Class II Special Controls Guideline: *In Vitro* Diagnostic Devices for *Bacillus* spp. Detection

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

Preface

Public Comment

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Table of Contents

I. INTRODUCTION **5**

II. *BACILLUS* SPP. - BACKGROUND **5**

III. SPECIAL CONTROLS - BACKGROUND **6**

IV. SCOPE **6**

V. RISKS TO HEALTH **7**

VI. SPECIFIC DEVICE DESCRIPTION REQUIREMENTS **8**

VI(A). Intended Use 8

VI(B). Reagents and Other Device Components 9

VI(C). Testing Procedures Using Your Device 9

VI(D). Specimen Storage and Shipping Conditions 9

VI(E). Interpreting Test Results/Reporting 9

VII. PERFORMANCE STUDIES **10**

VII(A). Analytical/Laboratory Performance Studies 10

VII(A)(1). Determination of Assay/Reagent Specificity 10

VII(A)(2). Precision/Reproducibility 11

VII(A)(3). Interfering/Inhibitory Substances 11

VII(A)(4). Effect of Culture Inoculation Density on Results With Bacteriophage Reagents (Bacteriophage Assays Using Culture Plating Methods) 12

VII(B). Clinical Information 12

VIII. LABELING **13**

VIII(A). Intended Use 13

VIII(B). Directions for Use 13

VIII(C). Precautions 14

VIII(D). Interpretation and Reporting of Assay Results 14

VIII(E). Performance Characteristics 15

IX. SPECIFIC REQUIREMENTS FOR *IN VITRO* DIAGNOSTIC DEVICES FOR *BACILLUS* SPP. DETECTION THAT USE NUCLEIC ACID AMPLIFICATION **15**

IX(A). Reagents and Other Device Components 16

IX(B). Testing Procedures 16

IX(C). Controls 17
IX(C)(1). Negative Controls 17
IX(C)(2). Positive Controls 17
IX(C)(3). Internal Control 18
IX(D). Performance Studies 18
IX(D)(1). Nucleic Acid Extraction 18
IX(D)(2). Assay Cut-off 19
IX(D)(3). Interpreting Test Results/Reporting 19
IX(D)(4). Analytical Sensitivity (Limit of Detection) 20
IX(D)(5). Carry-Over and Cross-Contamination Studies (For Multi-sample Assays and Devices That Require Instrumentation) 20
IX(D)(6). Interference/Inhibitory Substances 21
IX(D)(7). Precision/Reproducibility/Repeatability 21
X. REFERENCES 21

Class II Special Controls Guideline: In Vitro Diagnostic Devices for *Bacillus* spp. Detection

Guideline for Industry and Food and Drug Administration Staff

I. Introduction

This special controls guideline was developed to establish special controls for in vitro diagnostic devices for *Bacillus* species (spp.) detection.

This guideline identifies measures that FDA believes are necessary to mitigate the risks to health associated with devices of this type and provide a reasonable assurance of safety and effectiveness. Following the effective date of the final rule classifying the device,[1] manufacturers of in vitro diagnostic devices for *Bacillus* spp. detection[2] will need either to (1) comply with the particular mitigation measures set forth in the special controls guideline or (2) use alternative mitigation measures, which 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.

II. *Bacillus* Spp. - Background

An in vitro diagnostic device for *Bacillus* spp. detection is used to detect and differentiate among *Bacillus* spp. and presumptively identify *Bacillus anthracis* (*B. anthracis*) and other *Bacillus* spp. from cultured isolates or clinical specimens, as an aid in the diagnosis of anthrax and other diseases caused by *Bacillus* spp. This device may consist of *Bacillus* spp. antisera conjugated

[1] See [Microbiology Devices: Classification of In Vitro Diagnostic Devices for Bacillus Species Detection - published April 1, 2019 in the *FEDERAL REGISTER*]. In this final rule, FDA also limited distribution of the device to laboratories following public health guidelines that address appropriate biosafety conditions, interpretation of test results, and coordination of findings with public health authorities (see 21 CFR 866.3045(c)). FDA also restricted this device to prescription use (see 21 CFR 866.3045(d)).

