biocare, M4U536)

1. Deparafinize the sections using 3 changes of NeoClear or Xilol for 3 min each. Dehydrate the tissue with 3 changes of absolute ethanol per 3 min each.
2. Wash with distilled water 3 times.
3. Perform enzymatic digestion of the sections using proteinase K (Roche, 03115879), in a ratio of 1:200 (Digestion Buffer) by applying 100 µl per sheet and incubate for 15 min.
4. Wash with TBS 3 times.
5. Block endogenous proteins with the Background Punisher (Biocare, BP974L) by applying 100 µl per sheet and incubate for 10 min.
6. Wash with TBS 3 times.
7. Incubate with 100 µl of the primary antibody: anti-Yellow Fever (1:1000), (diluted with Da Vinci Green Diluent – Biocare, PD900) for 30 min.
8. Wash with TBS 3 times.
9. Apply 100 µl of the probe (MACH4 Universal AP-Probe, Biocare) for 10 min.
10. Wash with TBS 3 times.
11. Apply 100 µl of the polymer (MACH4 MR AP-Polymer, Biocare) for 15 min.
12. Wash with TBS 3 times.
13. Apply 100 µl of the Warp Red chromogen (Biocare, WR806S) for 5 min.
14. Wash with distilled water.
15. Contrast the sections with Harris Hematoxylin for 5 min, make 3 passes in ammonia water, wash with plenty of running water and let the sections dry in the oven at 60°C.
16. Assemble the sheets with Neomount.

PREPARATION OF REAGENTS

- **Washed buffer**
  200ml of TBS 20X buffer + 3800 ml distilled water.

- **Buffer digestion**
  150 ml tbs buffer (2M, pH: 7.5) + 50ml CaCl2 (1%) + 300ml deionized water.

- **Chromogen Warp Red**
  1 drop of Warp Red chromogen + 2.5ml Warp Red buffer (use immediately)

Annex 5.

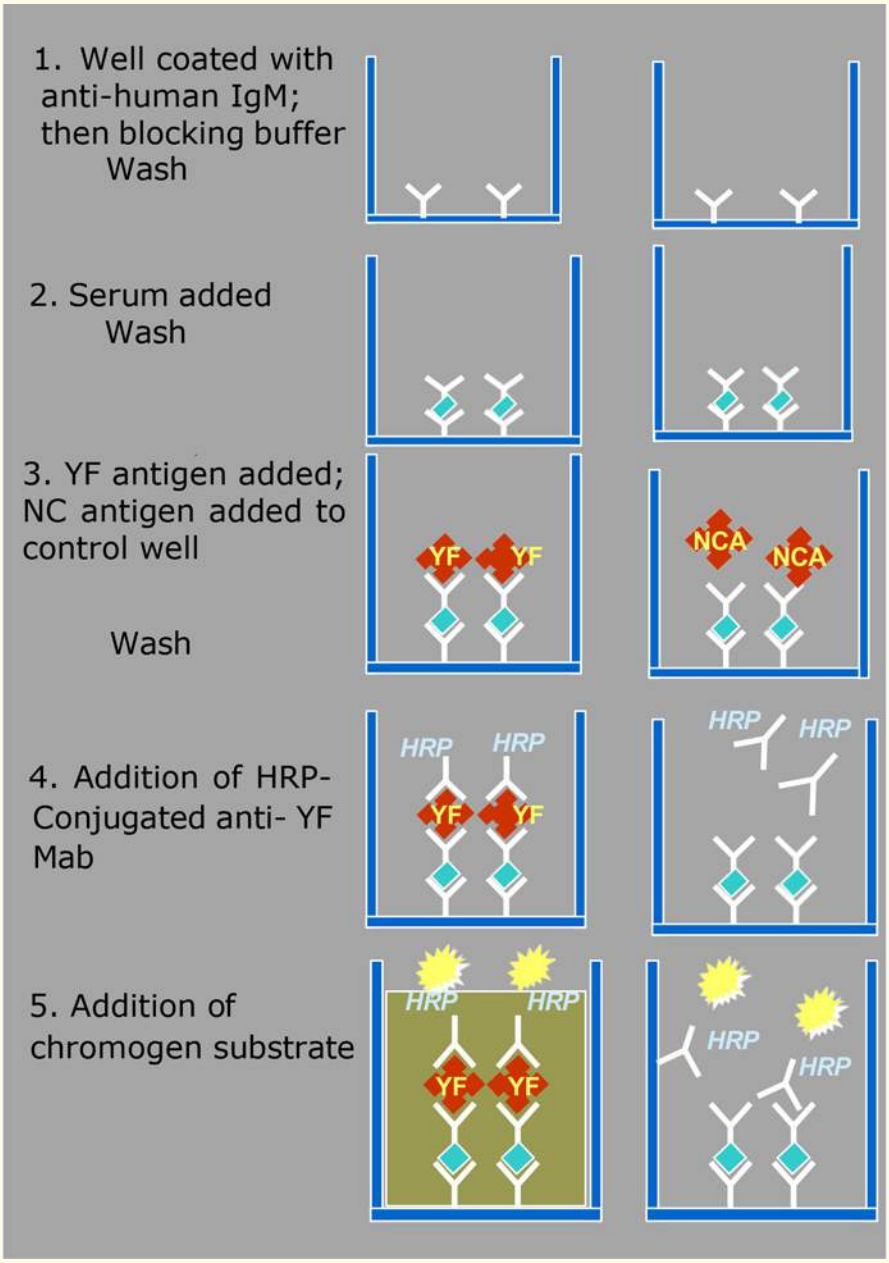
ANNEX 5.1A
Principle of detecting IgM antibodies for yellow fever virus in human sera using a plate-based IgM antibody capture technique

1. Anti-human IgM antibody is adsorbed onto the solid phase of the microtiter plate.
   - The excess anti-human IgM antibody plate is removed
2. A blocking buffer solution is added to block free binding sites in the well surface, preventing nonspecific binding of primary and secondary antibodies.
   - The blocking buffer is removed and the plate is washed.
3. Patient serum is added to the well. IgM antibody in the patient's serum is bound to anti-human IgM antibody. This step is non-virus-specific and eliminates competition with IgG.
   - The plate is then washed, removing other unbound immunoglobulins and serum proteins.
4. YF antigen is added and allowed to bind to the captured YF-specific IgM, if present.
   - The plate is then washed, removing unbound antigen.
5. Anti-viral antibody conjugate (flavivirus group-specific conjugate MAB 6B6C-1/HRP) is added and binds to the bound YF antigen
6. The presence of YF-specific IgM is visualized through addition of a fluorophore substrate

A colorimetric result is generated by the interaction of the enzyme and a chromogenic substrate. This colorimetric change is detected by a spectrophotometer (ELISA reader), and detection of colour above a threshold reveals the presence of YF IgM in the test sample.

FIGURE A5.1.1

Flow chart of a typical MAC-ELISA procedure for yellow fever.



Abbreviations: HRP, horseradish peroxidase; Mab, monoclonal antibody; NCA, non-viral control antigen; YF, yellow fever antigen.

***NOTE**: Assay controls must be run concurrently with all test samples.*

***Positive assay control**: Flavivirus IgM positive control or human clinical specimen with well-characterized YF IgM antibody levels*

***Negative assay control:** Normal human serum nonreactive to flavivirus antigens.*

***Non-viral control antigen (NCA)**: Non-viral control antigen has been prepared in the same manner as the viral antigen and contains cell lysate, cell culture media ingredients, fetal bovine serum etc. The positive control serum, negative control serum, and test serum are reacted concurrently with both viral and non-viral control antigens to compare specific versus nonspecific reactivity of the specimen, as shown in the Figure A5.1.1.*

CDC Yellow fever MAC-ELISA for use in the Global Yellow Fever Laboratory Network

Reagent and Reference Laboratory
Diagnostic and Reference Team
Arboviral Diseases Branch
Division of Vector-borne Diseases
Centers for Disease Control and Prevention

Fort Collins, Colorado USA

Protocol

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Table of Contents

PURPOSE

This document describes the use of an immunoglobulin M (IgM) antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) for the presumptive detection of antibodies to yellow fever (YF) virus.

INTENDED USE

The CDC YF MAC-ELISA (also known as the 3-day or 72h CDC YF MAC-ELISA) is intended for the presumptive detection of YF IgM antibodies in human serum specimens collected from suspected YF cases with clinical signs and symptoms associated with YF virus infection and history of residence in or travel to a geographic region with active YF virus transmission at the time of travel, or other epidemiologic criteria for which YF virus testing may be indicated per the WHO YF case definition. It is intended for use in surveillance of YF within the Global Yellow Fever Laboratory Network (GYFLN) according to the testing algorithm adopted by the network and consistent with individual laboratory procedures.

The protocol is intended to be used as a guide for laboratories to create their own standard operating procedure for the CDC YF MAC-ELISA.

The CDC YF MAC-ELISA should be validated for use under the specific laboratory conditions of the individual user prior to adopting the procedure.

Assay results are for the presumptive identification of IgM antibodies to YF virus. Positive and equivocal results are not definitive for diagnosis of YF virus infection. False-positive results are possible in patients with a history of infection with other flaviviruses. Confirmation of the presence of anti-YF IgM antibodies in equivocal or presumptive positive specimens requires additional testing [e.g., plaque reduction neutralization test (PRNT)]. Confirmed results must be combined with clinical observations, patient history, YF vaccine status, epidemiological information, and other laboratory evidence before being used to inform epidemiological responses.

Negative IgM results do not preclude the possibility of YF virus infection, past or present. Negative results may be seen in specimens collected too early in the illness (before antibodies develop) or after the window of detectable IgM closes.

The CDC YF MAC-ELISA is intended for use by trained laboratory personnel who are proficient in performing and interpreting immunoassays.

PROTOCOL USE LIMITATIONS

The YF MAC-ELISA assay described here has been optimized with the reagents listed below. Modifications of this assay (i.e., use of platforms or chemistries other than those described) has not been extensively studied and is not recommended.

YF IgM levels over the course of illness are not well-characterized. IgM levels are variable, but

generally are positive starting near day 4 post-onset of symptoms and continuing for 12 or more weeks following initial infection. YF IgM levels following YF vaccination have been documented to persist for years.

YF IgM levels may be low when the individual has had prior exposure to other flavivirus infections and thus false-negative results may occur.

The assay is not designed to differentiate antibodies produced by natural YFV infections from those produced in response to YF vaccine; therefore, vaccination history must be taken into consideration when interpreting a suspected YF case.

The assay is qualitative, and results are not a quantitative representation of YF antibodies. Results cannot be correlated to severity of disease.

ASSAY PRINCIPLE

Assays that detect viral specific IgM are advantageous because they detect antibodies produced during the first few days after onset of clinical symptoms in a primary infection, removing the need for convalescent-phase specimens in many cases. IgM capture is the optimum approach to IgM antibody detection because it is simple, sensitive, and applicable to serum.

The YF MAC-ELISA is a qualitative assay and provides a useful alternative to immunofluorescence for documentation of a serologic response to YF virus. ELISA is less subjective than immunofluorescence and large numbers of samples can be processed.

Anti-human IgM (the capture antibody) is coated on 96-well immunoassay plates. Unused binding sites on the plates are blocked, and the test specimen (human serum) is added. IgM present in the serum is bound to the capture antibody, and unbound substances including immunoglobulin G are washed away. Non-infectious YF viral antigen is added, which attaches to any captured IgM antibodies capable of binding YF antigen. The presence of antigen is detected by using enzyme-conjugated antiviral antibody. A colorimetric result is generated by the interaction of the enzyme and a chromogenic substrate. This colorimetric change is stopped using an acidic solution and the amount of color is read by a spectrophotometer (ELISA reader).

Specimens

ACCEPTABLE SPECIMENS

- Acute and convalescent human serum
  NOTE: Tube should be centrifuged, and serum decanted prior to shipment to avoid hemolysis.

The presence of hemoglobin in the specimens can result in reduction of signal in the YF MAC-ELISA.

SPECIMEN HANDLING AND STORAGE

At a minimum, store all diagnostic specimens at 2-8°C prior to testing, and ≤ -20°C after all anticipated testing has been completed. Avoid repeated freeze-thaw cycles. The YF Manual should be consulted for specific guidance on specimen handling.

Safety

SAFETY/PRECAUTIONS

Serum from suspected YF cases should be treated as potentially infectious. It is recommended that laboratories perform a risk assessment when conducting new tests and safety precautions should be based on the laboratory's risk assessment. See the Biosafety in Microbiological and Biomedical Laboratories (BMBL) for additional biosafety information about YF virus and laboratory biosafety practices.

The YF MAC-ELISA should be performed under laboratory safety conditions that take into consideration the potential infectious nature of the serum specimens involved. At a minimum, it is recommended that these procedures be performed using BSL-2 facilities and BSL-3 practices. To ensure safety of laboratory personnel, perform all sample manipulations within a Class II (or higher) biological safety cabinet (BSC).

Materials

DISCLAIMER: Names of vendors or manufacturers are provided as examples of suitable product sources. Use of trade names is for identification purposes only and does not constitute endorsement by CDC or the Department of Health and Human Services.

MATERIALS PRODUCED BY CDC

NOTE: Contact your WHO YF Regional Coordinator for information on obtaining these reagents. Refer to the product inserts for reconstitution and storage conditions.

- **Flavivirus IgM positive control:** Chimeric monoclonal antibody specific for Flavivirus; lyophilized.
- **YF viral antigen (any of the following products is acceptable for use in the assay):**
  - **YF Vero Tissue Culture Antigen:** This antigen is produced from attenuated YF vaccine strain YF 17D, inactivated by gamma irradiation and beta-propiolactone (BPL), concentrated for use in the CDC YF MAC-ELISA, and lyophilized.

- **YF COS-1 Recombinant Antigen:** This antigen is non-infectious YF virus-like particles prepared for use in the CDC YF MAC-ELISA, and lyophilized.

- **Normal antigen (culture source must match that of viral antigen)**
  - **Normal Vero Antigen:** Lyophilized normal antigen for use with YF Vero Tissue Culture Antigen.
  - **Normal COS-1 Recombinant Antigen:** Lyophilized normal antigen for use with YF COS-1 Recombinant Antigen.

- **Flavivirus group-specific conjugate MAB 6B6C-1/HRP:** Horseradish peroxidase conjugated monoclonal antibody 6B6C-1; lyophilized

MATERIALS REQUIRED BUT NOT PRODUCED BY CDC

NOTE: for materials requiring dilution/titration, see **Formulations** below.

- **Goat anti-human IgM coating antibody** (KPL catalog #01-10-03 sourced from LGC/Seracare catalog #5210-0157)
- **Deionized water**
- **Hydrochloric acid** (to adjust pH of coating buffer, if needed)
- **Coating buffer – tablets**
  - **0.05M Carbonate-Bicarbonate buffer pH 9.6 tablets** (available from multiple commercial sources, e.g., Sigma)
- **Coating buffer – individual reagents (if used instead of tablets):**
  - **Sodium carbonate** (anhydrous) (Na_2CO_3); (available from multiple commercial sources, e.g., Sigma, Thermo Fisher, etc.)
  - **Sodium bicarbonate** ($NaHCO_3$); (available from multiple commercial sources, e.g., Sigma, Thermo Fisher, etc.)
- **Glycerol** (recommended but not critical, available from multiple commercial sources, e.g., Sigma, Thermo Fisher, etc.)
- **Phosphate buffered saline** (PBS); (available in powdered or tablet form from multiple commercial sources, e.g., Sigma, Thermo Fisher, etc.)
- **Tween 20** (available from multiple commercial sources, e.g., Sigma, Thermo Fisher, etc.)
- **Nonfat dry milk** (available from multiple commercial sources, e.g., Sigma, Thermo Fisher, etc.)
- **Sulfuric acid** (H_2SO_4); (available from multiple commercial sources, e.g., Sigma, Thermo Fisher) or **TMB Stop Solution** (LGC/Seracare catalog #5150-0020)
- **Immulon II HB flat-bottomed 96 well plates**, Dynatech Technologies catalog #3455 (available from multiple commercial sources, e.g., Sigma, Thermo Fisher, etc.)
  NOTE: This is the only 96-well plate approved for this assay.
- **Enhanced K-Blue TMB substrate** (3,3',5,5'tetramethylbenzidine base; Neogen Corp, catalog#308175)
  NOTE: The use of alternative brands of TMB have not been investigated.
- **Negative control** (normal human serum) known to be IgM-negative for YF and other

flaviviruses.

NOTE: New lots of normal human serum should be tested using this protocol as if they were test specimens at 1:400. Diluted negative control should ideally have an OD between 0.05<0.2 but preferably closer to 0.1 and MUST be <0.2 when reacted on YF antigen.

- **In-house YF IgM-positive control** (recommended): a well-characterized YF IgM-positive human serum specimen with an OD of +/- 1.0 when reacted on YF antigen

EQUIPMENT AND CONSUMABLES

- Microplate washer
- Microplate reader with 450 nm filter
- Biosafety cabinet (BSC)
- Incubator set at 37°C
- Single and multi-channel pipettors (20 µL, 100 µL, 200 µL and/or 1000 µL Single Channel, 100 µL and/or 200 µL 8- or 12-channel)
- Pipet tips for listed pipettors
- Reagent reservoirs
- Timer
- Reagent mixing bottles; sterile 1L glass or plastic bottles; Gibco or alternate vendor
- Microfuge tubes to dilute patient serum; purchase sterile or autoclave and cool before use; Corning or alternate vendor
- Weigh boats for measuring dry chemical components, chemical resistant
- Permanent marker

Formulations

Buffers (made ahead of time)

- **Coating buffer made from tablets –** follow manufacturer's instructions for generation and storage conditions
- **Coating buffer made from individual reagents (if used instead of tablets)**: 0.015M sodium carbonate/0.035M sodium bicarbonate (0.05M Carbonate/bicarbonate buffer), pH 9.6

  1.59 g Na_2CO_3 + 2.93 g $NaHCO_3$ diluted in 1L water. Adjust pH to 9.6 with concentrated hydrochloric acid (if needed)
  - Store at 2-8 °C NOTE: Store for up to a year if sterile-filtered; storage time has not been determined for non-sterile coating buffer
- **50% glycerol** (recommended but not critical for reconstitution of coating antibody)

  Add equal quantities of glycerol and dH_2O
  - Store at room temperature for up to 6 months
- **Wash buffer**: Phosphate buffered saline (PBS); 0.05% Tween 20, pH 7.2. PBS is available in powdered or tablet form from multiple commercial sources

  For one liter, add specified weight or tablet number of PBS/L indicated by manufacturer,

add dH_2O to nearly 1L, dissolve, and add 0.5 ml Tween 20. Mix and adjust to 1L with dH_2O

- Store large volumes at room temperature for up to 1 week.

NOTE: for convenience, if sterile filtration devices are available, a 10X solution of PBS can be made and sterile filtered, and stored at room temperature for up to 1 year

- **Blocking buffer**: PBS/ 5% milk / 0.5% Tween 20
  For 500 ml, add specified weight or tablet number of PBS/500 ml indicated by manufacturer, add dH_2O to approximately 350 ml; add 25 g non-fat dry milk and dissolve; add 2.5 ml Tween 20 and adjust to 500 ml with dH_2O
  - Store at 2-8 °C for up to 2 weeks
- **Stop solution**: 0.5M H_2SO_4 (if not using commercial stop solution)
  Add 26.8 ml sulfuric acid to 973.2 ml dH_2O.
  - Store in glass up to 1 year at room temperature

Critical components requiring dilution

NOTE: Dilutions given are an indication of working dilutions only. Laboratories MUST determine the optimum dilution of each reagent for use in their individual laboratory. See additional information in Assay Standardization on page 8 below regarding how to optimize the critical components.

- **Goat anti-human IgM**: Diluted 1:2000 in coating buffer (titration is unlikely to be required if using KPL product)
- **Flavivirus IgM positive control**: Dilute up to 1:1000 in wash buffer
- **Negative control**: Diluted 1:400 in wash buffer (no titration required)
- **Patient serum**: Diluted 1:400 in wash buffer (no titration required)
- **YF viral antigen**:
  - YF Vero Antigen: Dilute up to 1:160 in wash buffer (1:100 for WHO labs)
  - YF COS-1 Recombinant Antigen: working dilution lot specific; dilute in wash buffer
- **Normal antigen**:
  - Normal Vero Antigen: Dilute in same manner as YF Vero antigen
  - Normal COS-1 Recombinant Antigen: Dilute in same manner as YF COS-1 antigen
- **Flavivirus group-specific conjugate 6B6C-1/HRP**: Dilute up to 1:4000 in blocking buffer

Assay Standardization

ASSAY STANDARDIZATION

The MAC-ELISA should be standardized and validated prior to use in the laboratory and re-standardization is required periodically. This should occur when new lot numbers of reagents are introduced, and at the very least, once a year. It is recommended that the mean optical density of the positive control serum reacted with the YF viral antigen be set to approx. 1.0. The mean OD of the

normal control serum reacted with the YF viral antigen should be between 0.05 and 0.2 though preferably closer to 0.1. The standardization of reagents is normally achieved via 2-fold titrations, always comparing the optical densities of the reagents when reacted on viral and normal antigens. Standardization and re-standardization may be confirmed by testing verification panels.

CDC YF MAC-ELISA PROCEDURE

GENERAL CONSIDERATIONS

- Personnel must be trained in the use of the protocol and instruments.
- Wear clean gowns and new, powder-free gloves during assay reagent setup and handling. Change gloves whenever you suspect they may be contaminated.
- Store all reagents at appropriate temperatures (see product inserts). Do not use reagents beyond their expiration dates.
- Keep reagent tubes capped as much as possible.
- Use aerosol barrier (filter) pipette tips only.
- Empty all trash daily.

ASSAY CONTROLS

- Positive and negative assay controls must be included on each assay plate.

NOTES REGARDING THE PROCEDURE:

- Coated plates can be kept at 2-8° C for up to a week. (See Step 2: Coating the Plates, below).
- Undiluted flavivirus IgM positive control aliquots can be stored at 2-8° C for up to 6 months.
- Flavivirus IgM positive control working dilution should be prepared immediately prior to use.
- Working dilutions of patient samples and negative control sera should be prepared the day of test.
- Reconstituted, undiluted viral and normal antigens MUST be stored at ≤ -20° C and can be used for up to one year. Aliquots can be made to limit freeze-thaw cycles.
- Reconstituted Flavivirus group-specific MAB 6B6C-1/HRP MUST NOT be frozen.
- Antigens and conjugate must be diluted to the working dilutions immediately prior to use.
- To keep timing of reagent addition consistent, process plates in the order that they are numbered during all steps of the procedure.
- ASSURE THE PLATES DO NOT DRY OUT DURING THE ENTIRE MAC-ELISA PROCEDURE. Plates should be kept covered in an enclosed, humidified environment during all incubation times except for the coating, blocking, substrate, and stop solution steps. A large Ziploc-type bag containing a moist paper towel works well for this humidified environment purpose.

THE FOLLOWING PROCEDURE INCLUDES INFORMATION ON QUALITY CONTROL AND INTERPRETATION. THREE REPLICATES OF POSITIVE AND NEGATIVE CONTROLS AND THREE REPLICATES OF EACH SERUM SPECIMEN SHOULD BE TESTED ON BOTH YF AND NORMAL ANTIGENS. EIGHT (8) TEST SPECIMENS CAN BE ANALYZED PER PLATE.

METHOD

1. **MARK THE PLATE**
   Determine the number of ELISA plates needed. Using a fine-tipped permanent marker, number and label the 96-well plates. Using the inner 60 wells of the plate, identify the location of each clinical specimen (S1-S8) by using a corresponding template (see Fig. 1).
   NOTE: If you are using the CDC standard ELISA calculations workbook, placement of the specimens and controls according to Fig. 1 is imperative.
2. **COAT THE PLATE**
   - Dilute goat anti-human IgM 1:2000 in coating buffer, pH 9.6.
   - Coat the inner 60 wells of the 96-well plate with 75 µL per well of diluted goat anti-human IgM. Leave outer rows/columns empty (see Fig. 1).
   - Cover the plate with an extra plate and incubate at **2-8°C overnight**.
3. **BLOCK THE PLATE**
   - After overnight incubation, decant the coating antibody.
   - Blot plates on paper towels or other absorbent material.
   - Block plates with 200 µL blocking buffer per well.
   - Cover the plate with an extra plate and incubate at **room temperature for 30 minutes**.
4. **WASH THE PLATE**
   - Wash wells 5X with wash buffer using an automatic plate washer. No dwell time is necessary.
   - Wells should be filled to the top during each cycle (i.e. 250 µL).
5. **ADD SAMPLE AND CONTROLS**
   - Dilute patient serum 1:400 in wash buffer.
   - Dilute positive control in wash buffer at the dilution determined during assay standardization.
   - Dilute negative human serum control 1:400 in wash buffer
   - Briefly vortex or mix diluted patient serum and controls
   - Add 50 µL per well of each diluted patient serum (S) to a block of 6 wells
   - Add 50 µL per well of diluted positive control to a block of 6 wells.
   - Add 50 µL per well of diluted negative human serum control to a block of 6 wells.
   - Cover and incubate plates for **1 hour at 37° C** in a humidified chamber.
6. **WASH THE PLATE**
   - Wash wells 5X with wash buffer by using an automatic plate washer.
7. **ADDITION OF ANTIGENS**
   - Dilute **YF** viral antigen in wash buffer at the dilution determined during assay standardization.
   - Dilute **Normal** antigen in wash buffer at the same dilution as the YF viral antigen.
   - Add 50 µL per well of diluted **YF** viral antigen to the left three wells of each serum

block (see Fig 1).

- Add 50 µL per well of diluted **Normal** antigen to the right three wells of each block (see Fig. 1).
- Cover and incubate plates **overnight at 2-8°C** in a humidified chamber.

