Guidance for Industry and FDA Staff

Class II Special Controls Guidance Document: Testing for Detection and Differentiation of Influenza A Virus Subtypes Using Multiplex Nucleic Acid Assays

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Food and Drug Administration
Center for Devices and Radiological Health
Office of In Vitro Diagnostic Device Evaluation and Safety
Division of Microbiology Devices

Preface

Public Comment

Written comments and suggestions may be submitted at any time for Agency consideration to the Division of Dockets Management (HFA-305), Food and Drug Administration, 5630 Fishers Lane, rm. 1061, Rockville, MD 20852. Alternatively, electronic comments may be submitted to http://www.regulations.gov. All comments should be identified with the docket number of the notice of availability that publishes in the *Federal Register*. Comments may not be acted upon by the Agency until the document is next revised or updated.

Additional Copies

Additional copies are available from the Internet at: http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/default.htm. You may also send an e-mail request to dsmica@fda.hhs.gov to receive an electronic copy of the guidance or send a FAX request to 301-847-8149 to receive a hard copy. Please use the document number 1672 to identify the guidance you are requesting.

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Class II Special Controls Guidance Document: Testing for Detection and Differentiation of Influenza A Virus Subtypes Using Multiplex Assays

1. Introduction

This document was developed as a special control to support the classification into class II (special controls) of respiratory viral panel multiplex[1] nucleic acid assays that include detection and differentiation of Influenza A virus subtypes. This guidance addresses only testing for detection and differentiation of influenza A virus nucleic acids using multiplex panels of respiratory viruses. A respiratory viral panel multiplex nucleic acid assay is a qualitative in vitro diagnostic device intended to simultaneously detect and identify multiple viral nucleic acids extracted from human respiratory specimens or viral culture. Multiplex nucleic acid assays testing for detection and differentiation of Influenza A virus subtypes are intended to detect influenza A RNA and specific Influenza A subtype RNA extracted from human respiratory specimens or viral culture, and differentiate between these subtypes. The detection and identification of a specific viral nucleic acid from individuals exhibiting signs and symptoms of respiratory infection aids in the diagnosis of respiratory viral infection when used in conjunction with other clinical and laboratory findings. In addition, differentiation of specific subtype RNA aids in the presumptive laboratory identification of Influenza A virus subtypes to provide diagnostic and epidemiological information on influenza. The device is intended for detection and identification of a combination of at least all of the following viruses:

(1) Influenza A
(2) Influenza A subtype H1

[1] In this guidance, multiplex assays are defined as those assays in which two or more targets are assayed through a common process of sample preparation, amplification and/or detection, and interpretation.

(3) Influenza A subtype H3

This guidance provides recommendations to manufacturers regarding preparation of premarket notifications for respiratory viral panel multiplex nucleic acid assays that include detection and differentiation of Influenza A virus subtypes. The recommendations in this document are applicable to assays that employ technologies such as polymerase chain reaction (PCR), reverse-transcriptase polymerase chain reaction (RT-PCR), and bead-based liquid arrays and microarrays.

This guidance addresses devices that are used in conjunction with clinical presentation and other laboratory tests (e.g., immunofluorescence, bacterial culture, chest x-rays/radiography) to aid in the diagnosis of Influenza A infection. This guidance does not address assays intended for use as the sole basis for diagnosis nor does it address assays meant to differentially diagnose viral from non-viral infections. For the assays addressed by this guidance, positive results do not rule out bacterial infection, or co-infection with other viruses.

This guidance is issued in conjunction with a *Federal Register* notice announcing the classification of a respiratory viral panel multiplex nucleic acid assay. Designation of this document as a special control means that any firm submitting a 510(k) for a respiratory viral panel multiplex nucleic acid assay that includes detection and differentiation of Influenza A subtypes, will need to address the issues covered in this special control guidance, as well as the issues covered in another special control guidance identified in the classification regulation, **"Class II Special Controls Guidance Document: Respiratory Viral Panel Multiplex Nucleic Acid Assay."** In addition to these guidance documents, for respiratory viral panels that include detection and identification of Human Metapneumovirus(hMPV), an additional special control guidance is **"Class II Special Controls Guidance Document: Testing for Human Metapneumovirus (hMPV) Using Nucleic Acid Assays."** See 21 CFR 866.3980(b). The firm must show that its device addresses the issues of safety and effectiveness identified in this guidance (and the other special control guidances referenced, as applicable) either by meeting the recommendations of this guidance or by some other means that provides equivalent assurances of safety and effectiveness.

Section 3 of this guidance document identifies the classification regulation and product code for Influenza A virus subtype differentiation using multiplex nucleic acid assays. Other sections of this guidance document identify risks related to this device type and provide recommendations to address these risks.

If you want to discuss an alternative means of satisfying the requirement of special controls for this device, you may contact the FDA staff responsible for implementing this guidance. If you cannot identify the appropriate FDA staff, call the appropriate number listed on the title page of this guidance.

The Least Burdensome Approach

The issues identified in this guidance document represent those that we believe should be addressed before your device can be marketed. In developing the guidance, we carefully considered the relevant statutory criteria for Agency decision-making. We also considered the burden that may be incurred in your attempt to comply with the guidance and address the issues we have identified. We believe that we have considered the least burdensome approach to resolving the issues presented in the guidance document. If, however, you believe that there is a less burdensome way to address the issues, you should follow the procedures outlined in the "**A Suggested Approach to Resolving Least Burdensome Issues**" document. It is available on our Center web page at: http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm085994.htm.

2. Background – Premarket Notifications

A manufacturer who intends to market a device of this generic type must

- conform to the general controls of the Federal Food, Drug, and Cosmetic Act (the act), including the premarket notification requirements described in 21 CFR 807 Subpart E,
- conform to the special control by addressing the specific risks to health identified in this guidance (see Section 4, below).
- satisfy other applicable special controls designated in 21 CFR 866.3980, the classification regulation for this type of device, and
- obtain a substantial equivalence determination from FDA prior to marketing the device. (See also 21 CFR 807.81 and 807.87).

FDA believes that special controls, when combined with the general controls, will be sufficient to provide reasonable assurance of the safety and effectiveness of multiplex nucleic acid assays that detect and differentiate influenza A subtypes.

This document supplements other FDA documents regarding the specific content requirements of a premarket notification submission. You should also refer to 21 CFR 807.87, the guidance, **Format for Traditional and Abbreviated 510(k)s**[2] and the section of CDRH's Device Advice webpage, **Premarket Notification 510(k)**.[3]

As described in **The New 510(k) Paradigm - Alternate Approaches to Demonstrating Substantial Equivalence in Premarket Notifications; Final Guidance**,[4] a manufacturer may submit a Traditional 510(k), an Abbreviated 510(k), or a Special 510(k). A manufacturer may choose to submit an abbreviated 510(k) when a guidance document exists, when special controls have been established, or when FDA has recognized a

[2] http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm084365.htm
[3] http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/HowtoMarketYourDevice/PremarketSubmissions/PremarketNotification510k/default.htm
[4] http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm080187.htm

relevant consensus standard. Manufacturers considering certain modifications to their own cleared devices may lessen their regulatory burden by submitting a Special 510(k). For more information on types of Premarket Notification 510(k)s that may be submitted to FDA, see CDRH's Device Advice webpage, **Premarket Notification 510(k).**

3. Devices within the Scope of this Document

The scope of this document is limited to the following devices, described in 21 CFR 866.3980:

> **21 CFR 866.3980 Respiratory viral panel multiplex nucleic acid assay.** A respiratory viral panel multiplex nucleic acid assay is a qualitative in vitro diagnostic device intended to simultaneously detect and identify multiple viral nucleic acids extracted from human respiratory specimens or viral culture. The detection and identification of a specific viral nucleic acid from individuals exhibiting signs and symptoms of respiratory infection aids in the diagnosis of respiratory viral infection when used in conjunction with other clinical and laboratory findings. The device is intended for detection and identification of a combination of the following viruses:
>
> (1) Influenza A and Influenza B
> (2) Influenza A subtype H1 and Influenza A subtype H3
> (3) Respiratory Syncytial Virus subtype A and Respiratory Syncytial Virus subtype B
> (4) Parainfluenza 1, Parainfluenza 2, and Parainfluenza 3 virus
> (5) Human Metapneumovirus
> (6) Rhinovirus
> (7) Adenovirus

Product codes applicable for devices described in this guidance and cleared under 21 CFR 866.3980:

OCC – Respiratory virus panel nucleic acid assay system
OEP – Influenza A virus subtype differentiation nucleic acid assay

As already noted, devices subject to this special control guidance document are also subject to the special control guidance, "Class II Special Controls Guidance Document: Respiratory Viral Panel Multiplex Nucleic Acid Assay," and, if they include testing for hMPV, to the special control guidance **"Class II Special Controls Guidance Document: Testing for Human Metapneumovirus (hMPV) Using Nucleic Acid Assays."** See 21 CFR 866.3980(b). Both of these special controls guidances contain additional information relevant to premarket notification submissions for these specific devices.