[2] As stated in the preamble to the final rule, for currently marketed devices, FDA does not expect submission of documentation to FDA demonstrating compliance with the special controls set forth in sections VI, VII, and IX of the special controls guideline. Manufacturers of such devices must comply with the labeling special controls set forth in section VIII of the special controls guideline. FDA does not intend to enforce compliance with section VIII of the special controls guideline until April 1, 2020.

with a fluorescent dye (immunofluorescent reagents) used to presumptively identify bacillus-like organisms in clinical specimens; bacteriophage used to differentiate *B. anthracis* from other *Bacillus* spp. based on susceptibility to lysis by the phage; or antigens used to identify antibodies to *B. anthracis* in serum.

This guideline sets forth special controls for all devices of this type, including both the technologies described above and nucleic acid amplification-based *B. anthracis* assays. FDA has determined that several nucleic acid amplification-based *B. anthracis* assays are substantially equivalent to other devices within this preamendments type through the 510(k) process, and thus these nucleic acid amplification-based *B. anthracis* assays are also classified as class II devices and subject to the special controls and restrictions identified in FDA's final rule.[3]

Anthrax caused by *B. anthracis* is a disease of humans and animals. Human infections differ in clinical presentation depending on the portal of entry. The most common form is cutaneous anthrax, which results from entry through cuts or abrasions in the skin. Gastrointestinal anthrax is caused by ingestion of contaminated meat or other food products. Inhalation anthrax results from inhalation of spores and is fatal if untreated. *B. cereus* is a causative agent of gastrointestinal disease that is generally self-limiting; non-gastrointestinal infections also occur and are usually associated with trauma or surgery, implants, catheters and shunts.

III. Special Controls - Background

FDA believes that special controls, combined with 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 *in vitro* diagnostic devices for *Bacillus* spp. detection. Thus, 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,[4] (2) comply with the special controls identified in this guideline, and (3) obtain a substantial equivalence determination from FDA prior to marketing the device.

IV. Scope

The scope of this document is limited to devices as described in 21 CFR 866.3045:

> "(a) Identification. An *in vitro* diagnostic device for *Bacillus* spp. detection is used to detect and differentiate among *Bacillus* spp. and presumptively identify *B. anthracis* and other *Bacillus* spp. from cultured isolates or clinical specimens as an aid in the diagnosis of anthrax and other diseases caused by *Bacillus* spp. This device may consist of *Bacillus* spp. antisera conjugated with a fluorescent dye (immunofluorescent reagents) used to presumptively identify bacillus-like organisms in clinical specimens; bacteriophage used for differentiating *B. anthracis* from other *Bacillus* spp. based on susceptibility to lysis by the phage; or antigens used to identify antibodies to *B. anthracis* (anti-toxin and anti-

[3] See [Microbiology Devices: Classification of In Vitro Diagnostic Devices for Bacillus Species Detection - published April 1, 2019 in the *FEDERAL REGISTER*].
[4] For additional information regarding 510(k) submissions, refer to 21 CFR 807.87.

capsular) in serum. Bacillus infections include anthrax (cutaneous, inhalational, or gastrointestinal) caused by *B. anthracis,* and gastrointestinal disease and non-gastrointestinal infections caused by *B. cereus*."

This proposed classification currently consists of the following product codes:

NVQ [Bacteriophage and controls, *B. anthracis* lysis]

NPO [Kit, Immunochromatographic, *B. anthracis* differential antibody]

NRL [Enzyme linked immunoabsorbent assay, antibody, *B. anthracis*]

NHT [Assay, Nucleic Acid Amplification, *B. anthracis*]

NWZ [Gas chromatography, *B. anthracis* membrane fatty acids]

The special controls set forth in this guideline will apply to all devices classified under 21 CFR 866.3045 including those falling under product codes not yet established.

This guideline is *not* intended to address specific issues for testing environmental samples, or for samples collected to assess exposure by the presence/absence of spores on mucosal, skin or other surfaces. Methods and approaches for specific detection of spores alone are beyond the scope of this guideline.

V. Risks to Health

FDA has identified four risks to health associated with the use of in vitro diagnostic devices for *Bacillus* spp. detection. The risks are false negative test results, false positive test results, risks to laboratory workers from handling specimens and control materials, and unique device specific risks. The measures to mitigate these identified risks are summarized in Table 1.