8. **WASH THE PLATE**
   - Wash wells 5X with wash buffer by using an automatic plate washer.
9. **ADDITION OF CONJUGATE**
   - Dilute Flavivirus group-specific MAB 6B6C-1/HRP in blocking buffer at the dilution determined during assay standardization.
   - Add 50 µL per well of diluted conjugate
   - Cover and incubate plates for **1 hour at 37° C** in a humidified chamber.
10. Turn on plate reader to warm up
11. Remove TMB-ELISA from refrigerator and let warm to room temperature.
12. **WASH THE PLATE**
    - Wash wells 5X **twice (for a total of 10 washes)** with wash buffer by using an automatic plate washer.
    - Turn the plates 180º in the washer after the first series of 5 cycles. This promotes consistent results.
13. **ADD SUBSTRATE**
    - With the plate and TMB substrate at room temperature (20-25°C), add 75 µL per well of TMB substrate to the inner 60 wells.
    - Place plate uncovered in the dark to block out light. Incubate at room temperature for 10 minutes.
    - A blue color will develop in antibody-positive wells.
14. **ADD STOP SOLUTION**
    - Add 50 µL 0.5M sulfuric acid (H_2SO_4) **OR** 75 µL TMB Stop Solution per well to **all wells, including the outer rows of wells on the plate**.
    - The wells that were blue will now change to a yellow color.
    - Allow plates to sit at room temperature for 1 minute.
15. **READ PLATE**
    - Read plate in microtiter plate reader by using a 450 nm filter.
    - NOTE: Programming the plate reader to blank on some of the outer wells should be done if manually calculating results; blanking of outer wells is not necessary if using the Standard CDC MAC-ELISA calculations workbook, which subtracts the average optical density of wells A1-D1 from the raw results.

Figure 1: Plate layout for 8 specimens (S) and controls

Analysis of Test Results

TEST VALIDITY DETERMINATION

Before the results can be calculated for the clinical specimens, the test must be determined to be **valid** (see Table 1).

For the test to be valid, the following criteria **MUST** be met:

1. The Positive Control P/N value must be ≥ 2.0:
P/N = Mean optical density (OD) POS Control on YF antigen (P) ÷ Mean OD NEG Control on YF antigen (N)

2. The Positive Control Nonspecific background (NBR) value must be ≥ 2.0
NBR = Mean OD POS Control on YF antigen ÷ Mean OD POS Control on Normal antigen

3. The Negative Control OD must be < 0.2
Negative Control OD: Mean OD of the Negative Control reacted with YF viral antigen (N)

Test validity must be determined for each plate. Results for clinical specimens may **only** be determined if the test is valid. If the test is not valid, then the assay must be repeated. If the Positive Control P/N value, Positive Control NBR value, or Negative Control OD criteria still fail after repeat, then one or more of the reagent or test parameters was likely in error, and troubleshooting should be performed.

Table 1: Validity criteria for positive and negative controls

Criteria	Definition	Result
Positive control P/N	Mean OD of the positive control reacted with YF viral antigen (P) / Mean OD of the negative control reacted with YF viral antigen (N)	**< 2 Plate IS NOT Valid**
		≥ 2 Plate IS Valid
Positive control NBR (non-specific background)	Mean OD of the positive control reacted with YF viral antigen / Mean OD of the positive control reacted with Normal antigen	**< 2 Plate IS NOT Valid**
		≥ 2 Plate IS Valid
Negative control OD	Mean OD of the negative control reacted with YF viral antigen	**≥ 0.2 Plate IS NOT Valid**
		< 0.2 Plate IS Valid

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SPECIMEN RESULT DETERMINATION

1. SPECIMEN P/N

To determine whether the clinical specimens (S1-S8) contain IgM to YF virus (which could indicate recent infection with YF virus) the **Specimen P/N** value must be calculated:

Specimen P/N = Mean OD Specimen on YF antigen (P) ÷ Mean OD NEG Control on YF antigen (N)

2. SPECIMEN NON-SPECIFIC BACKGROUND REACTION (NBR)

For each specimen with a **Specimen P/N ≥ 2**, determine whether a non-specific background reaction (NBR) is being generated.

NBR = Mean OD Specimen on YF antigen ÷ Mean OD Specimen on Normal antigen

NOTE: NBR does not need determining for specimens with a P/N <2.0

INTERPRETATION OF SPECIMEN RESULTS

All specimens that have a P/N value ≥3.0 **AND** a NBR value ≥2.0 should be reported as **presumptive YF IgM-positive**.
Specimens that have a P/N value ≥3.0 **AND** a NBR value <2.0 should be reported as **YF IgM inconclusive.**
All specimens that have a value of 2.0 ≤ P/N < 3.0 **AND** a NBR value ≥2.0 should be reported as **YF IgM equivocal**.
Specimens that have a value of 2.0 ≤ P/N < 3.0 **AND** a NBR value <2.0 should be reported as **YF IgM inconclusive**.
Specimens that have a P/N value of <2.0 should be reported as **YF IgM negative** (see Table 2).

Table 2: YF MAC-ELISA Specimen Results Interpretation

Specimen P/N value	Specimen NBR value	Result Interpretation	Report	Action
< 2	Any	Negative	No evidence of recent YF virus infection detected.	Report results*
2 ≤ P/N < 3	**≥ 2**	Equivocal	YF MAC-ELISA results were equivocal for the presence of anti-YF virus IgM antibodies.	Refer to regional guidelines for confirmatory testing
≥ 3	**≥ 2**	Presumptive Positive	Serological evidence of recent YF virus infection.	Refer to regional guidelines for confirmatory testing
≥2	**<2**	Inconclusive	Inconclusive	Refer to regional guidelines for confirmatory testing

* If a very early serum specimen results as negative, a serum specimen taken after day 4 post-onset of symptoms should be requested and tested before the case is reported as negative for serological evidence of recent YF viral infection. Without testing of a later specimen, a negative result may reflect testing of an acute-phase specimen obtained before YF IgM antibody has risen to detectable levels.

Assay Limitations

Interpretation of YF MAC-ELISA results must account for the possibility of false-negative and false-positive results.

False-negative results can arise from:

- Specimen collection conducted before YF IgM has reached detectable levels (typically around 4

days post-onset of symptoms)
- Specimen collection conducted after YF IgM levels have decreased below detectable levels (typically months post-onset of symptoms)
- Exposure of the patient to previous flavivirus infections causing low YF IgM levels
- Failure to follow the assay procedures

False-positive results can arise from:
- Cross reactivity with IgM produced in response to other flaviviruses such as dengue virus

Positive results in patients with history of vaccination is not considered false-positive, but interpretation of results must take this history into consideration.

No evaluation of cross-reactivity with Rheumatoid Factor has been conducted.

Negative results do not preclude infection with YF virus. All results should be interpreted by a trained professional in conjunction with review of the patient's history and clinical signs and symptoms.

Proper collection, storage and transport of specimens are essential for correct results.

Performance has only been established with the specimen types listed in the Intended Use (p.3).

Performance Characteristics

Cross Reactivity

Flavivirus Cross-reactivity

Only limited evaluation of cross-reactivity with flaviviruses or arboviruses has been conducted. Of 106 individuals with evidence of recent dengue virus infection based on clinical, epidemiologic, and test results, samples from 40% (N=43) cross-reacted causing YF IgM MAC-ELISA to be positive or equivocal. There was less cross-reactivity seen with other recent flavivirus infections (CDC, unpublished data).

Cross-reactivity with anti-dengue virus antibodies is likely. Follow-up testing is necessary to rule-out a false-positive result. Confirmation of the presence of anti-YF IgM requires testing by a reference laboratory. The gold-standard method for confirmation of the presence of anti-YF antibodies is the PRNT.

Contact

For questions related to the protocol, reagents, or troubleshooting, please contact the CDC Fort Collins Reference and Reagent Laboratory at reagents2@cdc.gov.

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DAY 1

1. Using a fine-tipped permanent marker, number and label 96-well plates. Identify the location of each clinical specimen (S1-S8) by using the appropriate laboratory code number. Label the template sheets to match the plates.
2. Calculate dilutions for test (patient) serum and positive and negative controls. Always prepare extra due to volume loss during pipetting! (Extra volume included in calculations below)

	Goat anti-human IgM	***Flavivirus IgM positive control Humanized 6B6C-1***	***Normal human serum negative control***	***Patient serum***
Lot #		TC01067	M NHS	
Dilute in	Coating buffer	Wash buffer	Wash buffer	Wash buffer
Volume per well	75 µL	50 µL	50 µL	50 µL
Number of wells	60	6	6	6
Dilution	1:2000	1:1000*	1:400	1:400
Volume diluent	5 mL/plate X n plates =	400 µL/plate X n plates=	400 µL/plate X n plates=	400 µL
Volume reagent	2.5 µL/plate X n plates=	4 µL/plate 1:10 dilution X n plates=	1 µL/plate X n plates=	1 µL

** In order to dilute this reagent, you will be provided with a 1:10 dilution. Each team will then do a 1:100 dilution of that stock to make the final working dilution.*

3. Coat the 96-well plates
   - » Dilute goat anti-human IgM 1:2000 in coating buffer, pH 9.6 as calculated above.
   - » Pipet 75 µL diluted goat anti-human IgM solution into each of the inner 60 wells of the 96-well plate.
   - » Leave wells in outer rows/columns empty.
   - » Cover plates with extra plate, parafilm or plate sealer.
   - » Incubate at 2-8°C overnight.

DAY 2

1. Dump out the coating antibody and blot plates on paper towels.
2. Block plates
   - » Pipet 200 µL blocking buffer into each of the inner 60 wells of the plate.
   - » Cover plate with extra plate, parafilm or plate sealer.
   - » Incubate at room temperature for 30 minutes.
3. Dilute patient serum, Flavivirus positive control and negative human serum control in wash buffer as calculated above.
4. Wash plates 5X with wash buffer (250 µL/well).
5. Add patient serum and controls
   - » Pipet 50 µL per well of each diluted patient serum to a block of 6 wells (refer to setup template on pg. 4).
   - » Pipet 50 µL per well of diluted positive control to the block of 6 wells in the REF section of the plate.
   - » Pipet 50 µL per well of diluted negative control to the block of 6 wells in the N section of the plate.
   - » Incubate plates for 1 hour at 37°C in a plastic ziplock bag with damp paper towels.
6. Wash plates 5X with wash buffer (250 µL/well).
7. Calculate and prepare dilutions of viral and normal antigens.

	Yellow fever 17D Vero antigen	***Normal Vero antigen***
Lot #	TC00599	TC01124
Dilute in	Wash buffer	Wash buffer
Volume per well	50 µL	50 µL
Number of wells	30	30
Dilution*	1:160	1:160
Volume diluent	2 mL/plate X n plates =	2 mL/plate X n plates =
Volume reagent	12.5 µL/plate X n plates=	12.5 µL/plate X n plates=

** Note that antigen dilutions are lot specific and should be determined ahead of time.*

8. Add antigen
   - » Pipet 50 µL of diluted Yellow fever 17D Vero antigen per well to the LEFT three wells of each serum and control block (see plate setup template).
   - » Pipet 50 µL of diluted normal Vero antigen the RIGHT three wells of each serum and control block.
   - » Incubate overnight at 4°C in a plastic ziplock bag with damp paper towels.

DAY 3

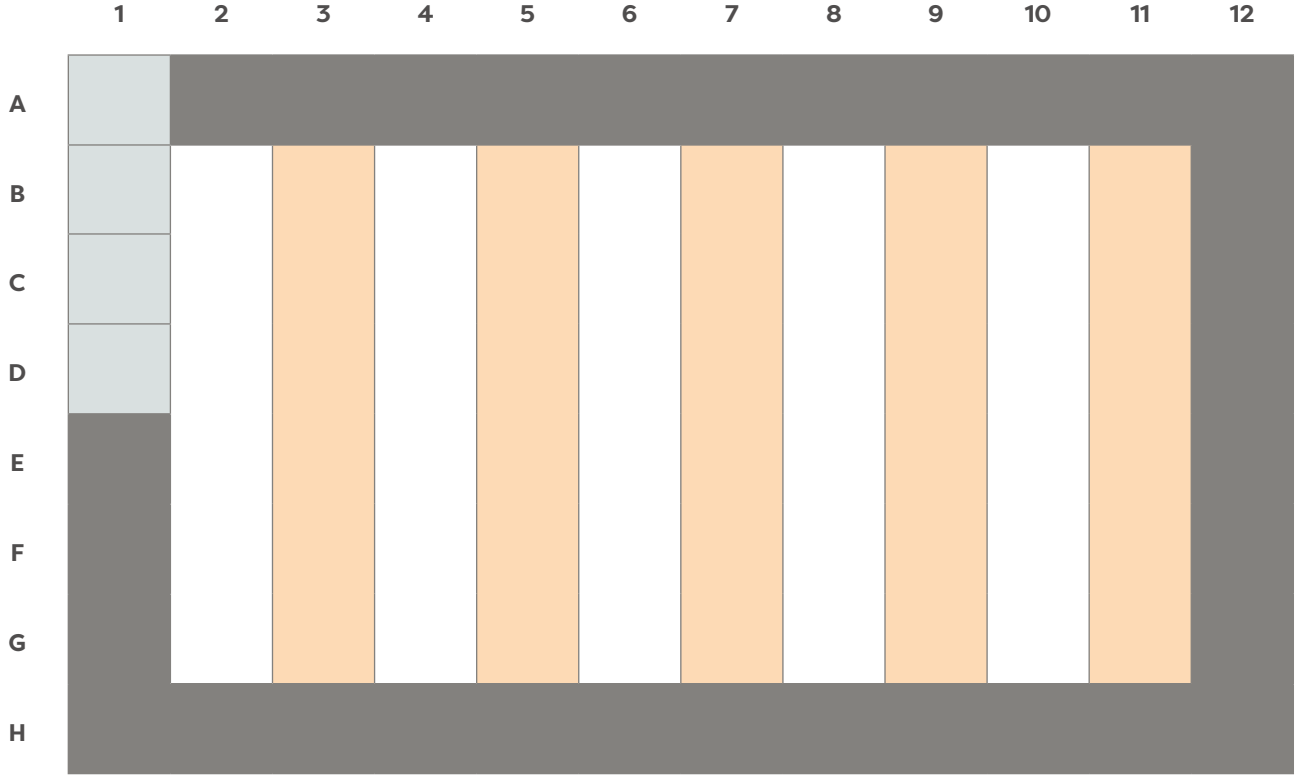
1. Calculate and prepare dilution of conjugate immediately before washing plate.

	Flavivirus group- specific conjugate MAB 6B6C-1/H
Lot #	JIR 39147
Dilute in	Blocking buffer
Volume per well	50 µL
Number of wells	60
Dilution	1:4000
Volume diluent	4 mL/plate X n plates =
Volume reagent	1 µL/plate X n plates =

2. Wash plates 5X with wash buffer (250 µL/well).
3. Add conjugate
   - » Pipet 50 µL per well of diluted conjugate to each of the inner 60 wells of the plate.
   - » Incubate 1 hour at 37°C in a plastic ziplock bag with damp paper towels.
4. Turn on plate reader to warm up, and remove TMB from refrigerator.
5. Wash plates 5X twice with wash buffer (250 µL/well).
   - » Turn the plates 180° in the washer after the first series of 5 cycles.
6. Add substrate
   - » Pipet 75 µL per well of TMB substrate to the inner 60 wells of the plate.
   - » Immediately cover plates to block out light.
   - » Incubate at room temperature for 10 minutes.
7. Add stop solution
   - » Pipet 75 µL per well of stop solution to ALL wells, including the outer rows of wells on the plate.
   - » Allow plates to sit at room temperature for 1 minute.
   - » Read plates in microtiter plate reader by using a 450 nm filter, blanking on well A1 to D1.
8. Enter results into CDC ELISA Excel Spreadsheet for calculations and result interpretation.

Plate Setup:

	1	2	3	4	5	6	7	8	9	10	11	12
A	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY
B	EMPTY	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	EMPTY
C	EMPTY	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	EMPTY
D	EMPTY	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	EMPTY
E	EMPTY	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	EMPTY
F	EMPTY	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	EMPTY
G	EMPTY	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	Viral Antigen	Normal Antigen	EMPTY
H	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY	EMPTY



Calculations and interpretation of results:

TEST VALIDITY DETERMINATION (as updated from the CDC Yellow fever MAC-ELISA for us in the Global Yellow Fever Laboratory Network, Annex 5.1B)

Before the results can be calculated for the clinical specimens, the test must be determined to be valid (see Table below).

For the test to be valid, the following criteria MUST be met:

1. The Positive Control P/N value must be ≥ 2.0:

P/N = Mean optical density (OD) POS Control on YF antigen (P) ÷ Mean OD NEG Control on YF antigen (N)

2. The Positive Control Nonspecific background (NBR) value must be ≥ 2.0

NBR = Mean OD POS Control on YF antigen ÷ Mean OD POS Control on Normal antigen

3. The Negative Control OD must be < 0.2

Negative Control OD: Mean OD of the Negative Control reacted with YF viral antigen (N)

Test validity must be determined for each plate. Results for clinical specimens may only be determined if the test is valid. If the test is not valid, then the assay must be repeated. If the Positive Control P/N value, Positive Control NBR value, or Negative Control OD criteria still fail after repeat, then one or more of the reagent or test parameters was likely in error, and troubleshooting should be performed.

Criteria	*Definition*	*Result*
Positive control P/N	Mean OD of the positive control reacted with YF viral antigen (P) / Mean OD of the negative control reacted with YF viral antigen (N)	**< 2 Plate IS NOT Valid**
		≥ 2 Plate IS Valid
Positive control NBR (non-specific background)	Mean OD of the positive control reacted with YF viral antigen / Mean OD of the positive control reacted with Normal antigen	**< 2 Plate IS NOT Valid**
		≥ 2 Plate IS Valid
Negative control OD	Mean OD of the negative control reacted with YF viral antigen	**≥0.2 Plate IS NOT Valid**
		<0.2 Plate IS Valid

Read plate as :

Well	OD		
B10			
C10			
D10			
Mean = P			
E10			
F10			
G10			
Mean = N			
P/N		YES	NO
Test Valid?			

SPECIMEN RESULT DETERMINATION

1. SPECIMEN P/N

To determine whether the clinical specimens (S1-S8) contain IgM to YF virus (which could indicate recent infection with YF virus) the Specimen P/N value must be calculated:

Specimen P/N = Mean OD Specimen on YF antigen (P) ÷ Mean OD NEG Control on YF antigen (N)

Read plate as:

	S1	S2	S3	S4	S5	S6	S7	S8
OD viral agn								
OD viral agn								
OD viral agn								
Mean = P specimen								
Specimen P/N								
Negative?								

2. SPECIMEN NON-SPECIFIC BACKGROUND REACTION (NBR)

For each specimen with a **Specimen P/N ≥ 2**, determine whether a non-specific background reaction (NBR) is being generated.

NBR = Mean OD Specimen on YF antigen ÷ Mean OD Specimen on Normal antigen

NOTE: NBR does not need determining for specimens with a P/N <2.0

Read plate as :

	S1	S2	S3	S4	S5	S6	S7	S8
OD normal agn								
OD normal agn								
OD normal agn								
Mean normal agn								
2X mean normal agn								
Specimen P ≥ 2X								
Specimen P ≥ 2X mean normal agn?								
Inconclusive?								

INTERPRETATION OF SPECIMEN RESULTS

All specimens that have a P/N value ≥3.0 AND a NBR value ≥2.0 should be reported as presumptive YF IgM-positive.

Specimens that have a P/N value ≥3.0 AND a NBR value <2.0 should be reported as YF IgM inconclusive.

All specimens that have a value of 2.0 ≤ P/N < 3.0 AND a NBR value ≥2.0 should be reported as YF IgM equivocal.

Specimens that have a value of 2.0 ≤ P/N < 3.0 AND a NBR value <2.0 should be reported as YF IgM inconclusive.

Specimens that have a P/N value of <2.0 should be reported as YF IgM negative (see Table 2).

YF MAC-ELISA Specimen Results interpretation

Specimen P/N value	*Specimen NBR value*	*Result Interpretation*	*Report*	*Action*
< 2	Any	Negative	No evidence of recent YF virus infection detected.	Report results*
2 ≤ P/N < 3	≥ 2	Equivocal	YF MAC-ELISA results were equivocal for the presence of anti-YF virus IgM antibodies.	Refer to regional guidelines for confirmatory testing
≥ 3	≥ 2	Presumptive Positive	Serological evidence of recent YF virus infection.	Refer to regional guidelines for confirmatory testing
≥2	<2	Inconclusive	Inconclusive	Refer to regional guidelines for confirmatory testing

** If a very early serum specimen results as negative, a serum specimen taken after day 4 post-onset of symptoms should be requested and tested before the case is reported as negative for serological evidence of recent YF viral infection. Without testing of a later specimen, a negative result may reflect testing of an acute-phase specimen obtained before YF IgM antibody has risen to detectable levels.*

ANNEX 5.1D
Standard CDC MAC-ELISA results calculations

Please use the Excel worksheet for the Standard CDC MAC-ELISA results calculations available at *https://www.who.int/publications/i/item/9789240084476* or see details below.

CDC MAC-ELISA Calculations workbook

Notes to users:
If needed, unprotect sheet using password: yf
Enter information requested in orange cells; the sheet is not set up to calculate CSF or titration results; these should be performed manually
Copy raw OD's into cells A10:L17 of Raw Data Table; delete "spotty" wells (results will adjust)
Worksheet assumes blanks are calculated using wells A1-D1 on the microtiter plate. If needed, unprotect and adjust formula in cell A19

Raw Data Table

Test name: YF MAC-ELISA Date: Plate: Operator:

#DIV/0!	Avg Blank

Blank-adjusted Values Table

#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Plate set-up Enter sample name in upper left (orange) cell of each set of 6 wells (table will auto-populate)

Viral Ag	Norm Ag	Viral Ag	Norm Ag	Viral Ag	Norm Ag	Viral Ag	Norm Ag	Viral Ag	Norm Ag
1	1	3	3	5	5	7	7	POS CTRL	POS CTRL
1	1	3	3	5	5	7	7	POS CTRL	POS CTRL
1	1	3	3	5	5	7	7	POS CTRL	POS CTRL
2	2	4	4	6	6	8	8	NEG CTRL	NEG CTRL
2	2	4	4	6	6	8	8	NEG CTRL	NEG CTRL
2	2	4	4	6	6	8	8	NEG CTRL	NEG CTRL

QC		
Avg Neg control viral Ag A450	#DIV/0!	#DIV/0!
Avg Pos control viral Ag A450	#DIV/0!	
Avg Pos control normal Ag A450	#DIV/0!	
P/N for Pos control	#DIV/0!	#DIV/0!
NBR for Pos control	#DIV/0!	#DIV/0!
Is test valid and OK to report results?		#DIV/0!

Interpretation of results

P/N	NBR	RESULT
≥3.0	≥2.0	Positive (POS)
2.0-<3.0	≥2.0	Equivocal (EQ)
P/N <2.0	ANY	Negative (NEG)
P/N ≥2.0	<2.0	Uninterpretable (UI)

IF test is not valid, do not report results; repeat the test

Results

Sample	Viral Ag A450	Normal Ag A450	Avg Viral Ag A450	Avg Norm Ag A450	P/N	Avg NBR	Ind NBR	NBR QC Pass/Fail*	Result
1	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
	#DIV/0!	#DIV/0!					#DIV/0!	#DIV/0!	
	#DIV/0!	#DIV/0!					#DIV/0!	#DIV/0!	
2	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
	#DIV/0!	#DIV/0!					#DIV/0!	#DIV/0!	
	#DIV/0!	#DIV/0!					#DIV/0!	#DIV/0!	
3	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
	#DIV/0!	#DIV/0!					#DIV/0!	#DIV/0!	
	#DIV/0!	#DIV/0!					#DIV/0!	#DIV/0!	
4	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
	#DIV/0!	#DIV/0!					#DIV/0!	#DIV/0!	
	#DIV/0!	#DIV/0!					#DIV/0!	#DIV/0!	
5	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
	#DIV/0!	#DIV/0!					#DIV/0!	#DIV/0!	
	#DIV/0!	#DIV/0!					#DIV/0!	#DIV/0!	
6	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
	#DIV/0!	#DIV/0!					#DIV/0!	#DIV/0!	
	#DIV/0!	#DIV/0!					#DIV/0!	#DIV/0!	
7	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
	#DIV/0!	#DIV/0!					#DIV/0!	#DIV/0!	
	#DIV/0!	#DIV/0!					#DIV/0!	#DIV/0!	
8	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
	#DIV/0!	#DIV/0!					#DIV/0!	#DIV/0!	
	#DIV/0!	#DIV/0!					#DIV/0!	#DIV/0!	

