Class II Special Controls Guideline: Dengue Virus Nucleic Acid Amplification Test Reagents

Guideline for Industry and Food and Drug Administration Staff

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U.S. Department of Health and Human Services
Food and Drug Administration
Center for Devices and Radiological Health
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Division of Microbiology Devices

Preface

Public Comment

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1. Introduction

This special controls document was developed to support the classification into class II (special controls) of dengue (DEN) virus (DENV) nucleic acid amplification test (NAAT) reagents. The DENV NAAT reagents are devices using procedures such as real-time Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) methods that detect the DENV serotypes 1, 2, 3, or 4 from viral RNA in human serum and plasma from individuals who have signs and symptoms consistent with dengue (mild or severe). These devices consist of primers, probes, enzymes and controls for the amplification and detection of DENV serotypes 1, 2, 3, or 4 viral RNA. The device is intended for use in the diagnosis of patients in conjunction with other clinical and laboratory findings.

This guideline identifies measures that FDA believes will mitigate the risks to health associated with these devices and provide a reasonable assurance of safety and effectiveness. Following the effective date of a final rule reclassifying the device, firms submitting a 510(k) for a DENV NAAT reagents will need either to (1) comply with the particular mitigation measures set forth in the special controls guideline or (2) use alternative mitigation measures, but demonstrate to the Agency's satisfaction that those alternative measures identified by the firm will provide at least an equivalent assurance of safety and effectiveness.

2. Dengue Virus Background

DENVs are a significant cause of febrile illness in the tropical and subtropical regions of the world. DENVs, members of the *Flaviviridae* family, are single positive-stranded RNA viruses. Dengue fever and dengue hemorrhagic fever/dengue shock syndrome are caused by infection with one of four closely related but serologically distinct DEN viruses, designated DEN-1, DEN-2, DEN-3, and DEN-4 viruses. DENV is transmitted to humans by the bite of an infected *Aedes* mosquito. The incubation period after a

mosquito bite, ranges from three to eight days before symptoms occur. Primary or classical dengue fever is characterized by high fever and two or more of the following symptoms: severe headache, retro-orbital eye pain, myalgia, maculo-papular rash, arthralgia, lymphadenopathy, and leukopenia, which normally resolves itself in five days. A more severe form of DENV disease is called dengue hemorrhagic fever (DHF) which in some patients progresses to dengue shock syndrome (DSS). Although DHF may occur in primary DENV infections, it is usually found in individuals with secondary DENV infections caused by a different DENV serotype. Individuals with DHF display significant thrombocytopenia, which can cause severe hemorrhagic and shock manifestations (DHF/DSS).

Diagnosis of acute (on-going) or recent DEN infection can be established by testing serum samples during the first 5 days of symptoms and/or early convalescent phase (more than 5 days of symptoms). Anti-DENV IgM antibodies are produced and detectable at days 3-5 after onset of fever during the acute phase of the illness. The detection of DEN viral RNA genome in the blood serum or plasma collected from individuals with signs and symptoms determines DENV infection (mild or severe).

3. Premarket Notifications – Background

FDA concludes that special controls, when combined with the general controls of the Federal Food, Drug & Cosmetic Act (the FD&C Act), are necessary to provide reasonable assurance of the safety and effectiveness of DENV NAAT reagents A manufacturer who intends to market a device of this type must (1) conform to the general controls of the FD&C Act, including the premarket notification requirements described in 21 CFR 807 Subpart E, (2) address the specific issues of safety and effectiveness identified in this guideline, and (3) obtain a substantial equivalence determination from FDA prior to marketing the device.

This guideline identifies the classification regulation for DENV NAAT reagents. In addition, other sections of this guideline list the risks to health and describe mitigation measures that, if followed by manufacturers and combined with the general controls, will address the risks associated with these devices and will generally lead to a timely premarket notification (510(k)) review. This document, will supplement other FDA documents regarding the specific content requirements of a premarket notification submission for DENV NAAT reagents. For additional information regarding 510(k) submissions, refer to 21 CFR 807.87 and the Center for Devices and Radiological Health (CDRH) Device Advice: Comprehensive Regulatory Assistance.[1]

[1] http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/default.htm.

4. Scope

The scope of this document is limited to devices, identified and classified under 21 CFR 866.3946.

21 CFR 866.3946 – Dengue virus nucleic acid amplification test reagents

> (a) Identification. Dengue virus nucleic acid amplification test reagents are devices that consist of primers, probes, enzymes and controls for the amplification and detection of dengue virus serotypes 1, 2, 3, or 4 from viral RNA in human serum and plasma from individuals who have signs and symptoms consistent with dengue (mild or severe). The identification of dengue virus serotypes 1, 2, 3 or 4 in human serum and plasma aids in the clinical laboratory diagnosis of dengue virus infection in conjunction with other clinical and laboratory findings.
>
> (b) Classification. Class II (Special Controls). The special control is FDA's guideline entitled "Class II Special Controls Guideline: Dengue Virus Nucleic Acid Amplification Test Reagents." For availability of the guideline document, see §866.1(e).

5. Risks to Health

FDA has identified the risks of false negative test and false positive test results, both of which can lead to individual and/or public health consequences, as risks to health associated with this device that require special controls. These risks, and the location of recommendations for addressing them, are summarized in the table below.