Failure of *Bacillus* spp. devices to perform as indicated or an error in interpretation of results may lead to false negative and false positive test results. A false positive result may result in a patient undergoing unnecessary or ineffective treatment, and also could result in inaccurate epidemiological information on the presence of anthrax disease being publicized in a community, potentially leading to unnecessary prophylaxis and management of others. A false negative result may lead to delayed recognition by the physician of the presence or progression of disease and also could result in a failure to promptly recognize, control, and prevent additional infections. A false negative result could also potentially delay diagnosis and treatment of infection caused by *B. anthracis* or other *Bacillus* spp.

Exposure to organisms potentially present in test specimens and those used as control materials poses a risk of infection to laboratory workers.

Under this guideline, manufacturers submitting a 510(k) for a device of this type must conduct a risk analysis prior to submitting a 510(k) to identify any other risks specific to their device. The 510(k) 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 and Mitigation Measures

Identified Risks	Mitigation Measures
A false negative test result may lead to delay of therapy and progression of disease and failure to promptly recognize, control, and prevent disease in the community	• Section VI (Device Description Containing the Information Specified in the Special Controls Guideline) • Section VII (Performance Studies) • Section VIII (Labeling) • Section IX (Specific Measures for Nucleic Acid Amplification Devices)
A false positive test result may lead to unnecessary or ineffective treatment and incorrect epidemiological information being publicized, potentially leading to unnecessary prophylaxis and management of others	• Section VI (Device Description Containing the Information Specified in the Special Controls Guideline) • Section VII (Performance Studies) • Section VIII (Labeling) • Section IX (Specific Measures for Nucleic Acid Amplification Devices)
Biosafety risks to laboratory workers handling test specimens and control materials	• Section VI (Device Description Containing the Information Specified in the Special Controls Guideline) • Section VIII (Labeling)
Unique device specific risks	• Section V (Risks to Health)

VI. Specific Device Description Requirements

In your 510(k) submission, you must provide, as discussed more fully below, certain detailed information regarding the intended use of your device, the reagents and other device components, the testing procedures for your device, specimen storage and shipping conditions for the device, and interpreting tests results and reporting. 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.

VI(A). Intended Use

Your 510(k) must include labeling that describes the intended use of your product. Your stated intended use must specify the following:

- what the assay measures (e.g., *B. anthracis* cell surface protein or target DNA sequences from specific *B. anthracis* plasmids);

- the clinical indications for which the test is to be used;
- the specific population for which the test is intended, including clinical and demographic description of patients (e.g., sex, age, symptoms) for whom clinical performance has been demonstrated;
- whether the test is qualitative or quantitative; and
- the type(s) of specimens to be tested, for example, whole blood collected in sodium citrate from individuals suspected of having anthrax, positive blood cultures, or cultured organisms grown on blood agar.

You must also prominently provide the following statement at the end of your intended use statement:

"The distribution of in vitro diagnostic devices for Bacillus spp. detection is limited to laboratories that follow public health guidelines that address appropriate biosafety conditions, interpretation of test results, and coordination of findings with public health authorities."

VI(B). Reagents and Other Device Components

You must describe with particularity the reagents and other device components in your 510(k).

VI(C). Testing Procedures Using Your Device

In your 510(k), describe, in detail, the principles of operation applicable to your device for its intended use. Specifically describe testing conditions, procedures and controls designed to provide safeguards for conditions that can cause false positive and false negative results, or present a biosafety hazard. These include, but are not limited to, procedures, methods, and practices incorporated into your directions for use (see Section VIII) to mitigate risks associated with testing [Reference 1].

VI(D). Specimen Storage and Shipping Conditions

If you recommend specimen storage conditions in your labeling, demonstrate that your device generates equivalent results for the stored specimens at several time points throughout the duration of the recommended storage and at both ends of your recommended temperature range. If a transport medium is recommended in your labeling for storage or shipping, conduct appropriate studies to demonstrate that the device performs as described when the specimen is preserved in the transport medium.