In addition, manufacturers who seek to establish the substantial equivalence of their devices to devices classified under 21 CFR 866.3332, Reagents for Detection of Specific Novel Influenza A Viruses, should consult the special control guidance document

designated in that classification, "Class II Special Controls Guidance Document: Reagents for Detection of Specific Novel Influenza A Viruses," (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm078583.htm) for relevant information on their premarket submission, as well as being prepared to satisfy the additional special control identified in that classification.

Finally, FDA has also made available the following guidance documents, which are not special control documents but also address influenza IVDs: "In Vitro Diagnostic Devices to Detect Influenza A Viruses: Labeling and Regulatory Path," (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm078538.htm) ("labeling guidance"), and the draft guidance document, "Establishing the Performance Characteristics of In Vitro Diagnostic Devices for the Detection or Detection and Differentiation of Influenza Viruses" (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm079171.htm). The recommendations of those documents are consistent with this special control, and FDA recommends that the labeling guidance, and when finalized, the draft guidance on establishing performance characteristics, be consulted for additional information on FDA's current thinking about the regulation of influenza diagnostics.

4. Risks to Health

Human influenza is a highly contagious acute respiratory tract disease. There are three genera of human Influenza viruses: A, B and C. Infection with Influenza A virus is the most severe, with several notable pandemics during the past century. Influenza A viruses are classified into subtypes according to the antigenic composition of their hemagglutinin (HA) and neuraminidase (NA) glycoproteins on the viral envelope.

Only some Influenza A subtypes (i.e., H1N1, H1N2, and H3N2) are currently in general circulation in humans. Other subtypes are found most commonly in other animal species. Avian influenza viruses are of great concern because some subtypes may be highly pathogenic, may cause sporadic human infection, and have the potential of causing a pandemic. Currently, avian influenza strains implicated in human disease include the highly pathogenic avian influenza (HPAI) H5N1 and H7N7 strains and the low pathogenic avian influenza (LPAI) strains H9N2, H7N2, and H7N3.

Influenza illness caused by commonly circulating Influenza A viruses can have high morbidity and mortality, particularly in special populations like the elderly and the very young. Acquired immunity to seasonal influenza viruses is limited because influenza viruses mutate in small but important ways from year to year (a process known as antigenic drift). Novel influenza viruses[5] present an even greater likelihood of morbidity and mortality, with the potential to cause widespread disease and/or disease of unusually high severity, because few people (or none at all) have prior immunologic exposure to

[5] Specific risks to health associated with reagents for detection of novel influenza viruses are identified in the special control guidance document applicable to devices classified under 21 CFR 866.3332, "Class II Special Controls Guidance Document: Reagents for Detection of Specific Novel Influenza A Viruses."

surface glycoproteins of these viruses. In addition, other pathogenicity factors may increase virulence.

Devices for the detection of Influenza A subtypes may be used to differentiate seasonal influenza subtypes currently in circulation (A/H1 and A/H3). However, a specimen from a patient who has symptoms consistent with influenza and is positive for Influenza A and negative for A/H1 and A/H3 will be considered suspected of being positive for novel influenza virus and should be further tested (see CDC/MMWR instructions (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5613a4.htm and http://www.cste.org/ps/2007pdfs/novelfluanndssjan10final23.pdf).[6] The potential ability of these devices to differentiate seasonal from pandemic influenza viruses has great public health implications in addition to their clinical utility in aiding patient management. Therefore, FDA has identified the following potential risks to health associated with this type of device, i.e., issues that may impact safety or effectiveness of a respiratory viral panel multiplex nucleic acid assay that detects and differentiates Influenza A subtypes. These include failure of the device to perform as indicated, leading to inaccurate results or lack of results, and incorrect interpretation of results; both of these potential risks may lead to incorrect patient management decisions.

Failure of the device to perform as indicated:

A failure to detect Influenza A nucleic acid sequences when a patient is infected with these viruses (a false negative result) could result in clinical misdiagnosis, withholding appropriate treatment, and not instituting prevention/control efforts. Detecting specific Influenza A nucleic acids when a patient is not infected (a false positive result), could lead to unnecessary treatment with potentially toxic drugs or failure to appropriately diagnose and treat influenza. A false positive result can lead to unnecessary isolation procedures or contact tracing. Failure of the device to produce a test result may lead to delay in patient diagnosis and treatment.

Failure to interpret results correctly:

A respiratory viral panel multiplex nucleic acid assay that detects and differentiates Influenza A subtypes is intended to aid in the diagnosis of Influenza A infection when used in conjunction with other clinical and laboratory findings. Therefore, failure to interpret assay results in the context of the other laboratory results and the clinical presentation could lead to inappropriate or delayed treatment. For example, positive assay results do not rule out bacterial co-infection, or co-infection with other viruses, and the Influenza A subtype detected by the assay may not necessarily be the cause of the clinical symptoms or disease. In addition, detection of the nucleic acid does not necessarily indicate active infection because Influenza A RNA sequences can persist *in vivo* independent of virus viability. Distinguishing non-viable viruses from infective viral particles can be discerned by cell culture. The erroneous perception that there is active or

[6] Centers for Disease Control and Prevention. 2006-07 Influenza vaccine composition in, "MMWR Recommendations and Reports: Prevention and Control of Influenza: Recommendations of the Advisory Committee on Immunization Practices (ACIP)." 2006; July 28; 55(RR10):1-42. www.cdc.gov/flu/professionals/vaccination/composition0607.htm

persistent infection when the virus may have already been killed may lead to unnecessary treatment or extended treatment. Therefore, additional testing (e.g., bacterial culture, immunofluorescence, and chest x T rays/radiography) is needed in order to obtain the final diagnosis of Influenza A infection, including the identification of the specific viral subtype.

This special control guidance makes recommendations for mitigating the risks associated with this type of assay by addressing the following specific sources of error:

Inaccurate results (i.e., false positive or false negative results) or lack of result may be attributed to the following:

- Failure or improper use of reagents, instrumentation, data management, or software included with the assay.
- Failure or improper use of ancillary reagents or problems with the quality of ancillary reagents.
- Improper testing when performed by laboratory personnel lacking expertise in molecular testing.

False positive results can be caused by the following:

- The potential of assay primers and probes to cross-react with nucleic acid sequences from non-influenza viruses also detected by the device (as appropriate), from other pathogens that may be present in patient specimens, or other endogenous nucleic acids sequences.
- In the case of an open assay system, there is a possibility of cross-contamination and amplicon contamination if proper control measures are not implemented.

False negative results can be caused by the following:

- RNA degradation due to improper storage or transport of specimens and extracted nucleic acid, or inadequate extraction of the nucleic acid material.
- The propensity of influenza viruses to mutate or the emergence of new subtypes. Primers and probes are generally selected for their homology with highly conserved regions within viral RNA segments. Primers and probes might fail to react with a newly isolated genetic subtype or emerging genetic mutant reducing assay performance.
- Competitive inhibition or interference by other substances present in patient specimens or introduced into the analytical system during sample processing/handling.

Failure to properly interpret test results due to:

- Possibility of bacterial co-infection.
- Inaccurate interpretation and reporting of testing results by laboratory personnel lacking expertise in viral diagnosis.