Failure of dengue virus (DENV) nucleic acid amplification test (NAAT) reagents devices to perform as indicated or an error in interpretation of the results may lead to misdiagnosis with significant implications on patient management.

A false positive test result for an individual may lead to unnecessary treatment and possibly a less thorough laboratory evaluation for the true cause of illness; in the setting of an outbreak investigation, a false positive result may lead to unnecessary initiation of mosquito vector control measures.

A false negative result may lead to inappropriate use of antibiotics or not being treated with the appropriate intravenous fluids or platelet transfusion, or a false negative result may lead to delay in recognizing the cause of a potential outbreak and the initiation of adequate mosquito vector control measures.

The symptoms of dengue infection, i.e., fever, headache, arthralgia, retro-orbital pain, rash, lymphadenopathy and leukopenia, overlap with other causes of acute febrile illnesses. In the absence of clear symptoms or signs that separate DENV infection from

other etiologies of febrile illnesses, it is likely that the results of a DENV diagnostic test would strongly influence ascribing the cause of febrile illness to DENV infection.

In the table below, FDA has identified the risks generally associated with the use of DENV NAAT reagents that require special controls. The measures to mitigate these identified issues are in this guideline, as shown in the table below, in combination with proposed subsection 21 CFR 866.3946. Under this guideline, manufacturers who intend to market a device of this type must conduct a risk analysis prior to submitting a premarket notification to identify any other risks specific to their device. The premarket notification must describe the risk analysis method used. If you elect to use an alternative approach to mitigate a particular risk identified in this guideline, or if you or others identify additional potential risks from use of a device of this type, you must provide sufficient detail regarding the approaches used to mitigate these risks and a justification for your approach.

Table 1 – Identified Risks to Health and Mitigation Measures

Identified Risks to Health	Mitigation Measures
A false positive test result for an individual may lead to unnecessary treatment and possibly a less thorough laboratory evaluation for the true cause of illness; a false positive result may lead to unnecessary initiation of mosquito vector control measures.	Device Description Containing the Information Specified in the Special Control Guideline (Section 6) Performance Characteristics (Section 7) Labeling (Section 8) Postmarket Measures (Section 9)
A false negative test result may lead to inappropriate use of antibiotics or a delay in treatment to prevent death due to dengue hemorrhagic fever or dengue shock syndrome or a false negative result may lead to delay in initiation of mosquito vector control measures.	Device Description Containing the Information Specified in the Special Control Guideline (Section 6) Performance Characteristics (Section 7) Labeling (Section 8) Postmarket Measures (Section 9)
An error in the interpretation of the results	Labeling (Section 8)

6. Device Description Containing the Information Specified in the Special Control Guideline

In your 510(k) submission, you must include a device description that meets the requirements of 21 CFR 807.87(a) and (f) and you must identify the legally marketed predicate device as required by 21 CFR 807.92(a)(3). Further, you must also identify the applicable regulation and the product code(s) for your device; you must include a table

that outlines the similarities and differences between the predicate device (or another legally marketed device for the same intended use) and your device. You may reference appropriate peer-reviewed articles that support the use of your device for its intended diagnostic use and the specific test principles incorporated into the device design. You must describe each of these device elements in detail.

In addition, you must include the following descriptive information to adequately characterize your device for the detection of DENV RNA in human serum and plasma or other human clinical specimens.

a. Intended Use

The intended use must specify the nucleic acid target (e.g., DENV RNA region detected by the device), specimen type for which testing will be indicated (i.e., serum or plasma), the clinical indication(s) for which the test is to be used, and the specific population(s) for which the test is intended. The intended use must state that the test is qualitative and any specific conditions of use. The intended use must also specifically state as part of the clinical indication whether the test is to be used in the setting of diagnosing individual patients (i.e., symptomatic individuals who have returned from DEN endemic regions) and/or for diagnosis of individuals during outbreak investigations.

In your 510(k), you must clearly describe the following information related to the intended use of your product:

- The identity of the different DENV serotypes and strains that your device will be able detect or not detect.
- How the device test results will be used to aid in laboratory identification of dengue viral RNA in clinical specimens from symptomatic patients.

b. Reagents and Other Device Components

You must describe design requirements for your device that address or mitigate risks associated with primers, probes, instruments, and controls used in a nucleic-acid test procedure to detect targeted RNA regions from DENV. Some examples are given below:

- Designing your freeze-dried sets of reagents or any other closed tube test system (e.g., self contained cartridge) to minimize false positives due to contamination or carryovers.
- Designing one or more than one assay for targeting different RNA sequences unique to DENV.
- Developing positive controls, negative controls, and inhibition controls to ensure accurate test results.
- Developing methods for extraction and purification that yield suitable quality and quantity of DEN RNA from human serum and plasma from clinical specimens for use in the test system with your reagents.

- Optimizing your reagents and test procedures for recommended instruments.
- Including illustrations or photographs of any non-standard equipment or methods if applicable.

In your 510(k), you must provide performance information supporting the conclusion that your design requirements have been met. You must provide the rationale for selection of specific RNA target sequences and selection of primers and probes.