*Denotes NBR <2; NBR QC Fail indicates background reaction and only applies to P/N's that otherwise would be POS or EQ

Run notes

CDC MAC-ELISA Calculations workbook example

Notes to users:

If needed, unprotect sheet using password: yf

Enter information requested in orange cells; the sheet is not set up to calculate CSF or titration results; these should be performed manually

Copy raw OD's into cells A10:L17 of Raw Data Table; delete "spotty" wells (results will adjust)

Worksheet assumes blanks are calculated using wells A1-D1 on the microtiter plate. If needed, unprotect and adjust formula in cell A19

Raw Data Table

Test name:	WN MAC-ELISA		Date:	########		Plate:	1		Operator:	JB	
0,041	0,040	0,041	0,045	0,042	0,043	0,034	0,039	0,040	0,041	0,043	0,040
0,042	0,202	0,18	0,078	0,075	0,069	0,07	0,764	0,084	1,101	0,077	0,039
0,042	0,216	0,188	0,077	0,076	0,07	0,093	0,709	0,146	1,212	0,079	0,042
0,041	0,218	0,189	0,075	0,074	0,071	0,068	0,793	0,097	1,193	0,079	0,042
0,043	0,077	0,065	0,344	0,084	0,095	0,068	0,786	0,073	0,098	0,072	0,041
0,039	0,079	0,071	0,365	0,146	0,09	0,068	0,783	0,077	0,089	0,074	0,04
0,041	0,094	0,072	0,328	0,097	0,091	0,071	0,781	0,079	0,091	0,069	0,039
0,041	0,040	0,041	0,045	0,042	0,043	0,034	0,039	0,040	0,041	0,043	0,040

0,0415	Avg Blank

Blank-adjusted Values Table

	0,161	0,139	0,037	0,034	0,028	0,029	0,723	0,043	1,060	0,036
	0,175	0,147	0,036	0,035	0,029	0,052	0,668	0,105	1,171	0,038
	0,177	0,148	0,034	0,033	0,030	0,027	0,752	0,056	1,152	0,038
	0,036	0,024	0,303	0,043	0,054	0,027	0,745	0,032	0,057	0,031
	0,038	0,030	0,324	0,105	0,049	0,027	0,742	0,036	0,048	0,033
	0,053	0,031	0,287	0,056	0,050	0,030	0,740	0,038	0,050	0,028

Plate set-up

Enter sample name in upper left (orange) cell of each set of 6 wells (table will auto-populate)

Viral Ag	Norm Ag	Viral Ag	Norm Ag	Viral Ag	Norm Ag	Viral Ag	Norm Ag	Viral Ag	Norm Ag
1	1	3	3	5	5	7	7	POS CTRL	POS CTRL
1	1	3	3	5	5	7	7	POS CTRL	POS CTRL
1	1	3	3	5	5	7	7	POS CTRL	POS CTRL
2	2	4	4	6	6	8	8	NEG CTRL	NEG CTRL
2	2	4	4	6	6	8	8	NEG CTRL	NEG CTRL
2	2	4	4	6	6	8	8	NEG CTRL	NEG CTRL

QC		
Avg Neg control viral Ag A450	0,051	PASS
Avg Pos control viral Ag A450	1,127	
Avg Pos control normal Ag A450	0,037	
P/N for Pos control	22,029	PASS
NBR for Pos control	30,602	PASS
Is test valid and OK to report results?		YES

Interpretation of results

P/N	NBR	RESULT
≥3.0	≥2.0	Positive (POS)
2.0-<3.0	≥2.0	Equivocal (EQ)
P/N <2.0	ANY	Negative (NEG)
P/N ≥2.0	<2.0	Uninterpretable (UI)

IF test is not valid, do not report results; repeat the test

Results

Sample	Viral Ag A450	Normal Ag A450	Avg Viral Ag A450	Avg Norm Ag A450	P/N	Avg NBR	Ind NBR	NBR QC Pass/Fail*	Result
1	0,161	0,139	0,171	0,144	3,332	1,183	1,159	Fail	UI
	0,175	0,147					1,191	Fail	
	0,177	0,148					1,197	Fail	
2	0,036	0,024	0,042	0,028	0,818	1,503	1,511	Fail	NEG
	0,038	0,030					1,271	Fail	
	0,053	0,031					1,721	Fail	
3	0,037	0,034	0,035	0,034	0,687	1,050	1,090	Fail	NEG
	0,036	0,035					1,029	Fail	
	0,034	0,033					1,031	Fail	
4	0,303	0,043	0,304	0,068	5,945	4,506	7,118	Pass	POS
	0,324	0,105					3,096	Pass	
	0,287	0,056					5,162	Pass	
5	0,028	0,029	0,029	0,036	0,557	0,803	0,965	Fail	NEG
	0,029	0,052					0,553	Fail	
	0,030	0,027					1,113	Fail	
6	0,054	0,027	0,051	0,028	0,987	1,836	2,019	Pass	NEG
	0,049	0,027					1,830	Fail	
	0,050	0,030					1,678	Fail	
7	0,723	0,043	0,714	0,068	13,951	10,575	17,000	Pass	POS
	0,668	0,105					6,388	Pass	
	0,752	0,056					13,541	Pass	
8	0,745	0,032	0,742	0,035	14,498	21,297	23,635	Pass	POS
	0,742	0,036					20,887	Pass	
	0,740	0,038					19,720	Pass	

*Denotes NBR <2; NBR QC Fail indicates background reaction and only applies to P/N's that otherwise would be POS or EQ

Run notes

ANNEX 5.2
List of reagents required to perform the in-house CDC yellow fever MAC-ELISA method

The list of reagents below include sets of items required for the performing the CDC MAC- ELISA method, as previously bundled through the UNICEF procurement mechanism. Newer commercial assays evaluated and recommended for use by WHO are now available through the UNICEF procurement mechanism.

General Description: Clinical Laboratory Bundle for YF Diagnostics, 400 tests

Supplier's product reference: AMEX-YF Dx

Product description/ Bundle composition

Module 1

- 75 x S0001738 Immulon 2HB Plates (ref. 3455, Manufacturer: Thermo Fisher, USA)
- 2 x S0001732 3,3 ,5,5-Tetramethylbenzidine-TMB,200ml (ref. 308175, Manufacturer: Neogen Life Sciences)
- 1 x S0001746 TMB Stop Solution,400ml (ref. 5150-0020, Manufacturer: Seracare, USA)
- 1 x S0001737 Goat anti-human IgM coating antibody,1mg (ref. 5210-0157, Manufacturer: Seracare, USA)

Module 2

- 1 x S0001736 Glycerol for molecular biology,100ml (ref. 356352, Manufacturer: Merck Millipore, Germany)
- 1 x S0001747 Tween 20,100ml (ref. BP337-100, Manufacturer: Thermo Fisher, USA)
- 1 x S0001745 Skim milk,500g (ref. T145.2, Manufacturer: Carl Roth, Germany)
- 5 x S0001739 Phosphate Buffer Saline-PBS tablet,box/100 (ref. 1107.1, Manufacturer: Carl Roth, Germany)
- 1 x S0001731 Carbonate Bicarbonate Buffer Capsules/100 (ref. 1027.3, Manufacturer: Carl Roth, Germany)

Module 3

- 530 x S0001744 Reagent reservoir 25ml (ref. 1550/SG, Manufacturer: Aptaca, Italy)
- 4 x S0001743 Racks (standard micro tubes, format 4x6 (ref. 456335C, Manufacturer: Globe Scientific, USA)
- 1 x S0001734 Cryotube,2ml, sterile,screw cap, box/1000 (ref. 6311 , Manufacturer: Aptaca, Italy)
- 1 x S0001735 Dilutiontube2m, non-stersnapcap,box/1000 (ref. 1303 , Manufacturer: Aptaca, Italy)
- 12 x S0001740 Pipette tip, 2 - 20 ul, Rack/96 (ref. 5-118-C5-0, Manufacturer: AHN Biotechnologie, Germany)
- 63 x S0001741 Pipette tip, 20 - 200 ul, Rack/96 (ref. 5-128-C5-0, Manufacturer: AHN Biotechnologie, Germany)
- 9 x S0001742 Pipette tip, 100 - 1000 ul, Rack/96 (ref. 5-203-C4-0, Manufacturer: AHN Biotechnologie, Germany)

Module 4 (formally donated by CDC in a pre-packed modular form; (Lyophilized materials: - 20 deg. C, storage 5+ years)

- 1 x Flavivirus IgM +ve control 0.25ml
- 4 x YF vero antigen 0.25ml
- 4 x Normal vero antigen 0.25ml
- 1 x HRP conjugated 6B6C 1/HRP anti-flavivirus 0.25ml

Packaging and labelling

Unit presentation: 1 (one) Clinical Laboratory Bundle for YF Diagnostics, 400 tests

ANNEX 5.3
Example of protocol for verification of a new serological assay in the laboratory: Template draft

Note: This document is intended to be used as a template for developing a method verification protocol and summarizing results.

Verification plan and protocol

Method Verification Protocol for: serology assay, anti-yellow fever IgM MAC-ELISA (YF MAC-HD)
Protocol Revision Number: *(assign a unique revision number for the verification)*
Branch/Laboratory: *(branch and laboratory name)*
Purpose and Rationale: To provide objective evidence that the method used meet the laboratories' acceptance criteria and intended use.

Method description/intended use:

Describe the diagnostic method and test strategy, type of results quantitative or qualitative and matrix intended to be used serum, plasma, or other patient samples according to the manufacturer recommendation.
If applicable, include any limiting factors, such as volume of samples that will impact the verification parameters. Also include any application(s) of the method that will not be utilized and thus will not be verified.
Also describe the acceptance criteria of results defined by the manufacturer.

Assay materials used:

Material/Matrix	Source/Manufacturer	Characterization	Lot #	Expiration

Sample required for verification:

Sample's reactivity	Number	Characterization of sample, matrix (serum, plasma, ….)
high positive	3 - 5	
medium positive	3 - 5	
low positive	3 - 5	
negative	3 - 5	

The sera used for the verification should be prequalified by another laboratory with the similar serology assay. Preferably 3 – 5 high, medium, and low reactive positive and some negative tested sera should be used. Aliquots of this samples should be tested according to the manufacturer's instructions.

Reactivity of sample for verification:

Sample's reactivity	expected OD value	OD value sample 1	OD value sample 2	OD value sample 3			% agreement
high positive							
medium positive							
low positive							
negative							
Positive control							
Negative control							

Acceptance criteria:
The assay performance followed the instructions for users should show the expected reactivity regarding the positive and negative controls.
Depending on the prequalification of the sera used for the verification the acceptance criteria should be defined. There should be an 80 - 100% agreement for the high, medium, and negative reactive samples regarding the rating of a qualitative assay for positive, negative, or equivocal results. Discrepant results should be further investigated and analysed for possible errors.
In case of significant differences of the results between the pre-tested samples and the obtained results a general examination of the laboratory performance should be performed.

Summary of the assay verification:
Describe the performance of the new implemented serology assay according to the verification performed. And if the assay fulfills the expectations in accordance with the manufacturer's instruction.

Report approval

Date:	Name	Title: Team Leader
Date:	Name	Title: Quality Manager
Date:	Name	Title: Laboratory Leader

All parts in *italic* requires completion by the diagnostic laboratory.

ANNEX 5.4
CDC MAC-ELISA TITRATION SOP

Please use the Excel spreadsheet for CDC MAC-ELISA TITRATION SOP available at *https://www.who.int/publications/i/item/9789240084476* or see details below.

Annex **Antigen titration for CDC _____ MAC-ELISA**

Test name: **Operator name:** **Date:**

	1	2	3	4	5	6	7	8	9	10	11	12
A		Viral Agn Dilution	Normal Agn Dilution	Viral Agn Dilution	Normal Agn Dilution							
B		1:25	1:25	1:25	1:25							
C		1:50	1:50	1:50	1:50							
D		1:100 P	1:100	1:100 N	1:100							
E		1:200	1:200	1:200	1:200							
F		1:400	1:400	1:400	1:400							
G		1:800	1:800	1:800	1:800							
H												

	Day 1	**Dilution**	**Amt/vol**	**Lot #**	**Added?**
1	**Coat plate:** Goat anti-hu IgM in carbonate buffer (1:2000). Add 75 ul/well	**1:2000**	_____		Yes/No
	Cover top plate with an empty plate				
	Incubate 16-24h at 4°C				
	Day 2				
2	Dump off capture Ab. Do not wash; blot dry.				
3	**Blocking:** 200 µl/well of PBS/5%milk/0.5% Tween-20				Yes/No
	Incubate 30 min at room temperature				
4	**Wash** 5X with PBS/0.05% Tween-20, 250 ul/well/cycle				
5	Add 50 ul **chimeric flavi positive control** to wells **B2:G3**				Yes/No
	Add 50 ul **negative control (1:400)** to wells **B4:G5**	**1:400**	_____		Yes/No
	Incubate 1 hr at 37°C/humid chamber				
6	**Wash** 5X with PBS/0.05% Tween-20, 250 ul/well/cycle				
7	**Antigens**: dilute YF viral and normal antigens in **wash buffer**				
	Add 50 ul wash buffer to wells in rows C to G				Yes/No
	Add 100 ul/well viral Ag (1:25) to **B2 and B4**	**1:25**	_____		Yes/No
	Add 100 ul/well normal Ag (1:25) to **B3 and B5**	**1:25**	_____		Yes/No
	Using a multichannel pipettor, transfer 50 ul antigens from wells B2:B5 to wells C2:C5.				
	Mix 3 times (avoid bubbles), and serially dilute to wells G2:G5, discarding 50 ul from G2:G5				Yes/No
	Incubate 16-24 hr at 4°C/humid chamber				
	Day 3				
8	**Wash** 5X with PBS/0.05% Tween-20, 250 ul/well/cycle				
9	**Conjugate**: Add 50 ul/well **6B6C-1 HRP-conjugate** diluted in **milk block**				Yes/No
	Incubate 1hr at 37°C/humid chamber				
10	**Wash** 10X with PBS/0.05% Tween-20, 250 ul/well/cycle, **rotate plate after 5 washes.**				
11	**Substrate:** Add 75 ul/well **Enhanced K-Blue TMB substrate** undiluted				Yes/No
	Incubate 10 min at room temperature in the dark				
12	**Stop Solution**: **TMB Stop Solution**; add 75 ul/well including blanks				Yes/No
13	Read at 450 nm in 1-5 min				

Analysis of results:

1 Calculate average blank OD

2 Subtract average blank OD from all test wells

3 Identify the row where the positive control on viral antigen is closest to 1.0 or slightly above

4 For this dilution, check that the NBR of the positive control is ≥2.0 (eg D2/D3)

5 For this dilution, check that the P/N is ≥2.0 (eg D2/D4)

6 For this dilution, check that the OD of the negative control reacted on viral antigen is <0.2

The ideal dilution is an OD of 1.0 for the positive control on viral antigen
and an OD of <0.1 for the negative control on viral antigen

Example

after blanks subtracted

		2	3	4	5
		POS ctrl		NEG ctrl	
	Dilution	Viral Agn	Normal Agn	Viral Agn	Normal Agn
A	1:25	2,561	0,445	0,551	0,101
B	1:50	1,783	0,255	0,334	0,085
C	1:100	1,239	0,103	0,125	0,077
D	1:200	0,859	0,099	0,071	0,055
E	1:400	0,521	0,078	0,068	0,049
F	1:800	0,337	0,079	0,061	0,051
G	1:1600	0,205	0,068	0,055	0,049
H					

OD of positive control reacted on viral antigen closest to or slightly above 1.0 is in D2 (1.239; dilution 1:100)

NBR is 12.029

P/N is 9.912

OD of Neg ctrl reacted on viral antigen is 0.125

Here, the OD of the negative control is good but not ideal (>0.1)
Check the closest lower dilution, row E, 1:200

OD of Pos Ctrl on viral antigen = 0.859
NBR is 8.677
P/N is 12.098
OD of Neg ctrl reacted on viral antigen = 0.071
Working dilution of the antigens best between 1:100 and 1:200 eg 1:160

ALWAYS try out working dilution in a regular test with some known samples/controls to confirm dilution
Note that if it is impossible to obtain a dilution that gives ideal results, you may need to use the closest dilution to ideal and titrate the conjugate

ANNEX 5.5A CDC MAC-ELISA technical guide

The following information has been taken from the CDC MAC-ELISA technical guide.

Plates	Cover plate with empty plate during all incubations Use Immulon 2HB. Other brands have not been validated for this test Ideal to not stack the plates to keep incubation temperature consistent, but they can be stacked (no more than 5) Do not allow wells to dry out between washing and adding reagents - this will cause inconsistent results
Coat	Use @ 1:2000- should be no need to vary Coating buffer kept @ 4°C for up to 1 year. Avoid contamination by using sterile pipettes or pouring from the bottle. Ideal to 0.2u filter but not critical. Coating buffer is approx. pH 9.6 and should not need adjusting Coating antibody: Reconstitute in 1 ml 50% glycerol and store at 4°C up to one year. Acetic acid has not been validated for reconstitution of the antibody for this test. Coated plates should be kept at 4°C and plates should be covered with an empty plate or with plate sealer to prevent drying out (causes reduced OD's) Coated plates can be kept at 4°C for no more than 7 days Accidentally using antibody diluted in wash buffer will cause signal to be absent
Block	Keep up to 3 weeks @ 4°C 5% non-fat dry milk in 1X PBS and 0.05% Tween20 (a 0.1X PBS will cause low OD…some labs have made this mistake)
Samples	Do not use highly hemolysed serum or whole blood Dilute serum at 1:400 in 1X wash buffer Make serum dilutions the same day you perform the test (IgM signal diminishes overnight) Mix serum dilution just prior to adding to plate (helps with consistency) Freeze serum if not being tested within 7 days CSF can be used undiluted or up to 1:5. Use normal CSF at the same dilution as a control for calculating results

Chimeric Flavivirus Positive control	Use per package insert. Stable @ 4°C for up to 6 months. Good to reconstitute, aliquot and freeze and then keep aliquot @ 4°C until finished up to 6 months Max freeze-thaws have not been determined but at least 3 has been verified as OK Make control dilutions no more than 1 hour before adding to the test
Negative control	Provided by user. Use @ 1:400 in 1X wash buffer Should represent your population and best to use a pool of sera that have previously tested negative in arbo tests
Viral antigen	Antigens must be kept frozen at -20°C. They do not survive @ 4°C Best to reconstitute and freeze aliquots. Make working dilution in 1X wash buffer and use within 1 h **Do not** make working dilution ahead of time; cannot be stored at any temperature Neat, reconstituted antigen can be freeze-thawed at least 10X for up to a year Normal Ag used @ same dilution as viral antigen After the overnight antigen incubation there may be condensation on the underside of the plate. Blot this with a paper towel before continuing. Make sure the plate is not upside down when antigens are added.
Conjugate	Reconstitute in sterile water according to insert instructions Store at 4°C for up to 1 year. May need to retitrate after several months if activity decreases Dilute in blocking buffer. Make dilution <1 hour before use Do not freeze the conjugate; it will lose activity Accidentally diluting the conjugate in wash buffer rather than blocking buffer will cause high background
Substrate	Bring to room temperature before use Careful to avoid leaving drops of substrate on rims of wells…will cause spotty wells. Only pour enough substrate into reagent reservoir for one plate at a time in case it turns blue Discard substrate bottle or reagent reservoir if contents are blue Avoid putting pipettes into bottle of substrate

Stop solution	Try not to introduce bubbles into the wells at this stage. If you get bubbles, use a needle or clean 10 ul pipette tip (finger over end) to burst them before reading the plate
	Extremely high titered samples may occasionally give a green colour as all the substrate has not been quenched. This is rare.
	Watch out for bubbles in the "blank" well--they will alter your results by giving false high OD's
	Avoid generation of bubbles - recommend to aspirate pipette to first "stop", not all the way
Wash buffer	Make sure 1XPBS/0.05% Tween-20. The pH should be 7.0-7.4 and should not need adjusting when using tablets
	Usually can store wash buffer at RT for several weeks; CDC makes 10X PBS and makes 1X wash buffer as needed
	Wash buffer at 0.1X will cause lowered OD's
	Use a reliable source of water in the wash buffer
Plate washer	Best to use fast aspiration speed and moderate dispense speed
	Make sure no water or cleaner in lines
	Wells should be washed with 250 ul buffer in each cycle
	Flush the probes with de-ionized or mineral-free water after use each day to prevent build-up of minerals and clogged probes
Plate reader	Calibrate and check lamp. If fails calibration, replace lamp.
	The lamp is OK but printed OD's are lower than they seem when I look at the plate - probably means there are bubbles in the "blank" wells
	Frequently asked questions
	Should results be used after blank OD removed? Yes. Blank OD's are usually around 0.04
	My results are not as good as they were when originally titrated. What to do? Answer: If this situation repeats, you may need to retitrate a reagent
	My results (controls) are inconsistent run to run. Why? Answer: In our experience, inconsistency improves once test is run regularly, but check for bubbles and check your pipetting technique. Check that the wash head on plate washer has been cleaned regularly, especially if not used frequently. Consult section "Inconsistent results"

	If my replicates have inconsistent OD's what do I do? Answer: "Spotty wells" with obviously inconsistent high OD's should be eliminated from calculations and use average of 2 replicates. If all 3 replicates are vastly different, retest the sample.
Frequently asked questions continued	Will condensation on the underside of the plates cause problems? Answer: Maybe. When reading plates, be sure that there is no condensation on the under surface of the plate; blot the underside of the plate with a paper towel
	My OD readings are inconsistent within the 3 replicates of the sample. Why? This is often due to forgetting to vortex samples and reagents prior to adding to the plate.
	Are the reagent containers important? We recommend using new plasticware for storage of buffers, except if using a sulfuric acid-stop which should be be kept in glass.
	What is "overnight" incubation? 16-24 h
	Does it matter what time of day you read the plates? We recommend the morning, as OD's tend to raise in the afternoon. Above all, be consistent
	When reading the plates, how many significant figures should I use? Generally 3. Rounding up is OK except for samples that would become positive if rounded up.
	Why turn a plate 180 degrees for the 10X wash? This ensures even washing of the plate
	Will heat inactivation of the samples affect the results? In our experience, no.
	A new technician is getting strange results. Any ideas? Make sure they know how to operate the pipettes correctly. This is a common source of error.
	Our plate washer has broken. Can I wash plates by hand? Answer: Yes, discard plate contents, blot on paper towel, then add 200 ul wash buffer per well using a pipettor, dump and repeat for a total of 3 washes except for the wash step before the substrate addition (6 washes). Note that while the OD's may be lower with manual washing, the P/N's will be very similar (or a little higher) than when using a plate washer. Be sure to note the change in protocol if you switch wash methods.
	Why do the viral antigen wells have no OD but some of the normal antigen wells have high OD, including the positive control? Answer: the plate was likely upside down when you added the antigens. Correct the position of the numbers to calculate results.

	Troubleshooting by symptom
No signal	Accidentally using coating antibody diluted in wash buffer will cause signal to be absent
	Forgot to add coating antibody to the buffer
	Forgot to add conjugate to buffer
Low than expected OD's	Coated plates should be kept at 4°C and plates should be covered with an empty plate or with plate sealer to prevent drying out (causes reduced OD's)
	Using plate coated more than 7 days ago
	Make serum dilutions the same day you perform the test (IgM signal diminishes overnight)
	Make control dilutions no more than 1 hour before adding to the test
	Antigens must be kept frozen at -20°C. They do not survive @ 4°C
	Make antigen working dilution in 1X wash buffer and use within 1 h
	Diluted antigen in coating buffer
	Store conjugate at 4°C for up to 1 year. May need to retitrate after several months if activity decreases
	Do not freeze the conjugate; it will lose activity
	Bring substrate to RT before use
	Wash buffer at 0.1X will cause lowered OD's
	Make sure no water or cleaner in lines
	Make sure your reader is set on 450 nm (405 will give low readings)
	Forgot to add serum samples or antigen
	Used coating antibody, antigen or conjugate at the wrong dilution
	Used water to wash the plates
Unexpectedly high OD's	Insufficient washing; check wash programme
	Make sure cross-contamination does not occur
	Incorrect reagent dilutions; check calculations
	Longer than recommended incubation time especially conjugate and substrate steps
	Bubbles in wells
	Incubation of antigen overnight at 37°C
High background	Incorrect coating antibody dilution; check calculations
	Incorrect conjugate dilution
	Conjugate diluted in incorrect buffer such as wash buffer
	Reagents incorrectly titrated especially if a new lot number recently received; retitrate the new reagent.
	Buffers may be contaminated
	Assay may have been performed at the wrong temperature. Check thermometers and make sure antigen is incubated at 4°C not 37°C
	Water quality may be poor
	Substrate may have deteriorated; check that it is not blue
	Incubate substrate for exactly 10 minutes protected from dark at room temperature
Inconsistent results	Bubbles in some wells
	Results may differ if test performed at different times of day (especially if there is fluctuation of temperature)
	Incorrect use of pipettors; this is common among new technicians
	Plate washer malfunction; check that all wells are being aspirated completely and filled properly
	Try to avoid stacking plates and always keep top of plate covered
	Briefly vortex or mix all samples and reagents immediately before use
	Drops of substrate deposited on the edge of wells may fall in and contaminate wells; check technique
	Lack of use of the test; frequent practice will improve consistency of results
	Plates drying out between steps will cause inconsistent results; do not allow to dry out

National Center for Emerging and Zoonotic Infectious Diseases

What can possibly go wrong? Troubleshooting the MAC-ELISA

Jane Basile, US CDC

Regional combined molecular & serology training workshop for English-speaking African countries in Entebbe, Uganda, hosted by the **Uganda Virus Research Institute (UVRI)**
2021

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"There is no such thing as a failed experiment – the trick is to figure out what experiment you just did"

- Prof. Phil J. Squire

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CDC MAC-ELISA is a laboratory developed test (LDT)

What is a LDT?

- A test that requires careful attention to detail at all stages
- A test that is more challenging to use than a kit
- A test that may require adjustments
- A test that may not work identically in all laboratories
- A test that may require troubleshooting

Try to prevent having to troubleshoot!