- Misinterpretation due to performance variability related to Influenza A virus prevalence and specific patient populations.

Prior to submitting your premarket notification you should conduct a risk analysis and identify any other risks specific to your device. Risks may vary depending on the type of nucleic acid assay, the specific intended use of the test, the specimen type, and how the result will be used. The premarket notification should describe the method utilized to conduct the risk analysis.

In the table below, FDA has identified the risks to health generally associated with the use of this device. Measures recommended to mitigate the identified risks are described in this guidance document, as shown in the table below. If you elect to use an alternative approach to address the risks identified in this document, or have identified risks additional to those in this document, you should provide sufficient detail to support the approach you have used to address that risk.

Identified risk	Recommended mitigation measures
Failure of the device to perform as indicated: Inaccurate results (i.e., false positive or false negative results), or lack of results	Labeling (Section 7) Performance Characteristics (Sections 6) Device Description (Section 5)
Failure to properly interpret test results	Labeling (Section 7)

5. Device Description

In your 510(k) submission, you should identify the regulation, the product code, and a legally marketed predicate device. We recommend that you include a table that outlines the similarities and differences between the predicate and your device.

You should include the following descriptive information to adequately characterize your respiratory viral panel multiplex nucleic acid assay that is intended to detect and differentiate influenza A viruses.

5.A Intended Use

The intended use should specify the Influenza A virus types and subtypes the device detects and identifies, the nature of the analyte (e.g., RNA), test platform, specimen types for which testing will be indicated, the clinical indications for which the test is to be used, and the specific population(s) for which the test is intended. The intended use should state that the test is qualitative, whether analyte detection is presumptive, and any specific conditions of use.

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

- The identity, phylogenetic relationship, or other recognized characterization of the Influenza viruses that your device is designed to detect.
- How the device results might be used in a diagnostic algorithm and other measures that might be needed for a laboratory identification of Influenza A virus and of specific Influenza A virus subtypes.
- Clinical and epidemiological parameters that are relevant to a patient case diagnosis, as applicable. The World Health Organization (WHO) and other public health entities provide criteria that may be used as a guide for defining patient cases.

5.B Test Methodology

You should describe in detail the methodology used by your device. For example, you should describe the following elements, as applicable to your device:

- Test platform (e.g., RT-PCR, bead arrays).
- Specificity of probes for the Influenza sequences of interest.
- Information and rationale for selection of specific target sequences and the methods used to design primers and probes.
- Limiting factors of the assay (e.g., saturation level of hybridization, maximum cycle number).
- Specimen type (e.g., swabs, aspirates, and viral culture media), collection and handling methods.
- Reagent components provided or recommended for use, and their function within the system (e.g., buffers, enzymes, fluorescent dyes, chemiluminescent reagents, other signaling/amplification reagents).
- The potential for specific and non-specific probe cross-hybridization.
- Internal controls and a description of their specific function in the system.
- External controls that you recommend or provide to users.
- Instrumentation required for your device, including the components and their function within the system.
- Types of output generated by the instrumentation and system parameters (e.g., measurement ranges).
- The computational path from raw data to the reported result (e.g., how raw signals are converted into a signal). This would include sufficient software controls for identifying and dealing with obvious problems in the dataset. Describe adjustment to the background signal for normalization, if applicable.
- Illustrations or photographs of non-standard equipment or methods, if available.

Where applicable for your device, you should describe design control specifications that address or mitigate risks associated with primers, probes, and controls used to detect viral RNA segments, such as the following examples:

- Prevention of probe cross-contamination for multiplexed tests in which many of the probes are handled during the manufacturing process.
- Correct placement and identity of assay features (e.g., probes).
- Minimization of false positives due to contamination or carryover of sample.
- Use of multiple probes for a single analyte to enable detection of virus variants appearing due to mutations within the target RNA segment(s), or variants within a designated Influenza virus strain (or lineage).
- Developing or recommending validated methods for nucleic acid extraction and purification that yield suitable quality and quantity of viral nucleic acid for use in the test system with your reagents. You should address suitable validated extraction method(s) for different specimen types claimed in your device's intended use.

In your 510(k), you should provide performance information that supports the conclusion that your design requirements have been met. You should also provide information to verify the design of your reagents, e.g., rationale for selection of specific conserved target sequences and the methods used to design primers and probes (see Section 6 – Performance Characteristics).

5.C Ancillary Reagents

Ancillary reagents are those reagents that an assay manufacturer specifies 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 manufacturer and catalog or product number, or other specific designation, in order for your device to achieve its labeled performance characteristics. For example, if your device labeling specifies the use of Brand X DNA amplification enzyme, and use of any other DNA amplification enzyme may alter the performance characteristics of your device from that reported in your labeling, then Brand X DNA amplification enzyme is an ancillary reagent of concern for the purposes of this document.[7]

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.

[7] 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. If you are unsure whether this aspect of the special controls applies to your device, we recommend you consult with the FDA.

If the instructions for use of your device specify one or more ancillary reagents of concern, you should address 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 should address the elements 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 should 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 for use provided directly with the ancillary reagent and those supplied by you with your device, and any other issues that could present a risk of obtaining incorrect results with your device.

2. Using your risk assessment as a basis for applicability, you should 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 your device.
    - Any other issues that must be addressed in order to assure safe and effective use of your test in combination with named ancillary reagents, in accordance with your device's instructions for use.

In addition, you should 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 should contact FDA for advice.

5.D Controls

Controls should provide information about (1) sample quality, (2) nucleic acid quality, and (3) process quality. We generally recommend that you include the following types of controls:

5.D.i Negative Controls

Blanks 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. These controls are used to rule out contamination with target nucleic acid or increased background in the amplification reaction. It may not be applicable for assays performed in single-test, disposable cartridges or tubes.

Negative sample control

The negative sample control contains non-target nucleic acid or, if used to evaluate extraction procedures, it contains the whole organism not targeted by the assay. 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-respiratory virus infected individual
- Samples containing a non-target organism (e.g., cell line infected with non- respiratory virus)
- Surrogate negative control, e.g., packaged RNA

5.D.ii Positive Controls

Positive control for complete assay

The positive control is designed to mimic a patient specimen, contains target nucleic acids, and is used to control the entire assay process, including nucleic acid extraction, amplification, and detection. Acceptable positive assay control materials include cell lines infected with a non-pathogenic strain of virus detected by the assay in the appropriate matrix mimicking the recommended assay specimen type.

Positive control for amplification/detection

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

5.D.iii 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 (e.g., polymerase, primers), equipment function (e.g., thermal cycler), and the presence of inhibitors in the samples. Examples of acceptable internal control materials include human nucleic acid co-extracted with the influenza virus and primers amplifying human housekeeping genes (e.g., RNaseP, β-actin). The need for this control is determined on a device case-by-case basis.

Controls should approximate the composition and nucleic acid concentration of the recommended specimen in order to adequately challenge the system, as well as address reproducibility around the cut-off.

In your 510(k), we recommend that you describe the following items concerning quality control and calibration:

- The nature and function of the various controls that you include, or recommend for use, with your device. These controls should enable the user to determine if all steps and critical reactions have proceeded properly without contamination or cross-hybridization.
- Your methods for value assignment (relative or absolute) and validation of control and calibrator material, if applicable.
- The control parameters that could be used to detect failure of the instrumentation to meet required specifications.

We recommend that you consult with FDA when designing specific controls for your device.

5.E Interpreting Test Results/Reporting

In your 510(k), you should describe how positive, negative, equivocal (if applicable), or invalid results are determined and how they should be interpreted. In your 510(k) submission, we recommend that you indicate the cut-off values for all outputs of the assay.

- Specifically, you should 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 is also for defining a positive result of the assay.
- If the assay has an equivocal zone, we recommend that you provide cut-off values (limits) for the equivocal zone.
- If your interpretation of the initial equivocal results requires re-testing, you should provide (1) a recommendation whether re-testing 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 confirms the significance of the cut-offs).

