World Health Organization

Manual for the Laboratory Diagnosis of Japanese Encephalitis Virus Infection

For Evaluation Purposes

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Contents

Glossary

Antibody capture technique: laboratory process for detecting virus-specific antibodies in patient's cerebrospinal fluid or blood by first capturing patient's antibodies in the wells of microtitration plate and then testing with virus specific antigen.

CSF: Cerebrospinal fluid

Endemic: The constant presence of a disease or infectious agent within a given geographical area or population group.

Enzootic: The constant presence of a disease within a given animal population.

Epidemic: An outbreak of disease in human population.

Genotype: Distinct familial cluster of viruses based on genetic similarities and implying evolution from a common ancestor virus.

IgG (immunoglobulin G): The major class of circulating antibody that is produced several days to weeks after infection but remains to provide protection for months to years.

IgM (immunoglobulin M): The class of antibody that is the first to be produced in response to infection, and the first to disappear from the blood.

JE: Japanese Encephalitis

JEV: Japanese Encephalitis Virus

Quality Assessment: A system for testing the proficiency of a laboratory.

Quality Assurance: The process that guarantees the quality of laboratory results and encompasses both quality control and quality assessment.

Quality Control: The process of continually monitoring working practices, equipment and reagents.

Viraemia: Spread of virus throughout the body via the bloodstream.

1. Purpose

The purpose of this manual is to support the surveillance and control of Japanese Encephalitis (JE) by:

- Presenting accurate information on the epidemiology, pathology and clinical features of the disease
- Describing the expected role of the laboratory in disease surveillance;
- Presenting detailed and general descriptions of procedures recommended for effective laboratory diagnosis of JE infection

The manual is intended for use by virologists and technologists working in laboratories collaborating in JE control. It may also be of interest to managers of JE control programmes and field staff, who will be better able to appreciate the role of the laboratory and use it appropriately.

2. Introduction

Japanese encephalitis (JE) is a major cause of childhood mortality and morbidity in countries of Southeast Asia and Western Pacific regions. It is the most important cause of arboviral encephalitis: approximately 3 billion people live in endemic regions and, even with current levels of inconsistent and sporadic surveillance, it is reported to cause at least 50 000 cases with 10 000 deaths annually.

A laboratory diagnosis of JE is essential for accurate diagnosis and surveillance. Enhanced surveillance activities are needed to determine the disease burden and trends, substantiate the need for vaccination, monitor impact of vaccination programs, and to warn of or confirm the cause of outbreaks.

The Southeast Asia WHO region has added encephalitis to its list of reportable diseases and is beginning to collect data. A network of 30 laboratories will likely be established throughout Southeast Asia and Western Pacific regions. Laboratories with advanced diagnostic capabilities will be designated to serve as regional reference facilities. The guidelines in this manual are designed to ensure quality control of diagnostic and testing activities in order to facilitate training and help standardize laboratory procedures.

This JE Laboratory manual is a first edition. It is anticipated that revisions will be made reflecting comments received from users in the field; development and availability of new techniques; and with further understanding of the virus.

All countries at-risk of JE should have access to a qualified national level laboratory capable of confirming JE by a validated IgM test. All these countries should also be aware of the regional reference laboratories that can aid them in confirmatory testing. Procedures for specimen transport between peripheral and regional reference laboratories and the process for testing of those specimens should be formally put in place.

This manual provides guidelines on the establishment and maintenance of an effective laboratory network capable of reliably providing confirmation of JE infection.

The diagnosis of JE is usually made serologically using IgM-capture enzyme-linked immunosorbent assay (ELISA). Most patients either have antibody at presentation at a health facility or a few days later. CSF is the preferred sample for diagnosis of JE because if anti JE IgM is detected in the CSF this confirms infection of the central nervous system with JEV.[1] This is in contrast to asymptomatic infection with JEV or vaccination with live attenuated JE vaccines, which may cause an antibody rise in the serum, but are not thought to lead to antibody production in the CSF. Occasionally the CSF sample may be taken too early in the illness for IgM to be present at a diagnostic titre.[1] For example in one 1995 study only 75% of CSF samples taken on days 3-4 of the illness were positive; whereas more than 95% of those taken beyond day 10 of illness were positive.[2] A second serum samples collected approximately one week after the first sample can be very helpful in accurate diagnosis, if the first one is IgM negative,[3] but most patients have antibody detectable one week after admission. In one study, 20% of JE patients had a negative serum sample at admission, but later seroconverted.[4] Even if the first serum sample is positive, a second sample can be useful because it will usually show an increased titre compared to the first sample, which aids in confirming an acute infection and related disease, rather than the consequence of a recent coincidental infection.

Commercial kits are now available for the detection of JE IgM using capture ELISA, dot-blot or immunoprecipitation assays. These tests can be readily performed at many peripheral laboratories. Polymerase chain reaction (PCR) tests have been developed and may have a role to play in JE diagnosis in the future. PCR requires laboratory facilities and special equipment not routinely available in peripheral laboratories. The short period of viraemia in infected humans may also limit the usefulness of PCR as a clinical diagnostic tool. Viral isolation can also be performed, but it is slow and technically difficult, and is often negative because virus has cleared by the time patients present to hospital. This manual is intended to be useful to laboratories with limited facilities, but may also be useful to laboratories undertaking reference and research work.

JE epidemiology

Japanese encephalitis virus is transmitted between animals by *Culex* species of mosquitoes, and occurs in countries across eastern and southern Asia and the Pacific. Related neurotropic flaviviruses are found across the globe and share many virological, epidemiological, and clinical features.[5] Molecular virological studies suggest that all flaviviruses originated from a common ancestor some 10-20 000 years ago, and are rapidly evolving to fill ecological niches.[6]

Enzootic cycle

Japanese encephalitis virus is transmitted naturally between wild and domestic birds and pigs by the Culex mosquitoes, the most important for human infection being *Culex tritaeniorrhynchus* which breeds in pools of stagnant water such as paddy fields.[7] Although many animals can be infected with the virus, only those which develop high viraemias are important in the natural cycle. As well as maintaining and amplifying Japanese encephalitis virus in the environment, birds may also be responsible for the spread to new geographical areas. Pigs are the most important natural host for transmission to humans. They are often

kept close to humans, have prolonged and high viraemias, and produce many offspring, providing a continuous supply of uninfected new hosts. The virus does not typically cause encephalitis in these natural hosts, although abortions can occur in pregnant sows.

Epidemiology of human disease

Humans become infected with Japanese encephalitis virus coincidentally when living or travelling in close proximity to animals and birds infected with JE. Although most cases occur in rural areas, Japanese encephalitis virus is also found on the edge of cities. Epidemiological studies have shown that after the monsoon rains mosquitoes breed prolifically. As mosquito numbers grow, so does their carriage of Japanese encephalitis virus and the infection rate of pigs.[8 9] Human infection soon follows.

Although the virus has occasionally been isolated from human peripheral blood,[10] viraemias are usually brief and titres low; as a result humans are considered a dead end host from which transmission does not normally occur. Serological surveys have shown that in rural Asia most of the population are infected with Japanese encephalitis virus during childhood or early adulthood.[11] Most infections of humans are asymptomatic or result in a non-specific flu-like illness. Estimates of the ratio of symptomatic disease to asymptomatic infection vary between 1 in 25,[12] and 1 in 1 000.[13]

Broadly speaking two epidemiological patterns of Japanese encephalitis are recognized.[14] In northern areas (northern Viet Nam, northern Thailand, Taiwan China, Republic of Korea, Japan, China, Nepal, and northern India) large epidemics occur during the summer months, whereas in southern areas (southern Viet Nam, southern Thailand, Indonesia, Malaysia, Philippines, Sri Lanka, and southern India) Japanese encephalitis tends to be endemic, and cases occur sporadically throughout the year with a peak after the start of the rainy season.[14]

Geographical distribution

In the past 50 years the geographical area affected by Japanese encephalitis virus has expanded (figure 1). Differences in diagnostic capabilities and in reporting of encephalitis make it impossible to plot this expansion precisely. In China outbreaks of summer encephalitis occurred from 1935, and the virus was first isolated there in 1940. In 1949, large epidemics were reported from the Republic of Korea for the first time. Epidemics in northern Viet Nam followed in 1965 (currently 1 000-3 000 cases occur nationally each year), and in Chiang Mai in northern Thailand in 1969 (currently 1 500-2 500 cases occur nationally each year). Japanese encephalitis was recognized in southern India in 1955, but was confined to the south until the 1970s. The fact that adults and children were equally affected in these Indian states strongly supports the idea that the virus was introduced here for the first time in the 1970s. More recent outbreaks have seen a predominance of paediatric cases, with almost 5 000 cases and 1 300 deaths in the 2005 outbreak in Gorakhpur, northern India. The late 1970s also saw the first cases in Burma and Bangladesh, and large epidemics in south western Nepal. In 1985 Sri Lanka experienced its first epidemic with 410 cases and 75 deaths. Japanese encephalitis virus continues to spread west with cases occurring in Pakistan[15] and now annual epidemics in the Terai (low-lying) region as well as the Kathmandu valley of Nepal.[16]

Figure 1: Countries at risk for JE

In Malaysia the disease is endemic; the virus was first isolated in the 1960s and about 100 cases are recorded annually. Japanese encephalitis is endemic in Indonesia, and 1 000-2 500 cases of encephalitis are reported annually, although in most the aetiological agent is not confirmed.[17]

The reasons for the spread of Japanese encephalitis are incompletely understood, but probably include changing agricultural practices, such as increasing irrigation (which allows mosquito breeding over longer periods), and animal husbandry (which provides a steady supply of host animals). Windborne mosquitoes are thought to have been important in the spread of JE to the Torres Strait islands and the Australian mainland in 1998.[18]

In developed countries such as Japan, Taiwan China, and the Republic of Korea the number of cases has fallen, probably due to a combination of mass vaccination of children, spraying of pesticides, changing pig rearing practices, separation of housing from farming, better housing with air conditioning, and less availability of mosquito breeding pools.[7] However, in the Republic of Korea the widespread use of vaccine in children has been associated with a higher incidence of Japanese encephalitis in those over 15 years of age, possibly indicating waning immunity many years after vaccination.[14]

Virology of JE

In common with all flaviviruses, Japanese encephalitis virus has a small (50 nm) lipoprotein envelope surrounding a nucleocapsid comprising of core protein and 11 kb single stranded RNA (3 800 kD). At least four genotypes of Japanese encephalitis virus occur in Asia, which relate roughly to the geographical area of isolation.[19, 20]

Clinical features

Clinical JE follows an incubation period of 4-14 days and is mostly characterized by sudden onset of fever, chills and aches, including headaches and sometimes meningismus, particularly in adults. In children, gastrointestinal pain and dysfunction may dominate the initial stage of the disease. Convulsions are also very common in paediatric patients. Although JE is often a mild disease, leading to an uneventful recovery, some cases rapidly progress to severe encephalitis with mental disturbances, general or focal motor abnormalities, and progressive coma. Of the approximately 50 000 cases of JE officially reported each year, about 10 000 end fatally, and a high percentage (about half) of the survivors are left with neurological and psychiatric sequelae, requiring extensive long-term care. Most fatalities and residual sequelae occur in children under 10 years of age.

Acute Flaccid Paralysis: In 1995 a subgroup of patients infected with Japanese encephalitis virus were identified who presented with a poliomyelitis-like acute flaccid paralysis.[21] After a short febrile illness there was a rapid onset of flaccid paralysis in one or more limbs, despite a normal level of consciousness. Weakness occurred more often in the legs than the arms, and was usually asymmetric. Electromyography (EMG) was suggestive of anterior horn cell damage.[21] Flaccid paralysis also occurs in comatose patients with "classic" Japanese encephalitis, being reported in 5%-20%. [22, 23] Occasionally respiratory muscle paralysis may be the presenting feature.[24]

3. Role and function of the laboratory in JE control and prevention

Role of the laboratory in JE surveillance

Surveillance and rapid response to identified disease threats are at the core of preventive medicine. A well-designed and well-implemented infectious disease surveillance programme can provide a means to detect unusual clusters of disease, document the geographical and demographic spread of an outbreak, estimate the magnitude of the problem, describe the natural history of the disease, identify factors responsible for emergence, facilitate laboratory and epidemiological research, and assess the success of specific intervention efforts. The effectiveness of surveillance depends on the speed of reporting and analysing the results.