How to avoid QC failures:

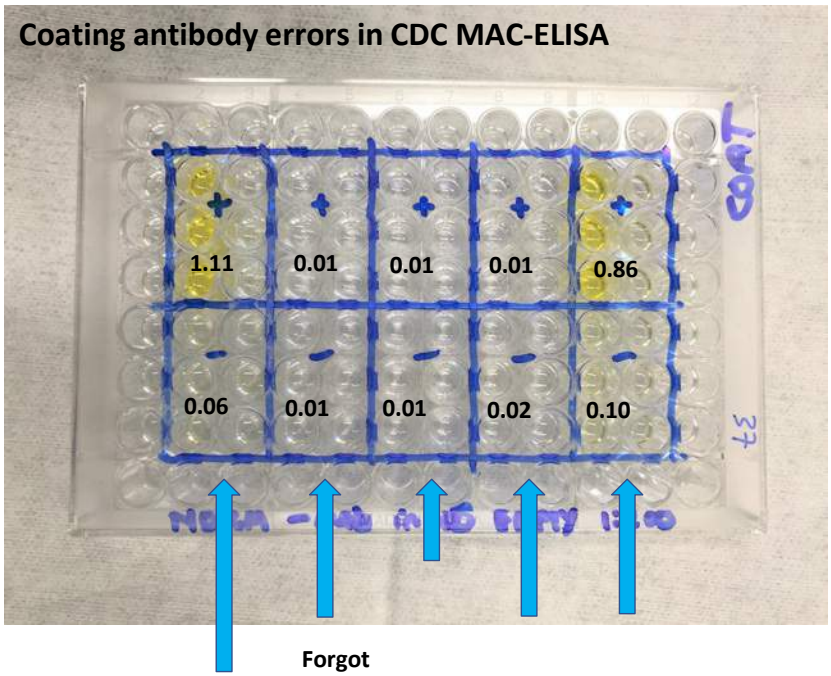
- ✓ Check the expiration dates of reagents
- ✓ Make sure reagents are reconstituted and stored according to package insert
- ✓ Label and date solutions
- ✓ Create an SOP that you understand, with checkboxes for each stage
- ✓ Follow the SOP!
- ✓ Use good quality de-ionized or molecular grade water for making solutions
- ✓ Perform visual check that plate washer is working correctly
- ✓ Never reuse pipette tips
- ✓ Calibrate the plate reader and pipettors
- ✓ Keep notes about what you did
- ✓ Be consistent; run the test frequently

Reality…..

Are you a technician?
Are you a scientist?

Run the test like a technician
Troubleshoot problems like a scientist

(also refer to the technical guide)



OD's after blanks subtracted

c
o
a

Diluted coating antibody in wash

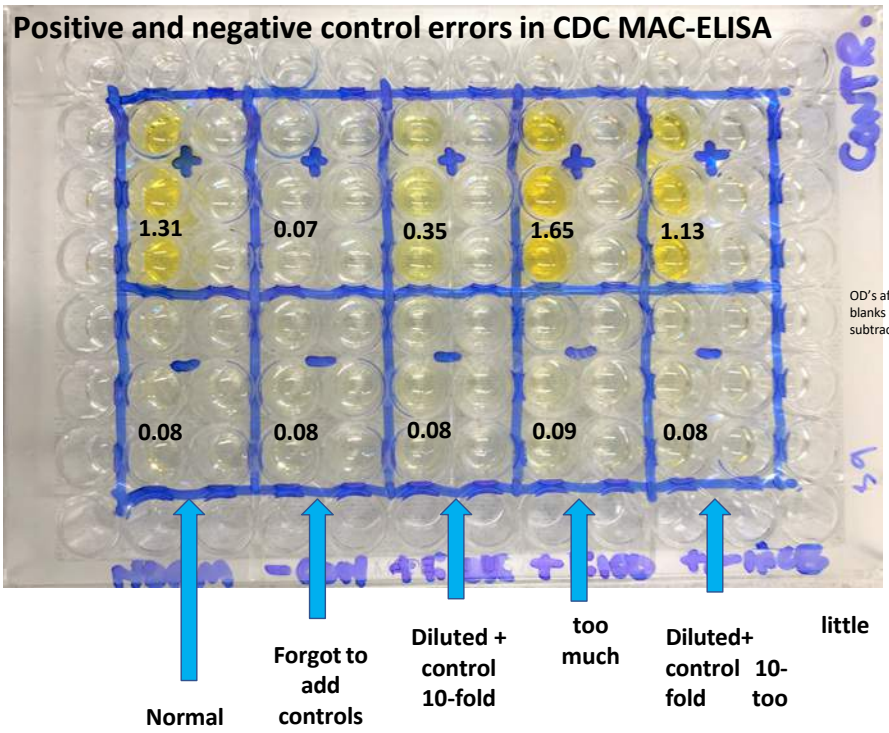
Wells not

1 0 X

a n t i b o d y

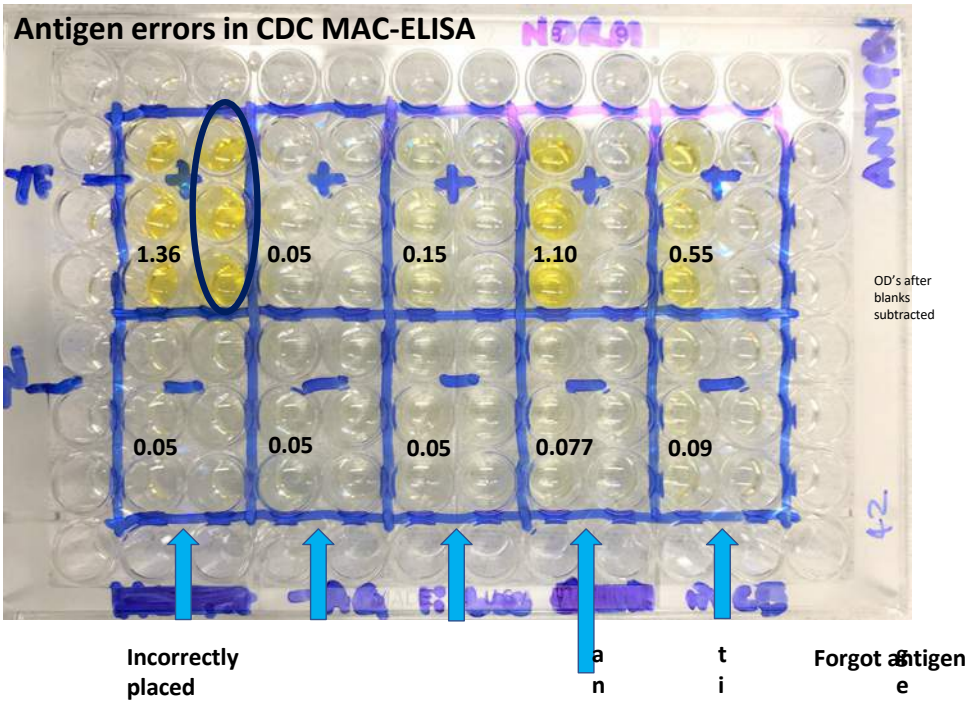
Normal antibody buffer filled conc

Positive and negative control errors in CDC MAC-ELISA



Diluted+/- controls in coating buffer

2
0
1



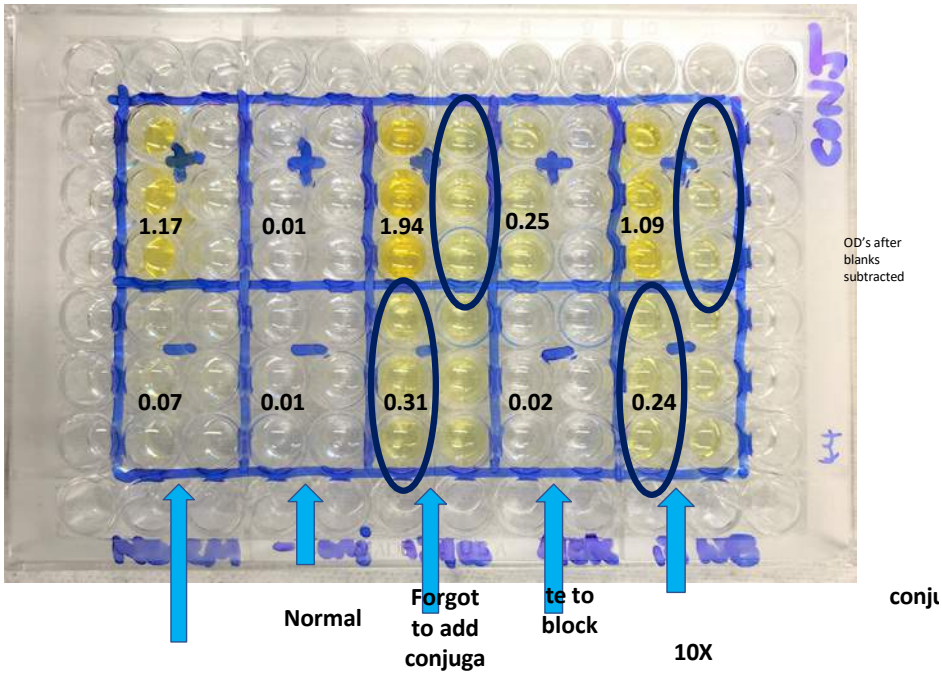
Diluted antigens 10- fold too much

Normal

Antigens diluted in CB

2022

Conjugate errors in CDC MAC-ELISA



0.1X conjugate

In wash buffer (not block)

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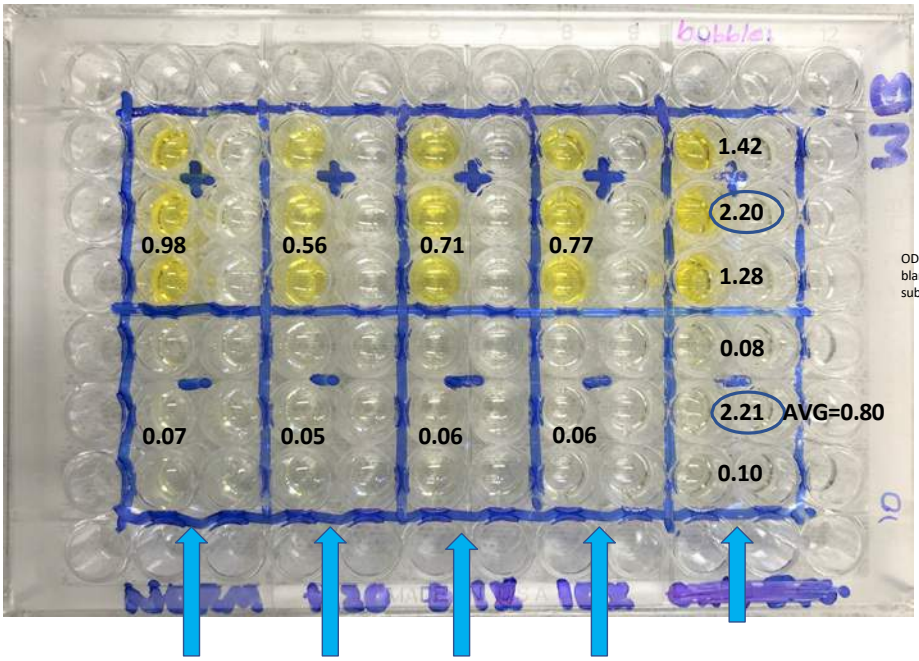
Wash buffer/stop solution errors in CDC MAC-ELISA

Watch for bubbles in blank wells!



rmal	di H2O	0.1X WB	10X WB	Bubbles in stop

For every run, **BEFORE DISCARDING THE PLATE** perform a visual check…

Does the color you see correspond with the printed results?
If no, what could the problem be?
Possible answers:
a. Bubbles in wells (test samples, controls or blanks)
Remedy: Burst any bubbles and re-read plate; adjust technique
b. Reader set to wrong wavelength
Remedy: Correct the wavelength
c. Lamp is failing
Remedy: Calibrate reader and replace lamp if needed

Does the color you see correspond with the calculated results?
If no, what could the problem be?
Possible answers:
a. Inconsistent wells due to bubbles
Remedy: Burst bubbles and re-read
b. Incorrectly-calculated results
Remedy: Check calculations and spreadsheet formulae (lock sheet)

A test can be valid and yet still have a problem

How do you know when a test looks "wrong"?

- ✓ Experience
- ✓ Keep records of control values/track trends
- ✓ Use an in-house positive control and track trends
- ✓ Use an in-house negative control and track trends

General strategy is to repeat the test before making changes
Most ELISA problems are due to operator error

Try to figure out what happened
Note if any buffers have been changed or water suppliers etc. since last good run.

For a correctly titrated test that was previously OK……

Try to diagnose the problem by symptom

Strategy: Does problem affect whole plate including controls? If yes...

No signal

Possible reasons:

- Diluted coating antibody in wash buffer
- Forgot to add coating antibody or conjugate to buffer

Low signal

Many possible reasons including….

- Used plates coated >7 days ago
- Stored reagents at wrong temperature
- Made working dilutions too far in advance
- Diluted reagents in wrong buffer
- Diluted reagent incorrectly (too dilute)
- Used cold substrate
- Made wash buffer incorrectly
- Set reader to wrong wavelength
- Used water to wash plates

Unexpectedly high OD's

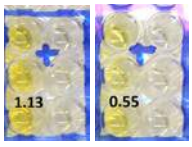
Possible reasons:

- Washed plates insufficiently
- Diluted reagents incorrectly (too concentrated)
- Used longer than recommended incubation times (especially conjugate an
- Overnight antigen incubation at 37 °C
- High laboratory temperature

High background

Possible reasons:

- Diluted coating antibody or conjugate incorrectly
- Diluted conjugate in wash buffer
- Used incorrect incubation temperatures
- Used deteriorated substrate (blue)
- Used longer than recommended incubation times
- Poor water quality



Inconsistency between plates on different days

Possible reasons:

- Laboratory temperature fluctuations/time of day
- Infrequent use of the test

Inconsistent control or sample OD's or "spotty" wells

Possible reasons:

- Bubbles may be in some wells (remove bubbles and re-read)
- Pipettors being used incorrectly or may be broken/wrong tips
- Samples may not have been mixed after dilution – VORTEX!
- Substrate may be on the edge of wells
- Plates may have dried out between steps
- Plate washer may have clogged probes
- Forgot to add the control or sample to the wells
- Cross-contamination of wells

Immediate remedy: Use average of 2 consistent wells if possible or repeat affected samples (or whole plate if controls are invalid).

Pay attention to the above list when repeating the test

Effect of vortexing the sample after dilution

Samples and controls *not* vortexed

0.052	0.016	0.252	0.029	0.758	0.021	1.110	0.014	1.370	0.016
0.050	0.012	0.386	0.010	0.880	0.017	1.374	0.015	1.603	0.012
0.061	0.010	0.418	0.009	0.966	0.009	1.454	0.009	1.537	0.010
1.311	0.007	0.822	0.008	0.407	0.008	0.066	0.008	0.050	0.010
1.299	0.008	0.861	0.009	0.403	0.009	0.069	0.008	0.044	0.010
1.462	0.007	0.799	0.009	0.391	0.007	0.068	0.009	0.049	0.009

Samples and controls vortexed

0.029	0.013	0.284	0.016	0.574	0.016	0.891	0.008	1.113	0.010
0.034	0.008	0.246	0.006	0.586	0.012	0.889	0.016	1.161	0.010
0.049	0.008	0.312	0.006	0.632	0.014	1.041	0.017	1.171	0.014
1.034	0.005	0.629	0.006	0.329	0.012	0.046	0.013	0.037	0.017
0.956	0.007	0.642	0.008	0.312	0.012	0.052	0.014	0.037	0.016
1.035	0.010	0.642	0.011	0.298	0.009	0.054	0.017	0.034	0.015

If there is *no other reason* for the problem

Retitrate the reagents generally one at a time

And if that doesn't work

Obtain new reagents

Remember that **CONSISTENCY** is key

Thank you

For more information, contact CDC
1-800-CDC-INFO (232-4636)
TTY: 1-888-232-6348 www.cdc.gov

Annex 6.

ANNEX 6.1
Example of RNA extraction protocol for clinical specimens using Qiagen QIAamp Viral RNA Mini Kit

1. Prepare kit reagents per manufacturer's instructions:

- Addition of carrier RNA to Buffer AVL.
  - » Add 310 µL Buffer AVE to tube containing lyophilized carrier RNA. Dissolve thoroughly and divide into 50 µL aliquots and store at -30 to -15°C.
  - » Calculate volume of Buffer AVL-carrier RNA mix needed:

# samples x 0.56 mL =	mL Buffer AVL
mL Buffer AVL x 10 µL/mL =	µL carrier RNA-Buffer AVE

  - » Mix by inverting tube 10 times; do not vortex.
- Addition of ethanol to wash buffers AW1 and AW2.
  - » Add the appropriate amount of 96-100% ethanol to wash buffer solutions as indicated on each bottle

2. Extraction/purification of RNA from samples and controls:

- Before beginning, equilibrate samples and Buffer AVE to room temperature.
- Mix 140 µL serum with 560 µL Buffer AVL-carrier RNA mixture and incubate at room temperature for 10 min.
- Briefly centrifuge sample tubes to remove drops from inside lid.
- Add 560 µL ethanol (add 200 µL to proficiency panel samples) to each sample and mix by pulse-vortexing for 15 seconds.
  - » Briefly centrifuge to remove drops from inside lid.
- Apply 630 µL each sample to a labeled QIAamp Mini column in a 2 mL collection tube.
  - » Take care to avoid wetting the rim when adding liquid to columns.
  - » Close column cap and centrifuge at 6000 x g (8000 rpm) for 1 minute.
  - » Move column to a clean 2 mL collection tube.
    - (a) Discard old collection tube containing filtrate.
- Repeat step (d).
- Add 500 µL Buffer AW1 to column.
  - » Close cap and centrifuge at 6000 x g (8000 rpm) for 1 minute.
  - » Move column to a clean 2 mL collection tube.
    - (a) Discard old collection tube containing filtrate.

- Add 500 µL Buffer AW2 to each column.
  - Close cap and centrifuge at FULL SPEED (20,000 x g; 14,000 rpm) for 3 minutes.
  - Move column to a clean 2 mL collection tube.
    (a) Discard old collection tube containing filtrate.
  - Centrifuge at FULL SPEED for 1 minute to remove any residual wash buffer.
  - Move column to a labeled 1.5 mL microcentrifuge tube.
    (a) Discard old collection tube containing filtrate.
- Add 60 µL Buffer AVE to column.
  - Close cap and incubate at room temperature for 1 minute.
  - Centrifuge at 6000 x g (8000 rpm) for 1 minute.
  - Discard column.
- Store RNA -30 to -15°C.

A6.1.1 PROTOCOL FOR THE YELLOW FEVER REAL-TIME RT-PCR

(Domingo, et al., 2012 J Clin Microbiol 50:4054)

1. Rehydrate Primers and Probes

- Using sterile, nuclease-free water, rehydrate primers and probes per the following table.
  - Store probe in the dark.
  - Allow to rehydrate for 15 minutes at room temperature.
  - Vortex and briefly centrifuge.
  - Aliquot and store at -20°C

Domingo YF primer/probe set

Primer			*Volume H2O*	*Concentration*
Forward	YF15-38FDom	GCTAATTGAGGTGYATTGGTCTGC		100 µM
Probe	YF41-64FAMDom	ATCGAGTTGCTAGGCAATAAACAC		25 µM
Reverse	YF83-103RDom	CTG CTA ATC GCT CAA MGA ACG		100 µM

** Domingo, et al., 2012 J Clin Microbiol 50:4054*

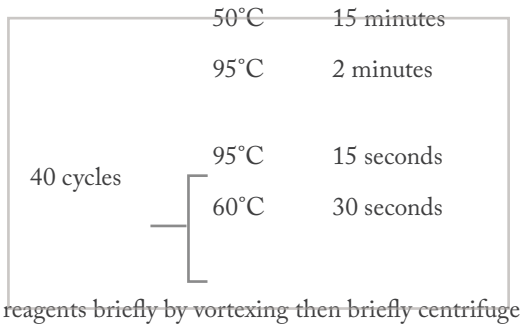
Real Time RT-PCR using SuperScript III Platinum One-Step qRT-PCR Kit

> **Note:** RT-PCR setup should be performed in a clean biosafety cabinet.

1. Thaw on ice:

   RNA samples
   Primers
   Probe
   2X Reaction Mix

2. Program ABI 7500 thermal cycler:



3. Mix reagents briefly by vortexing then briefly centrifuge and return to ice.
4. Prepare reaction mix on ice per table below.

Component	*Volume per reaction*	*Volume x (n+1)**
Nuclease-free H2O	1.35 µL	
2X Reaction Mix	12.5 µL	
Forward primer (YF15-38F) (100 µM)	0.25 µL	
Reverse primer (YF83-103R) (100 µM)	0.25 µL	
Probe (YF41-64FAM) (25 µM)	0.15 µL	
SuperScript III Taq mix	0.5 µL	

** n = number of reactions, (n+1) allows for extra reaction mix volume for pipetting error.*

5. Place PCR plate on ice or in cold block.
6. According to the plate setup diagram below, add the following to plate wells:
   - 15 µL reaction mix to all wells being utilized by your group.
   - 10 µL nuclease-free H2O to No Template Control (NTC) wells.
   - 10 µL sample RNA to sample wells.
   - 10 µL control RNA to positive (+) and negative (-) control wells.

Example Plate set up												
	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	(-) CTRL									
B	S2	S2	(-) CTRL									
C	S3	S3	NTC									
D	S4	S4	NTC									
E	S5	S5	(+) CTRL									
F	S6	S6	(+) CTRL									
G	S7	S7										
H	S8	S8										

7. Seal plate with optical strip caps and load plate on Applied Biosystems 7500 Real-Time PCR instrument.
8. After completion of the run, save and analyze the data.

➤ Test validation

- Positive control should be positive (reaction generates a curve that crosses the threshold at less than 38 cycles). If negative, repeat the plate.
- Negative control should be negative (reaction generates a curve that crosses the threshold at or above 38 cycles, OR, that fails to cross the threshold). If positive, repeat the plate.
- No-template control should be negative. If positive, look for possible sources of contamination in reagents and primers and repeat the test.

➤ If all control results are acceptable proceed to analyze each sample result.

- A result is interpreted as negative if the reaction generates a curve that crosses the threshold at or above 38 cycles, OR, that fails to cross the threshold.
- A result is interpreted as positive if the reaction generates a curve that crosses the threshold at less than 38 cycles.

Record your results here:

	S1	S2	S3	S4	S5	S6	S7	S8	(-) CTRL	NTC	(+) CTRL
Replicate 1											
Replicate 2											
INTERPRETATION											

YELLOW FEVER GENOME (WILD TYPE AND VACCINE STRAINS) DETECTION BY REAL-TIME RT-PCR (RT-qPCR): LABORATORY PROTOCOL

Graciously provided by Dr Cristina Domingo Carrasco, from the Robert Koch Institute, Berlin, Germany

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1. INTRODUCTION

1.1 Purpose of the activity

To detect the presence of yellow fever virus (YFV) RNA from wild type and/or vaccine strains from clinical samples.

1.2 Background

Yellow fever is a notifiable disease caused by the YFV. The detection of the YFV genome in a patient sample confirms unequivocally the diagnosis in natural infections. Likewise, in the case of adverse events after yellow fever vaccination with the strains YF-17D or YF-17DD, the detection of the viral genome longer than one week after vaccination, at any day in high viral load, or the detection of YF genome in tissue, is needed to confirm the causality of the adverse event. For YFV diagnosis purposes a reliable molecular diagnosis assay with suitable sensitivity and proved specificity regarding other flaviviral infections is mandatory.

This RT-qPCR is based on the detection of a conserved region within the 5´-non coding region (5´-UTR) of the YFV genome [1]. This protocol has been developed at the Robert Koch Institute and was selected for its good performance compared to different YFV molecular test evaluated during a YFV diagnosis external quality assurance activity [2]. The protocol has been routinely used for the screening of sera and urine samples of YFV vaccinees for the detection of YFV-17D genome and yielded an excellent profile of sensitivity [3].

2. PERSONNEL

The person(s) performing the procedure should be fully trained and competent in the molecular diagnosis of flavivirus infections, including sample management, viral RNA extraction from clinical samples, cDNA synthesis, DNA amplification techniques, and results interpretation.

3. SAFETY

Containment Biosafety Level 2 should be used for clinical samples. Handling of YFV suspected samples should be carried out under a laminar air flow (Biosafety level II). The person(s) manipulating YFV suspected samples must be vaccinated against the virus at least 15 days prior the activity.

4. TARGET

YFV 5´-UTR region

5. PRINCIPLE OF THE METHOD

The goal of this procedure is the transcription of the viral RNA into cDNA, and the amplification of an 88 bp length fragment of the YF genome with specific primers for YFV by a real time quantitative RT-PCR (RT-qPCR). The evidence of a specific amplification is revealed by a sequence specific hydrolysis probe (FAM-TAMRA or FAM-BHQ) through the fluorometric measurement of the probe signal.

6. MATERIALS

6.1 Equipment and Materials

Microcentrifuge

Vortex

Real time thermocycler (validated using a ABI7500 cycler but use of other cyclers is possible)

Racks for 1.5 ml and 0.2 ml tubes

Micropipettes: ranging from 0.1 to 1000 µl

Sterile filter tips

Sterile nuclease-free micro-centrifuge tubes: 1.5 ml and 200 µl

Optical Tubes

Protective Personal Equipment (Laboratory coat and gloves)

Cooling blocks

6.2 Reagents

Description	Specifications	Company*	Reference
Water	Molecular biology grade	Sigma	95284
RT-qPCR kit	AgPath-ID™ One-Step RT-PCR Reagents Ambion (Thermo Fisher)	Thermo Fisher	100 rxn ref: AM1005
Specific primers	Lyophilized, 25 nmol, desalt	Metabion	-
Probe	1.0OD, lyophilized, HPLC purified; dye: 5´- FAM, quencher: TAMRA or BHQ	Metabion	-

* Other reagents of similar characteristics could be used

7. OPERATIONAL PROCEDURES

All the protocol described will be carried out following strictly good laboratory practices for performing molecular amplification assays. For this purpose, a physical separation between pre-PCR and post-PCR areas will be kept including material, equipment, reagents and coats. The workflow will be unidirectional from clean to "contaminated" areas. The previous RNA extraction step will be carried out in a different area where the amplification process is done and using a biosafety (level II) laminar flow. The amplification area (thermocycling area) will be isolated from the extraction, pre-PCR and PCR areas.