- If one of the reported outputs of your assay can be an equivocal result, you should provide the interpretation and recommendation for how the user should follow- up the equivocal results for each pathogen on your panel.
- If the assay has an invalid result, you should describe how an invalid result is defined. If internal controls are part of the determination of invalid results, you should provide the interpretation of each possible combination of control results for defining the invalid result. Provide recommendation on how to follow up any invalid result, i.e., whether the result should be reported as invalid or re-testing is recommended. If the re-testing is recommended, provide the information similar to the one for re-testing of equivocal results (whether re-testing should be repeated from the same nucleic acid preparation, a new extraction, or a new patient specimen).

6. Performance Characteristics

In your 510(k), we recommend that you detail the study design you used to evaluate each of the performance characteristics outlined below.

If your product labeling calls for the use of ancillary reagents, the premarket performance testing submitted to support your 510(k) should use the ancillary reagents referenced in your instructions. The performance claims you establish through premarket testing, which will be reflected in your labeling under 21 CFR 809.10(b), should be based on the particular test configuration you describe in your labeling.

6.A Preanalytical Factors

Consideration of preanalytical factors is critical for high-quality respiratory viral panel nucleic acid tests. In your 510(k), we recommend that you address the following issues regarding preanalytical factors.

6.A.i Specimen Collection and Handling

You should specify the specimen type(s) your assay is intended to measure. Different types of respiratory specimens can be used; however, nasal washes and nasopharyngeal aspirates tend to be more sensitive than pharyngeal swabs. The timing of specimen collection is very important because the viral yield is the highest for respiratory specimens obtained within four days of onset of influenza-like symptoms.

The quality and quantity of extracted target can be highly dependent on multiple factors such as specimen source, collection method, and handling (e.g., transport and storage times and temperatures). The testing information you provide in your 510(k) should validate that (1) your system provides adequate and appropriate nucleic acid for all analytes detected by your assay (i.e., different virus types and subtypes,), and (2) the device maintains acceptable performance (e.g., accuracy, reproducibility) under all the various conditions you recommend in your labeling.

For example, you should assess the effect of recommended storage times and temperatures on sample stability and recovery using an analysis of specimen aliquots stored and/or transported under your recommended conditions of time and temperature. You should state your acceptance criteria for all specimen stability parameters.

Specimens for pathogen identification should be collected and handled using all applicable state and federal biosafety guidelines. For standard precautions for handling of specimens, refer to the most current editions of the related CLSI documents. [8]

6.A.ii Fresh vs. Frozen Samples (stability)

Sensitivity for detection of some viruses changes depending whether the specimen is fresh or frozen. In developing your test, we recommend that you conduct an adequate assessment as to whether this is a concern for your device. We recommend that you assess the effect of repeated freeze/thaw cycles on the yield of the viral nucleic acid and its influence on the assay performance.

6.A.iii Nucleic Acid Extraction

Different extraction methods may yield nucleic acids of varying quantity and quality, and therefore the extraction method can be crucial to a successful result. You should evaluate the effect of your chosen extraction method on the performance of the assay with respect to satisfactory nucleic acid quantity and quality for the intended use of the assay. We recommend that you evaluate your assay's analytical and clinical performance characteristics for each virus type and subtype using the entire pre-analytical process (including extraction procedures) that you recommend for use with your assay. Specifically you should demonstrate the reproducibility and limit of detection (LoD) of your assay with recommended extraction procedure(s). In addition, external site studies (including reproducibility and clinical studies) should include the extraction procedures prescribed in your labeling. You should describe the design and results of these evaluations in your 510(k).

We recommend that you perform these evaluations whether you intend to actually provide reagents for extraction and preparation of nucleic acid in your test kit, or whether you simply instruct users concerning appropriate reagents that can be used with your device.

If you include or recommend multiple extraction methods for use with your assay, you should demonstrate extraction quality and efficiency, as well as analytical and

[8] *Biosafety in Microbiological and Biomedical Laboratories* 1999. Richmond, J.Y. and McKinney, R.W. eds. HHS Publication Number (CDC) 93-8395; and CLSI (formerly NCCLS) document M29-A, Protection of Laboratory Workers from Infectious Disease Transmitted by Blood, Body Fluids, and Tissue, Wayne, PA. Clinical and Laboratory Standards Institute; 1997.

clinical performance of your assay with each extraction method and each virus type or subtype. Specifically, you should demonstrate LoD and reproducibility for each method. You may be able to combine the extraction method variable with each site performance variable. 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 sites: test extraction method A at site 1, method B at site 2, and method C at site 3. However, if the studies from the three sites indicate statistically significant differences in assay performance, the reproducibility study should be expanded to include testing each extraction method at all three study sites (e.g., site 1 extraction methods 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 at external sites), each extraction method should 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 should not be pooled, but rather should be analyzed separately. As a consequence, additional prospective clinical samples may be called for in order to support the claimed extraction method.

We recommend that you provide your recommendations for assuring specimen adequacy for the different specimen types for which your assay is indicated. For example, the quality of the nucleic acid can be assessed using internal controls that determine the presence and/or quality of the nucleic acid. When using contrived samples for analytical validation, you should ensure that the specimens used for spiking are derived from more than one patient to account for biological variability.

6.A.iv Well-to-Well Cross Contamination with Automated Extraction Systems

If automated systems are used or recommended for nucleic acid extraction, you should assess the potential for well-to-well cross contamination as part of the performance qualification of the extraction instrument. You should provide a software hazard analysis for automated extraction systems as part of your 510(k). A validation study of the extraction process can be designed in a grid such that a nucleic acid-containing sample with a concentration at the highest anticipated clinical level is surrounded on all sides by a "no template control." The results should demonstrate that well-to-well cross-contamination does not occur.

6.A.v Performance Study Quality Controls

Evaluation of assay performance should include appropriate controls for the duration of the analytical and clinical studies. This includes any positive and negative controls provided with your assay as well as appropriate external controls recommended, but not necessarily provided, with the assay.

The external positive control contains target nucleic acids and is used to control the entire assay process including nucleic acid 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). Some examples of acceptable external positive assay controls include:

- Cell lines infected with a non-pathogenic strain of virus detected by the assay
- Vaccine or prototypic vaccine strains
- Low pathogenic viruses
- Inactivated viruses
- Packaged viral RNA

If your test requires the use of ancillary reagents, you should 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.

6.B Analytical Performance

The following are analytical performance characteristics you should establish for your assay.

6.B.i Limit of Detection (LoD)

LoD is defined as the lowest concentration of analyte that can be consistently detected (typically in ≥95% of samples tested under routine clinical laboratory conditions) in a defined type of specimen. This concentration must yield an assay value that can be reproducibly distinguished from values obtained with samples that do not contain the analyte.

Determination of LoD for multiplex assays follows the same principles as for single analyte assays (described in CLSI document EP17-A, *Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline*. Clinical and Laboratory Standards Institute; Wayne, PA: 2004). During the validation of a test system, you should determine the LoD for each specimen type and each analyte that will be tested in a respiratory viral panel multiplex assay. This can be accomplished by limiting dilutions of regrown and retitered viral stocks. The reference methods we recommend for determination of the viral titers prior to use in the study are tissue culture infectious dose 50 ($TCID_{50}$) or plaque assay, expressed in plaque forming units/mL (PFU/mL). You should prepare serial dilutions using appropriate pooled negative sample matrixes as diluents that include 3-5 replicates for each dilution. You should report the LoD as the level of virus that gives a 95% detection rate. Depending on the assay, it might not be necessary to perform a separate LoD determination at the whole range of concentrations for every single specimen type; LoD should be determined for at least the most common and most problematic ones. The LoD may be confirmed by preparing at least 20 additional replicates at the proposed LoD concentration and demonstrating that the virus can be detected 95% of the

time. You should utilize the entire test system from sample preparation to amplicon detection when evaluating assay LoD.

6.B.ii Analytical Reactivity (Inclusivity)

We recommend that you demonstrate that the test can detect at least ten virus strains representing temporal and geographical diversity for each claimed influenza subtype at viral levels at or near the LoD. For subtypes for which it is difficult to obtain sufficient samples to demonstrate detection of ten strains, we recommend that you contact FDA to discuss your study. All virus identities and titers should be confirmed.