Infection with JE virus may be asymptomatic, or may cause febrile illness, meningitis, myelitis or encephalitis. Encephalitis is the most commonly recognized presentation, and is clinically indistinguishable from other causes of an acute encephalitis syndrome (AES). Syndromic surveillance therefore aims to identify patients with AES and among these confirm JE virus infection using standardized laboratory techniques. So a definitive

diagnosis of JE infection cannot be based on clinical impressions alone, but must rely on laboratory confirmation.

The WHO recommended case definition for suspect Japanese Encephalitis[25] is:

Clinical case definition

Clinically, a case of acute encephalitis syndrome is defined as a person of any age, at any time of year with the acute onset of fever and a change in mental status (including symptoms such as confusion, disorientation, coma, or inability to talk) AND/OR new onset of seizures (excluding simple febrile seizures*). Other early clinical findings may include an increase in irritability, somnolence or abnormal behaviour greater than that seen with usual febrile illness.

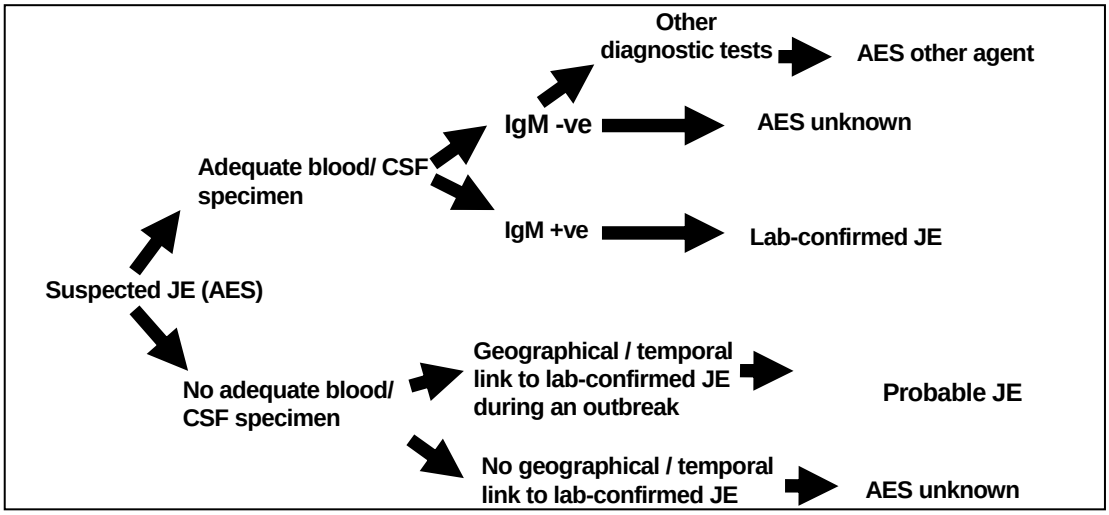
Case classification

Suspected case : A case that meets the clinical case definition for AES. Suspected cases are further classified in one of the following four ways (figure 2).

> **Laboratory-confirmed JE:** A suspected case that has been laboratory-confirmed as JE.
>
> **Probable JE:** A suspected case that occurs in close geographical and temporal relationship to a laboratory-confirmed case of JE, in the context of an outbreak.
>
> **"Acute encephalitis syndrome" – other agent:** A suspected case in which diagnostic testing is performed and an etiological agent other than JE virus is identified.
>
> **"Acute encephalitis syndrome" – unknown:** A suspected case in which no diagnostic testing is performed or in which testing was performed but no etiological agent was identified or in which the test results were indeterminate.

*A simple febrile seizure is defined as a seizure that occurs in a child aged 6 months to less than 6 years old, whose only finding is fever and a single generalized convulsion lasting less than 15 minutes, and who recovers consciousness within 60 minutes of the seizure

Figure 2 Final classification scheme for AES cases†



Laboratory criteria for confirmation

The recommended method for laboratory confirmation of a JE virus infection is:

- **Presence of JE virus-specific IgM antibody in a single sample of cerebrospinal fluid (CSF) or serum, as detected by an IgM-capture ELISA specifically for JE virus;**

Further confirmatory tests (e.g. looking for cross-reactivity with other flaviviruses circulating in the geographical area, or repeating the test with a more specific assay) should be carried out when:
(a) there is an ongoing dengue or other flavivirus outbreak;
(b) vaccination coverage is very high;
(c) there are no epidemiological and entomological data supportive of JE transmission;
(d) a non validated assay is used at the primary testing laboratory

† A suspected case of JE can also be a suspected case of bacterial meningitis. In this event, a CSF/blood sample should be sent to both the bacteriology and JE virology laboratories to allow rapid diagnosis and appropriate case management and classification.

Note: A serum sample should be obtained at admission. Because it may not yet be positive in a JE-infected person, a second serum sample should be collected at discharge or on the 10th day of illness onset or at the time of death and tested for presence of JE virus specific IgM. However, CSF is the preferred sample to collect because antibody in the CSF confirms the virus has infected the nervous system. Antibody in the serum only could simply reflect recent coincidental asymptomatic JEV infection, or recent vaccination with a live attenuated JE vaccine.

In addition, any one or more of the following laboratory criteria are confirmatory for JE:

- Detection of JE virus antigens in tissue by immunofluorescence or immunohistochemistry;

- Detection of JE virus genome in serum, plasma, blood, CSF, or tissue by reverse transcriptase polymerase chain reaction (RT-PCR) or an equally sensitive and specific nucleic acid amplification test;

- Isolation of JE virus in serum, plasma, blood, CSF, or tissue;

Note: Detection of virus genome or virus isolation in serum, plasma or blood is very specific for JE diagnosis; however, sensitivity is poor as virus levels are usually undetectable in a clinically ill JE case. Therefore a negative result by these methods should not be used to rule out JE in a suspected case. Similarly detection of virus genome or virus isolation in CSF is usually only found in fatal cases and therefore not very sensitive and should not be used for ruling out a diagnosis of JE.

- Detection of a four-fold or greater rise in JE virus-specific antibody as measured by haemagglutination inhibition (HI) or plaque reduction neutralization assay (PRNT) in serum collected during the acute and convalescent phase of illness. For these two tests, two specimens for IgG should be collected at least 14 days apart. The IgG test should be performed in parallel with other confirmatory tests to eliminate the possibility of cross-reactivity, as indicated in footnote.

Note:

To confirm that a seasonal outbreak is due to JE, suspected cases should be tested until 5-10 are laboratory-confirmed as JE. If the outbreak is not an expected seasonal outbreak, or there are unusual epidemiological features (e.g. age distribution of cases not consistent with pattern of JE infection), testing of CSF is especially important as an encephalitis outbreak could be due to other aetiologies (e.g. Nipah virus). In a non-JE outbreak, sporadic cases of JE are still likely to occur, so the percentage of specimens confirmed as JE positive also should be considered. For example, in previous large JE outbreaks, typically about 30% cases have been JE positive, so confirmation of a much smaller percentage of cases may prompt further investigations into the aetiology of the outbreak. As a JE outbreak continues,

all samples may not need to be tested. During epidemics, laboratory testing can be limited to the confirmation of 5-10 cases detected early in the outbreak, for each geographical area and approximately 5-10% of cases could be tested on an ongoing basis, until the end of the outbreak.

The gap between reporting of a suspect case and case confirmation is a function of the case investigation system and the availability of, and access to, a viral diagnostic laboratory. There is a network of WHO laboratories for arboviruses and haemorrhagic fevers that is supplemented by national laboratories, many of which have been involved in other vaccine preventable disease control initiatives.

The laboratory is the foundation for all epidemiological and clinical procedures. Just as an excellent laboratory can provide invaluable information to clinicians and public health workers attempting to control an illness or an expanding epidemic, a deficient laboratory can provide misleading or incorrect information, effectively delaying the possibility of early control or activating control measures that are not warranted

Structure and activities of the Laboratory network in JE surveillance

There are five main objectives in setting up a network of laboratories that support various aspects of JE surveillance

- To develop standards for the laboratory diagnosis of JE and provide the necessary support as JE control evolves;
- To establish mechanisms for reference and support for regional, national and sub-national laboratories in the diagnosis of JE;
- To provide training resources and facilities for staff of regional, national and sub-national laboratories;
- To provide a source of reference materials and expertise for the development and quality control of improved diagnostic tests;
- To serve as a bank of JE virus isolates for molecular epidemiology and reference sera for quality control.

Individual laboratories are not expected to undertake the full range of tasks listed above, but will perform specific duties according to the needs of the national or regional control initiatives. The performance of Network Laboratories will be monitored by proficiency testing in selected techniques and by routine performance evaluation. These are described in more detail in the Performance and Quality Assurance sections below.

It is essential that the laboratory network be planned in tandem with regional control initiatives, such as immunization programmes, and established with properly trained personnel, suitable equipment and reagents. The JE laboratory network is being organized on four levels:

Global Specialized Laboratories

These laboratories help develop and refine laboratory diagnostic techniques and set the technical standards for the Laboratory Network. Their responsibilities extend to JE and other flavivirus laboratories globally. Strengthening quality assurance and developing proficiency testing programmes will be a key function. These laboratories also play a major role in providing molecular analysis of JE virus genome and interpreting molecular epidemiological data.

Regional Reference Laboratories

These are centres of excellence in each Region able to undertake international responsibilities. They provide confirmation of the results from National Laboratories, and characterization of virus strains. These laboratories are expected to be able to be capable of detecting JE-specific IgM and IgG, performing appropriate differential diagnosis assays and identifying JE virus genome using RT-PCR. They also help coordinate the Network by providing training and support to National and Sub-National Laboratories, helping strengthen quality assurance and quality control procedures, and may play a role in distribution of proficiency panels, essential reagents and laboratory consumables. They serve as reference laboratories for national laboratories in neighbouring countries and serve as national laboratories in their own countries.

National Laboratories

These laboratories will have the closest links with national immunization and surveillance staff. They will test specimens from suspected cases by IgM ELISA and report directly to the immunization and surveillance authorities. Due to possible cross reactivity with other flaviviruses it is essential that a representative number of IgM positive JE sera be tested for other regionally relevant flaviviruses and/or confirmed by the relevant reference laboratory. The national laboratory has the responsibility of forwarding representative specimens and materials to the designated reference laboratory for confirmation and further analysis.

Sub-National Laboratories

In some countries sub-national laboratories will be established for logistic, geographical or political reasons. The number of sub-national laboratories established will depend on the epidemiological priorities and resources available. These laboratories will have the responsibility for testing samples by IgM ELISA and will report directly to the national surveillance authorities. Representative samples should be forwarded to the designated national laboratory for confirmation and further analysis.

Generic laboratory capability

To be effective and efficient members of the Japanese Encephalitis Laboratory Network national and sub-national laboratories should have:

- Established links to the immunization and surveillance units at the Ministry of Health;
- Proven capability to perform testing;
- 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 feed back of data.

WHO has established an extensive laboratory network for measles and rubella diagnosis which has standardized on ELISA IgM testing for case confirmation. All laboratories are equipped with ELISA readers, washers and other appropriate serological equipment, staff are trained in ELISA technology and close collaboration with disease control programmes has been established. It will therefore often be appropriate that the laboratories in the measles and rubella network are considered when selecting the laboratories that conduct JE testing.

Coordination of the network

Coordination of the Japanese Encephalitis Laboratory Network will be carried out by WHO. Each of the WHO Regions has a Regional Laboratory Coordinator, responsible for the laboratories within their Region. Each of the Regions reports to the Global Laboratory Coordinator in WHO Headquarters, Geneva. Effective coordination is achieved through regular feed-forward of results, requests and queries, and feedback of analysis, comments and technical advice. Procurement and distribution of essential laboratory equipment and reagents is also effected through WHO mechanisms.

The smooth functioning of the Laboratory Network depends on the establishment of a system of communication within the network and with the programme. Standard referral and reporting forms have been developed to ensure that all essential patient information is transmitted (see Annex 1 for sample form). The format and timing of result reporting must be agreed upon in consultation with programme managers.

Monitoring indicators of field and laboratory performance will be routinely evaluated and include:

- The proportion of samples received in good condition;
- The proportion with properly completed laboratory forms;
- The proportion of results reported within a month of receipt of specimen in the laboratory.
- The proportion of positive samples referred to the regional reference laboratory within 7 days of being available. (Positive samples detected from any newly infected district should be sent immediately)

Virologists and epidemiologists at all levels must establish mechanisms to exchange information on a regular basis and evaluate performance indicators of the surveillance system. For example, the regional reference laboratories should meet at least once a year and the national and sub-national laboratories should hold meetings with surveillance/immunization authorities at least once a month.