The specific extraction method you believe most appropriate for each specific specimen type must be listed by name and catalog number in the package insert of your device.

c. Ancillary Reagents

Ancillary reagents are reagents specified in device labeling as "required but not provided" in order to carry out the assay as indicated in its instructions for use and to achieve the test performance claimed in labeling for the assay. For the purposes of this document, ancillary reagents of concern are those that must be specified according to specific designation, in order for your device to achieve its labeled performance characteristics. For example, if your device labeling specifies the use of a specific reagent (e.g., 'Brand X extraction buffer' or other buffers shown to be equivalent), and use of any other extraction buffer may alter the performance characteristics of your device from that reported in your labeling, then Brand X extraction buffer or other buffers shown to be equivalent are ancillary reagents of concern for the purposes of this document.[2]

By contrast, if your device requires the use of 95% ethanol, and any brand of 95% ethanol will allow your device to achieve the performance characteristics provided in your labeling, then 95% ethanol is not an ancillary reagent of concern for the purposes of this document.

If the instructions for use of your device specify one or more ancillary reagents of concern, you should describe how you will ensure that the results of testing with your device and these ancillary reagents, in accordance with your instructions, will be consistent with the performance established in your premarket submission. Your plan may include application of quality systems approaches, product labeling, and other measures.

In order to address this aspect of the special control, your 510(k) submission must include the information described below. FDA will evaluate whether your plan will help to mitigate the risks presented by the device to offer reasonable assurance of the safety and effectiveness of the device and establish its substantial equivalence.

1. You must include in your 510(k) a risk assessment addressing the use of ancillary reagents, including risks associated with management of reagent quality and variability, risks associated with inconsistency between instructions

[2] Even if you establish that one or more alternative ancillary reagents may be used in your assay, each of those named alternatives may still be an ancillary reagent of concern. We recommend you consult with FDA if you are unsure whether this aspect of the special controls applies to your device.

for use provided directly with the ancillary reagent and those supplied by you with your assay, and any other issues that could present a risk of obtaining incorrect results with your assay.

2. Using your risk assessment as a basis for applicability, you must describe in your 510(k) how you intend to mitigate risks through implementation of any necessary controls over ancillary reagents. These may include, where applicable:
    - User labeling to assure appropriate use of ancillary reagents (see "Labeling" for further discussion).
    - Plans for assessing user compliance with labeling instructions regarding ancillary reagents.
    - Material specifications for ancillary reagents.
    - Identification of reagent lots that will allow appropriate performance of your device.
    - Stability testing.
    - Complaint handling.
    - Corrective and preventive actions.
    - Plans for alerting users in the event of an issue involving ancillary reagents that would impact the performance of the assay.
    - Any other issues that must be addressed in order to assure safe and effective use of your test in combination with identified ancillary reagents, in accordance with your device's instructions for use.

In addition, you must provide testing data to establish that the quality controls you supply or recommend are adequate to detect performance or stability problems with the ancillary reagents.

If you have questions regarding identification, use, or control of ancillary reagents, you may contact FDA for advice.

d. Testing Methodology

You must provide in your 510(k) submission a detailed description of the principles of operation of your device for detecting and differentiating viral nucleic acids (RNA) from four DENV serotypes 1, 2, 3 or 4 in serum and plasma. You must specifically describe testing conditions, procedures and controls designed to provide safeguards for conditions that can cause false positive and false negative results, or that may present a biosafety hazard. These include, but are not limited to:

- Description of or recommendations for, any external controls and/or internal controls that monitor for contamination and extraction efficiency (e.g., internal control that generates a positive signal in the assay and demonstrates successful recovery of RNA as well as the integrity of the RNA extraction reagent).
- Overall design of the testing procedure, including control elements incorporated into the recommended testing procedures.

- Features and additional controls that monitor procedural errors or factors (e.g., degradation of master mix) that may adversely affect assay performance and detection.

You must include a description for all additional procedures, methods, and practices incorporated into your directions for use (See Section 8 - Labeling) that mitigate risks associated with DEN testing.

e. Specimen Storage and Shipping Conditions

If you recommend specimen storage and/ or shipping conditions, you must demonstrate that your device generates equivalent results for the stored specimens at several time points throughout the duration of the recommended storage period and at both ends of your recommended temperature range. You may use the methods described in the Clinical and Laboratory Standards Institute (CLSI) document H18-A4, Procedures for the Handling and Processing of Blood Specimens for further guidance [Ref. 1].

f. Interpretation of Test Results

You must describe how positive, negative, equivocal, or invalid results are determined and how they should be interpreted, if applicable. There should be clear explanations for how interpretative algorithms have been determined.

You must provide the cut-off value for defining a negative result of the assay. If the assay has only two output results (negative/positive), this cut-off also defines a positive result of the assay.

If the assay has an equivocal zone, you must provide cut-off values (limits) for the equivocal zone. If one of the reported outputs of your assay can be an equivocal result, you must provide the interpretation and recommendation for how the user should follow up on the equivocal result. If your interpretation of the initial equivocal result requires retesting, you must provide (1) a recommendation whether retesting should be repeated from the same nucleic acid preparation, a new extraction, or a new patient specimen, and (2) an algorithm for defining a final result by combining the initial equivocal result and the results after re-testing (note that this algorithm should be developed before the pivotal clinical study that evaluates the clinical performance of the assay).

If the assay can have an invalid result, you must describe how an invalid result is defined. If internal controls are part of the determination of invalid results, you must provide recommendations on the interpretation of each possible combination of control results for defining the invalid result. You must provide recommendations for how to follow up any invalid result, i.e., whether the result should be reported as invalid or whether retesting is recommended. If retesting is recommended, you must provide information similar to that for retesting of equivocal results (i.e., whether retesting should be repeated from the same nucleic acid preparation, a new extraction, or a new patient specimen).