VI(E). Interpreting Test Results/Reporting

In your 510(k), describe how presumptive positive, equivocal, and negative results are determined and how they should be interpreted, if applicable. Provide clear explanations for

how interpretative algorithms have been determined. Your labeling must indicate that test results should be reported as outlined in the current laboratory guidelines.

VII. Performance Studies

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

You must provide specific information in your 510(k) submission describing the protocols used during your assay development to allow FDA to accurately interpret acceptance criteria and data summaries contained in your submission during our review. When referring to Clinical and Laboratory Standards Institute (CLSI) protocols or guidelines, indicate which specific aspects of the protocols or guidelines were followed. Specific additional guidance for devices used in molecular diagnostic test methods and for devices used in immunological test methods can be found in CLSI MM3-A2 [Reference 2]. Relevant findings in published literature may also be cited. These specifics are also helpful to aid users in interpreting performance data in your labeling.

You may 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.

VII(A). Analytical/Laboratory Performance Studies

The appropriate types of analytical studies will depend on the applied technology, principles of operation and scientific evidence available. Where appropriate, you must establish the following performance characteristics for your device type in your 510(k). Additional types of analytical studies may be required, depending on the device type.

VII(A)(1). Determination of Assay/Reagent Specificity

For devices used to identify *B. anthracis* from cultures, characterize assay performance for bacteriophage and immunofluorescent antibody reagents using at least 25 different strains of *B. anthracis* (representing geographic and temporal diversity, including known genotypic and phenotypic variants), 10 strains of *B. thuringiensis*, and 10 strains of *B. cereus*, along with other representative *Bacillus* spp. and non-*Bacillus* Gram positive rods. If your device is indicated for direct specimen testing methods, also include organisms that could be expected to be found at the sampling site (e.g., both normal flora and other potential pathogens). Strains used for characterization of assay/reagent specificity can be selected from well-characterized archives or repositories. Definitive species identification of *Bacillus* spp. may call for a combination of phenotypic and genotypic methods (e.g., biochemical, antigenic, morphology, plasmid characterization, genotyping).

Evaluate antigens for anti-*Bacillus anthracis* antibody testing using human sera from naturally infected humans and those immunized with anthrax vaccine. Test sera from at least 200 human

samples, including those from individuals with compatible diseases and conditions, for specificity.

VII(A)(2). Precision/Reproducibility

Characterize within-day, day-to-day, intra-laboratory, and inter-laboratory reproducibility. Include in the reproducibility panel samples around the cutoff of the assay, at low and moderate positive concentrations, and at high negative concentrations. If results of your device are interpreted by a test platform, also conduct precision studies with all instruments recommended for use with your device (see Reference 3).

VII(A)(3). Interfering/Inhibitory Substances

Provide information and data to demonstrate that potentially interfering or inhibitory substances encountered in specific specimen types do not affect results. See Table 2 for a list of potentially endogenous and exogenous interfering substances that could be present in clinical specimens (e.g., blood, sputum, culture, etc.). If interference or inhibition has been reported in the literature or is evident in your studies, provide validated procedures or methods that can be used to avoid erroneous results.

Table 2 – List of Evaluated Potentially Interfering Substances

Endogenous Substances	Exogenous Substances	Solvents*	Technique-specific Substances
• Hemoglobin • Albumin • Bilirubin • Triglycerides • Cholesterol (total) • Immunoglobulins • Glucose	• Acetaminophen • Amoxicillin • Ascorbic acid • Aspirin • Cefotaxime • Chloroquine • Ciprofloxacin • Doxycycline • Erythromycin • Gentamicin sulfate • Ibuprofen • Naproxen sodium • Rifampin • Streptomycin • Sulfamethoxazole • Tetracycline • Tobramycin • Trimethoprim	• Acetone • DMSO • Ethanol • Ammonia Solution • Bleach	• DTT • B-acetylcysteine • Guanidine HCL • Triton X-100 • Isopropanol • Methanol

Endogenous Substances	Exogenous Substances	Solvents*	Technique-specific Substances
	• Acid-citrate-dextrose • Citrate (sodium) • EDTA • Heparin • Sodium polyanethol • sulfonate (SPS) • Albuterol (Salbutamol) • Cromolyn sodium • Flunisolide • N-acetylcysteine • Blood culture media • Sheep blood agar media		

*These are solvents used to dissolve potentially interfering substances in preparation for testing.