Performance indicators of surveillance and laboratory quality

It is important to regularly review laboratory quality systems to determine if any improvements can be made. Indicators can be helpful as management tools to identify areas where corrective action is needed. The WHO-recommended standards for surveillance of JE [25] propose the following indicator of good laboratory and surveillance

management practices (table 1). Annex 5 outlines some key quality assurance activities to strengthen laboratory quality.

Table 1 Laboratory and surveillance performance indicators

Indicator	Target
For all tests, laboratory results reported within 1 month after receipt of specimen (however, samples collected to identify the cause of an outbreak should be tested and reported within a minimum of 7 days after receipt)	≥ 80%
Percentage of CSF/serum samples reaching laboratory in adequate condition (i.e. transported under cold chain conditions)	≥ 80%

For the purposes of quality assurance, laboratories are requested to store all positive samples at -20°C for a period of at least 12 months and negative samples for a period of 3 months, but longer if there is a programme for obtaining a differential diagnosis for negative samples. If storage space is limited then the laboratory should contact the WHO laboratory coordinator for advice. No positive samples should be discarded without first consulting the WHO Laboratory coordinator.

Quality Assurance and Accreditation Programme for JE Laboratory Network

At the time of writing this laboratory manual it is anticipated that an accreditation programme will be developed for the JE Laboratory Network. Accreditation provides documentation that a laboratory has the capability and the capacity to detect, identify accurately, and promptly report confirmed JE cases. The accreditation process provides a learning opportunity, a mechanism for identifying resource and training needs and a measure of progress.

It is anticipated that the accreditation process of JE Laboratories would be an annual assessment by WHO, reviewing laboratory performance during the immediately preceding 12 months. Accreditation would be given for the forthcoming calendar year.

The performance criteria for accrediting JE Laboratories would likely include:

- Timeliness of reporting results within the specified timeframe (≥80%)
- Minimum workload as measured by the number of samples tested per year
- The accuracy of a defined percentage of test results as determined by the concordance of test results on sera submitted by the National Laboratory to the Regional Reference Laboratory; (≥80%)
- Appropriate QC procedures are in place and followed
- Passing an annual WHO approved proficiency test (≥80%)
- Onsite review of laboratory operating procedures and practices (≥80%)

The first 5 criteria would be assessed annually but the onsite review may occur less frequently, but no less than once every 2-3 years.

4. Specimen collection and handling

Choice of specimen

Blood and cerebrospinal fluid (CSF) are the most likely specimens to be referred for detection of IgM antibodies to Japanese Encephalitis Virus (JEV). These samples should be collected as soon as possible after admission of the patient. CSF is also an important diagnostic specimen to differentiate bacterial from viral infection and an aliquot should be also sent to a competent bacterial laboratory for appropriate testing. As JE IgM may take up to 10 days to develop after symptoms first develop, a second serum sample should be collected at discharge or on the 10th day of illness onset or at the time of death and tested for presence of JE virus specific IgM.

The large majority of JE infections are asymptomatic. Therefore, in areas that are highly endemic for JE, it is possible to have encephalitis due to a cause other than JE virus and have JE virus-specific IgM antibody present in serum. IgM antibody may also be present in serum after JE vaccination. Therefore testing of a CSF sample from all persons with encephalitis is recommended when feasible. However it may not be technically feasible, or may be contraindicated because of nosocomial infection concerns in settings where appropriately sterilized equipment may be unavailable.

Care should be taken to minimize risks to the patient as well as to health care providers. Blood or CSF specimens may contain infectious agents e.g. hepatitis or human immunodeficiency viruses in blood.

Laboratory personnel will not normally be required to perform venepuncture or lumbar puncture and details of these procedures are therefore not included here.

Patient information should be recorded on a test request form (see Annex 1) that must accompany any specimen when it is referred to the laboratory. Information should include: patient name, age (or date of birth), province/district/town of residence, JE vaccination history (including date of last JE vaccination), date of onset of first symptom, types of specimens and date of specimen collection. All specimen tubes should be labelled with the patient's name or identifier number, date of collection and specimen type.

Cerebrospinal fluid (CSF) collection

- The collection of CSF is an invasive technique that should only be performed by experienced personnel using appropriate equipment under aseptic conditions.
- CSF should be collected in a dry, sterile, screw cap container. The CSF can be aseptically divided into separate aliquots for examination for cells, biochemistry, microbiology and virology. For virological investigations, a minimum of 0.5ml of CSF is required.

- CSF should be transported to the local hospital laboratory as soon as possible (ideally within 1 hour). Before arrival at the laboratory, the CSF specimens for routine investigations should not be refrigerated or exposed to extreme cold, excessive heat or sunlight. However, if there will be a delay beyond 1 hour, specimens for virology should be refrigerated. In the laboratory priority should be given to ruling out treatable (usually bacterial) aetiological agents. *Note:* If the hospital laboratory does not have the capacity for bacterial testing, samples should be shipped to the JE laboratory as soon as possible.
- CSF samples for virological testing should be sent to the designated laboratory as soon as possible. Before transport, in the hospital laboratory, they should be held at 4°C for short term storage (1 to 3 days) or at or below –20°C for longer term storage. If a –20°C freezer is not available, they should be stored in the freezer section of the refrigerator. If specimens have been frozen, they should be transported frozen. Repeated freezing and thawing of CSF should be avoided as this may lead to instability of IgM antibodies.
- **See annex 2 for an algorithm summarizing CSF and serum collection procedures**.

Blood specimen collection

- A serum sample should be obtained at admission. Because it may not be positive in a JE-infected person, a second serum sample should be collected at discharge or on the 10^{th} day of illness onset or at the time of death.
- Blood should be collected by venepuncture and placed in a dry, sterile vial. The volume of specimen should be approximately 5 ml for older children and adults and 1 ml for infants and younger children.
- Whole blood is allowed to clot at room temperature and then stored in a cold box or refrigerator and maintained at 4−8 °C (and not frozen). It should be transported to the hospital laboratory within 24 hours.
- In the hospital laboratory, clotted whole blood should then be centrifuged at 1000 × g for 10 minutes to separate the serum. *Note:* If there is no centrifuge, the blood should be kept in a refrigerator until there is complete retraction of the clot from the serum (but no longer than 24 hours). The serum should be carefully removed with a fine-bore pipette, avoiding collection of red blood cells.
- Serum should be transferred aseptically to a dry, sterile vial labelled with the patient's name and identifier number, date of collection, and specimen type and transported to a designated laboratory for IgM testing.
- Serum samples received for IgM analysis should be tested as soon as possible after receipt in the laboratory. Short-term storage of serum (1-3 days) should be at 4^{o}C. Longer term storage of serum should be at or below –20^{o}C. Repeated freezing and thawing of serum should be avoided as this may lead to instability of IgM antibodies.
- See annex 2 for an algorithm summarizing the serum and CSF collection procedure.

Safe Specimen transportation

Transport of CSF and serum samples

Proper transport is necessary to protect the integrity of specimens, minimize the risk of transmission of infectious agents to anyone who may come in contact with the specimens (e.g. transport personnel, the sender, the receiver or the public), and to comply with national and international regulations for transport of diagnostic specimens. The safe transport of diagnostic specimens and infectious materials is therefore of concern to all who are involved in the process. Proper transport is also important to avoid inaccuracies in diagnosis due to degraded or mishandled specimens. The following is necessary at the national level:

- Ensure the availability of specimen collection containers, laboratory referral forms and secondary transport containers at health care facilities.
- Identify the personnel to be responsible for collection of specimens and for arranging for their transport to the laboratory.
- Laboratory personnel should provide authorities at the health care facility with full documented procedures relevant to specimen collection and packaging e.g. type and volume of specimen to be collected for JEV IgM detection, timing of specimen collection, how specimens are to be packed (see below), storage conditions to be maintained during specimen transportation, and, the mechanisms established for transport and delivery of samples to the laboratory.

Packaging of specimens for transport

In principle a "triple packaging system" (figure 3) is always required when specimens are to be moved from one location to another. Specimens should be packaged as follows:

- *Primary receptacle.* Specimens should be placed within a primary receptacle which is usually a labelled, watertight, leak-proof receptacle containing the specimen. The receptacle should be wrapped in enough absorbent material to absorb all fluid in case of breakage or leakage.
- *Secondary receptacle.* A second durable, watertight, leak-proof receptacle should be used to enclose and protect the primary receptacle(s). Several wrapped primary receptacles may be placed in one secondary receptacle. Sufficient additional absorbent material must be used to cushion multiple primary receptacles and absorb fluids in case of breakage or leakage.
- *Outer shipping package or transport container*. The secondary receptacle is placed in an outer transport or shipping container which protects it and its contents from outside influences such as physical damage and water while in transit.

- Specimen data forms, letters, and information to identify the specimen, the sender, and the receiver should be placed in a waterproof bag and taped either to the outside of the secondary receptacle or to the inner lid of the outer transport/shipping container.
- Ice packs can be placed between the secondary and outer transport/shipping package, should they be required for keeping specimens cool during transport.

- The outer shipping or transport container should be labelled with the name of the receiver, indication of storage conditions required during transport, and bear any

additional labels or stickers that may be required to fulfil local or international regulations (see below).

Figure 3A Serum specimen within primary receptacle

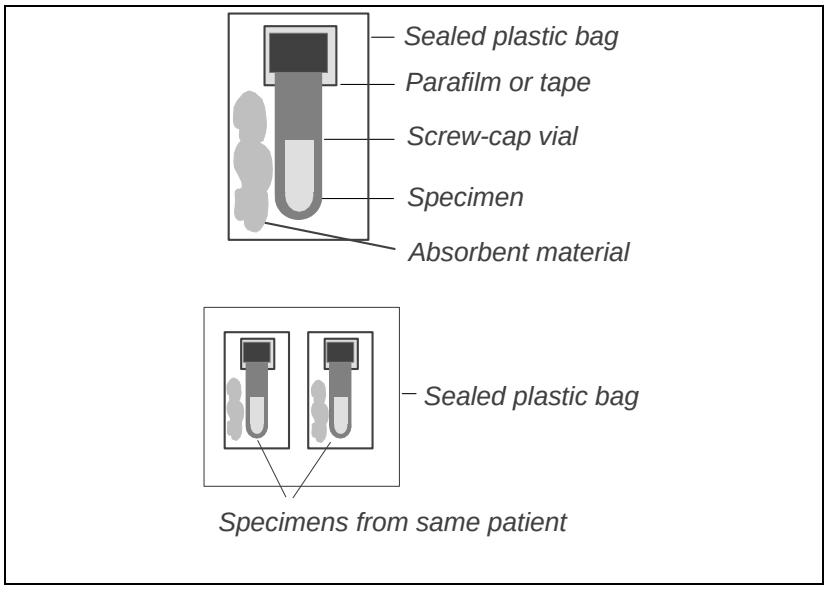


Figure 3B Several serum specimens within secondary receptacle

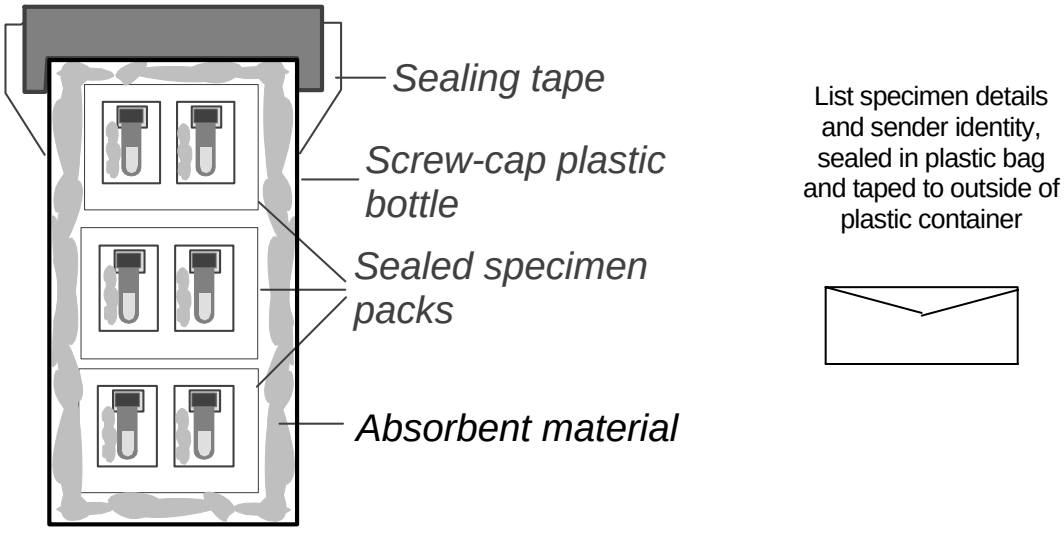
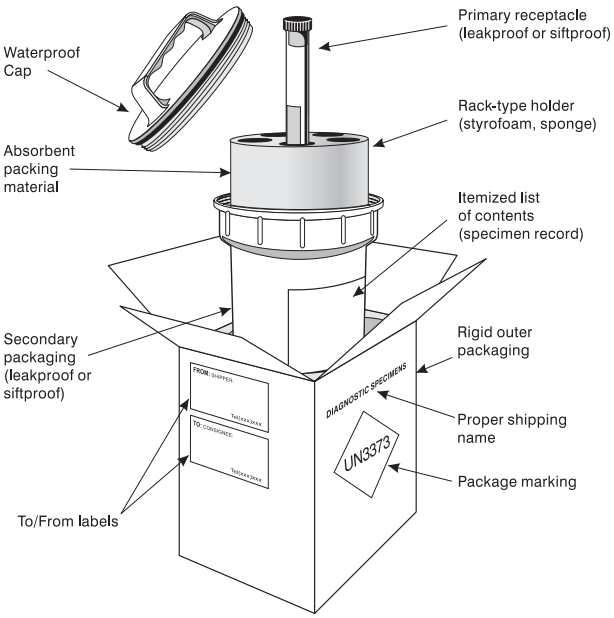