In addition, you must describe how you monitor results over time to identify changes in performance due to nucleic acid variations in the DENV or when a new DENV strain becomes known or emerges, or any changes in performance due to prevalence deviation from the existing prevalence at the time your product was evaluated.

7. Performance Characteristics

a. General Study Recommendations

Your 510(k) submission must include detailed descriptive information regarding the studies that you conducted to establish each of the performance characteristics outlined below.

Prospective clinical studies must be included to determine the performance of your device in conditions similar to the proposed intended use. In general, for both clinical studies and reproducibility studies, you must conduct testing at three (3) sites, representative of where you intend to market the device (e.g., clinical laboratory sites).

You must provide appropriate specific information in your 510(k) submission describing the protocols used during your assay development in order for FDA to accurately interpret acceptance criteria and data summaries contained in your application during our review. This information is also important to aid users in understanding the information in your labeling. When referring to CLSI (Clinical and Laboratory Standards Institute) protocols or guidelines, you must indicate which specific aspects of the protocols or guidelines were followed.

We recommend that you contact FDA prior to initiating your clinical studies program to obtain feedback regarding your planned studies and the intended uses that are planned for inclusion in your 510(k) submission.

b. Analytical Studies

You must establish the following performance characteristics for your DENV NAAT reagents in your 510(k).

(1) Analytical Sensitivity

(a) Limit of Detection

You must determine the limit of detection (LoD) of your assay for all four DEN-1, -2, -3, and -4 serotypes using cultured and quantified (pfu/ml) stocks of whole virus. The study must include testing of serial dilutions of viable (live) DENV for all four serotypes and 3-5 replicates for each dilution made in DENV negative human serum or plasma or equivalent matrix. You must report the LoD as the level of virus that gives a 95% detection rate. The LoD must be confirmed by preparing at least 20 additional replicates at the LoD concentration and demonstrating that the virus was detected 95% of the time. The LoD of your assay must be correlated to pfu/ml and RNA copy numbers.

You must determine the LoD for each analyte in the most commonly used or most challenging matrix tested by the device. We suggest that you refer to CLSI document EP17-A [Ref. 2] when designing your studies.

(b) Analytical Reactivity

You must further confirm the LoD of your assay by testing additional strains for all four DEN-1, -2, -3, and -4 serotypes, i.e., a panel of well characterized DEN samples should be tested by your assay to establish reactivity with different circulating strains of DENV serotypes.

You may cite literature and/or other evidence for DEN strains excluded from your study; additional DEN strains may be appropriate for inclusion based on clinical and epidemiological trends at the time the device is being developed.

The results of strain reactivity testing, i.e., strains that are not detected by the device, must be listed in the device labeling.

(2) Analytical Specificity

(a) Cross-Reactivity:

You must test for potential cross-reactivity against the pathogens listed in Table 2 that can cause febrile illness. In particular, studies must be conducted to characterize performance in the presence of other flaviviruses (e.g., St. Louis encephalitis, West Nile, yellow fever, Japanese encephalitis), alphaviruses (e.g., eastern equine encephalitis), and other viruses and bacteria that cause fever and rash symptoms (e.g., enteroviruses, herpes simplex). Microorganisms must be tested at medically relevant viral and bacterial levels (usually 10^6 cfu/ml or higher for bacteria and 10^5 pfu/ml or higher for viruses). The identities and titers of viral and bacterial isolates used for cross-reactivity studies must be confirmed prior to testing.

Table 2. Microorganisms for Cross-Reactivity Studies

Test Organism
West Nile virus
Japanese encephalitis virus
Saint Louis encephalitis virus
Yellow fever virus
Hepatitis A virus
Hepatitis B virus
Hepatitis C virus
Epstein Barr virus
Borrelia burgdorferi
Leptospirosis
Cytomegalovirus
VZV
Herpes
Eastern equine encephalitis virus
Chikungunya virus
Influenza A and B virus
Measles virus

(b) Interference

You must conduct a comprehensive set of interference studies with your device. Potentially interfering substances include, but are not limited to, other constituents of the specimen of choice e.g., white blood cells, protein, whole blood, hemoglobin, and controls or reagents spiked into the specimen for control purposes. You must test interference at or near the assay LoD. Each interfering substance must be evaluated at its potentially highest concentration ("the worst case"). If no significant clinical effect is observed, no further testing is necessary. We recommend you refer to the CLSI document, "Interference Testing in Clinical Chemistry," EP7-A2 [Ref. 3] for additional information. Other potentially interfering substances include, but are not limited to, the following:

Table 3. Substances for Interference Studies

Substances
Bilirubin
Cholesterol
Lipids

Heparin
Genomic DNA
Sodium Citrate
EDTA
Albumin
Hemoglobin

(3) Carry-Over and Cross-contamination Studies (for Multi-sample Assays and Devices that Require Instrumentation)

You must demonstrate that carry-over and cross-contamination do not occur with your device. In a carry-over and cross-contamination study, high positive samples must be used in series alternating with high negative samples in patterns dependent on the operational function of the device. You must perform at least five (5) runs with alternating high positive and high negative samples. The high positive samples in the study must be high enough to exceed 95% or more of the results obtained from specimens of diseased patients in the intended use population. The high negative samples contain the analyte concentration below the cut-off such that repeat testing of this sample is negative approximately 95% of the time. The carry-over and cross-contamination effect can then be estimated by the percent of negative results for the high negative sample in the carry-over study compared with expected 95%.