Include in your studies the effect of culture age and growth media with specific antibody and nucleic acid reagent testing from cultures (solid or liquid). Include the results in the package insert in the Performance Characteristics section and also as a limitation statement to inform the user that cultures older than a specified number of days may result in a false negative result.

VII(A)(4). Effect of Culture Inoculation Density on Results With Bacteriophage Reagents (Bacteriophage Assays Using Culture Plating Methods)

Conduct studies to demonstrate whether there is a risk of a false negative test result due to heavy culture inoculation when using bacteriophage-specific assays. One technique may be to streak a suspect culture for isolation and add bacteriophage to areas of the plate with varying amounts of inoculum. Also assess phage titer and stability of bacteriophage reagents.

VII(B). Clinical Information

Provide information to demonstrate the reliability of your device for detecting *Bacillus* spp. in each type of clinical specimen that you indicate as suitable for testing with your device. In general, when the number of human clinical samples available for clinical testing is very low or non-existent, the available evidence for FDA's premarket review may, of necessity, be obtained from analytical rather than clinical studies; spiked human samples and/or animal samples may be adequate. In this circumstance, it is critical to have well designed analytical studies. Animal

studies may be used to supplement analytical studies where appropriate.[5] Performance assessments are to be relative to the known presence or absence of a characterized *Bacillus* spp., or to the definitive identification of culture growth. Where appropriate, multiple tests and methods may be needed to appropriately identify/characterize a *Bacillus* spp. recovered from human specimens by culture methods or detected directly in human specimens.

Also provide data from testing specimens from the intended use population (e.g., patients with febrile illnesses or skin lesions). Because anthrax would not be expected in a prospective evaluation, do not represent these data as specificity, but rather as agreement with an expected negative result.

For devices used to identify culture isolates or growth, clinical evaluations are not applicable so long as studies with culture stocks reasonably represent fresh culture growth and conditions for testing (e.g., 12 -18 hours growth from 5% sheep blood agar plates).

VIII. Labeling

Your labeling for devices for *Bacillus* spp. detection must include the information described below to help to ensure that users understand the appropriate uses of the device.

VIII(A). Intended Use

The intended use statement must clearly specify the intended specimen type(s), whether the testing to be performed is qualitative, semi-quantitative, or quantitative, the testing methodology, along with the indicated patient population and other conditions for use as appropriate.

The following statement must be included at the end of your intended use statement:

"The distribution of in vitro diagnostic devices for Bacillus spp. detection is limited to laboratories that follow public health guidelines that address appropriate biosafety conditions, interpretation of test results, and coordination of findings with public health authorities."

VIII(B). 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 the use of additional control measures and testing of control materials to ensure use in a safe and effective manner.

You must address the following in the directions for use:

[5] FDA supports the principles of the "3Rs," to reduce, refine, and replace animal use in testing when feasible. We encourage sponsors to consult with us if they wish to use a non-animal testing method they believe is suitable, adequate, validated, and feasible. We will consider if such an alternative method could be assessed for equivalency to an animal test method.

- Emphasize appropriate storage conditions for reagents and identify reagents that are temperature, humidity, and/or light sensitive.

- When testing requires a culture of *Bacillus* spp., specify the appropriate type of culture media from which growth is to be tested, incubation conditions, and length of incubation (including minimum and maximum incubation times).

- Provide directions for using control reagents that are provided with the product, as well as required or recommended control materials that may be used but are not provided with the product. Describe each aspect of the testing procedure that is controlled. Provide acceptable values for the control reagent testing and justification for your selected values.

- Provide instructions for biosafety precautions with specimen handling and testing procedures. Specify at which procedural step the test sample is non-infectious.

- For products that rely on antigen/antibody reactions in the testing procedure, include recommendations for reducing the risk of a false negative test result due to prozone or Hook effect.

VIII(C). Precautions

The precaution section of the package insert must include a statement that reads:

"The interpretation of test results requires experienced clinical personnel who have training in principles and use of microbiological culture identification methods and infectious disease diagnostics and have the necessary awareness to report an identification of B. anthracis and coordinate with local or state public health directors."