Figure 3C. Example of Triple Packaging System



International transport of diagnostic specimens

Packaging of clinical specimens and virus isolates for transportation

The IATA Dangerous Goods Regulations governing the transportation of biological specimens were updated and become applicable January 2007. The following is a summary of the changes and the current regulations. More details can be found in the Guidance on regulations for The Transport of Infectious Substances, 2007-2008. http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_EPR_2007_2/en/index.html

Classification of specimens

There are 3 categories of specimens under the new regulations

1) Infectious Substance, Category A

Substances that contain **highly pathogenic agents**, also known as "Category A" infectious substances. Examples of Category A pathogens are listed in Annex 2 of Guidance on regulations for The Transport of Infectious Substances, 2007-2008.

Dangerous goods are assigned UN numbers and proper shipping names. For category A substances these are: **UN 2814, INFECTIOUS SUBSTANCE, AFFECTING HUMANS, or UN 2900, INFECTIOUS SUBSTANCE, AFFECTING ANIMALS. Live JEV cultures are included in this category.**

The following conditions are required to ship Category A Infectious Substances

- Shippers' Declaration for Dangerous Goods
- Full training and certification
- UN Specification Packaging must follow **Packing Instruction 620, (PI 602 for IATA)**

2) Biological Substance, Category B

Materials containing or suspected to contain infectious substances that **do not** meet Category A criteria..

UN number and proper shipping name for category B substances are: **UN 3373, BIOLOGICAL SUBSTANCE; CATEGORY B.** The conditions required to transport category B substances include:

- The triple component Packing Instruction 650 (see example below) to be followed
- Package labelled with "Biological Substance, Category B" in letters at least 6mm high
- Does not require Shipper's Declaration for Dangerous Goods
- Label UN3373 in diamond-shaped mark used

3) Exempt:

Human (or animal) specimens for which there is minimal likelihood that pathogens are present are not subject to dangerous goods requirements and regulations **if** they are transported in packaging which will prevent any leakage **and** are correctly labelled.

Patient diagnostic specimens (including serum and CSF) are in this category if there is minimal likelihood that pathogens are present and if they are packaged and labelled appropriately.

- The exterior packaging must be marked as "**Exempt human specimen**"
- The packaging must consist of three components (triple packing):
    - leak-proof primary receptacle
    - leak-proof secondary packaging
    - outer packaging of adequate strength for its capacity, mass and intended use
    - at least one side of the packaging must have minimum dimensions of 100 x 100 mm
    - For liquids: Absorbent material in sufficient quantity to absorb the entire contents (placed between the primary and secondary packaging to prevent leakage to the outer packaging, see figure 12)
    - When multiple "fragile" primary receptacles are used in a single package, they must be wrapped or separated so that contact between them is prevented (see figure 12)

NOTE: *Packing Instruction 650 (as for UN 3373/Category B shipments) can be used, except labelled with "***Exempt human specimen"** in place of **"UN3373".**

Exceptions

Because of the low hazard they present, the following substances are exempted from dangerous goods requirements and regulations.

- Substances that do not contain infectious substances or will not cause disease in humans or animals
- Substances containing microorganisms that are not pathogenic to humans or animals
- Substances in a form in which any pathogens present have been neutralized or inactivated such that they no longer pose a health risk
- Environmental samples that are not considered to pose a significant risk of infection
- Dried blood spots and faecal occult blood screening tests
- Decontaminated medical or clinical wastes

Dried blood samples do not need to meet the triple packaging requirement, but should be packaged in airtight containers to ensure no contact with personnel may occur during the shipping process.

Packaging

An example of the triple packaging meeting Packing Instructions 650 can be seen above (Figure 3c).

Marking

Each package shall display on the external surface of the outer packaging

- the shipper's (sender's, consignor's) name, address and telephone number
- the telephone number of a responsible person knowledgeable about the shipment
- the receiver's (consignee's) name, address and telephone number
- the proper shipping name ("BIOLOGICAL SUBSTANCE; CATEGORY B")
- temperature storage requirements (optional)
- marking UN 3373 as below

Refrigerants

Refrigerants may be used to stabilize infectious substances in Categories A and B during transit and should be used when shipping samples for attempted virus isolation or when virus cultures are being shipped for further investigation. Serum or CSF samples being sent for validation or confirmatory testing should be shipped at 4-8°C.

Ice or ice packs pre-frozen at -20°C are suitable for maintaining temperatures 4-8°C in a suitably well insulated shipping container for up to 3 days. For maintaining cold-chain conditions for longer than 3 days, dry ice may be considered, if supplies are readily available.

Ice or dry ice shall be placed **outside** the secondary receptacle. Wet ice shall be placed in a leak-proof container; the outer packaging or overpack shall also be leak-proof. **Dry ice must not be placed inside the primary or secondary receptacle because of the risk of explosions.** A specially designed insulated packaging may be used to contain dry ice. The packaging must permit the release of carbon dioxide gas if dry ice is used. ICAO/IATA Packing Instruction 904 shall be observed.

The secondary receptacle shall be secured within the outer package to maintain the original orientation of the inner packages after the refrigerant has melted or dissipated.
If dry ice is used to ship infectious substances in Category A, the details shall appear on the shipper's Declaration for Dangerous Goods (figure 5). In addition, the outermost packaging shall carry the hazard label for dry ice and the appropriate marking. If dry ice is used to ship infectious substances in Category B, the package shall be marked "Carbon dioxide, solid" or "Dry ice".

If liquid nitrogen is used as a refrigerant, special arrangements shall be made in advance with the carrier. Primary receptacles shall be capable of withstanding extremely low temperatures, and packaging and documentation requirements for liquid nitrogen shall be observed. In particular, the outermost packaging shall carry the hazard label for liquid nitrogen. For air transport, the handling label for cryogenic liquids shall also be affixed.

Transport Planning

Successful shipment of materials requires advanced planning, appropriate packaging, labelling, and documentation. Coordination and communication are required between all parties involved – the sender, carrier, and receiver. It is the responsibility of the sender to ensure the correct designation, packaging, labelling and documentation of all shipped

materials to ensure that the material is transported safely and arrives on time and in good condition.

Make advance arrangements with the receiver

Once it has been decided that materials need to be shipped from the laboratory, the receiver should be contacted and informed of the nature of the materials to be sent. The sender should enquire about any import permits required by the receiving laboratory's national government. If permits are needed, the receiving laboratory will need to obtain the CURRENT permit and send it (usually a faxed or emailed copy) to the shipping laboratory so that the permit can be given to the carrier. The sender should also enquire about carriers the receiver has good experience with. The sender and receiver should then make advance arrangements for a mutually convenient time for shipment to ensure that the appropriate staff are available to receive the shipment. It is recommended to plan to avoid weekend or public holiday arrivals.

Make advance arrangements with the carrier

Knowing that a shipment is necessary, the laboratory should contact a carrier familiar with handling infectious substances and diagnostic specimens and make arrangements to ensure that:

- The shipment will be accepted
- The shipment is undertaken by the most direct routing, avoiding weekend arrival
- Archives and documentation of the shipment progress will be kept
- The conditions of the shipment while in transit will be monitored
- The sender will be notified of any delays

The sender should request any necessary shipping documents that the carrier may require or any specific instructions necessary to ensure safe arrival of the shipment. The carrier may also provide advice on packaging.

Packaging for infectious material, including JE virus isolates

JEV isolates should be categorized as Category A infectious substances for shipment. Hand carriage of such Category A infectious substances on airlines is strictly prohibited by international carriers.

JEV isolates must be packaged in accordance with the "triple packaging system" outlined above and the primary receptacle should ideally be an outside thread, screw-cap container of suitable size, for example 1.8 ml cryovial. Furthermore the caps must be tightened and sealing tape, for example Parafilm, or other water-proof plastic tape, must be applied over the cap and top of the tube. Further packaging in secondary containers etc. should be as previously described. Either the primary or secondary receptacle must be capable of withstanding a pressure differential of not less than 95 kPa. Sealed secondary plastic containers should be surrounded by ice packs or additional plastic containers containing ice, and fitted into an insulated container (polystyrene) with a fibreboard outer packaging. Ice packs should be leak proof and wrapped in an outer package to prevent their contents from spilling out in the case of unintended melting. The maximum number of frozen icepacks which can be fitted around the secondary packaging container should be inserted. Additional materials can be added to prevent the secondary container from moving around during transport.

The insulated container and outer packaging must conform to *IATA Dangerous Goods Regulations Packaging Instruction P620* and UN6.2 specifications which require that standards are met for a 9-metre drop test, a puncture test and a pressure test.

The maximum volume of specimens that can be legally included in one package is 50 ml or 50g for passenger aircraft and 4 litres or 4 Kg for cargo aircraft. For road, rail and sea transport the maximum net quantity that can be contained in an outer shipping package is 400 Kg for solids and 450 litres for liquids

The outside of the package (figure 4) should be marked as follows:

- The sender's name, address and contact telephone / fax numbers
- The receiver's name, address and contact telephone / fax numbers
- Marking indicating "**UN2814 - INFECTIOUS SUBSTANCES AFFECTING HUMAN and UN2900 - INFECTIOUS SUBSTANCES AFFECTING ANIMALS"** for shipping of category A infectious substances.
- Marking indicating "**Packed in compliance with IATA packing instruction 620"**
- It may be of benefit to include al label requesting: **"Refrigerate package where possible"**.
- A label for "infectious substance".
- Orientation label on each side of the package to indicate the position of closures on the primary receptacle, accompanied by the words **"THIS SIDE UP"** or **"THIS END UP"**.
- If dry ice is used, this must be specified, along with the weight used and a UN1845 label for **"Miscellaneous dangerous substances"** must be added.

The box should be sealed using wide sealing tape, taking care not to obscure the labels with the tape. Specimens packaged in this way must be marked "Dangerous Goods", as is required for Category A "infectious substances affecting humans and animals". The airway bill must also bear similar words.

Preparing the documentation and sending the package

The documentation required to be completed for shipping materials is determined by the nature of the materials being sent. In general, each shipment should be accompanied with the following documents:

- Shipper's Declaration of Dangerous Goods (only those goods classified as infectious substances, see Figure 5).
- A packing list / proforma invoice / customs declaration / commercial invoice which includes the receivers address, the number of packages, detail of contents, weight, value (a minimal value should be indicated for customs purposes if the items are supplied free of charge).
- Airway bill if shipped by air
- Export / import permit if required

The airway bill should be marked with the following information:

- Name, address, telephone/telex/fax of receiver
- Number of specimens

- "Highly perishable"
- "Telephone receiver upon arrival" (repeat telephone number)
- Airway bill handling information: "URGENT: DO NOT DELAY: Biological specimens -- highly perishable -- store at 4^0C to 8^0C"

Notification of the receiver

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

- Number of specimens shipped
- Estimated number of cartons and weight
- Flight number and arrival date/time
- Airway bill number
- Need to inform the shipper if package is not received
- A copy of the airway bill has been mailed to the receiving laboratory

Notification of the sender

Once the package has been received, the receiver should immediately notify the sender of the receipt and condition of the shipment and any problems encountered.