(4) Precision/Reproducibility/Repeatability

Within-Laboratory Precision/Repeatability
You must conduct within-laboratory precision studies for devices that include instruments or automated components. You may perform these studies in-house, i.e., within your own company.

You must test sources of variability (such as operators, days, assay runs, etc) for a minimum of 12 days (not necessarily consecutive), with 2 runs per day, and 2 replicates of each sample per run. These test days must span at least two calibration cycles, if the calibration cycle is shorter than 2 months. The test panel must consist of 3-6 samples (1-2 viral strains) at four levels of viral load that include:

- A "negative" sample: a sample with an analyte concentration below the clinical cut off such that results of repeated tests of this sample are negative 100% of the time.
- A "high negative/low positive" sample (C_{20} to C_{80}) with a concentration of analyte just below the clinical cut-off so that retesting of this sample is negative approximately 20% to 80% of the time.
- A "low positive" sample with a concentration of analyte just above the clinical cut-off so that repeated test results is positive approximately 95% of the time.
- A "moderate positive" sample with a concentration that one can anticipate positive results approximately 100% of the time.

Reproducibility
The protocol for the reproducibility study may vary slightly depending on the test format. Generally you must the following protocol for DENV NAAT reagents:

- perform reproducibility studies at three sites (two external, one in-house site).
- a five day testing protocol, including, at a minimum, two runs per day (unless the assay design precludes multiple runs per day) and three replicates of each panel member per run.
- at least two operators each day at each facility performing the test. You must provide training only to the same extent that you intend to train users after marketing the test.
- The sample panel must be same as described in the repeatability study above.

You may refer to the CLSI documents, EP5-A2 [Ref. 4], EP12-A2 [Ref. 5], and EP15-A2 [Ref. 6] for guidance on precision and reproducibility study design.

c. Controls

When conducting the performance studies described below, you must run appropriate external controls every day of testing for the duration of the analytical and clinical studies. You may contact OIVD's Division of Microbiology Devices at FDA for further information regarding controls. Generally you must include the following types of controls:

(1) Negative Controls

Blank or no template control

The blank, or no-template control, contains buffer or sample transport media and all of the assay components except nucleic acid. This control is used to rule out contamination with target nucleic acid or increased background in the amplification reaction. For self-contained tests (i.e., a test where a single specimen is analyzed in a single-use consumable containing all pre-analytical and detection steps) negative controls should be run at some frequency (daily or weekly) to control for contamination.

Negative sample control

The negative sample control contains non-target nucleic acid or, if used to evaluate extraction procedures, it contains the whole organism (other than DENV). It reveals non-specific priming or detection and indicates that signals are not obtained in the absence of target sequences. Examples of acceptable negative sample control materials include:

- Patient specimen from a non- DENV infected individual
- Samples containing a non-target organism

- Surrogate negative control (e.g., packaged RNA)

(2) Positive Controls

Positive control for complete assay

The positive control contains target nucleic acids and is used to control the entire assay process including RNA extraction, amplification, and detection. It is designed to mimic a patient specimen and is run as a separate assay, concurrently with patient specimens, at a frequency determined by a laboratory's Quality System (QS). Examples of acceptable positive assay control materials include:

- Patient specimen from a dengue infected individual or spiked matrices with live DENV
- DENV tissue culture supernatants

Positive control for amplification/detection

The positive control for amplification/detection contains purified target nucleic acid at or near the limit of detection for a qualitative assay. It controls the integrity of the sample and the reaction components when negative results are obtained. It indicates that the target is detected if it is present in the sample.

(3) Internal Control

The internal control is a non-target nucleic acid sequence that is co-extracted and co-amplified with the target nucleic acid. It controls for integrity of the reagents (polymerase, primers, etc.), equipment function (thermal cycler), and the presence of amplification inhibitors in the samples. Examples of acceptable internal control materials include human nucleic acid co-extracted with the DENV RNA and primers amplifying human housekeeping genes (e.g., RNaseP). The internal control for a device is determined on a case-by-case basis [Ref. 7].

d. Nucleic Acid Extraction

Different extraction methods may yield DENV RNA of varying quantity and quality, and therefore the extraction method can be crucial to a successful result. Purification of DENV RNA from human blood serum or plasma can be challenging because biological samples may contain low viral loads in the background of human genomic DNA, as well as high levels of proteins and other contaminants.

For these reasons, you must evaluate the effect of your chosen extraction methods on the performance of the assay with respect to satisfactory DENV RNA quantity and quality for the intended use of the assay. In addition, you must evaluate your assay's analytical and clinical performance characteristics using the entire analytical process (including extraction procedures) that you recommend for use with your assay. This must include demonstrating the LoD and reproducibility of your assay with each extraction procedure.

In addition, external site studies (including reproducibility and clinical studies) must include the extraction procedures prescribed in your labeling.

You must perform these evaluations whether you intend to actually provide reagents in your test kit for extraction and preparation of nucleic acid or whether you instruct users to obtain appropriate reagents from commercial suppliers.