Suggested strains for the LoD and analytical reactivity studies are shown in Table 1. If vaccine strains are included, they should represent recent flu seasons. The information on the current vaccine strains is available from the Centers for Disease Control and Prevention (CDC) at www.cdc.gov/flu/professionals/vaccination/composition0607.htm. Vaccine strains may vary from one influenza season to another.

Table 1. Influenza strains recommended for analytical sensitivity (LoD) studies.

Type	Subtype	Influenza Viral Strain
A	H1N1-like	A/New Caledonia/20/1999
A	H3N2-like	A2/Wisconsin/67/2005 or Ag equiv A/Hiroshima/522005
B		B/Malaysia/2506/2004 or Ag equiv B/Ohio/1/2005
A	H1N1	A/PR/8/34
A	H1N1	A/FM/1/47
A	H1N1	A/NWS/33
A	H1N1	A1/Denver/1/57
A	H1N1	A/New Jersey/8/76
A	H3N2	A/Port Chalmers/1/73
A	H3N2	A/Hong Kong/8/68
A	H3N2	A2/Aichi2/68
A	H3N2	A/Victoria/3/75
A	H1	A/NY/55/2004
A	H3	A/Hawaii/15/2001
B		B/Lee/40
B		B/Allen/45
B		B/GL/1739/54
B		B/Taiwan/2/62
B		B/Hong Kong/5/72
B		B/Maryland/1/59

B		B/Florida/2006
A	H5N1	Human and /or Avian
A	H5N2	Avian
A	H7N2	Human and /or Avian
A	H7N7	Human and /or Avian
A	Other subtypes	Human and/or animal species

6.B.iii Analytical Specificity

Cross-reactivity

We recommend that you test for potential cross-reactivity with non-influenza respiratory pathogens and other microorganisms with which the majority of the population may have been infected e.g., Epstein Barr Virus (EBV) and cytomegalovirus (CMV). We recommend that you test medically relevant levels of viruses and bacteria (usually 10^6 cfu/ml or higher for bacteria and 10^5 pfu/ml or higher for viruses). We recommend that you reconfirm the virus and bacteria identities and titers. The microorganisms recommended for cross-reactivity studies are listed in Table 2.

Table 2. Microorganisms recommended for analytical specificity (cross-reactivity) studies.

Organism	Type
Adenovirus	Type 1
Adenovirus	Type 7
Human coronavirus*	
Cytomegalovirus	
Enterovirus	
Epstein Barr Virus	
Human parainfluenza	Type 1
Human parainfluenza	Type 2
Human parainfluenza	Type 3
Measles	
Human metapneumovirus	
Mumps virus	
Respiratory syncytial virus	Type B
Rhinovirus	Type 1A
Bordetella pertussis	
Chlamydia pneumoniae	
Corynebacterium sp.	
Escherichia coli	
Hemophilus influenzae	
Lactobacillus sp.	

Legionella sp	
Moraxella catarrhalis	
Mycobacterium tuberculosis avirulent	
Mycoplasma pneumoniae	
Neisseria meningitides	
Neisseria sp.	
Pseudomonas aeruginosa	
Staphylococcus aureus	Protein A producer
Staphylococcus epidermidis	
Streptococcus pneumoniae	
Streptococcus pyogenes	
Streptococcus salivarius	

*We recommend that you include the OC43 and 229E strains of Human coronavirus in your cross-reactivity study.

Additionally, we recommend testing cross-reactivity of your assay with vaccines such as live attenuated influenza virus vaccine (Nasal-Spray Flu Vaccine), considering there may be reactive patient testing results from individuals that have received the vaccine.

Interference

We recommend that you conduct a comprehensive interference study using medically relevant concentrations of the interferent and at least two strains for each influenza type to assess the potentially inhibitory effects of substances encountered in respiratory specimens.

Potentially interfering substances include, but are not limited to, the following: blood, nasal secretions or mucus, and nasal and throat medications used to relieve congestion, nasal dryness, irritation, or asthma and allergy symptoms. Examples of potentially interfering substances are presented in Table 3. We recommend that you test interference at the assay cut-off determined for each influenza virus type and for each of the interfering substances. We also recommend that you evaluate each interfering substance at its potentially highest concentration ("the worst case"). If no significant clinical effect is observed, no further testing is necessary. Please refer to the CLSI document EP7-A2 for additional information.

Table 3. Substances Recommended for Interference Studies

Substance	Active Ingredient
Mucin: bovine submaxillary gland, type I-S	Purified mucin protein
Blood (human)	
Nasal sprays or drops	Phenylephrine, Oxymetazoline,

	Sodium chloride with preservatives
Nasal corticosteroids	Beclomethasone, Dexamethasone, Flunisolide, Triamcinolone, Budesonide, Mometasone, Fluticasone
Nasal gel	Luffa opperculata, sulfur
Homeopathic allergy relief medicine	Galphimia glauca Histaminum hydrochloricum
FluMist©	Live, intranasal influenza virus vaccine
Throat lozenges, oral anesthetic and analgesic	Benzocaine, Menthol
Anti-viral drugs	Zanamivir
Antibiotic, nasal ointment	Mupirocin
Antibacterial, systemic	Tobramycin

6.B.iv Cut-off

In your submission, you should explain how the assay cut-off(s) was determined (see also Section 5.E) and how this cut-off value(s) was validated. The cut-off should be established using appropriate statistical methods. For example, you may provide a result distribution, 95th and 99th percentiles, percents of the non-negative (positive or equivocal) results, and so on, for the clinical samples without any respiratory viruses 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 document CLSI document GP10-A *Assessment of the Clinical Accuracy of Laboratory Tests Using Receiver Operating Characteristics (ROC) Plots; Approved Guideline*. Wayne, PA, Clinical and Laboratory Standards Institute; 1995). If the assay has an equivocal zone, you should explain how you determined the limits of the equivocal zone. The performance of your device using the established cut-off (and equivocal zone, if applicable) should be validated in an independent population consistent with the defined intended use of your device (your pivotal clinical study).

6.B.v Precision (Repeatability/Reproducibility)

We recommend that you provide data demonstrating the precision (i.e., repeatability and reproducibility) of your system. The CLSI documents, EP5-A2 *(Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline—Second Edition*. Wayne, PA. Clinical and Laboratory Standards Institute; 2004) and EP12-A2 (*User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline.* Wayne, PA. Clinical and Laboratory Standards Institute; 2008), include guidelines that may be helpful for

developing experimental design, computations, and a format for establishing performance claims.

We recommend you establish the precision for each Influenza virus type and subtype. Ideally, all sources of assay variability in the precision study should be identified. In general, any variable that changes from day to day or week to week should be examined for its impact on assay precision. While some sources of variability can be evaluated in an in-house precision study, the site-to-site reproducibility study should include an evaluation of the major sources of variability described below, for each virus:

- *Extraction-to-extraction reproducibility*: samples used in reproducibility testing are processed from clinical specimens (e.g., nasopharyngeal swabs) at the test site, using the extraction procedure you recommend in the test labeling.
- *Between-instrument reproducibility*
- *Site-to-site and operator-to-operator reproducibility:* include three or more sites (at least two external sites and one in-house site) with multiple operators at each site. Operators should reflect potential users of the assay in terms of education and experience. You should provide training only to the same extent that you intend to train users after marketing the test.
- A minimum of three-to-five non-consecutive days to cover day-to-day variability of each analyte tested by the respiratory viral panel (if applicable, spanning two instrument calibration cycles).
- A minimum of two runs per day (unless the assay design precludes multiple runs per day) and two replicates of each panel member per run is recommended to assess between-run component as well as within-run and within-day imprecision in your reproducibility study.
- *Lot-to-lot reproducibility*: evaluate multiple product lots (e.g., multiple lots of assay reagents and ancillary reagents, multiple lots of primers and probes for RT-PCR, multiple lots of beads or arrays), and multiple instruments.
- The Influenza A type and every subtype the test can detect should be represented by the test samples. For each analyte (target) detected by the device, we recommend including at least three levels of viral load, including analyte or output concentrations close to the assay cut-off:

    1. A "high negative" sample (C_5 concentration): a sample with an analyte concentration below the clinical cut-off such that results of repeated tests of this sample are negative approximately 95% of the time (and results are positive approximately 5% of the time).