Additional measures for contamination control will be implemented to minimize the risk of contamination as chemical decontamination with specific reagents, and UV-light exposure of material and cabins

8. SAMPLES AND CONTROLS

- Samples: Extracted RNA from samples of **suspected YF natural infections** or **suspected adverse event after vaccination cases.**
- Controls:
    - No-template control (NTC): Nuclease free water
    - Positive controls (PC): *In vitro* RNA controls with preestablished Cts (Controls around Ct 28 and Ct 32 are recommended

9. REAL-TIME qRT-PCR FOR THE DETECTION OF YFV GENOME

9.1 Primers and Probes (5´-3´):

Primer YFall Forward	5´- G CTA ATT GAG GTG **Y**AT TG GTC TGC -3´
Primer YFall Reverse	5´- C TGC TAA TCG CTC AA **M**GA ACG -3´
Probe YFall	5´- FAM-ATC GAG TTG CTA GGC AAT AAA CAC-TMR -3´

Note: degenerated primers are shown in bold

9.2 Procedure

The appropriate lab worksheet and plate layout should be filled in before starting the procedure. Set up the master mix according to the specific RT-qPCR kit recommended procedure, using the example below.

	[µl]	Factor	Excess (%)
RT-qPCR Protocol	1x	10x	10
H_2O	3,2	32	35,2
Buffer PT 2x	12,5	125	137,5
Primer YFALL Forward (10 µM)	1	10	11
Primer YFALL Reverse (10 µM)	1	10	11
Probe YFall (10µM)	0,3	3	3,3
Enhancer	1	10	11
Enzym Mix	1	10	11
Template	5		
Total	25		

The master mix should be thoroughly mixed and briefly spun down (5-10 seconds) before aliquoting 20 µl per well of the real-time plate/strip

Addition of 5 µl of RNA into each well should be done in a separate cabin and in the following order:

a. NTC

b. Samples

c. PC

Seal the plate with sealing foil or carefully cap the tubes to avoid contamination.

Briefly spin down the samples before introducing them in the thermocycler.

9.3 Cycling conditions

Place the plate/strips in the real-time thermocycler that has been pre-programmed with the following cycling parameters:

Time (min)	Temp	Comments
20	50°C	RT
5	95°C	
0:15	95°C	45 cycles
*0:45	60°C	*measurement

10. ANALYSIS AND INTERPRETATION OF RESULTS

The fluorescent data can be viewed during and after the PCR reaction. Analyze and save data according to the recommended procedure of the thermocycler used. Analyze the data by comparing the results obtained for the positive control and negative controls.

Check the Ct value for the positive controls. These should reproducibly amplify with a Ct value of 28±2 and 32±2 Ct. Greater deviation from the predetermined Ct values suggests that the positive controls may have degraded or the procedure did not perform properly. The NTC should not result in an increase in fluorescence above the baseline.

If all controls are within acceptable limits, analyze the data for the test samples. Otherwise consider the presence of inhibitors in the samples, or problems during the extraction procedure or PCR reaction set up.

An increase in fluorescence will be observed for positive samples.

11. FOLLOW UP TESTING

A second amplification technique directed to a different target within the YF genome (NS3 RT-qPCR, or NS5 panflavivirus RT-PCR) can be attempted for confirmation purposes. The positives samples might be subjected to viral cell culture.

12. REFERENCES

1. Domingo C, Patel P, Yillah J, et al. Advanced Yellow Fever Virus Genome Detection in Point-of-Care Facilities and Reference Laboratories. Journal of Clinical Microbiology. 2012;50 (12):4054-4060.

2. Domingo C, Escadafal C, Rumer L, Méndez JA, García P, Sall AA, et al. (2012) First International External Quality Assessment Study on Molecular and Serological Methods for Yellow Fever Diagnosis. PLoS ONE 7(5): e36291.

3. Domingo, C., S. Yactayo, E. Agbenu, et al., *Detection of yellow fever 17D genome in urine.* J Clin Microbiol, 2011. 49(2): p. 760-2.

DAY 1

RNA Extraction – Qiagen QIAamp Viral RNA Mini Kit

1. Prepare kit reagents per manufacturer's instructions:

- Addition of carrier RNA to Buffer AVL.
  - Add 310 µL Buffer AVE to tube containing lyophilized carrier RNA. Dissolve thoroughly and divide into 50 µL aliquots and store at -30 to -15°C.
  - Calculate volume of Buffer AVL-carrier RNA mix needed:

_____ # samples x 0.56 mL = _____ mL Buffer AVL

_____ mL Buffer AVL x 10 µL/mL = _____ µL carrier RNA-Buffer AVE

  - Mix by inverting tube 10 times; do not vortex.
- Addition of ethanol to wash buffers AW1 and AW2.
  - Add the appropriate amount of 96-100% ethanol to wash buffer solutions as indicated on each bottle

2. Extraction/purification of RNA from samples and controls:

- Before beginning, equilibrate samples and Buffer AVE to room temperature.

Note: Proficiency Panel samples (50 µL) used in this practicum have already been combined with 200 µL Buffer AVL-carrier RNA mixture. For RNA extraction from patient serum specimens you would begin by mixing 140 µL serum with 560 µL Buffer AVL-carrier RNA mixture and incubating at room temperature for 10 min.

- Briefly centrifuge sample tubes to remove drops from inside lid.
- Add 560 µL ethanol (add 200 µL to proficiency panel samples) to each sample and mix by pulse-vortexing for 15 seconds.
  - Briefly centrifuge to remove drops from inside lid.
- Apply 630 µL each sample to a labeled QIAamp Mini column in a 2 mL collection tube.
  - Take care to avoid wetting the rim when adding liquid to columns.
  - Close column cap and centrifuge at 6000 x g (8000 rpm) for 1 minute.
  - Move column to a clean 2 mL collection tube.
    (a) Discard old collection tube containing filtrate.

- Repeat step (d).
- Add 500 µL Buffer AW1 to column.
  - Close cap and centrifuge at 6000 x g (8000 rpm) for 1 minute.
  - Move column to a clean 2 mL collection tube.
    (a) Discard old collection tube containing filtrate.
- Add 500 µL Buffer AW2 to each column.
  - Close cap and centrifuge at FULL SPEED (20,000 x g; 14,000 rpm) for 3 minutes.
  - Move column to a clean 2 mL collection tube.
    (a) Discard old collection tube containing filtrate.
  - Centrifuge at FULL SPEED for 1 minute to remove any residual wash buffer.
  - Move column to a labeled 1.5 mL microcentrifuge tube.
    (a) Discard old collection tube containing filtrate.
- Add 60 µL Buffer AVE to column.
  - Close cap and incubate at room temperature for 1 minute.
  - Centrifuge at 6000 x g (8000 rpm) for 1 minute.
  - Discard column.
- Store RNA -30 to -15°C.

3. Rehydrate Primers and Probes

- Using sterile, nuclease-free water, rehydrate primers and probes per the following table.
  - Store probe in the dark.
  - Allow to rehydrate for 15 minutes at room temperature.
  - Vortex and briefly centrifuge.
  - Aliquot and store at -20°C.

Domingo YF primer/probe set

Primer			*Volume H2O*	*Concentration*
Forward	YF15-38FDom	GCTAATTGAGGTGYATTGGTCTGC		100 µM
Probe	YF41-64FAMDom	ATCGAGTTGCTAGGCAATAAACAC		25 µM
Reverse	YF83-103RDom	CTG CTA ATC GCT CAA MGA ACG		100 µM

** Domingo, et al., 2012 J Clin Microbiol 50:4054*

DAY 2

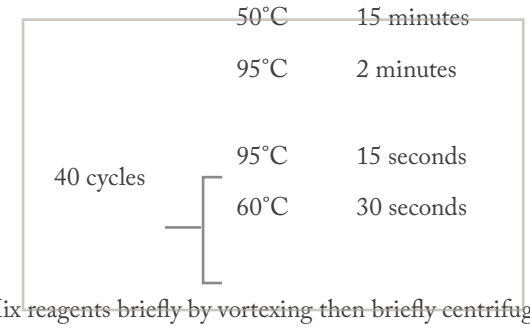
Real Time RT-PCR using SuperScript III Platinum One-Step qRT-PCR Kit

> **Note:** RT-PCR setup should be performed in a clean biosafety cabinet.

1. Thaw on ice:

   RNA samples
   Primers
   Probe
   2X Reaction Mix

2. Program ABI 7500 thermal cycler:



3. Mix reagents briefly by vortexing then briefly centrifuge and return to ice.
4. Prepare reaction mix on ice per table below.

Component	*Volume per reaction*	*Volume x (n+1)**
Nuclease-free H2O	1.35 µL	
2X Reaction Mix	12.5 µL	
Forward primer (YF14-34F) (100 µM)	0.25 µL	
Reverse primer (YF115C) (100 µM)	0.25 µL	
Probe (YF34-57FAM) (25 µM)	0.15 µL	
SuperScript III Taq mix	0.5 µL	

** n = number of reactions; (n+1) allows for extra reaction mix volume for pipetting error.*

5. Place PCR plate on ice or in cold block.
6. According to the plate setup diagram below, add the following to plate wells:
   - 15 µL reaction mix to all wells being utilized by your group.
   - 10 µL nuclease-free H2O to No Template Control (NTC) wells.
   - 10 µL sample RNA to sample wells.
   - 10 µL control RNA to positive (+) and negative (-) control wells.

Plate 1

	Group 1				Group 2				Group 3			
	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	(-) CTRL		S1	S1	(-) CTRL		S1	S1	(-) CTRL	
B	S2	S2	(-) CTRL		S2	S2	(-) CTRL		S2	S2	(-) CTRL	
C	S3	S3	NTC		S3	S3	NTC		S3	S3	NTC	
D	S4	S4	NTC		S4	S4	NTC		S4	S4	NTC	
E	S5	S5	(+) CTRL		S5	S5	(+) CTRL		S5	S5	(+) CTRL	
F	S6	S6	(+) CTRL		S6	S6	(+) CTRL		S6	S6	(+) CTRL	
G	S7	S7			S7	S7			S7	S7		
H	S8	S8			S8	S8			S8	S8		

Plate 2

	Group 4				Group 5							
	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	(-) CTRL		S1	S1	(-) CTRL					
B	S2	S2	(-) CTRL		S2	S2	(-) CTRL					
C	S3	S3	NTC		S3	S3	NTC					
D	S4	S4	NTC		S4	S4	NTC					
E	S5	S5	(+) CTRL		S5	S5	(+) CTRL					
F	S6	S6	(+) CTRL		S6	S6	(+) CTRL					
G	S7	S7			S7	S7						
H	S8	S8			S8	S8						

7. Seal plate with optical strip caps and load plate on Applied Biosystems 7500 Real-Time PCR instrument.
8. After completion of the run, save and analyze the data.

- Test validation
  - Positive control should be positive (reaction generates a curve that crosses the threshold at less than 38 cycles). If negative, repeat the plate.
  -
  - Negative control should be negative (reaction generates a curve that crosses the threshold at or above 38 cycles, OR, that fails to cross the threshold). If positive, repeat the plate.
  - No-template control should be negative. If positive, look for possible sources of contamination in reagents and primers and repeat the test.
- If all control results are acceptable proceed to analyze each sample result.
  - A result is interpreted as negative if the reaction generates a curve that crosses the threshold at or above 38 cycles, OR, that fails to cross the threshold.
  - A result is interpreted as positive if the reaction generates a curve that crosses the threshold at less than 38 cycles.

~~Record your results here:~~

	S1	S2	S3	S4	S5	S6	S7	S8	(-) CTRL	NTC	(+) CTRL
Replicate 1											
Replicate 2											
INTERPRETATION											

ANNEX 6.4
SOP template to be adapted to the respective diagnostic laboratory

Note: The following SOP template is provided to facilitate the quick implementation of the RealStar® Yellow Fever Virus RT-PCR assay in the diagnostic laboratory quality management system. Since the laboratory conditions vary in the different laboratories regarding extraction methods and type of cycler the SOP template should be adapted according to the respective laboratory methods and equipment.

Standard operating procedure (SOP)	Issued by:	ID:
Yellow Fever (YF) Nucleic Acid Testing (NAT) by PCR	Alison Jane Basile, Holly Hughes, Matthias Niedrig	2nd draft Revision: 08/06/2021

A6.4.1 BACKGROUND

Despite the execution of continuous YF vaccination campaigns in Africa and South America with the live attenuated vaccine, regular outbreaks or patients suspected to be infected with YF are a constant challenge for the health service and the public health system.

The clinical diagnosis of YF is difficult because symptoms are similar to a wide range of diseases, including dengue fever, other hemorrhagic viral diseases, leptospirosis, viral hepatitis, and malaria; hence laboratory confirmation is essential [2].

The majority of patients infected with YFV have no or only mild illness. In persons who develop symptoms, the incubation is typically 3 – 6 days. The initial symptoms include abrupt onset of fever, chills, severe headache, back pain, general body aches, nausea and vomiting, fatigue, and weakness. After a brief symptom remission which lasts hours to a day, approximately 15% of infected individuals progress to develop a more severe form of the disease. This severe form is characterized by high fever, jaundice, bleeding, and eventually shock and multiple organ failure [4,5]. Since no specific treatments for these patients are available, only supportive care to treat dehydration, respiratory failure, and fever can be used [1,3,4].

For patients clinically suspected of YFV infection, immediate laboratory diagnostic confirmation is essential. Besides the serological detection of YF-specific IgM antibodies, molecular detection of YF RNA is a highly reliable, rapid, and sensitive means to diagnose the disease if performed appropriately. YF viral RNA is fragile compared to antibodies; therefore, proper sample acquisition and handling techniques must be employed, followed by timely cold chain-maintained transport of the samples to the diagnostic laboratory. These are prerequisites for a reliable diagnosis. Because the clinical picture of such patients is complex, additional information regarding the patient´s course of disease, symptoms and clinical data are extremely helpful in facilitating at a conclusive diagnosis. A separate form providing relevant parameters like travel and vaccination history, course of disease, date of onset and symptoms, date of sample acquisition etc. should be part of the information provided when the sample is submitted to the laboratory. Immediate communication between the respective health service and the diagnostic laboratory before shipment is sent is highly recommended.

A6.4.2 PURPOSE

The RealStar® Yellow Fever Virus RT-PCR Kit 1.0 is an in vitro diagnostic test, based on real-time PCR technology, for the qualitative detection of YF virus specific RNA.

This kit was developed for the detection of all described yellow fever virus strains including the vaccine strain 17D.

The key features of the test are:

- Detection of Yellow Fever virus specific RNA
- Ready to use kit including Internal and Positive Control
- Compatible with various real-time PCR platforms
- CE-IVD marked in vitro Diagnostic Test

According to the manufacturer's instructions for use (IFU), the assay includes a heterologous amplification system (Internal Control) to identify possible RT-PCR inhibition and to confirm the integrity of the reagents of the kit.

Real-time RT-PCR technology utilizes reverse-transcriptase (RT) reaction to convert RNA into complementary DNA (cDNA), polymerase chain reaction (PCR) for the amplification of specific target sequences and target specific probes for the detection of the amplified DNA. The probes are labelled with fluorescent reporter and quencher dyes.

Probes specific for YFV RNA are labelled with the fluorophore FAM™. The probe specific for the Internal Control (IC) is labelled with the fluorophore JOE™.

Using probes linked to distinguishable dyes enables the parallel detection of YFV specific RNA and the Internal Control in corresponding detector channels of the real-time PCR instrument.

The test consists of three processes in a single tube assay:

- Reverse transcription of target and Internal Control RNA to cDNA
- PCR amplification of target and Internal Control cDNA
- Simultaneous detection of PCR amplicons by fluorescent dye labelled probes

Detailed information on assay performance evaluation, the analytical sensitivity, specificity and precision of the RealStar® Yellow Fever Virus RT-PCR Kit 1.0 can be found in the manufacturer's IFU.

A6.4.3 KIT COMPONENTS

LID COLOR	COMPONENT	NUMBER OF VIALS	VOLUME [µL/VIAL]
Blue	Master A	8	60
Purple	Master B	8	180
Green	Internal Control	1	1000
Red	Positive Control	1	250
White	Water (PCR grade)	1	500

A6.4.4 STORAGE CONDITION'S FOR KIT COMPONENTS

- The RealStar® Yellow Fever Virus RT-PCR Kit 1.0 is shipped on dry ice. The components of the kit should arrive frozen. If one or more components are not frozen upon receipt, or if the tubes have been compromised during shipment, contact Altona Diagnostics GmbH for assistance.
- All components should be stored between -25°C and -15°C upon arrival.
- Repeated thawing and freezing of Master reagents (more than twice) should be avoided, as this might affect the performance of the assay. The reagents should be frozen in aliquots, if they are to be used intermittently.
- Storage between +2°C and +8°C should not exceed a period of two hours.
- Protect Master A and Master B from light

A6.4.5 MATERIAL AND DEVICES REQUIRED FOR THE KIT PERFORMANCE

- Desktop centrifuge with a rotor for 2 ml reaction tubes
- Centrifuge with a rotor for microtiter plates, if using 96 well reaction plates
- Vortex mixer
- Appropriate 96 well reaction plates or reaction tubes with corresponding (optical) closing material
- Pipettes (adjustable)
- Pipette tips with filters (disposable)
- Powder-free gloves (disposable)
- Cold blocks or Ice-bath
- Appropriate nucleic acid extraction system or kit (see list in Supplementary information A6.4.8)
- Appropriate real-time PCR instrument (see list in Supplementary information)

If other nucleic acid extraction system or kits and/or other PCR cyclers are used that are not listed in Annex 1, the performance of the kit must be verified according to international standards. An SOP for such a verification is provided in a separate document

Please ensure that all instruments used have been installed, calibrated, checked and maintained according to the manufacturer 's instructions and recommendations.

General recommendations and precautions

Before first use check the product and its components for:

- Integrity
- Completeness with respect to number, type and filling
- Correct labelling
- Frozen upon arrival
- Use of this product is limited to personnel specially instructed and trained in the techniques of real-time PCR and in vitro diagnostic procedures.
- Specimens should always be treated as infectious and/or biohazardous in accordance with safe laboratory procedures.
- Wear protective disposable powder-free gloves, a laboratory coat and eye protection when handling specimens.
- Avoid microbial and nuclease (DNase/RNase) contamination of the specimens and the components of the kit.
- Always use DNase/RNase-free disposable pipette tips with aerosol barriers.
- Always wear protective disposable powder-free gloves when handling kit components.
- Use separated and segregated working areas for (i) sample preparation, (ii) reaction setup and (iii) amplification/ detection activities. The workflow in the laboratory should proceed in a unidirectional manner. Always wear disposable gloves in each area and change them before entering a different area.
- Dedicate supplies and equipment to the separate working areas and do not move them from one area to another.
- Store positive and/or potentially positive material separated from all other components of the kit.
- Do not open the reaction tubes/plates post amplification, to avoid contamination with amplicons.
- Additional controls may be tested according to guidelines or requirements of local, state and/or federal regulations or accrediting organizations. Do not autoclave reaction tubes after the PCR, since this will not degrade the amplified nucleic acid and will bear the risk to contaminate the laboratory area.
- Do not use components of the kit that have passed their expiration date.
- Discard sample and assay waste according to your local safety regulations

A6.4.6 TEST PROCEDURE

A6.4.6.1 SAMPLE PREPARATION

Extracted RNA is the starting material for the RealStar® Yellow Fever Virus RT-PCR Kit 1.0. The quality of the extracted RNA has a profound impact on the performance of the entire test system. It must be ensured that the system used for nucleic acid extraction is compatible with real-time PCR technology. Kits listed in Supplementary information are evaluated for nucleic acid extraction for this kit.

A6.4.6.2 MASTER MIX SETUP

All reagents and samples should be thawed completely, mixed (by pipetting or gentle vortexing) and centrifuged briefly before use. The RealStar® Yellow Fever Virus RT-PCR Kit 1.0 contains a heterologous Internal Control (IC), which can either be used as a RT-PCR inhibition control or as a control for the sample preparation procedure (nucleic acid extraction) and as a RT-PCR inhibition control.

1. If the IC is used as a RT-PCR inhibition control, but not as a control for the sample preparation procedure, set up the Master Mix according to the following pipetting scheme:

NUMBER OF REACTIONS (RUNS)	1 REACTION	12 REACTIONS
Master A	5 µl	60 µl
Master B	15 µl	180 µl
Internal Control	1 µl	12 µl
Volume Master Mix	**21 µl**	**252 µl**

2. If the IC is used as a control for the sample preparation procedure and as a RT-PCR inhibition control, add the IC during the nucleic acid extraction procedure.
3. No matter which method/system is used for nucleic acid extraction the IC must not be added directly to the specimen. The IC should always be added to the specimen/lysis buffer mixture. The volume of the IC is dependent on the elution volume and is equal to 10% of the elution volume. For instance, if the nucleic acid is going to be eluted in 60 µl of elution buffer or water, 6 µl of IC per sample must be added into the specimen/lysis buffer mixture.
4. If the IC was added during the sample preparation procedure, set up the Master Mix according to the following pipetting scheme:

NUMBER OF REACTIONS (RUNS)	1 REACTION	12 REACTIONS
Master A	5 µl	60 µl
Master B	15 µl	180 µl
Volume Master Mix	**20 µl**	**240 µl**

5. If the IC (Internal Control) was added during the sample preparation procedure, at least the negative control must include the IC.
6. No matter which method/system is used for nucleic acid extraction, never add the IC directly to the specimen.

A6.4.6.3 REACTION SETUP

1. Pipette 20 µl of the Master Mix into each required well of an appropriate optical 96-well reaction plate or an appropriate optical reaction tube.
2. Add 10 µl of the sample (eluate from the nucleic acid extraction) or 10 µl of the controls (Positive or Negative Control).

REACTION SETUP	
Master Mix	20 µl
Sample or Control	10 µl
Total Volume	**30µl**

3. Make sure that at least one Positive and one Negative Control is used per run.
4. Thoroughly mix the samples and controls with the Master Mix by pipetting up and down.
5. Close the 96-well reaction plate with appropriate lids or optical adhesive film and the reaction tubes with appropriate lids.
6. Centrifuge the 96-well reaction plate in a centrifuge with a microtiter plate rotor for 30 seconds at approximately 1000 x g (~ 3000 rpm).

A6.4.6.4 PROGRAMMING THE REAL-TIME PCR INSTRUMENT

For basic information regarding the setup and programming of different real-time PCR instruments, please refer to the user manual of the respective instrument.

For detailed programming instructions regarding the use of the RealStar® Yellow Fever Virus RT-PCR Kit 1.0 on specific real-time PCR instruments please contact our Technical Support (manufacturer IFU chapter 14: Technical Assistance).

1. Define the following settings:

SETTINGS	
Reaction Volume	30 µl
Ramp rate	Default
Passive Reference	**None**

2. Define the fluorescence detectors (dyes).

TARGET	DETECTOR NAME	REPORTER	QUENCHER
YFV specific RNA	YFV	FAMTM	(None)
Internal Control (IC)	IC	JOETM	(None)

3. Define the temperature profile and dye acquisition:

	STAGE	CYCLE REPEATS	ACQUISITION	TEMPERATURE [°C]	TIME [MIN, SEC]
Reverse Transcription	Hold	1	-	55	20:00
Denaturation	Hold	1	-	95	02:00
Amplification	Cycling		-	95	00:15
		45	-	55	00:45
			-	72	00:15

For basic information regarding data analysis on specific real-time PCR instruments, please refer to the user manual of the respective instrument.

For detailed instructions regarding the analysis of the data generated with the RealStar® Yellow Fever Virus RT-PCR Kit 1.0 on different real-time PCR instruments please contact Technical Support (manufacturer IFU chapter 14: Technical Assistance).

A6.4.7 ANALYSIS AND INTERPRETATION OF THE RESULTS

For interpretation of the molecular diagnosis the following criteria has to be fulfilled. A qualitative diagnostic test run is valid, if the control conditions are met:

CONTROL ID	DETECTION	CHANNEL
	FAM™	JOE™
Positive control	+	+/-*
Negative Control	-	+

** The presence or absence of a signal in the JOE™ channel is not relevant for the validity of the test run*

A **qualitative** diagnostic test run is **invalid**, (i) if the run has not been completed or (ii) if any of the control conditions for a **valid** diagnostic test run are not met.

In case of an invalid diagnostic test run, repeat testing by using the remaining purified nucleic acids or start from the original samples again.

DETECTION	CHANNEL	RESULTS INTERPRETATION
FAM™	JOE™	
+	+*	YFV specific RNA detected
-	+	No YFV specific RNA detected. Samples does not contain detectable amounts of YFV specific RNA
-	-	RT-PCR inhibition or reagent failure. Repeat testing from original sample or collect and test a new sample.

** Detection of the Internal Control in the JOE™ detection channel is not required for positive results in the FAM™ detection channel. A high YFV RNA load in the sample can lead to a reduced or absent Internal Control signal.*

Considering that the time and duration of the YFV viremia could vary considerably among different patients depending on the severity of the disease, the interpretation of the molecular test should be interpreted with caution. Specimens with high Ct positive curves (e.g. >38) should be interpreted with caution and re-extracted and retested as per internal Quality Assurance procedures.

A **positive** result is evidence that the patient is infected with YFV. In case of a full-blown YF infection of a severely ill patient the virus RNA may be detected in most body fluids including urine and tissues.

Also, in patients recovered a from the YF infection, RNA may be found in urine even after virus clearance from the blood.

Compared to the high predictive value of a positive PCR for a real YF infection, a negative result does not exclude a YFV infection completely.

Reasons for false negative results include poor quality of the patient sample, a timepoint of sampling too long after onset of disease, and inappropriate transport conditions. The results must be interpreted accordingly.

If suspected YF infection persists for a patient, a second sample should be analyzed if no other cause of the disease (hepatitis, malaria, Dengue, etc.) can be found.

Assaying for anti-YF IgM antibodies with confirmatory neutralization antibody testing for IgM- positive samples is another suitable method to confirm the YF infection in patients suspected of YF infection.

The diagnostic finding of a **positive** result should be communicated to the sender of the patient material **immediately**.