If a limitation statement is appropriate under Section VII(A)(3), then a limitation statement must also be added in the precaution section of the package insert to inform the user that cultures older than a specified number of days may result in a false negative result.

VIII(D). Interpretation and Reporting of Assay Results

Your discussion in the product labeling of interpretation and reporting of assay results must include:

- Identification of each of the possible device results (e.g., positive, equivocal or indeterminate, and negative). Describe how the operator is to interpret these test results, and give acceptance and rejection criteria for controls. You must include recommendations for how to proceed if control results are not acceptable.

- Clear and exact criteria for evaluating a test result as positive, negative, or equivocal/indeterminate. If appropriate, use photographs and/or diagrams to indicate how to interpret results for tests that give a qualitative result. Provide the likelihood of a *B. anthracis* identification (for culture identification reagents), the likelihood of infection

(for reagents detecting specific anti-*B. anthracis* antibodies), or the likelihood of *B. anthracis* presence (for reagents detecting *B. anthracis* directly in patient specimens) based on reliable information available from the literature or other sources.

- For tests that rely on antigen/antibody interactions to detect bacterial cell components or bacterial products, recommendations for reducing the risk of a false negative test result due to non-optimal initial inoculum density.

- Testing to detect intact bacteria or bacterial products may give different results with samples that contain spores versus those that contain vegetative cells only, or a mixture of spores and vegetative cells. Include the varying results in your labeling. If appropriate, use photographs and diagrams to show results that can be expected if a sample contains spores.

- For tests to detect an antibody response to *B. anthracis*, a warning statement concerning the interpretation of a positive test and how it does not by itself conclusively establish recent infection, as persons immunized with an anthrax vaccine may test positive with this test in the absence of any natural exposure to *B. anthracis*. Indicate in the labeling that antibiotic treatment early in natural infection with *B. anthracis* may decrease the antibody response and therefore may give a negative result with this test.

- A statement that *B. anthracis* is a nationally notifiable disease that must be reported to public health authorities in accordance with state and local law. Your labeling must indicate that the test should be used and reported as outlined in current laboratory guidelines.

VIII(E). Performance Characteristics

Your labeling must include a summary of the study designs and study results described in Section VII of this guideline that would aid the user in interpreting test results and understanding device performance. This section must include clinical and analytical performance characteristics. If the assay was not evaluated using specimens from individuals presenting signs and symptoms of anthrax, instruct users to establish the clinical sensitivity of this test on prospectively collected clinical specimens as these specimens become available. Data for negative agreement/clinical specificity must be included and indicated clearly to the users.

IX. Specific Requirements for In Vitro Diagnostic Devices for *Bacillus* spp. Detection That Use Nucleic Acid Amplification

In vitro diagnostic devices for *Bacillus* spp. detection that employ nucleic acid amplification are used to determine the presence of pathogenic *Bacillus* spp. directly in human specimens and/or blood or colony cultures derived from clinical specimens by detecting nucleic acid sequences or regions that are unique to *Bacillus* spp. and which discriminate *Bacillus* spp. pathogen from other microbial organisms. These devices include primers, probes, enzymes and specific controls for

amplification and are designed for use in specific instrument systems. Detection of *B. anthracis* by a nucleic acid amplification detection system aids in the definitive identification of *B. anthracis* in infected patients in conjunction with other laboratory results and clinical presentation. The following are additional specific requirements for this type of in vitro diagnostic device for *Bacillus* spp. detection.

IX(A). Reagents and Other Device Components

In your 510(k) for devices based on nucleic acid amplification that are intended for presumptive detection of *B. anthracis* DNA in human specimens or blood or colony cultures or liquid culture derived from clinical specimens, describe the device design of your device and explain how the design addresses or mitigate risks of false negative or false positive results associated with primers, probes, instruments, and controls used in a nucleic-acid test procedure to detect targeted DNA segments from *B. anthracis.* Some examples are given below:

- Designing your freeze-dried sets of reagents or any other closed tube test system (e.g., self-containing cartridge) to minimize false positives due to contamination or carryovers.
- Designing one or more assay for targeting different DNA sequences unique to *B. anthracis*.
- Developing positive, negative, and inhibition controls to ensure accurate test results.
- Developing methods for extraction and purification that yield suitable quality and quantity of DNA from human specimens, blood, colony cultures, or liquid culture derived 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), provide performance information supporting the conclusion that your device design meets the criteria set forth above. Provide the rationale for your selection of specific DNA target sequences and your selection of primers and probes (see Section VII).