2. A "low positive" sample (C_{95} concentration): a sample with a concentration of analyte just above the clinical cut-off such that results of repeated tests of this sample are positive approximately 95% of the time.

3. A "moderate positive" sample: a sample ideally reflecting clinically relevant concentration.[9] At this concentration one can anticipate positive results approximately 100% of the time (e.g., approximately two to three times the concentration of the clinical cut-off).

When the limit of blank (LoB) is used as a cut-off, then the concentration C_{95} is the same as the limit of detection (LoD) and the zero concentration (no analyte present in sample) is C_5 if LoB is established with Type I error of 5%.[10]

In the study design description in your 510(k), you should identify which factors (e.g., instrument calibration, reagent lots, and operators) were held constant and which were varied during the evaluation, and describe the computations and statistical analyses used to evaluate the data. In general, for qualitative tests, variance components should be estimated using the appropriate statistical method for each of the factors considered in the precision study, as well as overall variation. Particularly for qualitative tests that have underlying quantitative output, the component of precision is often measured for each source of variation, as well as the total variation, using analysis of variance. For each sample in the precision study, provide the mean value with variance components (standard deviation and percent CV). In addition, for each sample, provide percents of the values above and below the cut-off and percent of invalid results for each site separately and for all sites combined (if applicable, provide percents of equivocal results for each sample in the precision study for each site and for all sites combined).

6.B.vi Carryover studies and Cross-contamination Studies (for multi-sample assays and devices that require instrumentation)

For multi-sample assays and devices that require instrumentation, we recommend that you demonstrate that carryover and cross-contamination do not occur with your device. In a carryover and cross-contamination study, we recommend that high positive samples be used in series alternating with high negative samples in patterns dependent on the operational function of the device. At least 5 runs with alternating high positive and high negative samples should be performed. We recommend that the high positive samples in the study be high enough to exceed 95% or more of the results obtained from specimens of diseased patients in the

[9] Sample with a typical concentration of the infected subjects in the intended use population (for example, a median value of the concentrations from the infected subjects) .

[10] Type I error is the probability of having truly negative samples (those with zero analyte concentration) give values that indicate presence of analyte. Usually, Type I error is set as 5% or less.

intended use population. We recommend that 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 carryover 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 95%.

6.C Clinical Performance Studies

We recommend that you conduct prospective clinical studies to determine the performance of your device for all the Influenza subtypes as well as all specimen types you claim in your labeling.

6.C.i Study Protocol

We recommend that you develop a detailed study protocol that includes, for example, patient inclusion and exclusion criteria, type and number of specimens needed to demonstrate the performance of your device, directions for use, and a statistical analysis plan that accounts for variances to prevent data bias. We recommend that you include this and any other relevant protocol information in your premarket submission.

We encourage sponsors to contact the Division of Microbiology Devices at FDA, to request a review of their proposed studies and selection of specimen types. We particularly encourage manufacturers to seek this type of discussion when samples are difficult to obtain.

6.C.ii Study Population

We recommend that you conduct your studies on individuals presenting with influenza-like symptoms (e.g., cough, nasal congestion, rhinorrhea, sore throat, fever, headache, myalgia). The concentration of Influenza virus in nasal and tracheal secretions remains high for 24-48 hours after the onset of symptoms and may last longer in children. If your device is intended for screening individuals for influenza infection, you should also include asymptomatic individuals in your study population.

We recommend that you include samples from each age group in your clinical studies and that you present the data stratified by age (e.g., less than 5, 6–21, 22–59, and greater than 60 years old) in addition to the overall data summary table.

6.C.iii Reference Methods

We recommend that you compare your assay's performance to the performance of the established reference methods of viral culture or an FDA-cleared direct specimen fluorescent antibody (DSFA) assay. Viral culture should be performed on freshly collected specimens. For subtyping, after determination of Influenza A infection using viral culture, you may use a DFA or a subtype-specific well-characterized nucleic acid amplification method (e.g., PCR) followed by

bidirectional sequencing. The nucleic acid amplification method used for subtyping should target a different genomic region (i.e., incorporate different primers) from the one probed by your assay. We recommend that you provide published literature or laboratory data in your submission in support of the primers used for amplification. We recommend that you perform the sequencing reaction on both strands of the amplicon (bidirectional sequencing) and demonstrate that the generated sequence is at least 200 base pairs of an acceptable quality (e.g., a quality score of 40 or higher as measured by PHRED or similar software packages) and that it matches the reference or consensus sequence.

When using viral culture, you should provide the viral identification, e.g., staining with viral specific monoclonal antibodies, or PCR followed by sequencing of the amplicons as an alternative method for identification of the virus, in addition to a cytopathic effect (CPE). The CPE alone may not provide accurate viral identification. You should describe the performing laboratory procedure(s) for virus isolation in your submission, as well as specific data or literature to show that a particular cell line is validated to isolate a specific virus. You should not use previously frozen specimens for culture, as freeze-thawing results in loss of virus infectivity. We recommend that the viral culture method used in your study follow the CLSI document M41-A (Viral Culture; Approved Guideline Wayne, PA. Clinical and Laboratory Standards Institute; 2006), and the World Health Organization (WHO) Manual on Animal Influenza Diagnosis and Surveillance.[11] For DFA testing, you should provide the procedure description and data or literature to show that a particular antibody/fluorescent pattern and procedure are appropriate for a specific virus. If the DFA antibody used for virus detection in cultured cells is FDA-cleared, no validation information is needed in the submission, as long as the laboratory performing the test follows the package insert instructions. If the antibody used in the DFA is a preamendments device,[12] we recommend that you provide published literature or laboratory data in your premarket submission in support of the antibody validation for detection of influenza virus. If public health authorities recommend against culturing a novel virus, we recommend that you use nucleic acid amplification followed by sequencing of the amplicons to confirm the identity of the novel virus. The nucleic acid amplification method used in the comparator method should be targeted to the different genomic regions (i.e., incorporate different viral target) from the ones probed by your assay.

6.C.iv Specimen Type(s)

We recommend that you test clinical samples from each specimen type you claim in your intended use (e.g., nasal swabs, nasopharyngeal swabs, nasal aspirates) to

[11] WHO Manual on Animal Influenza Diagnosis and Surveillance. 2002, Geneva, (World Health Organization). (Complete document WHO/CDS/CSR/NCS/2002.5, available at: http://www.who.int/csr/resources/publications/influenza/en/whocdscsrncs20025rev.pdf

[12] Preamendments devices are those devices that were introduced or delivered for introduction into interstate commerce for commercial distribution prior to May 28, 1976 (the date of enactment of the Medical Device Amendments of 1976).

demonstrate that correct results can be obtained from clinical material. For the prospectively collected samples, the performance for each virus type or subtype is described by sensitivity and specificity. Sensitivity for an Influenza type or subtype is the ability of the test to obtain positive results for this virus in the samples with positive results obtained by the comparator method (reference method or composite reference method) for this Influenza virus type or subtype. Specificity for an Influenza type or subtype is the ability of the test to obtain negative results for this virus in the samples with negative results obtained by the comparator method for this Influenza virus type or subtype. For each Influenza viral type or subtype in the panel, sensitivity is calculated by dividing the number of true positive results by the sum of true positive and false negative results; and specificity is calculated by dividing the number of true negative results by the sum of true negative and false positive results (for additional details, see CLSI. Verification and Validation of Multiplex Nucleic Acid Assays; Approved Guideline. CLSI document MM17-A. Wayne, PA: Clinical and Laboratory Standards Institute; 2008). The estimation of sensitivity and specificity should be provided along with 95% two-sided confidence intervals (for more details about confidence intervals, see CLSI. User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline. CLSI document EP12-A2 Wayne, PA: Clinical and Laboratory Standards Institute; 2008).