Depending on the **national reporting** system the information of a positive finding should also be **immediately** forwarded to the respective public health institution to inform further public health measures.

According to the International Health Regulations (103) YF infections belong to the reportable infections of international public health concerns.

REFERENCES FOR ANNEX 6.4

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212::aid-jmv5] 3.0.co;2-b.

Related resources

RealStar® Yellow Fever Virus RT-PCR Kit 1.0. for 96 Order No. 671013 https://altona-diagnostics.com/en/products/reagents-140/reagents/realstar-real-time-pcr- reagents/realstar-yellow-fever-virus-rt-pcr-kit-ce.html

Validation of a real-time RT-PCR based detection system for Yellow Fever Virus specific RNA

https://altona-diagnostics.com/files/public/Content%20Homepage/-%2002%20RealStar/RealStar%20References/YFV_Poster_ASTMH_New%20Orleans%202018.pdf

Altona; Instructions for Use, RealStar®, Yellow Fever Virus RT-PCR Kit 1.0, 05/2018 EN https://altona-diagnostics.com/en/products/reagents-140/reagents/realstar-real-time-pcr- reagents/realstar-yellow-fever-virus-rt-pcr-kit-ce.html

Konformitätserklärung/Declaration of Conformity (DoC)/Déclaration de conformitéconformité RealStar® Yellow Fever Virus RT-PCR Kit 1.0 (DE, EN, FR) https://altona-diagnostics.com/files/public/Content%20Homepage/-%2002%20RealStar/DoC%20RealStar-DE-EN-FR/DoC_YFV%201.0_20200526_DE_EN_FR.pdf

A6.4.8 SUPPLEMENTARY INFORMATION FOR ANNEX 6.4

According to the instructions for use the following kits and systems are suitable for nucleic acid extraction:

- QIAamp® Viral RNA Mini Kit (QIAGEN)
- QIAsymphony® (QIAGEN)• NucliSENS® easyMag® (bioMérieux)
- MagNA Pure 96 System (Roche)
- m2000sp (Abbott)
- Maxwell® 16 IVD Instrument (Promega)
- VERSANT® kPCR Molecular System SP (Siemens Healthcare)

Alternative nucleic acid extraction systems and kits might also be appropriate. The suitability of the nucleic acid extraction procedure for use with RealStar® Yellow Fever Virus RT-PCR Kit 1.0 has to be validated by the user.

If using a spin column-based sample preparation procedures that include wash buffers containing ethanol, it is highly recommended to perform an additional centrifugation step for 10 min at approximately 17000 x g (~ 13000 rpm), using a new collection tube, prior to the elution of the nucleic acid.

If your sample preparation system is using wash buffers containing ethanol, make sure to eliminate any traces of ethanol prior to elution of the nucleic acid. Ethanol is a strong inhibitor of real-time PCR.

The use of carrier RNA is crucial for extraction efficiency and stability of the extracted nucleic acid.

The RealStar® Yellow Fever Virus RT-PCR Kit 1.0 was developed and validated to be used with the following real-time PCR instruments:

- Mx 3005P™ QPCR System (Stratagene)
- VERSANT® kPCR Molecular System AD (Siemens Healthcare Diagnostics)
- ABI Prism® 7500 SDS (Applied Biosystems)
- ABI Prism® 7500 Fast SDS (Applied Biosystems)
- LightCycler® 480 Instrument II (Roche)
- Rotor-Gene® 6000 (Corbett Research)
- If using the Rotor-Gene® 6000 it is highly recommended to use the 72-well rotor with the appropriate 0.1 ml reaction tubes, or the Rotor-Gene® Q 5/6 plex (QIAGEN)
- Rotor-Gene® Q5/6 plex Platform (QIAGEN)
- CFX96™ Real-Time PCR Detection System (Bio-Rad)
- CFX96™ Deep Well Real-Time PCR Detection System (Bio-Rad)

PD Dr. Andreas Nitsche
Robert Koch Institute
Centre for Biological Threats and Special Pathogens
Highly Pathogenic Viruses
Seestrasse 10
13353 Berlin
Germany

ROBERT KOCH INSTITUT

Yellow fever set of molecular standards for assay verification

This set of four standards serves as yellow fever virus positive control material with defined concentration to test and verify molecular diagnostics for yellow fever virus.

Use

The four vials you receive contain cell culture supernatant of yellow fever virus (strain 17-D) infected Vero E6 cells. The samples were adjusted to a range of detection of yellow fever genome (high, medium and low viral load), aliquoted and, freeze-dried. The virus in each preparation was inactivated by heat treatment and additional γ-irradiation. No remaining infectivity could be proven.

Based on an elution volume of 60 µl in the RNA-extraction protocol, quantification by real-time RT-PCR assays using a molecular standard curve revealed the following results*

Assay	Ct value*/5 µL RNA	Copies*/5 µl RNA
Yellow fever RT-PCR (Domingo et al, JCM 2012; 50:12)	Calibrator-1: 26± 0.5 (CV 0.88 %) Calibrator-2: 29± 0.5 (CV 0.88 %) Calibrator-3: 32± 0.5 (CV 0.51 %) Calibrator-4: 36± 1 (CV 1.31 %)	Calibrator-1: 3.9E+04 Calibrator-2: 5E+03 Calibrator-3: 8.9E+02 Calibrator-4: 8E+01
RealStar®Yellow Fever Virus RT-PCR Kit	Calibrator-1: 24± 0.5 (CV 0.68 %) Calibrator-2: 28± 0.5 (CV 0.42 %) Calibrator-3: 31± 0.5 (CV 0.78 %) Calibrator-4: 34.5± 0.5 (CV 0.6 %)	

*Average of six measurements

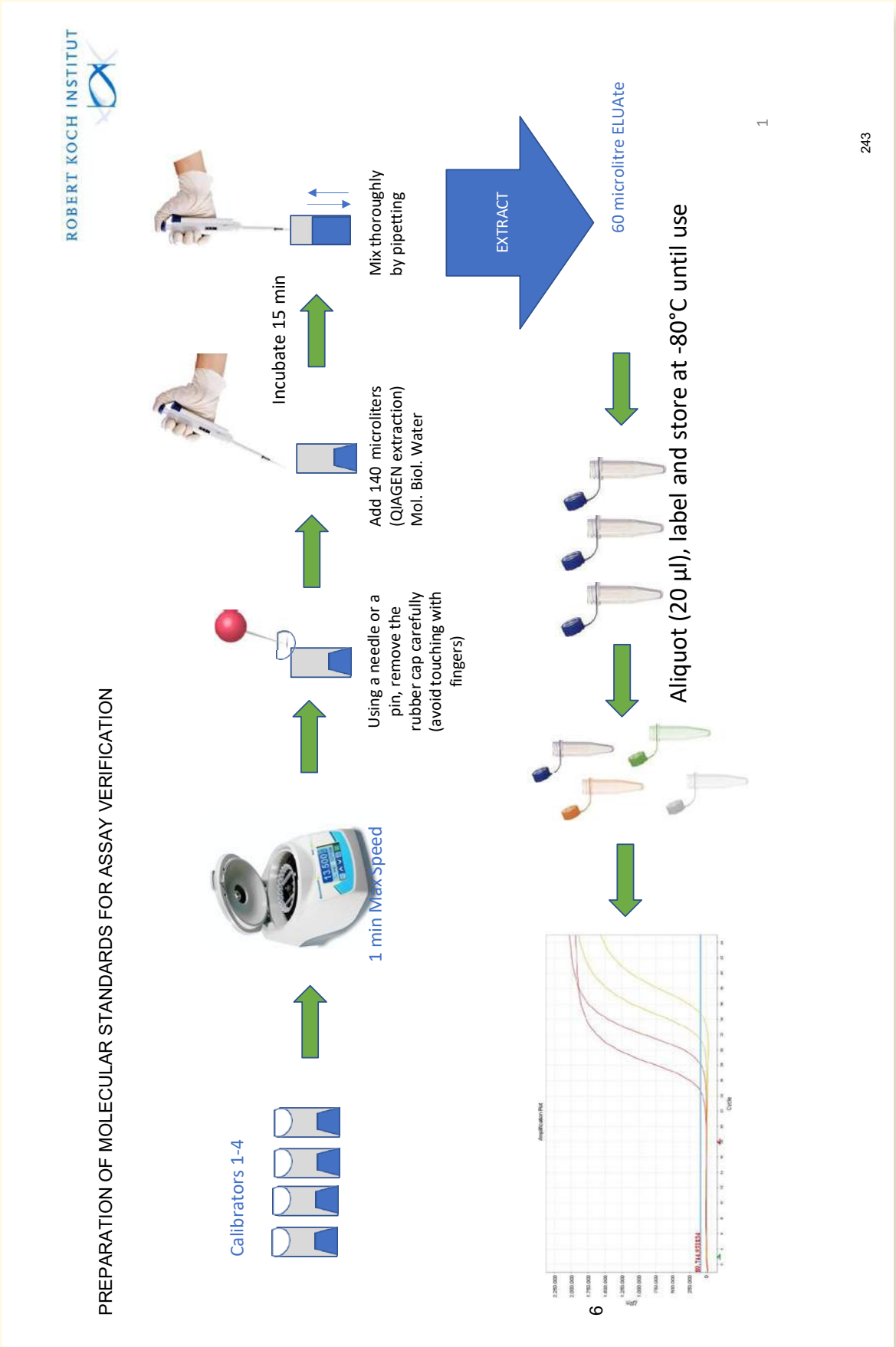
To prepare the **calibrators** for verification of your RT-PCR assays, resuspend the lyophilized material in 100 µL of molecular biology grade water. Alternatively, you can resuspend the material in the sample volume specified in your extraction protocol. The samples have to be extracted at once without preparing aliquots of the starting material.
Aliquot them (20 microliters aliquots) and store at -80°C until use.
For verification of your YF RT-PCR assays analyze the four samples in duplicate in the same run.

Contact:

Dr. Cristina Domingo
Robert Koch Institute
Centre for Biological Threats and Special Pathogens
Highly Pathogenic Viruses
Seestrasse 10
13353 Berlin
Germany
Phone: +49 30 18754 2954
Email: Domingo-CarrascoC@rki.de

Version 01 March 2021

ANNEX 6.4.9b
Preparation of molecular standards for assay verification (courtesy of Robert Koch Institut)



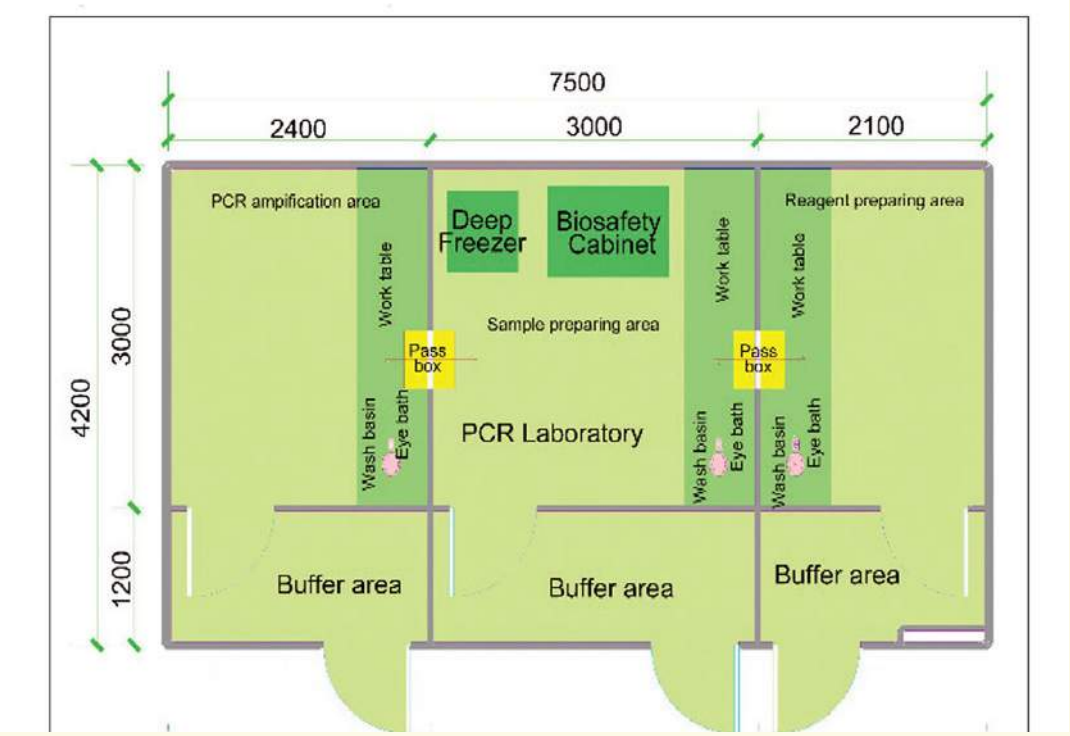
ANNEX 6.5
Molecular laboratory design and workflow

The following information is from the WHO SEARO, Establishment of PCR laboratory in developing countries, 2016. https://apps.who.int/iris/handle/10665/249549

A PCR laboratory should contain two functional work areas: a pre-amplification area and a post-amplification area. These two areas should ideally be in separate rooms, or when space constraints exist, separate workstations/ biosafety cabinets in a single room. Supplies and equipment should be dedicated to each work area and should not be interchanged between areas. A laboratory performing PCR analyses on diagnostic samples should be divided into at least three physically separate rooms (Figure A6.5.1):

- Reagent preparation (using positive pressure to prevent the introduction of contamination)
- Sample preparation (using negative pressure to keep template nucleic acids in the room)
- Amplification and product detection (using negative pressure to keep amplified nucleic acids in the room)

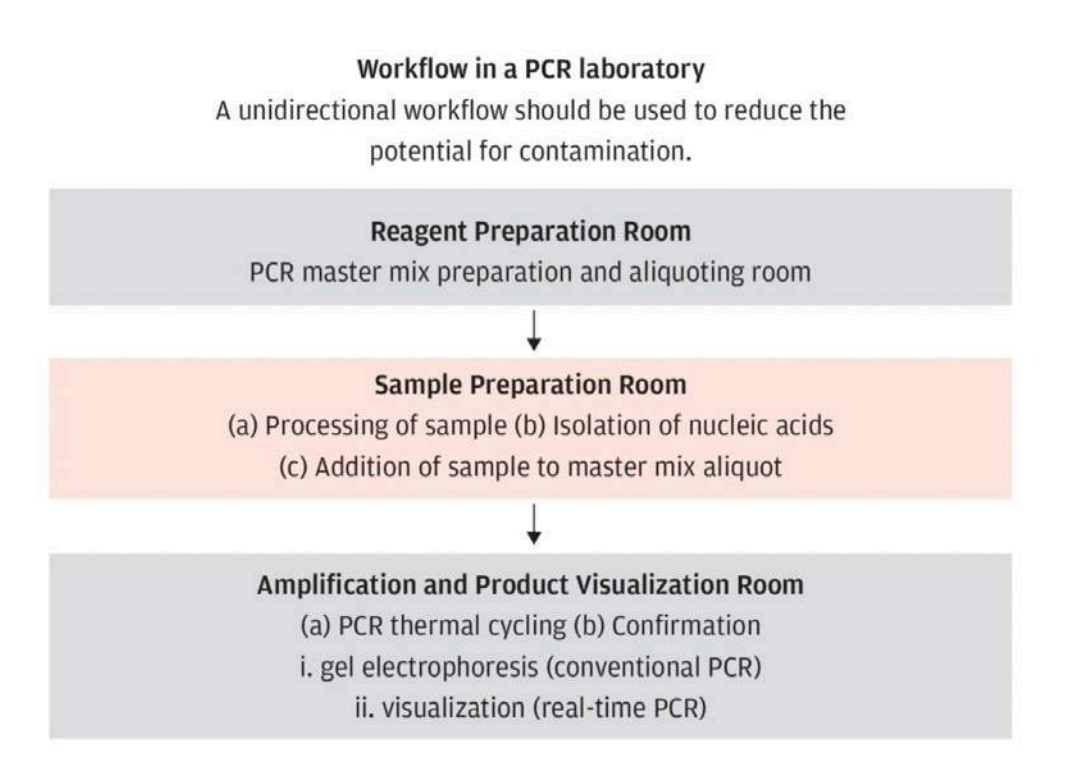
FIGURE A6.5.1 **A model layout for a PCR laboratory.**



Source: taken from the WHO SEARO, Establishment of PCR laboratory in developing countries, 2016. https://apps.who.int/iris/handle/10665/249549

A unidirectional workflow should be observed to reduce the chances for contamination to occur, as shown in Figure A6.5.2. No materials, supplies or equipment from the sample preparation room should be taken into the reagent preparation room. Similarly, nothing from the amplification and product detection room should be taken into the sample preparation room or the reagent preparation room.

FIGURE A6.5.2 **Flow diagram of unidirectional workflow in the molecular laboratory.**



Source: taken from the WHO SEARO, Establishment of PCR laboratory in developing countries, 2016.
https://apps.who.int/iris/handle/10665/249549

Annex 7.

Yellow fever confirmatory and reference testing at the regional reference laboratory

ANNEX 7.1 Example of PRNT SOP CDC UVRI

Approval Signatures- Current Version of Document			
Office	**Name**	**Signature**	**Date**
Author	Eric Mossel, CDC		
Quality Manager	Jocelyn Kiconco Kigozi, UVRI		
Laboratory Director	Julius Lutwama, UVRI		
Date Version Retired:			

Revision History		
Version	**Date**	**Brief description of the change**
Methods 003.0	14 Aug 2015	Creation of Document
Version 003.1	18 Aug 2017	Updated format for new requirements, changed ID Code to ProcessManagement from Methods.
Version 003.2	15 Oct 2019	Updated to reflect new Quality Manager
Version 003.3	21 Jun 2021	Clarified dilution series, corrected spelling of Quality Manager's name

Annual Quality Manager Review		
Reviewer	**Date**	**Recommendations**

Annual Staff Review		
Name	**Signature**	**Date**

☐ Additional Annual Review and Acknowledgment Form (FORM ARB DOCUMENTS006 – Annual Review and Acknowledgment Form) appended.

1. Purpose

This procedure describes the Plaque Reduction Neutralization Test procedure used in arbovirus diagnostic testing.

2. Application/Scope

The procedures below shall be used for the purpose of diagnostic testing of serum samples.

3. Abbreviations, definitions and terms

PRNT: Plaque Reduction Neutralization Test
PFU: Plaque forming unit

a. Responsibilities

Position	Responsibilities
Lab Director	Ensure that all laboratory personnel have read and understood the procedures described herein.
Lab Manager/Quality Manager	Ensure that all laboratory personnel have read and understood the procedures described herein. Monitor use of these procedures and implement changes as needed following non-conforming events related to these procedures.
Lab Technician	Remain familiar with the procedures described herein. Conduct these procedures as described and report any non-conforming events to Lab Manager. Maintain clear notes describing use of these procedures in laboratory notebook and report results to Laboratory Manager.

b. Safety Precautions

These procedures shall be performed in a certified biosafety cabinet. All personnel conducting these procedures must wear appropriate PPE as defined by the UVRI Safety Committee and specified in the SOP ARB FACILITIES-SAFETY003 – Personal Protective Equipment, including laboratory coat, gloves, and closed toe shoes. Procedures shall be conducted using universal laboratory best precautions and aseptic technique.

c. Principle

Neutralizing antibody is produced during arbovirus infection as part of the immune response to the virus. Neutralizing antibody blocks virus attachment to cells, thereby delaying or completely inhibiting virus infection. The action of neutralizing antibody is used in this test by reacting a known concentration of virus with test serum, alongside controls using antisera of know reactivity, to assess the neutralizing ability of antibody in

the test serum. The end point of this assay is calculated as the test serum titer at which 90% of the virus is neutralized.

d. Protocol

Before beginning you need to know:
Virus
Virus strain
Virus titer (in PFU/0.1 ml)
How many days to 2nd overlay specific for the virus

Calculate Virus dilution:
Want 200 PFU/0.1 ml for the test:

- Divide the titer of the virus (in PFU/0.1 ml) by desired concentration (200 PFU/0.1 ml) = dilution factor = total dilution

Calculate the dilution series you will use to achieve this dilution (use Form ARB Testing002.0 PRNT SetUp Worksheet).

Calculate how much diluted virus is needed:

- Calculate virus needed for test samples

  (# samples) x (# serum dilutions) x (0.06 ml virus per serum dilution)

- Also need virus for controls and virus back titration:

  (# controls [pos/neg] + # back titration plates) x (6 wells/plate) x (0.06ml/well)

  (Note: you do not need to use all 12 serum dilutions for the positive control – previous experiments should tell you what 6 dilutions will adequately bracket the optimum positive control serum dilution)

- add these together to get ml virus needed at 200 PFU/0.1 ml

- always make extra

Set up 96-well plate:

- Serum: (heat inactivate an aliquot of the patient serum at 56°C for 30 min prior to use)

  (Note: set up one 96 well plate row for each test serum and control serum)

  - pipet 24 µl serum or control antibody into 1st well of 96 well plate row
  - Add 96 µl BA-1 = 1:5 dilution
  - Add 60 µl BA-1 to other wells in the row
  - Serially dilute 1:2 through well 12 (60 µl previous dilution + 60 µl BA-1), discarding the extra 60 µl in last dilution well

- Positive control antiserum: dilute as for patient serum above, but select the 6 dilutions that bracket the known titer of the serum and use only those dilutions when plating onto 6 well plates. Add diluted virus to ONLY these 6 dilutions and plate ONLY these 6 dilutions on one control plate per positive serum

- Cell Control = BA-1 only

- Negative serum control (optional) (old labile serum factor sent in June 2012) = 1:5 through 1:160 dilutions of negative serum

  (Note: do not use the old labile serum factor as real Labile Serum Factor as referenced in some protocols for PRNT because it has been frozen/thawed too many times)

- Virus: Add 60 µl virus diluted to 200 PFU/0.1 ml virus to each serum dilution well (for positive control serum, add diluted virus to ONLY the 6 dilutions to be plated and plate ONLY these 6 dilutions on one control plate per positive serum). Note that this now makes the lowest dilution 1:10.

Virus Back Titration:

- dilute the 200 PFU/0.1 ml virus 1:10 *(= 10^{-1})* **(20 ul + 180 ul BA-1 = 10-1)**
- dilute the 10^{-1} virus 1:10 *(= 10^{-2})* **(20 ul + 180 ul BA-1 = 10-2)**
- add 125 ul each of the 200 PFU/0.1 ml, the 10^{-1}, and the 10^{-2} virus dilutions to 125 ul of BA-1 in wells of the 96-well plate and incubate along with the virus/serum mix

Incubate plate at 37ºC for 1 hour or overnight at 4ºC

Apply samples to the 6-well plates and overlay as described below :

- Need:
  - Confluent monolayers of Vero cells in 6-well plates
  - BA-1 or cell culture maintenance medium with 2% FBS, cold
  - Water bath at 42ºC
  - Small water bath to be used in BSC at 42ºC
  - Autoclave or microwave oven
  - Sterile 500 ml flask or bottle
  - Sterile graduated cylinder (100 or 250 ml) or sterile pipets
  - Sterile deionized water
  - Neutral Red Solution
  - SeaKem LE agarose powder
  - Barry's 2X YeLah nutrient medium without Sodium Bicarbonate, warmed to 42ºC.
    - See SOP ARB PROCESSMANAGEMENT020 - 2x Miller's Ye-Lah Media Preparation
    - add 60 ml sodium bicarbonate per liter before 1st use
  - Single and multi-channel pipets
  - P200 tips
  - P1000 tips
  - Autoclavable discard pan or bag

- Balance for weighing agaroTse

- Remove medium from 6-well plates by 'dumping' it into a waste container
- Pipet 100 µl each serum/virus mixture onto wells of the 6-well plate
  - do not pipet directly onto the cell sheet as this may dislodge cells and create 'false plaques'
  - after pipetting virus onto each plate, gently rock the plate twice to spread the inoculum over the cells
  - incubate for 1 hour at 37ºC, rocking the plates briefly every 15 minutes
- During incubation, prepare agarose overlay (see recipe below)
  - if using autoclave to melt the agarose you must start this before you begin the plate setup to be sure the agarose is ready when you need it. You will need to autoclave the agarose for 15 minutes (about a 1 hour autoclave run?) and then cool it for at least 10 minutes in the water bath to 42ºC before use
  - if using a microwave oven, begin preparing the agarose about 20 minutes before the end of the incubation time
    - microwave until all agarose is dissolved
    - cool in 42 ºC water bath for at least 10 min
- Add 1 volume of Barry's 2X YeLah medium (with sodium bicarbonate added if first use (see above), warmed to 42ºC) to 1 volume of cooled 2% agarose solution in the 42ºC water bath (need 3 ml/well x # plates + ~50-100 ml extra)
- Apply 3 ml overlay mixture to each well, being careful not to pipet directly onto the cell sheet and not to touch the pipet tip to the solution in the wells or to the plate
- Allow overlay to cool for 30 min at room temperature
- Invert plates and incubate at 37ºC in a CO2 incubator for appropriate number of days depending on the virus being titrated
- On appropriate day, apply 2 ml of second overlay containing neutral red solution (2.3 ml/100 ml overlay)
- Read and count plaques for 3 days, starting 1 day after application of the second overlay
  - record on FORM ARB PROCESSMANAGEMENT004.0 – PRNT Record Worksheet
  - make note of size and character (fuzzy, clear, etc) of the plaques for future reference

Overlay:
1 part Barry's 2X YeLah nutrient medium
1 part 2% SeaKem LE agarose in sterile water

(2nd overlay: also add neutral red to 2% [2.3 ml/100 ml final overlay volume])

Do not mix YeLah medium with agarose until agarose has cooled to 42ºC

How much 1st overlay?
(# plates) x 6 wells/plate x 3 ml/well = minimum volume needed (always make 50-100 ml more)

Make a minimum of 200 ml overlay, otherwise it will boil away during heating or cool too fast and solidify.