If you recommend or include multiple extraction methods, you must demonstrate the LoD and reproducibility for each method. With the assumption that the extraction method introduces minimum variability to the overall assay performance, you may be able to combine the extraction method variable with each site performance variable in the reproducibility study. For example if you recommend three different extraction methods, you can design a reproducibility study by evaluating one of the three extraction methods at each testing site: test extraction method A at site 1, method B at site 2, and method C at site 3. If the results generated from the test panel mentioned above do not show significant differences, no further reproducibility studies are needed. However, if the initial extraction equivalency studies from the three sites indicate statistically significant differences in assay performance, the reproducibility study must be expanded to include testing each extraction method at three study sites (e.g., site 1 extraction method A, B, and C; site 2 extraction methods A, B, and C; and site 3 extraction methods A, B, and C).

In addition to the analytical studies (LoD and Reproducibility), each extraction method must be utilized in at least one clinical site during the clinical trials to generate clinical performance data. If results from the expanded reproducibility testing indicate a significant difference in efficiency among the extraction methods, the data from each clinical testing site (using a different nucleic acid extraction method) are not considered equivalent and must not be pooled, but rather must be analyzed separately. As a result, additional prospective clinical samples may be called for in order to support the claimed extraction method.

e. Assay Cut-off

You must explain how the cut-off(s) was determined as well as how it was validated. The cut-off must be determined using appropriate statistical methods. To support the cut-off you determined, you may provide, for example, a result distribution, 95^{th} and 99^{th} percentiles, percents of the non-negative (positive or equivocal) results, and so on, for the clinical samples without any DENV RNA in your pilot studies. Selection of the appropriate cut-off can be justified by the relevant levels of sensitivity and specificity based on Receiver Operating Curve (ROC) analysis of the pilot studies with clinical samples (for details about ROC analysis, see CLSI document GP10-A [Ref. 8]). If the assay has an equivocal zone, you must explain how you determined the limits of the equivocal zone. The performance of your device using the pre-determined cut-off (and equivocal zone, if applicable) must be validated in an independent population consistent with the defined intended use of your device.

f. Specimen Collection and Handling

You must identify the specimen type(s) that your assay is intended to measure. A specimen has to be collected from the appropriate anatomical site or source at a time in the course of disease when DENV is likely to be isolated from the specimen.

The quality and quantity of the target analyte can be highly dependent on factors such as specimen source, collection method, handling (e.g., transport and storage times and temperatures). Testing results you provide in your 510(k) must validate that the device maintains acceptable performance (e.g., accuracy, reproducibility) under all the conditions recommended in your labeling. For example, you must assess the effect of recommended storage times and temperatures (including freeze-thaw cycles) on sample stability using an analysis of specimen aliquots stored and/or transported under your recommended conditions of time and temperature. You must state your acceptance criteria for all specimen collection and handling conditions and stability parameters.

Follow all applicable state and federal biosafety guidelines for collecting and handling specimens for pathogen identification. For standard precautions in specimen handling, refer to the most current editions of the related Clinical and Laboratory Standards Institute (CLSI) documents [Ref. 9]

g. Clinical Studies

You must conduct clinical studies to determine the performance of your device for the specific intended uses of your assay. The approach to specimen collection may differ depending on whether the intended use is as an aid in the diagnosis of specific individuals or as an aid in the investigation of suspected dengue virus outbreaks. For the diagnosis of individual patients, specimens must be prospectively collected and tested from individuals from the intended use population, i.e., patients with signs and symptoms consistent with dengue fever or dengue hemorrhagic fever. Fresh samples are preferred for these studies although it may be possible to supplement fresh samples with prospectively collected archived specimens.[3] To use prospectively collected archived specimens to evaluate dengue virus assays it must be demonstrated that sample freezing or other preservation techniques do not affect analyte stability, that appropriate archives are selected, and that appropriate measures are taken to identify and remove or mitigate any biases in the study set. If you evaluate the assay using specimens that were archived, you must ensure that the specimens are not selectively utilized, i.e., that all specimens are tested. Samples must be masked during testing to avoid possible bias. If both fresh and archived/frozen samples are tested, you must analyze the data of these two groups separately.

[3] In this guideline, we define prospectively collected archived specimens as specimens collected sequentially from all patients meeting study inclusion criteria; specimens must not be selectively included based on known results, and all testing must be conducted with investigators completely blinded to any previous results or patient characteristics. Specimens must be as fresh as possible or appropriately stored. .

The protocol for each clinical study performed must be included in the 510(k) submission. Sponsors are strongly encouraged to discuss study protocols with FDA prior to initiation of clinical studies.

The following issues must also be addressed during the design of your clinical trials:

(1) Reference Assays

You must assess and compare the performance of your device to an appropriate reference method or a predetermined algorithm based on a composite reference method (i.e., where the result of more than one assay, e.g., dengue IgM enzyme-linked immunosorbent assay (ELISA) and/or hemagglutination inhibition (HAI), and RT-PCR, is likely to be most appropriate).

The reference method or the composite reference method must be well-characterized and validated. You must provide published literature or laboratory data in support of the validation for detection and differentiation of the dengue serotypes. Validation must include LoD and analytical reactivity data. The LoD of the RT-PCR must be similar to the analytical sensitivity of the submitted device.