We recommend that you assess the ability of your device to detect and differentiate influenza viruses in fresh specimens from patients suspected of having an influenza infection. Frozen archived specimens may be useful for developing pre-clinical data but generally are not recommended for use to calculate clinical sensitivity or specificity because of a possible effect of freeze-thawing on the assay performance in comparison to fresh specimens. However, if you can demonstrate that freezing or other preservation techniques do not alter the performance of the device in comparison to testing of fresh specimens, for multiplex nucleic acid assays that detect and differentiate influenza viruses, analysis of prospectively collected archived specimens[13] may be acceptable if appropriate archives are selected and 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 after performing viral culture on fresh specimens, you should ensure that the specimens are not utilized selectively (i.e., you should still test all specimens in a prospective manner). Furthermore, samples should be masked to avoid testing bias. If both fresh and archived/frozen samples are tested, we recommend that you analyze the data of these two groups separately. We encourage you to contact the FDA to request a review of your proposed

[13] In this guidance, we define *prospectively collected archived specimens* as specimens collected sequentially from all patients meeting study inclusion criteria and representing assay intended use population (i.e., not pre-selected specimens with known results) coming in to a clinical testing facility between two pre-determined dates (e.g., from the beginning to the end of one flu season), so there is no bias and prevalence is preserved. These specimens should be appropriately stored (e.g., frozen at -70^{o}C) and, as noted in the text, the sponsor should show that there is no change in device performance due to banking/freezing/storage of the specimens.

studies.

In general, when the number of specimens available for clinical testing is very low (e.g., newly emerging strains), the available evidence for FDA's premarket review may, of necessity, be obtained from analytical rather than clinical studies. In this circumstance, it is particularly critical to have well designed analytical studies. Animal studies are optional and can be used to supplement analytical studies where appropriate.

The total number of samples you should include in your study for substantiating a claim for detection of Influenza A, or H/N subtypes of Influenza A, will depend on the prevalence of the virus and on assay performance. For devices detecting Influenza A virus, we recommend that you include a sufficient number of prospectively collected samples for each specimen type you claim to demonstrate at least 90% sensitivity with a lower bound of the two-sided 95% CI greater than 80%, irrespective of influenza virus type. We recommend that all influenza detecting devices demonstrate specificity with a lower bound of 95% (two-sided) confidence interval exceeding 90%.

If you have questions regarding the choice of appropriate specimen type(s) and numbers, please contact the Division of Microbiology Devices at FDA.

6.C.v Study Sites

We recommend that you conduct your studies at a minimum of three separate facilities, one of which may be in-house. The clinical dataset should consist of clinical samples collected from at least three different clinical sites in different geographical locations. Preferably, studies would be conducted using specimens obtained from a U.S. population. If the studies are conducted outside of the U.S, you should document the relevance of your studies to U.S. clinical practice and demographics.

In the rare cases when a particular Influenza virus subtype has been shown to have low prevalence in the intended use population during the available flu season, it may be appropriate to supplement prospectively collected specimens with banked specimens known to contain a particular Influenza virus subtype (i.e., pre-selected banked specimens).[14] In such cases, results from the banked specimens should be presented separately from the prospectively collected specimen results, and performance calculated as positive percent agreement and negative percent agreement.

[14] In this guidance, *pre-selected banked specimens* refers to banked or archived specimens that are selected by the sponsor for testing because they are known to contain a certain Influenza type or subtype. Because these specimens may **not** have been sequentially collected between two pre-determined dates and do not adequately represent the analyte prevalence in the intended use population, their use can result in biased test performance results.

6.C.vi Data Presentation

You should present separately the results of your analysis of sensitivity and specificity (with 95% CI) for each virus and subtype that your device identifies. Note that samples in the clinical study should be tested as described in the instructions for use of your device. For example, if the samples with initial equivocal or invalid results should be re-tested according to the instruction for use of your assay, then these samples should be re-tested in the clinical study and the final results for these samples should be used in your statistical analysis. For the samples in your clinical study, provide a percent of these re-tested equivocal or invalid samples where applicable for each Influenza virus type and subtype separately and for all combined. In addition, please present the percent of final invalid and final equivocal results (where applicable) for each respiratory virus separately and for all combined.

For the samples in the clinical study, provide signal (result) distributions (frequencies of signals) of your assay for the prospectively collected and archived samples separately for each virus type and subtype, and for all analytes combined. Also, provide signal distributions for the samples which are positive by the reference method for the prospectively collected and archived samples separately for each virus type and subtype and for all analytes combined. Similarly, provide the signal distributions of your assay for the samples in the clinical study that do not contain virus types or subtypes detected by your assay.

If your assay has an equivocal zone, you should provide the following in support of the validation of the equivocal zone, for the prospectively collected and archived samples for each respiratory virus:

a) total number of samples with initial values in the equivocal zone;

b) number of samples with initial values in the equivocal zone and positive results by the reference method; and

c) number of samples with initial values in the equivocal zone and negative results by the reference method.

If the equivocal zone values require re-testing, provide information about how the numbers described above changed after re-testing of the samples.

7. Labeling

Multiplex nucleic acid assays for detection and differentiation of Influenza A virus subtypes, like other devices, are subject to statutory requirements for labeling (sections 502(a), 201(n) of the Act; 21 USC § 352(a), 321(n)). These IVD devices must provide adequate directions for use and adequate warnings and precautions (section 502(f); 21 USC § 352(f)). Specific labeling requirements for all IVD devices are set forth in 21 CFR 809.10. See also 21 CFR § 801.119 (IVDs labeled in accordance with 21 CFR 809.10 are deemed to satisfy section 502(f)(1).)

Although final labeling is not required for 510(k) clearance, final labeling for in vitro diagnostic devices must comply with the requirements of 21 CFR 809.10 before an in vitro diagnostic device is introduced into interstate commerce.

To ensure compliance with section 502 of the Act and 21 CFR 809.10, FDA recommends that labeling for these devices address the items identified below. These labeling recommendations also help to mitigate the risks identified previously in this guidance to help ensure safe and effective use of these devices, particularly when a novel Influenza A virus may be emerging.

Your labeling should clearly describe the identity, phylogenetic relationship, or other recognized characterization of Influenza A viruses and specific Influenza A virus subtypes that your device is designed to detect, and the associated clinical aspects of human infection.

Intended Use

In addition to specific elements that describe the analyte detected, your intended use[i] should specify indications for testing respiratory specimens from patients with symptoms of respiratory illness and possibly a risk of exposure, and that the assay should be used in conjunction with other laboratory testing and clinical observations. FDA also recommends that your statement of intended use be clarified by a warning statement such as:

> *Negative results do not preclude influenza virus infection and should not be used as the sole basis for treatment or other patient management decisions*
>
> *Performance characteristics for influenza A were established when influenza A/H3 and A/H1 were the predominant influenza A viruses in circulation. When other Influenza A viruses are emerging, performance characteristics may vary.*
>
> *If infection with a novel Influenza A virus is suspected based on current clinical and epidemiological screening criteria recommended by public health authorities, specimens should be collected with appropriate infection control precautions for novel virulent influenza viruses and sent to state or local health departments for testing. Viral culture should not be attempted in these cases unless a BSL 3+ facility is available to receive and culture specimens.*

Directions for Use

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

The instructions for use provided with your test system should supply all instructions necessary to allow the test to achieve its claimed performance, as well as all limitations and warnings required for safe use of the test.

For test systems that call for ancillary reagents of concern (see Section 5.C) you should:

- Emphasize through conspicuous labeling that proper product performance requires use of specific ancillary reagents as directed. This labeling may include warnings against use of the device if specified ancillary reagents are not available.
- Assure that users can clearly identify which ancillary reagents are suitable for use with your test. For example, if only certain lots of a named ancillary reagent are appropriate for use, the labeling for your assay should identify those lots by number. (See 21 CFR 809.10(b)(8)(ii).)
- When your labeling calls for ancillary reagents that are supplied with instructions for use or other warnings or limitations by the ancillary reagent manufacturer, you should ensure that users of your assay will understand which instructions they should follow when using those ancillary reagents in your assay system. If there is a conflict between the directions and warnings provided by the manufacturer of the ancillary reagent and the instructions for use that you supply with your assay, you should assess and address the risk that users may mistakenly follow the labeling provided directly with the ancillary reagent and consequently obtain invalid results with your assay. We note that in some circumstances, statements in the labeling may not be sufficient to address the risks created by this conflict.