Safety in the receiving laboratory

The receiver should ensure that materials and equipment used to cool and ship specimens are treated as potentially contaminated with infectious agents. Therefore personal protective equipment (PPE) should be used when unpacking specimens and shipping materials should be autoclaved before discard. Re-usable shipping containers must be disinfected through thorough washing in hypochlorite solution and rinsed with water before being re-used.

Figure 5 Example of Shipper's declaration for dangerous goods

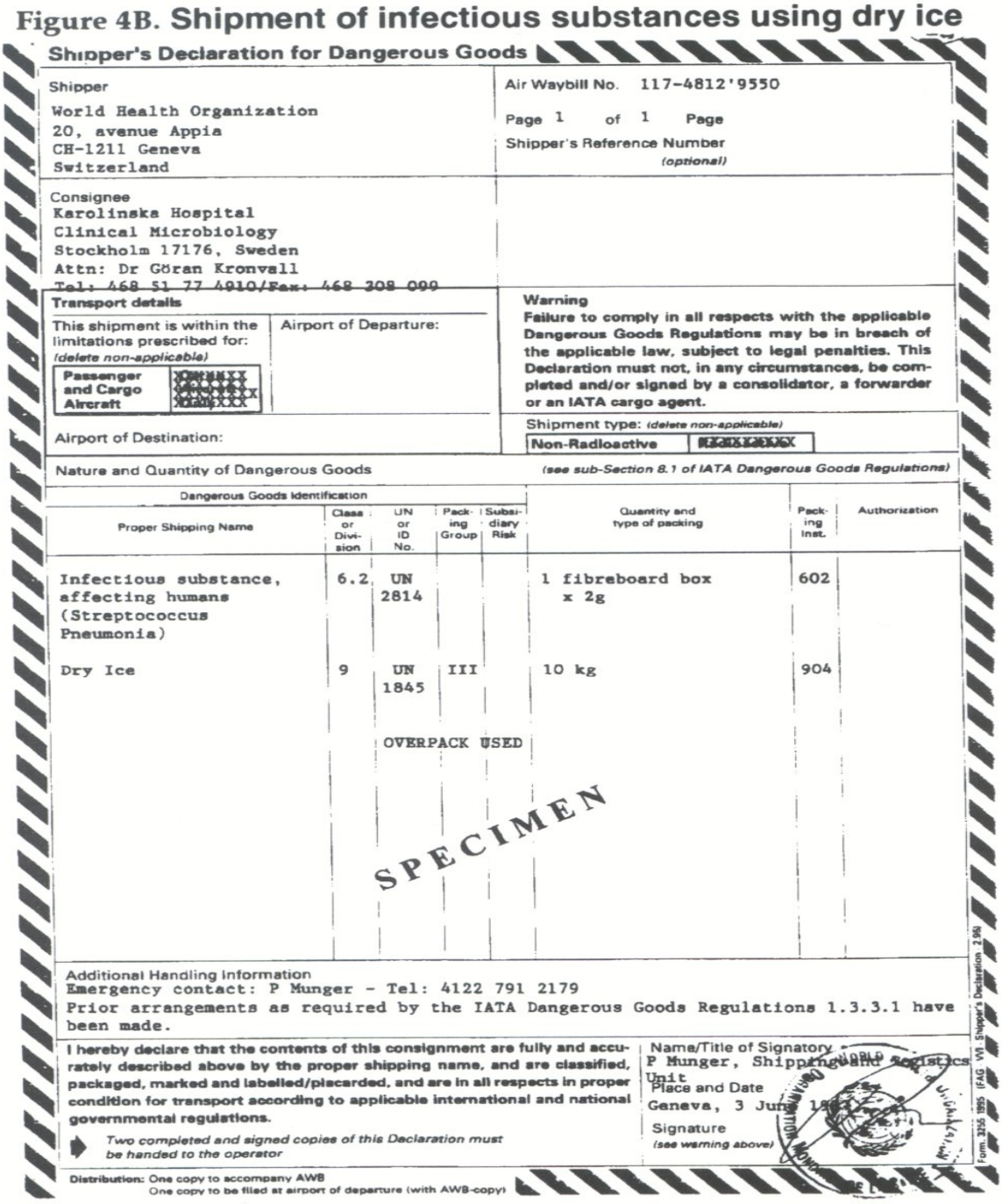


Figure 4B. Shipment of infectious substances using dry ice

Shipper's Declaration for Dangerous Goods

Shipper
World Health Organization
20, avenue Appia
CH-1211 Geneva
Switzerland

Air Waybill No. 117-4812'9550

Page 1 of 1 Page

Shipper's Reference Number (optional)

Consignee
Karolinska Hospital
Clinical Microbiology
Stockholm 17176, Sweden
Attn: Dr Göran Kronvall
Tel: 468 51 77 4910/Fax: 468 308 099

Transport details

This shipment is within the limitations prescribed for: (delete non-applicable)

Passenger and Cargo Aircraft

Airport of Departure:

Airport of Destination:

Warning
Failure to comply in all respects with the applicable Dangerous Goods Regulations may be in breach of the applicable law, subject to legal penalties. This Declaration must not, in any circumstances, be completed and/or signed by a consolidator, a forwarder or an IATA cargo agent.

Shipment type: (delete non-applicable)
Non-Radioactive

Nature and Quantity of Dangerous Goods (see sub-Section 8.1 of IATA Dangerous Goods Regulations)

Dangerous Goods Identification: Proper Shipping Name	Class or Division	UN or ID No.	Packing Group	Subsidiary Risk	Quantity and type of packing	Packing Inst.	Authorization
Infectious substance, affecting humans (Streptococcus Pneumonia)	6.2	UN 2814			1 fibreboard box x 2g	602	
Dry Ice	9	UN 1845	III		10 kg	904	
		OVERPACK USED					

SPECIMEN

Additional Handling Information
Emergency contact: P Munger - Tel: 4122 791 2179
Prior arrangements as required by the IATA Dangerous Goods Regulations 1.3.3.1 have been made.

I hereby declare that the contents of this consignment are fully and accurately described above by the proper shipping name, and are classified, packaged, marked and labelled/placarded, and are in all respects in proper condition for transport according to applicable international and national governmental regulations.

Two completed and signed copies of this Declaration must be handed to the operator

Name/Title of Signatory
P Munger, Shipping and Logistics Unit

Place and Date
Geneva, 3 Jun 19

Signature (see warning above)

Distribution: One copy to accompany AWB
One copy to be filed at airport of departure (with AWB-copy)

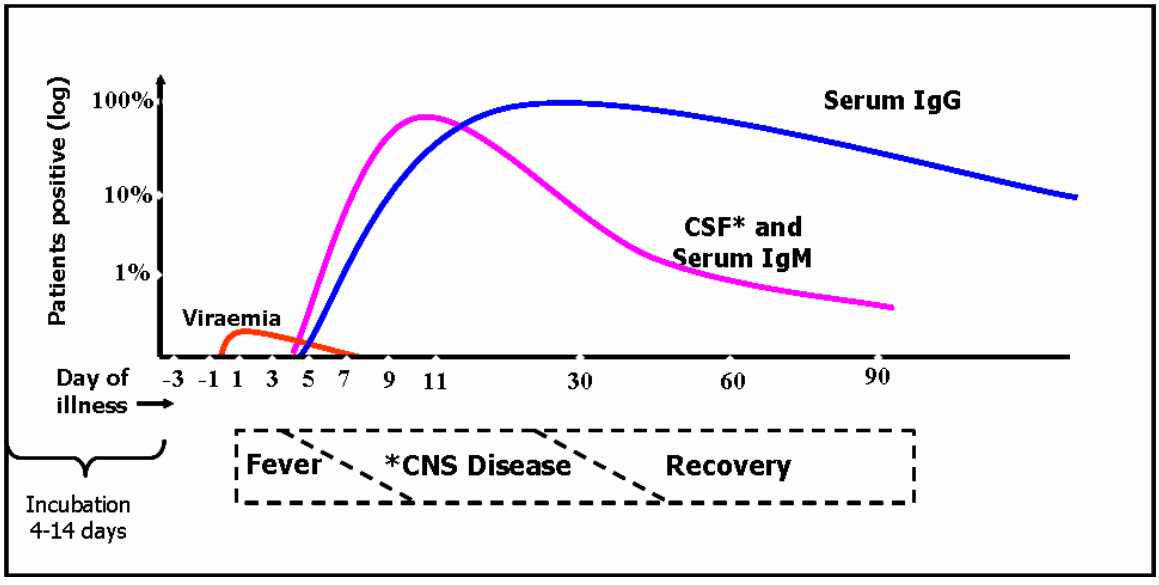
Form 3255 1995 (FAG VII Shipper's Declaration 2.96)

5. Laboratory diagnosis of JE Virus Infection

Attempts to isolate Japanese encephalitis virus from clinical specimens are usually unsuccessful, probably because of low viral titres and the rapid production of neutralizing antibodies. Isolates may sometimes be obtained from CSF or from brain tissue (either at necropsy or postmortem needle biopsy). Immunohistochemical staining of CSF cells or necropsy tissue with anti- Japanese encephalitis virus polyclonal antibodies may be positive.[26 27] However, for most practical purposes Japanese encephalitis is diagnosed serologically. The haemagglutination inhibition test was used for many years, but it had various practical limitations, and as it required paired serum, could not provide an early diagnosis.[28 71] In the 1980s IgM and IgG capture enzyme linked immunosorbent assays (ELISAs) were developed which have become the accepted standards for diagnosis of Japanese encephalitis.[29 30] After the first 9-10 days days of illness, the presence of anti-Japanese encephalitis virus IgM in the CSF has a sensitivity and specificity of >95% for CNS infection with the virus (before this, false negatives may occur)[31] (figure 6). The sensitivity for the detection of JE specific IgM in serum is approximately the same as for CSF.

Antibodies begin to appear soon after onset, but only about 70-75% of patients have IgM antibody in specimens collected up to 4 days after onset. However all patients will have antibody 7-10 days after onset. [31 32]

Figure 6 Schematic antibody responses in JE infection (after Solomon *et al* [33])



IgM assay

Although antigenic cross- reactivity in flaviviruses is common due to the large number of shared epitopes on the viral proteins, it was documented in 1982 that the presence of specific IgM antibodies could be used to diagnose Japanese encephalitis virus infections. The original methods have been modified for use to confirm other flavivirus infections, including Dengue which is often used for differential diagnosis in JE confirmatory testing.

The IgM diagnostic assay is based on the principle of IgM capture. Several JE research laboratories have developed their own in-house assays but these are generally not available to a wider market. Currently there are only a small number of commercial assays available. One commercial IgM assay currently uses separate JE and Dengue antigen wells to help differentiate between recent JE and Dengue infections.

The following procedure describes a generic outline for the detection of IgM antibodies to Japanese Encephalitis virus in human sera and CSF using an antibody capture technique.

IgM Test principle

- IgM antibody in the patient's serum or CSF is bound to anti-human IgM antibody adsorbed onto a solid phase, usually in a microtitre plate. This step is non virus-specific and eliminates competition with IgG;
- The plate is then washed, removing other immunoglobulins and serum proteins;
- JE antigen is then added and allowed to bind to any JE-specific IgM present;
- After washing, bound JE antigen is detected using anti-JE monoclonal antibody, following which a detector system with chromogen substrate reveals the presence or absence of JE IgM in the test sample.

A generic protocol for performing this assay and a trouble shooting guide are outlined in **annexes 2 and 3**

The procedures for performing assays for the confirmation of suspected JE cases should carefully follow the assay manufacturer's guidelines for both the operation and interpretation of the assay. It is recommended that in-house known positive and negative control samples are regularly tested to ensure that the assay and the operator are performing as expected.

An example of an algorithm summarizing the testing and reporting of samples tested with the PanBio assay is available in Annex 3.

Other test procedures

Plaque Reduction Neutralization Test

It is possible to confirm JE ELISA results using the sensitive plaque reduction neutralization assay (PRNT) method to differentiate JE antibody from other flaviviruses. The PRNT is a quantitative biological assay measuring neutralizing antibodies with the end-point determined by the neutralization of JE or other flavivirus plaques in cell monolayers, by the serum under test. This assay is considered more sensitive than ELISA for differentiating between different flaviviruses. However PRNT is time-consuming to perform, has a long incubation period and is labour intensive. It is recommended for use only in reference laboratories with experience in this assay and for samples which cannot be easily differentiated by ELISA methods.