If you compare your device to another molecular detection method (i. e., conventional RT-PCR assay followed by sequencing of the amplicons as a reference/comparator method for detection and differentiation of the dengue serotypes), the primer sequences for the reference/comparator PCR method must be different from the primer sequences included in your device. Sequencing must be performed on both strands of the amplicons (i.e., bi-directional sequencing), must demonstrate that the generated sequence is at least 200 base pairs of an acceptable quality (e.g., a quality score of 20 or higher as measured by PHRED or similar software packages), and must demonstrate that it matches the reference or consensus sequence [Ref. 10, 11].

FDA considers the nucleic acid extraction method (manual or automated), as well as reagents, assay conditions, and instrumentations, as important parts of the DENV NAAT reagents. Therefore the final formats of DENV NAAT reagents must include defined nucleic acid extraction methods, assay reagents, assay conditions, and instrumentation. Appropriate controls must be incorporated into each NAAT reference method to be used during the clinical studies.

You must contact the FDA for further information regarding the use of NAAT reference methods and/or establishing a predetermined algorithm that uses composite reference methods.

(2) Study Protocol

Clinical study protocols must be completed and reviewed by the investigators prior to the study's initiation. At a minimum, protocols must include complete patient

inclusion and exclusion criteria, the type and number of specimens needed, study procedures, and a detailed statistical analysis plan. Copies of the original study protocols, protocol modifications, and any other relevant study information must be included in your 510(k) submission.

We encourage sponsors to contact FDA to request a review of their proposed study protocols and the selection of specimen type(s) as part of the pre-Sub process. This is particularly recommended in a situation where different intended uses of the test may be studied or sponsors are planning to submit a 510(k) submission for the first time.

(3) Specimen Type(s)

Serum and plasma specimens are the sample matrix for this device. Specimens must be collected sequentially from all patients at each study site who meet the specific study inclusion criteria. The total number of specimens you must include in your study will depend on anticipated assay performance and the prevalence of DENV infection in the study population.

(4) Study Sites

For the intended use of individual patient diagnosis you must conduct your studies at a minimum of three different geographical sites representing testing environments where the device will ultimately be used (e.g., clinical laboratories) and by laboratory personnel likely to perform the test in clinical practice. At least one of the study sites must be in the United States to evaluate the specificity of the assay. It is recommended that sponsors discuss appropriate study sites for the intended use of outbreak investigation with FDA prior to initiating studies since these studies are more likely to need to use prospectively archived specimens.

(5) Study Population

You must conduct your studies on individuals presenting with signs and symptoms of DEN (e.g., high fever, severe headache, pain behind the eyes, joint pain, muscle and joint pain, rash, nausea and vomiting, nose or gum bleed, easy bruising, low white cell count). The majority of the samples must be collected as soon as possible after symptoms onset (to ensure a sufficient number of positive specimens) although later times of collection may be valuable for estimating device performance over time from symptom onset. Patients enrolled in clinical studies should meet the study inclusion and exclusion criteria for suspected DEN infection.

(6) Presentation of Clinical Study Results

Analysis must be based on the intended use, i.e., the unit of analysis must be by individual specimen or by testing an individual's acute and convalescent specimens,

Study analysis must account for all samples collected. Comparisons of device performance against the reference method must be included as 2 cell by 2 cell tables. Additional analyses must be included for device performance relative to patient characteristics, e.g., subject age, time of specimen collection relative to illness onset, study site, etc. In studies that combine fresh specimens and archived specimens, analyses must compare performance of each specimen type separately and then combined.

All study data must be included in the 510(k) submission as Microsoft Excel, delimited text, or as SAS transport files. Data files must include appropriate annotations or separate codebooks and must include all primary and derived variables, e.g., the result of the clinical reference algorithm for determining DEN diagnosis. Description of the statistical methods applied to the data set must be sufficiently detailed to allow interpretation of lower estimates of the positive agreement that may be acceptable and should be discussed with FDA prior to initiating clinical studies.

8. Labeling

DENV NAAT reagents, like other devices, are subject to statutory requirements for labeling (including sections 201(n) and 502(a) of the FD&C Act; 21 USC § 321(n) and 352(a)). These IVD devices must provide adequate directions for use and adequate warnings and precautions (Section 502(f) of the FD&C Act; 21 USC § 352(f). Specific labeling requirements for all IVD devices are set forth in 21 CFR parts 801 and 809.

Labeling for DENV NAAT reagents must also include the information described below. This labeling information helps to mitigate the risks identified previously in this guideline to ensure safe and effective use of these devices. Requirements in 21 CFR parts 801 and 809 must be addressed in device labeling even if not mentioned below.

a. Intended Use

The intended use must specify that the device is an aid in the diagnosis of DENV infection. You must also specify the DENV serotypes detected by your assay and any additional specific confirmatory measures that are needed to be taken to confirm your test result if your test is presumptive.

b. Device Description

In the device description, you must briefly describe the assay methodology used in this type of device.

c. Procedure

This section must include a general description of the entire analysis procedure, from the collections of patient samples to result reporting.

d. Directions for Use

You must provide clear and concise instructions that delineate the procedures for using the device, and the types of controls that will minimize risks of inaccurate results. Instructions must encourage use of additional control measures and testing of control materials to ensure use in a safe and effective manner.

e. Warnings, Precautions, and Limitations

In addition to any other limitations and warnings that are relevant to your specific assay, you must include statements, such as the following, under Limitations, as applicable:

- Testing should only be performed on patients with clinical symptoms consistent with Dengue fever or dengue hemorrhagic fever.
- Results from immunosuppressed patients must be interpreted with caution.
- The positive predictive value depends on the likelihood of the virus being present.
- Assay results should be interpreted only in the context of other laboratory findings and the total clinical status of the patient.
- Negative results do not preclude dengue virus infection and should not be used as the sole basis for treatment or other patient management decisions. A negative specimen collected between days 3-6 after onset of the febrile illness should be retested with an anti-DENV IgM test to increase the likelihood of making the diagnosis of dengue.
- A false negative result may occur if a specimen is improperly collected, transported or handled. False negative results may also occur if amplification inhibitors are present in the specimen or if inadequate numbers of organisms are present in the specimen.
- Detection of viral RNA may not indicate the presence of infectious virus or that dengue is the causative agent for clinical symptoms.
- This test cannot rule out diseases caused by other bacterial or viral pathogens.
- Assay performance characteristics have not been established for testing of cord blood for neonates, for prenatal screening, or for general population screening

without symptoms consistent with Dengue Fever. The test is not FDA cleared for the screening of blood or plasma donors.

- The performance of this test has not been established for monitoring treatment of dengue.

- This device is subject to a special control requiring that distribution be limited to laboratories with (i) experienced personnel who has training in standardized molecular testing procedures and expertise in viral diagnosis, and (ii) appropriate biosafety equipment and containment

f. Specimen Collection

You must provide guidance on what type of specimens should be used and the optimal time or window for collecting them (antigen or antibody). You must state if there are any inappropriate specimens that should not be used in this assay. You must also provided recommendations for specimen storage, number of freeze/thaw cycles and transport conditions that are optimal/acceptable for your device. For virus testing it must also be noted in labeling that samples should be collected as soon as possible after onset of symptoms.

g. Interpretation of Test Results

The interpretation of test results section in the package insert must list all possible assay outputs and determinations of the presence or absence of DENV RNA and the expected result of the assay controls. If internal controls are part of the determination of valid positive and negative results, you must provide the interpretation of each possible control result and a recommendation for how to follow up any invalid or indeterminate result.

If your assay has an equivocal zone, you must provide the interpretation and the recommendation for how to follow up with the equivocal result. (e.g., whether the equivocal result should be reported as such, or whether testing should be repeated). If your interpretation of the results requires repeat testing of an invalid or equivocal result, you must provide the recommendation whether testing should be repeated and how repeat testing should be performed (e.g., on the same or a different specimen from the same patient).

Final assay results must be reported as positive, negative, or equivocal (as appropriate). Depending on test performance or other device-specific factors, additional qualification may be necessary.

9. Postmarket Measures

As part of your good manufacturing practices performed as part of complying with the Quality Systems regulations under 21 CFR Part 820, you must anually obtain and

analyze postmarket data to ensure the continued reliability of your device for detecting different DENV strains and serotypes that may evolve over time. This is particularly true if new DENV strains emerge, or if DENVs that are less common at the time of your device clearance become more prevalent. Postmarket data must address the clinical performance of your device with new DENV strains.

To demonstrate how you will address this aspect of the special control, you must provide a plan with your 510(k) that describes how you intend to assure that the performance characteristics of your device remain unchanged over time. This plan is likely to include periodic testing of highly prevalent DENV strains at defined time intervals with your device. FDA will evaluate whether this plan will help to mitigate the risks presented by the device and therefore help to provide reasonable assurance of the safety and effectiveness of the device.

10. References

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2. Clinical Laboratory Standards Institute (CLSI). Protocols for Determination of Limits of Detection and limits of Quantitation; Approved guideline; CLSI document EP17-A (ISBN 1-56238-551-8) CLSI, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2004.

3. Clinical Laboratory Standards Institute (CLSI). Interference Testing in Clinical Chemistry; Approved Guideline; CLSI document EP07-A2 (ISBN 1-56238-584-4) CLSI, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2005.

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5. Clinical and Laboratory Standards Institute (CLSI). User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline. CLSI document EP12-A (ISBN 1-56238-468-6) CLSI, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2002.

6. Clinical Laboratory Standards Institute (CLSI). User Verification of Performance for Precision and Trueness; Approved Guideline; CLSI document EP15-A2 (ISBN 1-56238-574-7) CLSI, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2005.

7. Clinical and Laboratory Standards Institute (CLSI). Molecular Diagnostic Methods for Infectious Disease; Approved Guideline; CLSI document MM3-A2 [ISBN 1-56238-596-8] Clinical and Laboratory Standards Institute, Wayne, Pennsylvania 19087-1898 USA, 2006.

8. Clinical and Laboratory Standards Institute (CLSI). Assessment of the Clinical Accuracy of Laboratory Tests Using Receiver Operating Characteristics (ROC) Plots; Approved Guideline CLSI document GP10-A. CLSI, Wayne Pennsylvania 1997.

9. Clinical and Laboratory Standards Institute (CLSI). Protection of Laboratory Workers from Infectious Disease Transmitted by Blood, Body Fluids, and Tissue; CLSI document M29-A. CLSI, Wayne Pennsylvania 1997.

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11. Clinical and Laboratory Standards Institute. 2006. Molecular Diagnostic Methods for Infectious Disease; Approved Guideline. CLSI document MM3-A2 [ISBN 1-56238-596-8] CLSI, Wayne Pennsylvania 2006.