Quality Control

Quality control recommendations should include types of procedures and material that can be used as additional quality control measures, and the expected results for acceptability of control testing.

Precautions, Warnings, and Limitations

You should clearly describe any assay limitations in the labeling, including all appropriate limitations and warnings that a physician needs to know prior to ordering the test. We recommend that you incorporate directions for reporting results into the Results section, including a reminder to report results to state or local public health departments, if applicable.

Precautions

We recommend that you specify procedures for handling, storing, and disposing of specimens, including a reiteration and expansion of the procedures for working with specimens suspected to be infected with a novel influenza strain.

Limitations

In addition to any limitations and warnings that are relevant to your specific assay, we recommend providing statements, such as the following under Limitations (as applicable):

- *A trained health care professional should interpret assay results in conjunction with the patient's medical history, clinical signs and symptoms, and the results of other diagnostic tests.*
- *Analyte targets (viral sequences) may persist in vivo, independent of virus viability. Detection of analyte target(s) does not imply that the corresponding virus(es) are infectious, nor are the causative agents for clinical symptoms.*
- *The detection of viral sequences is dependent upon proper specimen collection, handling, transportation, storage and preparation (including extraction). Failure to observe proper procedures in any one of these steps can lead to incorrect results. There is a risk of false negative values resulting from improperly collected, transported, or handled specimens.*
- *There is a risk of false positive values resulting from cross-contamination by target organisms, their nucleic acids or amplified product, or from non-specific signals in the assay.*
- *There is a risk of false negative values due to the presence of sequence variants in the viral targets of the assay.*
- *It is recommended that specimens found to be negative after examination using this device be confirmed by an alternate method (e.g. cell culture). (Depending on the assay performance for specific analytes.) Additional testing for Influenza A, other Influenza A subtypes, or other respiratory infections may be required.*
- *Negative results (e.g., no Influenza A viral RNA detected and no specific Influenza A virus subtypes viral RNA detected) do not exclude influenza infection with other Influenza A viruses.*
- *The performance of the assay has not been established in individuals who received nasally administered Influenza A vaccine.*
- *Assay performance was not established in immunocompromised patients.*
- *Positive and negative predictive values are highly dependent on prevalence. The assay performance was established during the [e.g., 2006/2007 season]. The performance for some viruses may vary depending on the prevalence and population tested. False positive test results are likely when prevalence of disease due to Influenza A viruses or a specific Influenza A virus subtype is low or non-existent in a community.*
- *Additional testing is required to differentiate influenza type A and B viruses.* [If your device detects both Influenza A and B viruses, without distinguishing the two.]
- *Additional testing is required to differentiate any specific Influenza A subtypes or strains, in consultation with state or local public health departments.* [If your device detects influenza A and distinguishes it from influenza B viruses.]
- *If a specimen yields a positive test result for Influenza A, but produces negative test results for all specific influenza A subtypes intended to be*

differentiated (i.e., H1 or H3), this result requires notification of appropriate local, state, or federal public health authorities to determine necessary measures for verification in accordance with the MMWR notice (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5613a4.htm and http://www.cste.org/ps/2007pdfs/novelfluanndssjan10final23.pdf), to determine whether the specimen represents a novel strain of Influenza A.

If the pre-selected banked specimens were used for the estimation of the performance for any of the viruses or subtypes in the assay, there should be a limitation stating this, since the established performance of that specific virus or subtype does not reflect the performance or prevalence in the intended use population.

If positive or negative interference has been reported for any commonly used collection materials or substances that may be endogenously or exogenously introduced into a specimen prior to testing, you should advise users of the possibility of false negative or false positive results due to such interference.

Specimen Collection

We recommend that you state that inadequate or inappropriate specimen collection, storage, and transport are likely to yield false negative test results. We also recommend that you state that operator training in specimen collection is highly recommended because of the importance of specimen quality.

Performance Characteristics

We recommend that in your labeling you describe the population(s) (i.e., geographical location, specimen types, and age groups) whose specimens were tested to support performance characteristics. You should separately represent testing done on specimens from patient cases that are laboratory-confirmed with influenza due to the specific Influenza A virus subtype that your device is intended to detect.

You should include in the package insert a summary of study designs and the results from the studies (described in Section 6) that would aid users in interpreting test results. This section should include a description of the clinical (i.e., medical) and analytical (i.e., technical) performance characteristics. Clinical performance characteristics typically comprise prospective clinical study results summarizing performance (sensitivity, specificity or positive and negative percent agreement, 95% confidence intervals) for each virus type and subtype identified by your assay. In cases where some retrospective clinical samples were also used, these results should be presented separately from the prospective clinical study results, as positive and negative agreement for each virus type and subtype tested by the device. Analytical performance characteristics contain descriptions of the results and methodology used for the studies outlined in **Section 6**. In addition, analytical sensitivity levels (limits of detection) should be described in this section.

We recommend that the Performance Characteristics section describes the population(s) (i.e., geographical location, specimen types, and age groups) used to establish the

performance characteristics of the device and provide the season (e.g., calendar years of influenza season) when this evaluation took place, along with the predominant virus subtype(s) observed during that time.

We recommend that you stratify positive and negative test results from your submitted clinical study by specimen source(s) and age. We also recommend that you separate results for children <5 years of age, older children, and adults. If this information is not available, you should add a Warning statement such as "Differences in performance are expected when this test is used on specimens from adults versus children, but specific differences are not known."

If you represent results using standardized viral quantitation methods (such as WHO and CLSI) for various virus subtypes in the labeling, we also recommend qualifying the information with a statement such as "NOTE: Although the assay has been shown to detect cultured avian influenza viruses, including avian Influenza A subtype H5N1 virus, the performance characteristics of this test with specimens from humans infected with H5N1 or other avian influenza viruses are unknown." Such a statement may help avoid misleading users into thinking that this analytical information on the detection of specific cultured viruses applies to detection of these viruses in human clinical specimens.

Interpretation of Results

Your interpretation of the results section in the package insert should list all possible assay outputs and determinations of the presence or absence of each individual pathogen and assay control.

If internal controls are part of the determination of valid positive and negative results, you should provide the interpretation of each possible control result and recommendations on how to follow-up any invalid (i.e., no-call) result.

If your assay has an equivocal zone, you should provide the interpretation and the recommendation how to follow-up the equivocal result for each pathogen on your panel (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 invalid or equivocal result, you should provide the recommendation whether testing should be repeated from the same nucleic acid preparation, a new extraction, or a new patient specimen for each of these outputs.

If the assay results interpretation involves combining the outputs of several viruses and viral targets to get the results, as it would be the case in the respiratory viral panel assay that both detects Influenza A and differentiates Influenza A subtypes, there should be clear interpretation of valid and invalid output combinations, and recommendations for any required follow up or retesting in the case of the assay e.g., detecting Influenza A, but not any of the tested subtypes.

If your assay performance (i.e., sensitivity) for specific analyte(s) demonstrated a lower bound of the two-sided 95% CI as less than 90%, negative results for this analyte may need to be interpreted as presumptive prompting a recommendation for confirmation by an alternate method (e.g. cell culture).

Since your assay both detects Influenza A and differentiates between Influenza A subtypes, the interpretation of the results should direct the user that in the case a specimen yields a positive test result for Influenza A, but produces negative test results for all specific influenza A subtypes intended to be differentiated (i.e., H1 or H3), this result requires notification of appropriate local, state, or federal public health authorities to determine necessary measures for verification in accordance with the MMWR notice (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5613a4.htm and http://www.cste.org/ps/2007pdfs/novelfluanndssjan10final23.pdf), to determine whether the specimen represents a novel strain of Influenza A.

We recommend that you incorporate into the Results section directions for reporting results that include statements such as the following examples as applicable:

> *Report negative test results as "Influenza A virus not detected. This result does not exclude influenza viral infection."*
>
> *Report positive test results as "Positive for Influenza A virus. This result does not rule out co-infections with other pathogens or identify any specific influenza A virus subtype."*

Expected Values

This section should include the expected values using your test and the explanation of the result. It should also include the number of samples, age, gender, and demographics of the population used to determine the expected values.