How much 2nd overlay?
(# plates) x 6 wells/plate x 2 ml/well = minimum volume needed (always make 50-100 ml more)

e. Related documents

SOP ARB FACILITIES-SAFETY002 – General Laboratory Biosafety
SOP ARB FACILITIES-SAFETY003 – Personal Protective Equipment
FORM ARB PROCESSMANAGEMENT006 – PRNT SetUp Worksheet
FORM ARB PROCESSMANAGEMENT004 – PRNT Record Worksheet
SOP ARB PROCESSMANAGEMENT020 - 2x Miller's Ye-Lah Media Preparation
CDC-Fort Collins Arbovirus Diagnostic Instruction materials – Arbovirus Isolation and Identification.

f. References

N/A

g. Attachments/Annexes

N/A

Plaque Reduction Neutralization Test Worksheet

Virus: _____ Date of test:_____ Performed by:_____

Specimens tested:_____

_____

_____

Cell system: Vero Passage level: _____ Date seeded: _____

Virus Dilution to 200 PFU/0.1ml:

log Titer/0.1 ml:_____ Dilution series: _____

Titer/0.1 ml: _____

Titer ÷ 200 PFU/0.1 ml = total dilution

Volume of diluted virus to prepare:

(# samples) x (# serum dilutions) x (0.06 ml virus per serum dilution) = ml

_____ x _____ x 0.06 = _____ml for sample plates

(# +/-/cell CTRL plates) + (# back titration plates) x 6 wells/plate x 0.06 ml/well = ml

(_____ + _____) x 6 x 0.06 = _____ml for CTRLs and BT

ml for sample plates + ml for CTRLs and BT = ml diluted virus required (**make extra**)

_____ + _____ = _____ml (Make Extra) Total: _____ ml

Virus dilution plan:

_____µl virus seed + _____µl BA-1 = 1:_____ dilution

_____µl + _____µl BA-1 = 1:_____ dilution

_____µl + _____µl BA-1 = 1:_____ dilution

_____µl + _____µl BA-1 = 1:_____ dilution

_____µl + _____µl BA-1 = 1:_____ dilution

_____µl + _____ BA-1 = 1:_____ dilution

ANNEX 7.3
Example of PRNT plaque count and titer calculation worksheet

Test Date: _____ Laboratorian: _____

Date of 2nd overlay:_____ Incubation (Tick one): ☐ O/N @ 4°C ☐ 1hr @ 37°C

					Plaques counted on dates			
6-well plate	**Well #**	**Virus**	**Aby**	**Aby dilution**				**Titre**
1	1			10				
	2			20				
	3			40				
	4			80				
	5			160				
	6			320				
2	1			640				
	2			1280				
	3			2560				
	4			5120				
	5			10240				
	6			20480				
3	1			10				
	2			20				
	3			40				
	4			80				
	5			160				
	6			320				
4	1			640				
	2			1280				
	3			2560				
	4			5120				
	5			10240				
	6			20480				
5	1		Positive CTRL Antibody**					
	2							
	3							
	4							
	5							
	6							
6	1	None	Cell control					
	2		Medium only					
	3							
	4							
	5							
	6							
7	1		Back titration	0				
	2		(in duplicate)	0				
	3			10				
	4			10				
	5			100				
	6			100				

*For additional test sample plates, insert rows following Plate #4 and re-number control and BT plates.
**Specify antibody used; bracket known best working dilution.

Annex 8.

Data management and reporting of laboratory results

ANNEX 8.1A Case investigation form: Suspected yellow fever

Case investigation form : suspected yellow fever

As soon as Yellow fever is suspected, contact :
District communicable disease manager: *Telephone number:* _____ *Facsimile number:* _____
OR *District EPI programme manager:* *Telephone number:* _____ *Facsimile number:* _____

1. Record general information about the patient:							**Date of report:**
Patient's name and patient record number:							Sex: M [ ] F [ ]
							Patient's occupation:
Address:							Village or municipality:
District: State or Province:							Name of head of patient's household or village chief:
Patient's date of birth	dd		mm		y y		Patient's age (if date of birth unknown):
2. Does suspected case have:				**Date of onset**			**3. Record travel and yellow fever immunization history.**
Fever (>38°C or >101°F) That did not respond to antimalarial treatment	Y	N	U	dd	mm	y y	List names of other areas or districts that patient visited during the last two weeks:
Jaundice	Y	N	U	dd	mm	y y	
Slow pulse in relation to fever	Y	N	U	dd	mm	y y	Have cases of fever and jaundice been seen or reported in areas or districts that patient visited during the last two weeks? Y N U
Bleeding form the nose, gums or skin or gastrointestinal tract	Y	N	U	dd	mm	y y	
Reduced amount of urine	Y	N	U	dd	mm	y y	Has the patient ever received at least one dose of yellow fever vaccine? Y N U
Elevated level of protein in urine	Y	N	U	dd	mm	y y	What was final outcome for patient? (*circle one*) Living Dead Unknown If patient died, record date of death: (dd) ____ (mm) ____(yy) ____
Reported by: _____							**Contact number for health facility:** _____

Laboratory transmittal and final classification of the case

Specimens collected *(Circle one)*	Date specimens collected *(dd/mm/yy)*	Date received in laboratory *(dd/mm/yy)*	Type of test	Results				Date results sent to MOH *(dd/mm/yy)*	Date results received at MOH *(dd/mm/yy)*
				Pos	Neg	Not processed	Unk		
Blood			IgM						
			IgG (acute)						
			IgG (convalescent)						
Malaria slide			Microscopy						
Other									

1. Were specimens or isolates sent to another laboratory? *(circle one)* Yes No Unknown	**2. If YES, record laboratory's name, address, and telephone number:**
3. What is the final classification of the case? (*circle one*) Suspected Confirmed Discarded Unknown	
4. If case discarded as yellow fever, record diagnosis:	**5. What was final outcome for patient?** ***(circle one)*** Living Dead Unknown
	6. If patient died, record date of death: (dd) ____ (mm) ____ (yy) ____
Investigator's name: (*please print)*	**Signature:**
Address:	**Telephone number:**

ORGANIZACIÓN PANAMERICANA DE LA SALUD

Ficha de investigación epidemiológica de fiebre amarilla

1. Nombre del paciente:

2. Fecha de nacimiento: ____ / ____ / _____

3. Edad: | 4. Sexo: ☐ M-Masculino ☐ F-Femenino

5. Dirección: Calle:
Municipio:
Distrito:

6. Localidad: ☐ 1-Urbana ☐ 2-Rural ☐ 3-Urbana/Rural ☐ 9-Ignorado

7. Teléfono: ( ) - _____ -_____

Datos complementarios del caso:

8. Fecha de investigación: ____ / ____ / _____ | 9. Ocupación:

10. Descripción de fechas y lugares frecuentados en el período de 10 días antes del inicio de signos y síntomas

Fecha	Municipio	Estado	País

11. Vacunado contra la fiebre amarilla: ☐ 1-Sí ☐ 2-No ☐ 9-Ignorado

12. Fecha: ___ / ____ / _____

Datos clínicos

13. Signos y síntomas:

Fiebre	☐ 1-Sí ☐ 2-No ☐ 9-Ignorado	Dolor epigástrico	☐ 1-Sí ☐ 2-No ☐ 9-Ignorado
Cefalea	☐ 1-Sí ☐ 2-No ☐ 9-Ignorado	Signo de Faget	☐ 1-Sí ☐ 2-No ☐ 9-Ignorado
Escalofríos	☐ 1-Sí ☐ 2-No ☐ 9-Ignorado	Hematuria	☐ 1-Sí ☐ 2-No ☐ 9-Ignorado
Choque	☐ 1-Sí ☐ 2-No ☐ 9-Ignorado	Hematemesis	☐ 1-Sí ☐ 2-No ☐ 9-Ignorado
Vómitos	☐ 1-Sí ☐ 2-No ☐ 9-Ignorado	Oliguria	☐ 1-Sí ☐ 2-No ☐ 9-Ignorado
Ictericia	☐ 1-Sí ☐ 2-No ☐ 9-Ignorado	Anuria	☐ 1-Sí ☐ 2-No ☐ 9-Ignorado
Melena	☐ 1-Sí ☐ 2-No ☐ 9-Ignorado	Bradicardia	☐ 1-Sí ☐ 2-No ☐ 9-Ignorado
		Coma	☐ 1-Sí ☐ 2-No ☐ 9-Ignorado

Atención

14. Hospitalización: ☐ 1-Sí ☐ 2-No ☐ 9-Ignorado 15. Fecha: ____ / ____ / _____

16. Nombre del hospital:

17. Dirección:

Datos de laboratorio

18. Exámenes serológicos:

BT_____mg/dl
BD _____mg/dl
BI _____mg/dl
AST (TGO) _____UI
ALT (TGP) _____UI
Urea _____mg/dl
Creatinina _____mg/dl

Albúmina :
☐ 1 - cero
☐ 2 - +
☐ 3 - ++
☐ 4 - +++
☐ 5 - ++++

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atorio

19. Exámenes específicos:

Fecha de recolección de la muestra:

1ª ___/___/_____ 2ª ___/___/_____

Fecha de recepción de la muestra:

1ª ___/___/_____ 2ª ___/___/_____

Fecha de resultado de la muestra:

1ª ___/___/_____ 2ª ___/___/_____

Resultado

1 - Positivo
2 - Negativo
3 - Inconcluso
4 - No realizado

Títulos

☐ IgM ☐ IgG

S1_____ I: _____
S2_____ I: _____

20. Histopatología:

☐ 1 - Compatible ☐ 2 - Negativo ☐ 3 - No realizado ☐ 9 - Ignorado

Inmunohistoquímica

☐ 1 - Compatible ☐ 2 - Negativo ☐ 3 - No realizado ☐ 9 - Ignorado

21. Aislamiento viral:

Material recolectado

☐ 1 - Sí ☐ 2 - No ☐ 9 - Ignorado

En caso afirmativo, cuál

Suero: ☐ 1 - Sí ☐ 2 - No ☐ 9 - Ignorado

Tejidos: ☐ 1 - Sí ☐ 2 - No ☐ 9 - Ignorado

Resultado ☐ 1 - Aislado ☐ 2 - No Aislado

Medidas de control

22. Realizadas:

Bloqueo vacunal ☐ 1 - Sí ☐ 2 - No ☐ 3 - No aplica ☐ 9 - Ignorado

Control del vector ☐ 1 - Sí ☐ 2 - No ☐ 3 - No aplica ☐ 9 - Ignorado

23. Clasificación final:

☐ 1 - Fiebre amarilla urbana
☐ 2 - Fiebre amarilla selvática
☐ 3 - Descartado (especificar: _____ _____)

24. Criterios de confirmación/descarte:

☐ 1 - Laboratorio
☐ 2 - Vínculo epidemiológico
☐ 3 - Clínico

25. Probable lugar de infección:

País : Municipio:

Enfermedad relacionada

☐ 1 - Sí ☐ 2 - No ☐ 9 - Ignorado

Evolución del caso

☐ 1 - Recuperación ☐ 2 - Defunción ☐ 9 - Ignorado

Fecha de la muerte: ___/___/_____

Fecha de cierre: ___/___/_____

Observaciones

26. Estado/Municipio _____

Nombre: Firma:

Función:

262

Ministère de la Santé Publique 1.4.4 OMS **Centre Pasteur du Cameroun**

FORMULAIRE DE NOTIFICATION DES CAS DE FIEVRE JAUNE
NOTIFICATION OBLIGATOIRE
Prière de remplir toutes les cases

Utilisation officielle seulement | N0. EPID _____ | pays _____ | Région _____ | District Santé _____ | Année _____ | Nbre de cas _____ | Reçu le _____

1.17.7 IDENTIFICATION

District de Santé : _____ Région : _____ Nom de la Formation Sanitaire la plus proche _____

Village/Quartier _____ Ville : _____ Urbaine / Rurale ☐ 1 = Urbaine 2 = Rurale

Nom du patient : _____ Nom du Père / Mère _____
Adresse du atient _____
Date de naissance du patient : ___/___/_____ Age : _____ Sexe M / F ☐

1.17.8 NOTIFICATION

Date cas vu à la formation Sanitaire ___/___/_____ Date de la notification au District ___/___/_____

1.17.9 HISTORIQUE DE LA MALADIE

Date de début de l'ictère ___/___/_____ évolution du malade ☐ 1 = Oui, décédé 2 = Non, vivant 9 = Inconnu Nbre de dose 'accin Anti amaril ☐ Date de la dernière ___/___/_____ vaccination Antiamaril

1.17.9.1 ECHANTILLON DU PLASMA

Date de prélèvement ___/___/_____ Date d'expédition du prélèvement ___/___/_____ Vers le labo

Date de réception au labo ___/___/_____ Date de réception des ___/___/_____ CPC Résultats au PEV

☐ 1 = Positif 2 = Négatif 3 = Indéterminé Résultats de IgM Autres résultats Indirect de Amaril

☐ 1 = Cas confirmé par labo ou lien épidémiologique avec un autre cas confirmé
2 = Clinique (compatible/suspect). Test de labo non réalisés
3 = Exclu

Date d'expédition des résultats du labo
Au clinicien/district qui a envoyé le sang ___/___/_____

1.17.10 .1 INVESTIGATION DANS LA COMMUNAUTE

☐ Source de l'infection identifiée : 1 = Oui 2 = Non

☐ Si le test de confirmation IgM de fièvre jaune positif Investigation communautaire faite ? Si oui, décrire le résultat de l'investigation : 1 = Oui 2 = Non

1.17.11 INFORMATION SUR LA PERSONNE QUI A NOTIFIE

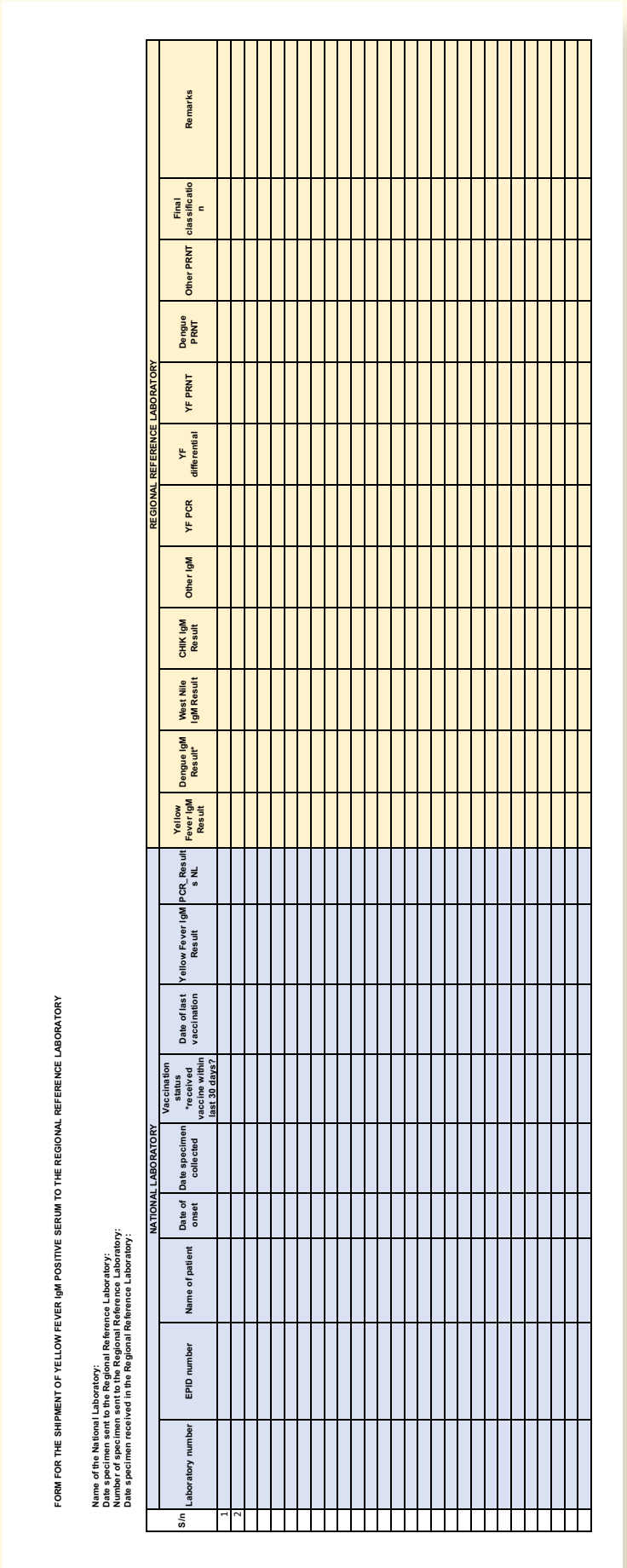
Nom _____ Titre _____

Unité : _____ Adresse _____ Tél : _____

Normes et Standards du Programme Élargi de Vaccination – PEV Cameroun 2009

ANNEX 8.2
Form for the shipment of yellow fever IgM positive serum to the regional reference laboratory

Please see Excel for the Form for the shipment of yellow fever IgM positive serum to the regional reference laboratory available at *https://www.who.int/publications/i/item/9789240084476* or see below



FORM FOR THE SHIPMENT OF YELLOW FEVER IgM POSITIVE SERUM TO THE REGIONAL REFERENCE LABORATORY

Name of the National Laboratory:
Date specimen sent to the Regional Reference Laboratory:
Number of specimen sent to the Regional Reference Laboratory:
Date specimen received in the Regional Reference Laboratory:

S/n	NATIONAL LABORATORY													REGIONAL REFERENCE LABORATORY														
	Laboratory number	EPID number	Name of patient	Date of onset	Date specimen collected	Vaccination status *received vaccine within last 30 days?	Date of last vaccination	Yellow Fever IgM Result	PCR, Results NL	Yellow Fever IgM Result	Dengue IgM Result*	West Nile IgM Result	CHIK IgM Result	Other IgM	YF PCR	YF differential	YF PRNT	Dengue PRNT	Other PRNT	Final classification	Remarks
1																					
2																					

Annex 9.

ANNEX 9.1
Levey-Jennings control charts and Westgard rules

The material below was modified from the WHO Quality Management Handbook (2011): chapter 7, pages 81-88.

LEVEY–JENNINGS CHARTS

To easily monitor and interpret quality control (QC) data the values of each positive control should be compared with the already established mean and standard deviations of the control sample.

The values of the mean, as well as the values of ±1, 2 and 3 SDs are needed to develop the chart used to plot the daily control values.

- To calculate 2 SDs, multiply the SD by 2 then add and subtract each result from the mean.
- To calculate 3 SDs, multiply the SD by 3, then add and subtract each result from the mean. For any given data point, 68.3% of values will fall between + 1 SD, 95.5% between ±2 SD and 99.7% between + 3 SD of the mean.

In order to develop Levey–Jennings charts for daily use in the laboratory, the first step is the calculation of the mean and SD of a set of 20 control values. Once the appropriate range of control values has been established, the laboratory will find it very useful to represent the range graphically for the purpose of daily monitoring. The common method for this graphing is the use of Levey– Jennings charts.

The control values are plotted on the Y-axis and date/time along the X-axis. The chart should be marked with the mean and one, two and three standard deviations above and below the mean value for easy interpretation.

Even when a control value falls within 2 SD, it can be a cause for concern. Levey– Jennings charts can help distinguish between normal variation and systematic error.

The first QC measurements for determining whether an assay is valid or not is whether the kit or assay is within the expiry date and that the kit/assay controls meet the stated limits. Rules, such as the Westgard rules can be applied to see whether the results from the in-house control sample are valid and sample results can be reported, or if they need to be repeated.

In order to improve efficiency and accuracy, a system using two or three controls for each run can be employed. Then another set of rules can be used to avoid rejecting runs that may be acceptable. These rules were applied to laboratory QC by a clinical chemist named James Westgard. This Westgard multirule system requires running two controls of different target values for each set of examinations, developing a Levey–Jennings chart for each, and applying the rules.

The use of three controls with each run gives even higher assurance of accuracy of the test run. When using three controls, choose a low, a normal and a high range value. There are also Westgard rules for a system with three controls.

Westgard rules are used to define specific performance limits for a particular assay and can be used to detect both random and systematic errors. Specifically, the assay run should be thoroughly investigated, and repeated if necessary, if:

1. The in-house positive control has three consecutive values more than one standard deviation either above or below the mean in-house positive control value.
2. The in-house positive control has two consecutive values more than two standard deviations either above or below the mean in-house positive control value
3. The in-house positive control has one value more than three standard deviations either above or below the mean in-house positive control value.

World Health Organization

Introduction

Accreditation of YF Laboratory is reviewed annually (if possible) by the WHO Regional Office and based on laboratory performance during the immediately preceding 12 months. Accreditation is given for the forthcoming 12 months.

There are eight Programmatic criteria that need to be met for accreditation in addition to the checklist minimal criteria (>80% in scoring points + all checklist mandatory criteria fully met). These eight Programmatic criteria are the following:

1. **The accuracy of YF and YF IgM detection is at least 90%.**
   Accuracy is determined by the agreement in test results on sera submitted by the National (or sub-national) Laboratory to the supervisory laboratory (National or Regional Reference Laboratory (RRL) during the 12-month review period. The percentage of specimens sent for validation is dependent on the quality of the laboratory and could range from 10-100% with the lower proportion for a fully accredited laboratory and 100% for a laboratory that has failed accreditation. Specimens for validation should be representative of all results (positive, negative, and equivocal) and outbreaks, and should be sent to the supervisory laboratory at regular intervals.
2. **IgM tests should be performed on at least 50 specimens annually.**
   To maintain skills in performing serological assays, virus laboratories should maintain appropriate reagents and assay kits to have capacity to test continually through the year. To maintain expertise, it is required that laboratories test a minimum of 50 specimens for EIA IgM or IgG detection annually, spread across the year. Where surveillance specimens are insufficient to meet this indicator then the lab may use positive control specimens for completing the minimum requirement.
3. **Internal quality control (QC) procedures are implemented.**
   Appropriate QC procedures are in place and followed, including appropriate serological and PCR controls (such as in-house positive and negative controls and assay controls), micro-pipettor calibration and temperature recording of incubators and refrigerators/freezers. Quality control data sheets and summaries of corrective action are retained and available for review.
4. **The score on the most recent WHO approved serological proficiency test is at least 90%.**
   Proficiency test results to be reported within 14 days of panel receipt to receive full credit. In the event of a "conditional pass" score is obtained (between 80% and 90%), the lab have implemented the recommended corrective actions.
5. **The score on the most recent WHO YF molecular proficiency test panel is at least 90%.**
   Molecular QA/QC panel results to be reported within 14 days of panel receipt to receive full credit. In the event of a "conditional pass" score is obtained (between 80% and 90%), the lab have implemented the recommended corrective actions This applies only to laboratories routinely performing molecular testing.
6. **Results from virus isolation/detection and genotyping (if performed) are completed within 2 months of receipt of specimen AND data reported to WHO monthly, for ≥80% of the specimens appropriate for genetic analysis.**
   Genotype information can assist national control programmes to determine transmission pathways and needs to be provided in a timely manner. Genetic data on appropriate specimens collected from separate chains of infection should be supplied to the national programme as soon as they become available. Laboratories are also encouraged to submit sequence data to GenBank, once sequencing is completed.
7. **The scoring points obtained in each of the relevant Checklist Assessments is at least 80%, along with ALL checklist mandatory criteria being fully met.**
   As evidenced in the General Lab sections G-V, the Serology section S-III, the Molecular section M-III, and the Virus Isolation section V-III. For Laboratories with consistently high-performance indicators, the Regional Laboratory Coordinator may waive the on-site review upon satisfactory completion of the annual checklist by the laboratory (see above).
8. **80% of YF IgM results are communicated within 7 days of receipt by the laboratory**.
   Subsequent YF IgM testing using the YF MAC-HD assay (ELISA), when needed are reported to the EPI programme promptly following efficient use of resources.

A Laboratory that achieves less than the passing score on any one of the applicable criteria will work with the Regional Laboratory Coordinator to:

- Identify areas where improvement is needed.
- Implement a work plan how to overcome the shortcomings.
- Estimate a time period for the laboratory progress.
- Provide for re-testing where required.
- Complete steps to achieve full accreditation.

A laboratory that fails to achieve a passing score in the most recent proficiency test panel(s) (serological and molecular if appropriate) and also a repeated proficiency test panel within 3 months of the failed panel, is deemed **Non-Accredited for that function (serological and/or molecular, if appropriate)** and arrangements must be made for another, fully accredited, Laboratory to perform duplicate tests on all appropriate specimens. Every effort will be made to bring any non-accredited laboratory to full accreditation status as soon as possible.

The Assessment contains four Checklists: **page**

All laboratories should be assessed annually but for laboratories with a consistently high accreditation score the WHO Regional Laboratory Coordinator may waive **annual** onsite reviews and determine accreditation status after review of criteria 1 to 7. In this situation, onsite reviews of well performing laboratories may be carried out every 2-3 accreditation cycles.

These checklists do not include all laboratory activities or cover all situations. It is intended to serve as a guide. The assessor is expected to ask detailed questions and make additional suggestions as appropriate to assure high quality laboratory performance.

National Laboratories that serve Sub-national Laboratories
Countries that have established sub-national laboratories for YF surveillance should endeavour to monitor the quality and performance of these laboratories. National laboratories should consider establishing a confirmatory testing and proficiency testing programme for such labs through appropriate referral mechanisms to WHO recognised confirmatory testing RRLs in order to monitor timeliness of reporting and ensure the performance of IgM assays, in a similar process to that used for determining the quality and performance across the entire YF Laboretary Network. WHO is willing to provide technical advice to National Laboratories planning to establish a sub-national laboratory monitoring programme.