RT-PCR

PCR assays are not recommended for routine diagnosis. Detection of virus genome is very specific for JE diagnosis; however, it is not sensitive. Virus is usually undetectable in a clinically ill JE case. Virus genome in CSF is usually only found in fatal cases. However PCR assays combined with sequencing can be useful for providing information about the molecular epidemiology and evolution of viruses. PCR testing is a function of the reference and specialized laboratories of the network. A laboratory should consult the JE Laboratory coordinator if they receive samples for RT-PCR or virus isolation (below) for details on how to proceed.

Virus Isolation

All arboviruses, including JE virus, are high-risk pathogens and laboratory procedures that amplify or concentrate the agent are potentially high-risk activities. All attempts at virus isolation must take account of these risks and appropriate laboratory biosafety practices observed. As a minimum, laboratory biosafety level 3 (BSL-3) requirements should be in place (WHO Biosafety Manual, Third edition, World Health Organization, 2004), and staff should be vaccinated against JE.

Isolation of JE virus from routine clinical samples is very challenging but may occasionally be successful from CSF or from brain tissue samples of fatal cases. After isolation, virus can be confirmed and identified using: appropriate polyclonal or monoclonal antibodies, by indirect immunofluorescence, by RT-PCR using JEV specific primers, or by nucleotide sequencing. As for PCR testing, virus isolation is a function of the reference and specialized laboratories of the JE network.

6. Data management

Introduction to data management

An essential part of the work of every laboratory is to record the details of all specimens tested, to record the results of testing, and to report the results. A good laboratory will also analyse the results it obtains, interpreting the results, looking for epidemiological patterns or trends, and summarizing results in the form of regular reports. The term "data management" covers all of these activities, and is an essential function of any disease surveillance system. Good laboratory data management is crucial to the JE control and prevention programme. Poor data management results in wasted time, effort and money, and makes it more difficult to reach the goal of disease control.

Good laboratory data management is essential to ensure accurate information is available for management and planning purposes. A laboratory request form should be completed at the time of specimen collection from each suspected JE case and should accompany each specimen sent to the laboratory. This form is additional to the case investigation form completed, or epidemiological data collected, from each suspected case. At the laboratory, there should be a system to record the receipt and condition of specimens that arrive, and to ensure test results are provided to the appropriate places.

Laboratory request form

The following fields should be included on the laboratory request form that accompanies the specimen (see annex 1):

Information to be completed by sender:
- Unique identifier (linked to clinical/epidemiological database with surveillance system);
- Patient name
- Age
- Province (or region)/district/town/village of residence
- Health facility name
- JE vaccination status
- Date of last JE vaccination
- Date of onset of first symptom of acute encephalitis
- Type of specimen (CSF, serum 1, serum 2)
- Date of specimen collection
- Date specimen sent to the laboratory

Information to be completed by laboratory on receipt of the specimen:
- Date specimen received in the laboratory
- Condition of specimen on receipt

Information to be completed by laboratory after completion of testing:
- Date specimen tested
- Result of testing
- Dates results sent to sender and to surveillance department

It may be convenient to establish a computer record system to store this information at the laboratory.

Receipt of specimens

A register should be kept at the laboratory listing clinical samples received. Information on the specimen label must be carefully checked to ensure that it matches information on the laboratory request form.

The condition of specimens when they arrive at the laboratory should also be checked. Improper handling, such as repeated freezing and thawing of a specimen, can destroy IgM antibody and should be recorded.

The following additional information should be recorded by the laboratory on receipt of a specimen:
- Specimen arrived with ice packs still cold or frozen (for feedback to programme) (y/n)
- Specimen arrived in adequate volume for full laboratory analysis (y/n)
- Specimen arrived with no evidence of leakage or desiccation (y/n)
- Specimen arrived with no sign of haemolysis (y/n)

Details of inadequate transport of specimens, and/or inadequate specimens (if the amount was not large enough for laboratory analysis), should be reported to the sender, to enable corrective action to be taken.

Reporting laboratory results

Laboratory results must be reported in a timely and accurate manner for several reasons. Reporting of laboratory results has a direct effect on the JE control programme through:

- Feedback to national surveillance/immunization authorities/teams for case follow up and planning immunization activities;
- Coordination of the JE control programme through WHO and other international agencies and bodies; and
- Monitoring of laboratory results and performance to identify possible problems and constraints.

Regular reporting of results will provide a continuous record demonstrating that recommended and acceptable procedures have been followed and laboratory accuracy has been at an acceptable level.

In addition to providing results to the person who submitted the specimen, the test results should be reported to the programme with responsibility for JE surveillance. It is important JE laboratory test results can be linked with the epidemiological data collected. Epidemiological data will reflect cases of acute encephalitis syndrome, but the proportion due to JE can only be determined from laboratory testing.

Details of how and when laboratories report should be arranged locally, in collaboration with the surveillance programme. The complete laboratory form may be copied and forwarded to the surveillance programme, or if a computer database has been established, a summary report can be prepared. No matter what system is used, reporting should be at least monthly, and the following information should be included:

- Unique identifier;
- Patient name
- Province (or region);
- District/town
- Hospital
- Specimen type
- Date of specimen collection
- Date specimen sent to lab
- Date specimen received in the laboratory
- Result of testing
- Date result provided to health care provider / surveillance unit

7. Laboratory safety

Each laboratory should have available the ***WHO Biosafety Manual (Third edition, World Health Organization, 2004)*** http://www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf which describes the essential biosafety, chemical, fire and electrical safety requirements to protect staff, the community and the environment. All staff should be aware of this manual and should proceed according to its contents. All new staff should be made aware of the risks involved in working in a JE laboratory before starting work in the laboratory and should be required to verify that they have read the Biosafety Manual. The director is responsible for implementation of and compliance with the provisions of the manual.

Laboratory manipulation of clinical JE specimens for virus detection and identification, and serologic testing are all associated with potential risks. Handling patient blood is potentially hazardous because of the risk of blood borne diseases. These risks must be recognized and appropriate safety measures taken to prevent laboratory infections. Ideally all activities involving handling of infectious and potentially infectious diagnostic materials should take place under BSL-2 conditions, and all activities involving the amplification of virus, either in vitro or in vivo, should take place under BSL-3 conditions (see WHO Biosafety Manual for detailed requirements).

The following rules should be observed as minimal safety precautions:

- People handling viruses must undergo appropriate training to minimize risk to laboratory staff and environs.
- Hepatitis B vaccine should be provided to all laboratory workers handling serum samples. Laboratory staff involved with handling potentially live JE virus should be vaccinated with an appropriate JE vaccine. Information regarding JE vaccine can be obtained from WHO.
- All laboratory procedures using infectious or potentially infectious materials must be carried out in a fully functioning Biological Safety Cabinet. Cabinets must be adequately maintained and periodically tested for correct operation.
- Mouth pipetting must not be permitted. All pipetting should be done using an automatic or manual safety pipetting device.
- Broken or chipped glassware should not be used. Contaminated glassware should be placed in an appropriate discard pan and the pan and contents sterilized. Never place your hands in discard pans. Never remove from the pan any material that has intentionally or unintentionally been discarded.
- Handle all cell cultures, both inoculated and uninoculated, with utmost precautions. Many infections have occurred among laboratory workers by improper handling of cell cultures.
- Every effort should be made to cover laboratory surfaces with adsorbent paper, which is to be discarded after each day's work. At the end of each workday, decontaminate workbenches with a suitable chemical. (Hypochlorite or VIRKON are suitable disinfectants)
- A clean laboratory coat should be worn when working in a laboratory where viruses or clinical materials are handled. The coat should fit properly and be buttoned or zipped closed. Torn coats should be repaired. Potentially contaminated coats should be sterilized by autoclaving, then washed and ironed. Laboratory coats are not to be worn outside the laboratory.
- Any laboratory accident should be reported to a supervisor **IMMEDIATELY**.
- Smoking, eating, or chewing any substance is absolutely forbidden.

8. Laboratory Quality Assurance

Laboratory Quality Assurance (LQA) is concerned with the organizational processes and the conditions under which laboratory activities are planned, performed, monitored, recorded and reported. Adherence by laboratories to the principles of LQA ensures the proper planning of activities and the provision of adequate means to carry them out. It promotes full and accurate reporting, and provides a means whereby the integrity of the activities can be verified.

Establishing LQA systems

Setting up a LQA system in a laboratory means defining the organizational structure, responsibilities, procedures, processes and resources necessary to achieve the following objectives:

- to prevent risks;
- to detect deviations;
- to correct errors;
- to improve efficiency;
- to ensure data quality and integrity.

It is the responsibility of the Director or Chief of the Laboratory to establish, implement and ensure compliance with LQA. However, LQA is the responsibility of all laboratory personnel. There are a number of elements that make up the LQA process, some of which are detailed below:

Standard operating procedures

Standard operating procedures (SOPs) describe in detail the activities performed in the laboratory so as to:

- provide uniformity, consistency and reliability in each of the activities performed in the laboratory;
- reduce systematic errors;
- provide training and guidance for new staff.

SOPs should be drawn up by specialized technical staff in the laboratory, revised by their immediate supervisor and approved by the Director of the laboratory. SOPs should be prepared for all general procedures and should closely follow the WHO recommended procedures. SOPs are especially important for the operation of assays should be developed closely following manufacturer's kit instructions.

Ideally SOPs should be drawn up under the following headings, as follows:

Title: Descriptive

Code: This code will identify:

- the laboratory;

- the number relating to each procedure;
- the number that identifies the revisions, with 00 being used for the original document.

Objective: The aim of the procedure being described should be expressed clearly and concisely.

Scope: the operating unit that will apply the procedure, and the field of application of the procedure.

Definitions: The meaning of the principal terms used in the procedure should be stated.

General description: Each SOP should be drawn up clearly, without ambiguity, so that it can be understood by staff with and without experience. Each step for performing the activity that is regulated by the procedure should be described in detail. Flow diagrams may be used to complement the description.

Safety conditions: These should reflect the safety measures and conditions to be kept in mind for the correct execution of the SOP. Material Safety Data Sheets should be included for hazardous chemicals used.

Documentation: the form or protocol in which the data and measurements involved in the procedures should be recorded.

References and documents: the references used to draw up the SOP.

Staff

The JE laboratory should have the necessary staff with suitable qualifications and experience to carry out safely and accurately all the functions and responsibilities required of the JE laboratory. The laboratory should prepare an organogram of the JE laboratory that reflects the hierarchy and lines of authority, and include the functions and responsibilities of each person.

Staff should include:

- director or chief of the laboratory;
- head of each section or unit if appropriate e.g. serology, cell culture or molecular biology' laboratories etc.;
- scientific, technical and auxiliary staff;
- administrative support, maintenance, cleaning and service staff;

Each post should have a job description including: functions and responsibilities, academic training required and experience necessary.

Staffing levels

Staffing levels should be adequate to enable all the functions expected of the JE laboratory to be carried out without compromising safety or the integrity of the processes performed in the laboratory. There are specialized activities within the laboratory that require staff with considerable experience, such as ELISA testing in national level laboratories, and cell and virus culture, performing virus detection procedures, RT-PCR and sequencing techniques at reference or specialized laboratory levels.

There should be at least one person with at least 12 months relevant experience to carry out these activities. It is advisable for at least one other person to work together with the experienced person to gain understanding of the activity and build capacity within the laboratory and allow for backup in the event of staff absence.

Human resources

The fundamental objective of the human resources policy is to have reliable staff with scientific and/or technological training to apply appropriate laboratory procedures correctly, and remunerated according to the labour market. The laboratory must regularly arrange and coordinate training courses to extend and update the skills of both technical and scientific staff according to needs identified and as proposed by the heads of department. This training is offered as a means of contributing to the success of the LQA process. A continuing education programme must be developed which includes on-site as well as external training. Documentation should be kept describing the staff training programme.

The human resources programme should include the technical evaluation of staff and follow-up of the performance of each staff member based on the job description. This system allows the correction of errors or weaknesses, and can also be used as a tool for promotion, where merited.