IMPORTANT NOTICE:

Sections G-I, G-II, and G-III of the General checklist, and Sections S-I, M-I, and V-I of the Serology, Molecular, and Virus isolation specific checklists respectively (when such method is used by the laboratory)

MUST BE FILLED BY THE LABORATORY AND RETURNED TO THE ASSESSOR AT LEAST 14 DAYS BEFORE THE IN-PERSON ASSESSMENT,

Along with electronic copies of all SOPs and other relevant QMS documents

World Health Organization

WHO Check-list for Annual Laboratory Accreditation of Yellow fever diagnostic laboratory

Part G-I: LABORATORY PROFILE:
(To be completed BY THE LAB and returned to the assessor before the assessment.)

Date and status of last accreditation:	…..... (dd/mm/yyyy): accredited, provisionally accredited, or not accredited (circle one)			
Dates of accreditation review:	From …….……. To..... (dd/mm/yyyy)			
Name of Institution:				
Website:				
Laboratory name:				
Type of laboratory:	Regional Reference laboratory: ☐ National laboratory: ☐ Sub-national laboratory: ☐			
Address:				
Street, # / P.O Box				
City, postal code				
Country				
Phone numbers: Landline:				
Phone numbers: Mobile:				
Email:				
Head of Institute:				
Head of Department:				
Head of Laboratory:				
Phone:				
Email:				
Laboratory Quality manager:				
Phone:				
Email:				
Laboratory focal point for Yellow fever testing:				
Reviewer(s) name:				
Part of laboratory accredited:	Virus diagnostic, YF diagnostic			

1	Description of the YF diagnostic laboratory facility	#	Comments
	Space (provide floor plan or sketch of laboratories if possible)		
	Total m^2 available:		
	Number of rooms:		
	Dedicated area for reagent preparation (media, buffers, etc.)		
1.2	**Description of the YF diagnostic laboratory equipment**	**#**	**Comments**
	Number of refrigerators		
	Number of -20°C freezers		
	Number of -80°C freezers		
	Number of liquid nitrogen tanks (-170°C)		
	Number of incubators		
	Number of biosafety cabinets class II		
	Number of biosafety cabinets class III		
	Number of automatic pipettes ≤ 20 µl		
	Number of automatic pipettes ≤ 200 µl		
	Number of automatic pipettes > 200 µl		
	Other equipment, please specify:		

1.3	Personal resources	#
	Number of scientific and technical staff assigned to YF laboratory: please provide details for each staff using the table below	

Names of staff	Position Title or Duties	Full-time or part-time in the lab	% of time working on YF	Years of experience in YF Lab	Undergone specific YF lab training

Part G-II: YELLOW FEVER LABORATORY TESTING
(To be completed BY THE LAB and returned to the assessor before the assessment.)

2	What tests for YF diagnostic are performed in the lab?	Please tick	Comments
	Anti-YF IgM ELISA		
	Anti-YF IgM Immunofluorescence (IFA)		
	Anti-YF IgM Rapid Test		
	Anti-YF IgM another Test: please specify:		
	Anti-YF IgG ELISA		
	Anti-YF IgG Immunofluorescence (IFA)		
	Anti-YF IgG Rapid Test		
	Anti-YF IgG another Test: please specify:		
	YF Plaque reduction neutralization test (PRNT)		
	YF neutralization test (NT)		
	YF molecular diagnostic assays – RT-PCR		
	YF molecular diagnostic assays - Recombinase polymerase amplification (RPA)		
	YF molecular diagnostic assays - Loop-mediated isothermal amplification (LAMP)		
	YF virus genome sequencing, (NGS)		
	Other test for differential diagnostic specific for IgM of other Flaviviruses (e.g.: Dengue, West Nile, Zika, …), please specify:		
	Other neutralization tests for differential diagnostic for other Flaviviruses (e.g.: Dengue, West Nile, Zika), please specify:		
2.1	**Standard operation procedure (SOP)**	**#**	**Comments**
	Number of SOPs for the different diagnostic methods		
	Number of SOPs for the equipment maintenance		
	Number of SOPs for personal training		
	Number of SOPs for the sample handling		

Part G-III: NATIONAL SURVEILLANCE DATA
(To be completed BY THE LAB and returned to the assessor before the assessment.)

	Country-specific data	#	Comments
	Estimated total Population		
	Estimated YF vaccination coverage in the population depending on the age, and region, please specify if possible		
	Number of suspected YF cases reported last year		
	Number of laboratory IgM presumptive positive YF cases		
	Number of confirmed YF cases by PCR		
	Number of confirmed YF cases by Plaque reduction neutralization assay (PRNT)		
	Incidence of all laboratories confirmed YF cases (per million)		
	Laboratory reporting	**#**	**Comments**
	Number of YF suspected specimens tested in lab		
	Median Number of days required to report YF IgM lab results.		

Overview of the concordance in testing data between the laboratory and the Epidemiological databases using the last complete calendar year register entries

	Number of Diagnostic specimen received by lab	Number of Suspects Tested	Number of IgM Pos	Number of IgM Equivocal	Number of IgM Neg	Number of specimen Not tested
Documented by laboratory						
Documented by surveillance program						

Part G-IV: EVALUATION AND SCORING OF THE GENERAL LABORATORY PROCESSES
(To be completed BY THE ASSSESSOR during the assessment.)

1.	**Evaluation of the management structure**	***Y/N/P****	**Score (/5)**
	Is the management structure clearly defined in an organigram?		/1
	Do each of the laboratory personnel have documented clear terms of references?		/1
	Supervisor or senior staff member critically reviews test worksheets and results for accuracy and completeness and indicate the need for any follow up actions.		/1
	Arrangements are in place for qualified back-up staff to maintain services during scheduled staff absences (e.g.: during vacation, study, maternity, or paternity leave).		/1
	Regular meetings and periodic on-site staff training are held with staff, and documented to review and improve laboratory performance.		/1
2.	**Infrastructure of the building and laboratory**	***Y/N/P****	**Score (/3)**
	Space is satisfactorily used with appropriate equipment placement.		/1
	Space configuration is adequate and consistent with good laboratory practices.		/1
	Space is clean and well kept.		/1
3.	**Personal infrastructure**	***Y/N/P****	**Score (/3)**
	Staff are effectively assigned.		/1
	The number of trained staff is adequate to handle the workload.		/1
	Staff have appropriate training for the activities they perform.		/1
4.	**Biosafety issues**	***Y/N/P****	**Score (/8)**
	Employees have been properly trained in biosafety (documented).		/1
	Appropriate SOPs are available to all employees:		/1
	Biosafety practices are enforced (0.5 pts/practice), including: a. Immunizing staff against yellow fever. b. Immunizing staff against hepatitis c. Hand washing. d. Routine use of gloves and laboratory coats. e. Pipetting with aid of mechanical device. f. No storage or eating, drinking, or smoking, in laboratory. g. Decontaminating lab work surfaces. h. Decontaminating all infectious waste before discarding.		/4
	Class II Biosafety cabinets are used for materials which are potentially infectious through an aerosol route.		/1
	Biosafety cabinets are maintained and certified annually, and dates recorded:		/1
5.	**Maintenance of the equipment and supplies**	***Y/N/P****	**Score (/5)**
	The equipment, like washer, ELISA plate reader, PCR machines, are in good condition appropriately maintained and functional.		**Mandatory for accreditation**
	Regular monitoring of temperatures of equipment, e.g. incubators, freezers & refrigerators is documented.		/1
	SOPs for maintenance are acceptable and available.		/0.5
	Equipment location is conducive to optimal performance.		/0.5
	Thermometer and Pipettes are calibrated		/0.5
	Current inventories are maintained		/0.5
	Is there a designated staff member responsible for regular replenishing of the diagnostic supplies stock?		/1
	No interruption to testing due to shortage of supplies has occurred.		/1

Y/N/P. = Yes/No/Partially

6.	**Preclinical information and sample transport**	***Y/N/P****	**Score (/4)**
	There is a clear recommendation for the medical institution which kind of diagnostic samples and volume should be taken from a patient suspected to be infected with YF.		**Mandatory for accreditation**
	There is a clear recommendation for the medical institution which kind of data (clinical presentation, vaccination and travel history) from a YF patient are requested. (see annex 1)		**Mandatory for accreditation**
	There are clear instructions how the samples should be transported to the laboratory considering temperature, biosafety issues, and time for the transport.		**Mandatory for accreditation**
	There is a clear information system (phone, email) whom to contact if a sample transport is planned.		**Mandatory for accreditation**
	Specimens for serological testing are appropriately labelled and stored at –20°C for at least 12 months		/1
	Specimens for virus isolation/detection are stored at –70°C if not tested within a day of receipt. If -70°C capacity is not available, shipped to the designated virus identification laboratory within 3 days of receipt with cold chain.		/1
	All potentially infectious materials are processed in a biological safety cabinet.		/1
	Specimens for virus isolation, and other potentially infectious materials are stored safely in designated freezers and refrigerators.		/0.5
	Positive specimens are stored for at least 12 months and only discarded after discussion with WHO.		/0.5
7.	**Cooperation with EPI and field Staff**	***Y/N/P****	**Score (/3)**
	Laboratory and EPI staff communicate/meet at least monthly, evidenced by meeting reports if available.		/1
	EPI staff are contacted if specimens arrive without adequate information or EPID numbers, and frequency of such events is recorded.		/1
	Laboratory staff have access to and are familiar with protocols for collecting and transporting of patient specimens and are able to advise field staff.		/1
8.	**Data and results reporting**	***Y/N/P****	**Score (/4)**
	All specimen data and testing results are systematically and promptly recorded in a register (electronic or paper-based) and backed up.		**Mandatory for accreditation**
	The communication of the results to the sender of the samples is clearly documented and communicated together with an interpretation of the findings, and consequences for the patient and next steps if necessary.		**Mandatory for accreditation**
	Coordination mechanism in place for regular update between laboratory and the in country Epidemiology/Surveillance team		/1
	Laboratory reports are submitted to the WHO regional office within the agreed frequency and format.		/2
	The follow-up data are available on all YF specimens.		/1

Part G-V: SUMMARY OF THE GENERAL LABORATORY PROCESSES REVIEW
(To be completed BY THE ASSSESSOR following the assessment)

Review summary score:

Section	Score obtained	Maximal score	% achieved	Mandatory criteria
1. Evaluation of the management structure		**5**	**%**	**-**
2. Infrastructure of the building and laboratory		**3**	**%**	**-**
3. Personal infrastructure		**3**	**%**	**-**
4. Biosafety issues		**8**	**%**	**-**
5. Maintenance of the equipment and supplies		**5**	**%**	**/1**
6. Preclinical information and sample transport		**4**	**%**	**/4**
7. Cooperation with EPI and field Staff		**3**	**%**	**-**
8. Data and results reporting		**4**	**%**	**/2**
TOTAL ACROSS ALL SECTIONS		**35**	**%§**	**/7***

- = not available, § = a minimum score of 80% must be obtained, * = all mandatory criteria must be met
IFA = immune fluorescence assay, PRNT = Plaque reduction neutralisation assay, RPA = Recombinase polymerase amplification, LAMP = Loop-mediated isothermal amplification, NGS = next generation sequencing
amplification, LAMP = Loop-mediated isothermal amplification, NGS = next generation sequencing

A minimum score across all sections of 80%
AND
All of the mandatory criteria (7/7) must be met within this section
(along with the other relevant testing sections) to be considered for accreditation.

Additional programmatic criteria listed on page 1 must ALSO be met for full accreditation.

***SUMMARY**, Comments, and recommendations:*

***NATURE OF DEFICIENCY, IF ANY, AND CORRECTIVE ACTION TAKEN**:*

COMMENTS AND RECOMMENDATIONS:

***CORRECTION SHOULD BE TAKEN UNTIL**:* *Comments and recommendations:*
CORRECTIONS ARE SATISFACTORY /NON-SATISFYING: *Comments and recommendations:*

Recommendations (check one)**:**

☐ **Accredited**: Laboratory meets necessary requirements.

☐ **Provisionally accredited**: Laboratory fulfilled most of the necessary requirements, but must improve on one or more relevant conditions to maintain good and reliable diagnostic testing.

☐ **Not accredited**: Laboratory did not fulfil the necessary requirements.

Comments and recommendations:

Place and date of the review

Reviewer:

Serology Checklist
Part S-1: Yellow Fever Serology Testing
(To be completed BY THE LAB and returned to the assessor before the assessment.)

1.	Description of the YF diagnostic laboratory equipment	#	Comments
	Number of ELISA plate shaker		
	Number of ELISA plate washer		
	Number of ELISA plate reader		
	Other equipment, please specify:		

2	Performance of YF diagnostic in the previous 12 months	#	Comments
	Dates from ___/___/____ (dd mm yyyy) to ___/___/____. (dd mm yyyy)		
	Number of specimens tested for YF IgM:		
	Number YF IgM results reported within 7 days of receipt:		
	Proportion of YF IgM results reported within 7 days of receipt (according to the region's recommendations) in %:		
	Number of samples forwarded to supervisory laboratory (NL/RRL):		
	Number of samples confirmed accurate for anti-YF IgM by supervisory laboratory (NL/RRL):		
	Number of times specimens sent to supervisory laboratory (NL/RRL) for anti-YF IgM confirmation during review period:		
	Number of samples forwarded to supervisory laboratory (NL/RRL) for quality control:		
	Number of samples confirmed accurate for anti-YF IgM by supervisory laboratory (NL/RRL):		
	Number of times specimens sent to supervisory laboratory (NL/RRL) for quality control during review period:		
2.1	**Result of most recent YF serology Proficiency Tests**	**#**	**Comments**
	Date of panel receipt:		
	Date of test report:		
	Proficiency test assessment score (passed, failed):		

Part S-II: EVALUATION AND SCORING OF YELLOW FEVER SEROLOGY TESTING
(To be completed BY THE ASSSESSOR during the assessment.)

1.	**Documented use of recommended assays**	***Y/N/P****	**Score (/2)**
	Records are maintained on all assays performed, including: a. Kit manufacturer and batch number b. Reagent expiry dates c. Use of Positive and Negative controls for all assays d. Specimens giving equivocal/indeterminate results are retested and appropriate action are taken. e. Optical density (OD) values and calculation details of specimens testing.		**Mandatory for accreditation**
	Is there a systematic adherence to the approved testing algorithms and national policies		**Mandatory for accreditation**
	Only validated assays are used for diagnostic.		**Mandatory for accreditation**
	Are the serology assay used are recommended by WHO and/or national authorities (list which one are in routine use)		/1
	SOPs for performing of the diagnostic tests are available.		/1
2.	**Serology diagnostic assays**	***Y/N/P****	**Score (/3)**
	Assay performed according to manufacturer's instructions.		**Mandatory for accreditation**
	All assays performance are evaluated by external quality assessment studies if available.		**Mandatory for accreditation**
	Efficient use of consumables (scheduled testing, waste monitoring, full use of kits, etc.)		/1
	Assay positive and negative controls are used correctly.		/1
	Levey-Jenning chart and Westgard rules are used for quality control		/0.5
	Patient samples are disposed appropriately.		/0.5

Part S-III: SUMMARY OF THE REVIEW
(To be completed BY THE ASSSESSOR following the assessment)

Review summary score:

Section	Score obtained	Maximal score	% achieved	Mandatory criteria
1. Diagnostic methods performed in the laboratory		**2**	**%**	**/3**
2. Serology diagnostic assays		**3**	**%**	**/2**
TOTAL ACROSS ALL SECTIONS		**5**	**%§**	**/5***

§ = a minimum score of 80% must be obtained, * = all mandatory criteria must be met

A minimum score across all sections of 80%
AND
All of the mandatory criteria (5/5) must be met within this section
(along with the General Laboratory section) to be considered for accreditation.

Additional programmatic criteria listed on page 1 and where findings
are summarized below must ALSO be met for full accreditation.

Findings on Programmatic Criteria:

1.	First available IgM results communicated promptly	80% of results <7 days:	
2.	IgM tests are performed on at least 50 specimens annually:		
3.	the accuracy of IgM detection is ≥ 90% (samples)		**%**
4.	Internal quality control (QC) procedures are implemented:		
5.	The score on the most recent WHO serology proficiency test is ≥ 90%: PT panel number: Date reported: dd/mm/yyyy		**%**
6.	The score on the most recent WHO Molecular proficiency test (if appropriate) is ≥ 90%: Panel number: Date reported: dd/mm/yyyy		**%**
8.	The score from the on-site review of laboratory operating procedures and practices is ≥ 80%:		**%**

***SUMMARY**, Comments, and recommendations:*
NATURE OF DEFICIENCY, IF ANY, AND CORRECTIVE ACTION TAKEN**:* ***COMMENTS AND RECOMMENDATIONS:

Molecular Checklist

Part M-I: Yellow Fever Molecular Testing
(To be completed BY THE LAB and returned to the assessor before the assessment.)

1	Description of the YF diagnostic laboratory equipment	#	Comments
	Number of PCR cycler		
	Number of PCs, notebooks		
	Number of centrifuges		
	Number of autoclaves		
	Number of precision scale		
	Number of water distiller		
	Other equipment, please specify:		

2	Performance of YF diagnostic in the previous 12 months	#	Comments
	Dates from __/__/____ (dd mm yyyy) to __/__/____. (dd mm yyyy)		
	Number of YF specimens tested by conventional PCR methods:		
	Number of YF specimens tested by real-time PCR methods:		
	Number of specimens appropriate for YF genotyping received:		
	Number of specimens genotyped:		
	Number of YF specimens genotyped within 2 months of receipt:		
	Total number of YF sequences reported to WHO during period of review:		
	Percentage of YF sequence data reported timely to WHO:		
2.1	**Result of most recent YF Molecular Proficiency Tests**	**#**	**Comments**
	Date of panel receipt:		
	Date of test report:		
	Proficiency test assessment score (passed, failed):		

Part M-II: EVALUATION AND SCORING OF YELLOW FEVER MOLECULAR TESTING
(To be completed BY THE ASSSESSOR during the assessment.)

1.	**Diagnostic methods performed in the laboratory**	***Y/N/P****	**Score (/1)**
	Only validated assays are used for diagnostic.		**Mandatory for accreditation**
	Records are maintained on all assays performed, including: **a. Kit manufacturer and batch number** **b. Reagent expiry dates** **c. Use of Positive and negative controls for all assays**		**Mandatory for accreditation**
	Is there a systematic adherence to the approved testing algorithms and national policies		**Mandatory for accreditation**
	SOPs for performing of the diagnostic tests are available.		/1
2.	**Molecular Techniques (if performed)**	***Y/N/P****	**Score (/8)**
	Assay performed according to manufacturer's instructions.		**Mandatory for accreditation**
	All assays performance are evaluated by external quality assessment studies if available.		**Mandatory for accreditation**
	Appropriate SOPs are available and used.		/1
	Staff received appropriate training in molecular diagnostic.		/1
	Source of molecular protocols are documented.		/0.5
	Thermocyclers are maintained and calibrated and properly.		/0.5
	Source of primers, probes and control RNA are documented		/0.5
	Appropriate RNA extraction preparation methods for PCR and testing procedures are established.		/1
	Records are maintained on all procedures and location of all specimens.		/0.5
	PCR results are documented adequately		/1
	Efficient use of resources (scheduled testing, waste monitoring, full use of kits, etc.)		/0.5
	Specimens are stored appropriately at –70°C or lower for at least 12 months:		/0.5
	Storage vials are clearly and permanently labelled.		/0.5
	Software for sequence alignment and phylogenetic analysis is adequate.		/0.5

Part M-III: SUMMARY OF THE REVIEW
(To be completed BY THE ASSSESSOR following the assessment)

Review summary score:

Section	Score obtained	Maximal score	% achieved	Mandatory criteria
1. Diagnostic methods performed in the laboratory		**1**	**%**	**/3**
2. Molecular Techniques (if performed)		**8**	**%**	**/2**
TOTAL ACROSS ALL SECTIONS		**9**	**%§**	**/5***

§ = a minimum score of 80% must be obtained, * = all mandatory criteria must be met

A minimum score across all sections of 80%
AND
All of the mandatory criteria (5/5) must be met within this section
(along with the General Laboratory section) to be considered for accreditation.

Additional programmatic criteria listed on page 1
must ALSO be met for full accreditation.

***SUMMARY**, Comments, and recommendations:*
NATURE OF DEFICIENCY, IF ANY, AND CORRECTIVE ACTION TAKEN**:* ***COMMENTS AND RECOMMENDATIONS:

Virus Isolation Checklist

Part V-I: Yellow Fever Virus Isolation Testing

(To be completed BY THE LAB and returned to the assessor before the assessment.)

1.	Description of the YF diagnostic laboratory equipment	#	Comments
	Number of fluorescence microscopes		
	Number of light microscopes (without fluorescence mode)		
	Number of cell culture CO_2-incubators		
	Other equipment, please specify:		

2.	Performance of YF diagnostic in the previous 12 months	#	Comments
	Dates from __/__/____ (dd mm yyyy) to __/__/____. (dd mm yyyy)		
	Number of specimens with virus isolation/detection attempted:		
	Number of YF viruses confirmed by virus isolation/detection:		
	Percentage of virus isolation/detection results reported within a month of being available:		
	Number of viruses forwarded to a sequencing laboratory:		
	Number of YF specimens tested by virus culture methods:		
	Total number of YF sequences reported to WHO during period of review:		
	Percentage of YF sequence data reported timely to WHO:		

Part V-II: EVALUATION AND SCORING OF YELLOW FEVER VIRUS ISOLATION
(To be completed BY THE ASSSESSOR during the assessment.)

1.	**Diagnostic methods performed in the laboratory**	***Y/N/P****	**Score (/1)**
	Only validated assays are used for diagnostic.		**Mandatory for accreditation**
	Records are maintained on all assays performed, including: **a. Kit manufacturer and batch number** **b. Reagent expiry dates** **c. Use of Positive and negative controls for all assays**		**Mandatory for accreditation**
	Is there a systematic adherence to the approved testing algorithms and national policies		**Mandatory for accreditation**
	SOPs for performing of the diagnostic tests are available.		/1
2.	**Cell-culture work for PRNT and virus isolation**	***Y/N/P****	**Score (/7)**
	Appropriate SOPs are available and used for: a. Freezing and recovery of cells: b. Routine passage of cells. c. Inoculation of cells d. Harvest and storage of viral isolates		/1
	Low passage cell stocks are labelled appropriately and stored in liquid nitrogen.		/0.5
	Permanent records are maintained on cell passage and storage histories.		/0.5
	Fetal calf serum is derived from certified, disease–free sources		/0.5
	Cells are routinely tested for the presence of mycoplasma.		/0.5
	Cells are cultured and maintained in a separate incubator from that used for specimen processing and virus inoculation.		/1
	Reagents and stock solutions are labelled appropriately, including dates of preparation and expiration, and stored at indicated temperatures.		/0.5
	Two sequential passages of 4-5 days are performed before recorded as negative (minimum time in culture is 8 days):		/0.5
	Isolates are confirmed with Immunofluorescence (IFA), RT-PCR, or other recommended method:		/0.5
	Aliquots of isolates are stored appropriately at –70ºC or lower for at least 12 months:		/0.5
	Storage vials are clearly and permanently labelled:		/0.5
	Permanent records are maintained on the identity and location of all isolates:		/0.5

Part V-III: SUMMARY OF THE REVIEW
(To be completed BY THE ASSSESSOR following the assessment)

Review summary score:

Section	Score obtained	Maximal score	% achieved	Mandatory criteria
1. Diagnostic methods performed in the laboratory		**1**	**%**	**/3**
2. Cell-culture work for PRNT and virus isolation		**7**	**%**	**-**
TOTAL ACROSS ALL SECTIONS		**8**	**%§**	**/3***

- = not available, § = a minimum score of 80% must be obtained, * = all mandatory criteria must be met

A minimum score across all sections of 80%
AND
All of the mandatory criteria (3/3) must be met within this section
(along with the General Laboratory section) to be considered for accreditation.

Additional programmatic criteria listed on page 1
must ALSO be met for full accreditation.

***SUMMARY**, Comments, and recommendations:*
NATURE OF DEFICIENCY, IF ANY, AND CORRECTIVE ACTION TAKEN**:* ***COMMENTS AND RECOMMENDATIONS:

Annex 1: Data checklist on the various important elements to be part in the YF Laboratory requisition form accompanying the diagnostioc specimens of a suspected YF case.

Epidemiology and case identification	**Comments**
Name of sender:	
Adress of sender:	
Contact phone/mobile N°:	
EPID no.	
Specimen no. from testing lab	
Name of patient	
gender	
age	
Profession, employment of the patient	
District/municipality code of patient	
Province/state code of patient	
Country of patient	
Documentation of last YF vaccination	
Travel history of the last 2 weeks	
Contact with other diseased patient in the last 2 weeks	
Date of disease onset	
Date of specimen collection	
Date of disease onset	
Clinical symptomes jaundice	
Clinical symptomes hepatitis	
Clinical symptomes fever	
Clinical symptomes hemorraghic symtomes	
Required emergency room/doctor visit	
Patient requiring hospitalization	
Life threatening illness	
Describe symptoms, signs of disease, time course and treatment, other diseases if any	
Specimen for serology and molecular testing	
Type of specimen (serum, plasma, blood, urine)	
Condition of specimen upon arrival	
Date specimen tested	
Date diagnostic results are reported	
Specimen for virus isolation/detection	
Condition of specimen upon arrival*	
Date specimen tested or stored (as appropriate)*	
Specimen source*	
Name of virus detection laboratory*	
Virus isolation/detection results	
Date specimen tested*	
YF virus detected*	
Date YF detection result reported*	
Date specimen sent to sequencing lab*	
Name of sequencing lab*	
Sequencing information	
Date YF virus specimen sent for sequencing*	
Date sequence result available*	
Name of sequencing laboratory*	

* If virus isolation/detection performed by National Laboratory

Department of Immunization, Vaccines, and Biologicals (IVB)
World Health Organization
20 Avenue Appia
1211 Geneva 27
Switzerland
E-mail: vaccines@who.int
Website: https://www.who.int/teams/immunization-vaccines-and-biologicals

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