Space allocation

The JE laboratory should have adequate space to safely perform all activities, store all necessary equipment and allow for easy cleaning and maintenance. There should be enough rooms to enable separation of infectious from non-infectious activities. Cell culture and media-making facilities should be separated as much as possible from all other activities and preferably be in a room(s) completely separated from the laboratory where viral or other microbiological activities are being carried out. There should be a clear delineation of different working areas to minimize the chances of contamination of clean areas. If possible, there should be a logical arrangement of activities in a laboratory or laboratories to minimize the distance infectious materials must be carried and to ensure that infectious materials are not being transported through clean areas. If space allows, specific areas and preferably specific rooms should be allocated for:

- reagents and consumables storage;
- instruments and equipment;
- washing, preparation and sterilization (clean and dirty);
- serology
- cell culture;
- specimen receipt and recording;
- specimen processing;
- inoculation, harvesting and typing;
- specialized activities;
- documentation, archiving and control;
- the administrative area;
- disposal of contaminated and medical wastes.

The following are the general characteristics with which the laboratory areas should comply:

- Lighting and ventilation should correspond to the needs of each working area, according to the specific requirements of the activity carried out. The surfaces of the workbenches should be smooth, easy to clean and made of material resistant to chemicals.
- Safety systems should cover fire, electrical emergencies, emergency shower and eyewash facilities.
- Hot and cold water, treated water, vacuum, gas, steam and electricity installations should be arranged so that they guarantee adequate use during the work and also facilitate maintenance and repair operations. Electrical installations should be arranged so that they do not pose any risk to workers, and electrical wires should not cross walkways. A standby generator is desirable for the support of essential equipment such as incubators, biological safety cabinets (BSCs), freezers etc, especially if power supply is erratic.
- Storage space must be adequate to hold supplies for immediate use and thus prevent clutter on bench tops and in aisles. Additional long-term storage space conveniently located outside the working areas should also be provided.
- Hand wash basins, with running water if possible, should be provided in each laboratory room, preferably near the door.
- An autoclave should be available in the same building as the laboratory.
- Facilities for storing outer garments and personal items, and for eating and drinking, should be provided outside the working areas.

Installation of equipment and organization of the laboratories should take biosafety and other safety standards into account.

Documentation

Documentation is defined as the set of quality manuals, standard operating procedures, instructions, forms, reports, analytical protocols and record of data that serve as evidence of the LQA and permit the traceability of data. Responsibility for the preparation and revision of documents should rest with the LQA or Quality Assurance department, or with the person appointed, depending on the complexity of the laboratory

Equipment and instruments

The laboratory should have the necessary equipment and instruments for the accurate performance of all tests performed. New instruments and equipment should be installed and calibrated if possible by the distributor or a suitably qualified person. All manuals and operating instructions should be stored in an area accessible to all users and a regular maintenance and calibration schedule established. All users should be completely familiar with the operating, maintenance and validation procedures to ensure correct functioning. Documentation of all malfunctions, maintenance and validation activities should be recorded in a central register.

The laboratory should have a list of equipment and instruments that include:

- the name;
- brand;

- donor or supplier;
- maintenance company;
- maintenance schedule;
- inventory number;
- serial number;
- model and year;
- location;
- date of purchase;
- date of first use;
- copy of manufacturer's handbook.

Supplies

Reference materials

These comprise material used to calibrate the test procedures and to guarantee uniformity in determining activity such as: reference positive and negative control sera.. A central registry or logbook should be kept containing the following:

- name of the reference material;
- supplier;
- origin;
- lot number;
- date of analysis to determine whether it complies with the stipulated requirements;
- place and conditions of storage;
- expiry date, where applicable;
- storage in an appropriate form (corresponding SOP).

This registry should contain all the information relating to the properties of the reference material. The quality of the reference material should be verified when the conditions have been altered and routinely once a year.

Reagents (including diagnostic kits)

Reagents can be defined as materials of chemical or biological origin used in laboratory assays. At least a six months' reserve stock of reagents should be held in the laboratory at all times. Given the long delivery times and difficulty of transport to some regions, reagents should be ordered six to 12 months ahead of need.

A central registry or logbook should be kept containing the following:

- name of the reference material;
- supplier;
- origin;
- lot number;

- date of analysis to determine whether it complies with the stipulated requirements;
- place and conditions of storage;
- expiry date, where applicable;
- storage in an appropriate form (corresponding SOP).

This registry should contain all the information relating to the properties of the reference material. The quality of the reference material should be verified when the conditions have been altered and routinely once a year.

Characteristics of reagents:

- they should be of appropriate quality.
- they should be obtained from recommended suppliers in their original packaging.
- a record should be kept of purchasing, reception and distribution to guarantee continuity, particularly with substances that need to be acquired in advance.
- they should be inspected to ensure that the seals are intact when received in 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.
- there should be a specific SOP for the transport, storage and handling of reagents and disposal.

Any changes to the composition of reagents or media or to the lot numbers of biological products (antisera, conjugates etc) should be fully documented in the central registry or logbook. Water should be considered a reagent and should comply with purity specifications or other technical requirements for use in the laboratory.

Reagents prepared in the laboratory should be prepared in conformity with written procedures and, where applicable, according to WHO standard recommendations, validated and labelled appropriately, stating the following:

- identification of the reagent;
- concentration;
- preparation and expiry date;
- storage conditions;
- initials of the technician responsible.

Annual accreditation

Accreditation provides documentation that the laboratory has the capability and the capacity to detect, identify, and promptly report JE positive samples using standardized and validated procedures. The accreditation process further provides a learning opportunity, a mechanism for identifying resource and training needs, a measure of progress, and a link to the JE WHO Laboratory Network. For full details see section 3 above.

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Annex 1. Example of a Japanese Encephalitis Virus Laboratory Request and Result form

	Patient number:	Date / /	
Patient name:		M	F
Date of birth / /	Age:		
Name of parent or guardian:			
Province:	District:		
Town/Village:	Health facility:		
Number of doses of Japanese Encephalitis Vaccine:	Date of last dose / /		
Date of onset of first symptom of disease: / /			
Provisional clinical diagnosis:			

Specimen type and ID No.	Date of collection	Date of shipment
(1)	/ /	/ /
(2)	/ /	/ /
(3)	/ /	/ /

Name of person to whom laboratory results should be sent:

Address:

Telephone number: Fax number: Email:

For use by the receiving laboratory:

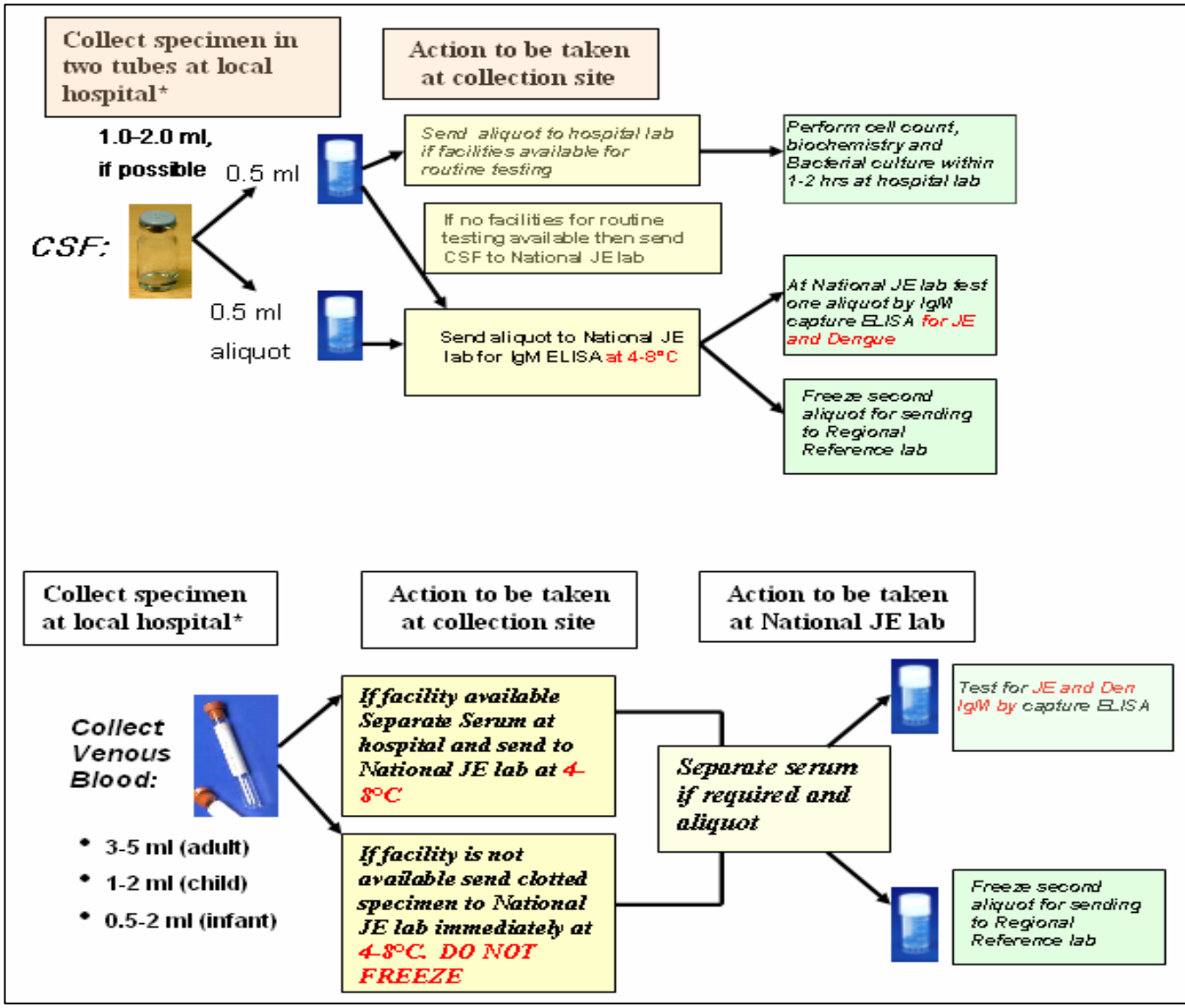
Name of laboratory:

Name of person receiving the specimen:

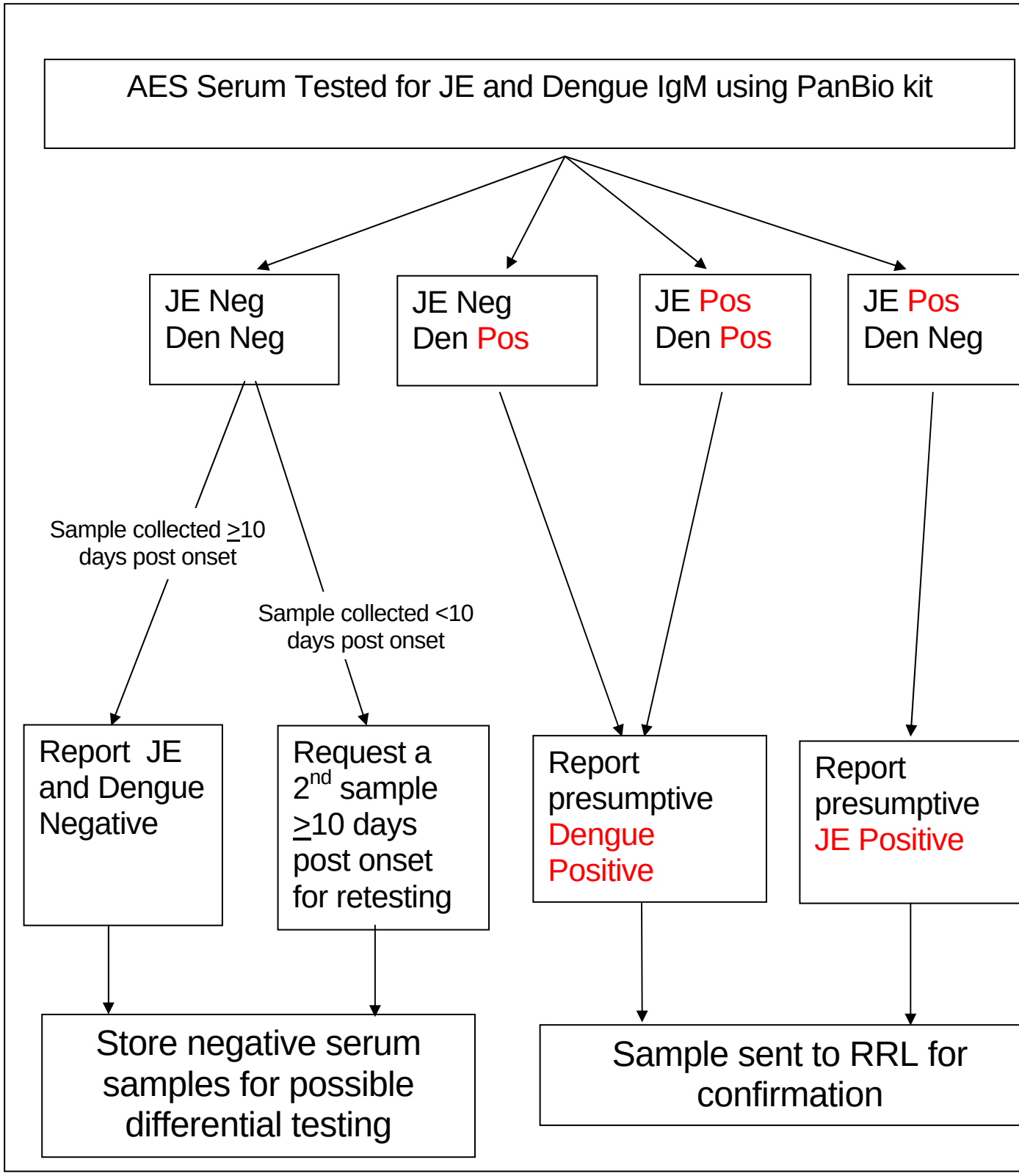
Specimen type	Date Received in Lab	Condition of specimen received*	Type of test	Date of test	Test result	Date result reported	Date sample sent to Reference Lab

* Sample is considered adequate if: no leakage, sufficient quantity, appropriate cold chain, complete documentation

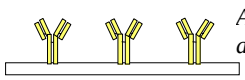
Annex 2. Sample algorithm for specimen collection, shipment and testing based on the PanBio Combination JE/DEN IgM assay



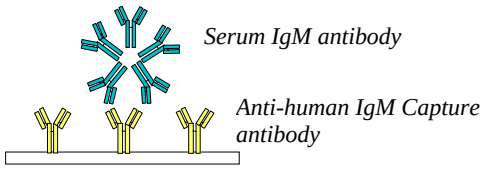
Annex 3. Algorithm for testing serum samples using PanBio Combination JE/DEN IgM assay



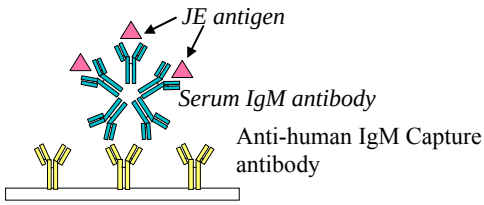
Annex 4. Generic schematic for JE IgM capture assay



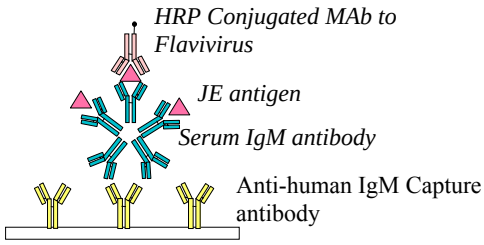
Coated plate with **anti human IgM**



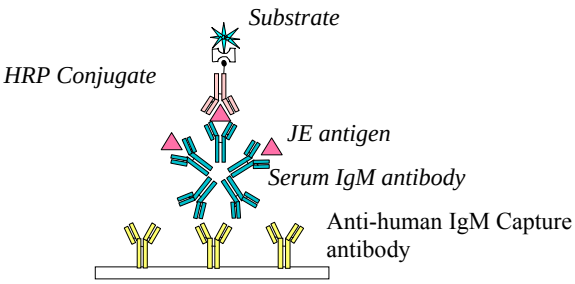
Add **serum** or **CSF** samples and **pos.** and neg. controls
Incubate
Wash



Add **JE virus antigen** and **control antigen** to appropriate wells
Incubate
Wash



Add **HRP-conjugated** anti-Flavivirus monoclonal antibody
Incubate
Wash



Add **substrate**
Incubate
Stop reaction
Read at appropriate wavelength

Annex 5. Trouble shooting guide for IgM capture assay

Quality Control

Each assay has a specific set of quality control criteria which must be met before the assay can be considered valid. It is essential that the quality control guidelines from the instructions provided by the manufacturer of the assay are followed for each assay run. It is also helpful to have a set of in-house positive and negative control sera which should be run in assays at regular intervals. These in-house samples can be helpful for comparing variation between batches of assays, helping trace assay problems and with trouble shooting. To ensure that in-house control sera remain stable, they should be aliquoted in single use volumes and stored at -20°C or lower. Ideally, in-house sera should be selected which are of sufficient volume to allow aliquots to be run at least every 2 to 4 weeks over a 12 month period. Presenting the absorbance values of kit and in-house controls in a graphic form can allow easy monitoring of the repeatability of ELISA assays being run in each laboratory and may be used to even monitor technical performance.

If an assay is invalid it is necessary to check all reagents and procedural steps in order to eliminate the problem. If the problem cannot be identified with any certainty then it is essential that the assay should be investigated step by step, examining or resolving each variable one at a time. The in-house control samples can be invaluable for this purpose. Below are some general guidelines for avoiding and resolving problems with ELISA assays.

Reagent problems

- Ensure that all reagents and samples have been stored correctly, that they have not been contaminated, and that they have not passed their expiry date.
- Always label all reagents and include the date of preparation/ reconstitution.
- During the assay keep the timing of reagent addition consistent, and process the plates in a consistent order during all steps of the procedure
- Regularly use an internal quality control sample with known absorbance value
- Avoid repeated freeze thawing cycles of sera, especially those used for internal quality controls. If internal control samples need to be frozen, make multiple aliquots of single use volumes.

Operator, mechanical or procedural errors

- Ensure the assay protocols are followed exactly, especially incubation temperatures and incubation times. Be aware that assay protocols may change. The manufacturer's instructions should be re-read with every new batch of assays and SOPs adjusted as appropriate.
- Monitor and record temperature of refrigerators, freezers and incubators daily.

- Ensure the correct washing procedures are followed. Under-washing can cause high background and over-washing can result in low absorbance. Follow exactly the recommended number of wash cycles and wash buffer dwell-time (time the wash buffer is left in the wells for each wash cycle).
- Check all channels on the ELISA washer are working correctly and not blocked. Use distilled water to rinse washer after every use to avoid salt crystallisation in the delivery head. High background absorbances in every well of a row or column may be caused by a blocked channel.
- Check that the correct filter in the ELISA reader is used for the appropriate substrate (for example; 405nm for ABTS, 450nm for TMB, 492nm for OPD). Read plate with another ELISA reader or try other filters if visual colour changes do not match the ELISA reader absorbance readings. The use of a dual wavelength ELISA reader reduces the chance of absorbance readings being influenced by non-substrate components. For a TMB substrate (450nm optimal filter wavelength) an appropriate reference filter wavelength would be 630nm.
- If no colour changes occur in the assay the problem may be in the conjugate, the substrate or both. One quick test to identify this problem can be to mix approximately 10 µl of the correctly diluted conjugate with 100 µl of the diluted substrate and observe whether colour develops. It is recommended to use low protein binding plastic containers for the dilution of both conjugate and substrate as some glass containers can be inhibitory to the enzymatic reaction as can residual chemicals from cleaning fluids.
- Confirm the pH of the dilution or wash buffer is optimal. Always use freshly prepared distilled water for preparation of reagents and wash buffers and check that the pH conforms to the protocol.

- Allowing a plate to dry out during the washing process can result in non-specific binding with a resultant high background problem.

- Ensure that the reagent dilutions have been correctly calculated by recalculating all of them and getting someone else to check them. Do not substitute reagents.

- Record assay performance data in a laboratory logbook, including:
    - kit batch number(s) and expiry date(s)
    - date and time of performing the assay
    - name of person performing the assay
    - name of person checking the results (usually the laboratory supervisor)
    - assay data calculations; dilutions; time of the start and end of each incubation
    - any other observations, errors, inadvertent changes
    - positive and negative control and QA data
    - raw absorbance data

Micropipette inaccuracies

- Regularly (at least every 3 months) clean and check the accuracy of the pipettes that are being used for the assay. Most inaccuracies related to micropipetting can be minimized by correctly using the micropipette (see micropipette instruction insert) and ensuring the barrel of the pipettor is kept clean, especially the area where the tip is fitted and preventing any sample entering the pipette barrel. The outside of the barrel should be cleaned after each use with a soft cloth wetted with 70% ethanol. The barrel and piston can be cleaned the same way every month, after being dismantled by an experienced technician.
- The volume delivery of a micropipette can be assessed by determining the weight of set volumes of distilled water with an analytical balance.
- Distilled water has a density of 1 µl/mg at 4°C and a barometric pressure of 1 atmosphere (or 1013.25 hectopascals (hPa)). The density of water at other temperatures and barometric pressures can be found using table 2.
- An accuracy of >98% in each micropipette's delivery should be attainable, but check with the manufacturers' instruction insert. Before proceeding it is essential that the balance has been calibrated and is accurate and preferably capable of measuring to 3 or 4 decimal places (i.e. 0.001g or 0.0001 g). Any micropipette with an inaccuracy greater than the acceptable range should not be used and either replaced or sent to a company with the specialization to repair and recalibrate it. Micropipettes should be decontaminated (check micropipette instruction insert) before sending to the repairer.
- Some laboratories may have the resources to have their micropipettes calibrated by a specialist company on a regular basis and this should occur regularly (e.g. every 3-6 months). Laboratories wishing to perform the calibration of micropipettes themselves may find the Gilson SOP for micropipettes helpful.[34] The regular cleaning and checking procedures outlined above should still be implemented, however.
- Use appropriate volume pipette for all measurements:
    - for 1–10 µl, use a Gilson P10 or equivalent;
    - for 5–20 µl, use a Gilson P20 or equivalent;
    - for 20–200 µl, use a Gilson P200 or equivalent;
    - for 200–1000 µl, use a Gilson P1000 or equivalent.

Table 2 Density of water at various temperatures and barometric pressures

Temperature °C	Air Pressure hPa					
	800	**853**	**907**	**960**	**1013**	**1067**
15	1.0018	1.0018	1.0019	1.0019	1.002	1.002
15.5	1.0018	1.0019	1.0019	1.002	1.002	1.0021
16	1.0019	1.002	1.002	1.0021	1.0021	1.0022
16.5	1.002	1.002	1.0021	1.0022	1.0022	1.0023
17	1.0021	1.0021	1.0022	1.0022	1.0023	1.0023
17.5	1.0022	1.0022	1.0023	1.0023	1.0024	1.0024
18	1.0022	1.0023	1.0024	1.0024	1.0025	1.0025
18.5	1.0023	1.0024	1.0025	1.0025	1.0026	1.0026
19	1.0024	1.0025	1.0025	1.0026	1.0027	1.0027
19.5	1.0025	1.0026	1.0026	1.0027	1.0028	1.0028
20	1.0026	1.0027	1.0027	1.0028	1.0029	1.0029
20.5	1.0027	1.0028	1.0028	1.0029	1.003	1.003
21	1.0028	1.0029	1.003	1.003	1.0031	1.0031
21.5	1.003	1.003	1.0031	1.0031	1.0032	1.0032
22	1.0031	1.0031	1.0032	1.0032	1.0033	1.0033
22.5	1.0032	1.0032	1.0033	1.0033	1.0034	1.0035
23	1.0033	1.0033	1.0034	1.0035	1.0035	1.0036
23.5	1.0034	1.0035	1.0035	1.0036	1.0036	1.0037
24	1.0035	1.0036	1.0036	1.0037	1.0038	1.0038
24.5	1.0037	1.0037	1.0038	1.0038	1.0039	1.0039
25	1.0038	1.0038	1.0039	1.0039	1.004	1.0041
25.5	1.0039	1.004	1.004	1.0041	1.0041	1.0042
26	1.004	1.0041	1.0042	1.0042	1.0043	1.0043
26.5	1.0042	1.0042	1.0043	1.0043	1.0044	1.0045
27	1.0043	1.0044	1.0044	1.0045	1.0045	1.0046
27.5	1.0044	1.0045	1.0046	1.0046	1.0047	1.0047
28	1.0046	1.0046	1.0047	1.0048	1.0048	1.0049
28.5	1.0047	1.0048	1.0048	1.0049	1.005	1.005
29	1.0049	1.0049	1.005	1.005	1.0051	1.0052
29.5	1.005	1.0051	1.0051	1.0052	1.0052	1.0053
30	1.0052	1.0052	1.0053	1.0053	1.0054	1.0055

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