List the specific extraction method recommended for each specimen type by name and catalog number in the package insert of your device (see Section VIII).

IX(B). Testing Procedures

Your 510(k) submission must include the following information:

- Overall design of the testing procedure, including control elements incorporated into the recommended testing procedures. These controls must approximate the lower range of clinically relevant *Bacillus* DNA levels and must be extracted as a clinical sample.

- Development, description, or recommendations for additional external or internal positive and negative controls that monitor for contamination and extraction efficiency (e.g., an internal control as a control for nucleic acid extraction and inhibition). Also include features and additional controls that reduce failure to recognize procedural errors or factors (e.g., degradation of master mix) that adversely affect amplification and detection conditions.

IX(C). Controls

When conducting the performance studies described below, run appropriate external controls every day of testing for the duration of the analytical and clinical studies. You may contact the Division of Microbiology Devices for further information regarding controls. For devices based on nucleic acid technology, include the following types of controls:

IX(C)(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. It may not be needed 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, the whole organism (other than *B. anthracis).* 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- *B. anthracis* infected individual.
- Samples containing a non-target organism.

IX(C)(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 DNA 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 *B. anthracis* infected individual or spiked matrices with live *B. anthracis.*

- *B. anthracis* culture isolates.

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.

IX(C)(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 (e.g., polymerase, primers, etc.), equipment function (e.g., 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 *B. anthracis* DNA and primers amplifying human housekeeping genes (e.g., RNaseP, β-actin). The need for this control is determined on a case-by-case basis by FDA.

IX(D). Performance Studies

For studies intended to determine the performance characteristics of a device based on nucleic acid amplification based device, include in the 510(k) submission the information described below. This section supplements the performance studies requirements described earlier in this document (see Section VII).

IX(D)(1). Nucleic Acid Extraction

Different extraction methods may yield *B. anthracis* DNA of varying quantity and quality, and therefore the extraction method can be crucial to a successful result. Purification of *B. anthracis* DNA from the clinical specimens or liquid blood culture or colony culture specimens can be challenging because biological samples may contain low bacterial loads in the background of human genomic DNA, as well as high levels of proteins and other contaminants.

For these reasons, evaluate the effect of your chosen extraction methods on the performance of the assay with respect to satisfactory *B. anthracis* DNA quantity and quality for the intended use of the assay. In addition, evaluate your assay's analytical and clinical performance characteristics using the entire analytical process (including extraction procedures) that you designate for use with your assay. Include demonstrating the Limit of Detection (LoD) and reproducibility of your assay with each extraction procedure. Instructions for conducting the LoD study are provided under "Limit of Detection" (see Section IX(D)(4) below). In addition, external site studies (including reproducibility and clinical studies) are to 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 you simply instruct users concerning appropriate reagents.

If you recommend or include multiple extraction methods, 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. 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 needs to 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 method A, B, and C, and site 3 extraction method A, B and C).

In addition to the analytical studies (LoD and Reproducibility), each extraction method is to 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 different nucleic acid extraction methods) are not considered equivalent and cannot be pooled, but rather must be analyzed separately. As a result, additional prospective clinical samples must be included in order to support the claimed extraction method.

IX(D)(2). Assay Cut-off

Explain how the cut-off(s) was determined (see also Section IX(D)(3) below) as well as how it was validated. You must determine the cut-off using appropriate statistical methods and provide support for your determined cut-off. To support the cut-off you determined, you may provide for example, 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 *B. anthracis* DNA 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 [Reference 4]). If the assay has an equivocal zone, 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) needs to be validated in an independent population consistent with the defined intended use of your device.

IX(D)(3). Interpreting Test Results/Reporting

Describe how presumptive positive, negative, equivocal, or invalid results are determined and how they are to be interpreted, if applicable. Clearly explain how interpretative algorithms have been determined. The information must include:

- The cut-off value for defining a negative result of the assay. If the assay has only two output results (negative or positive), this cut-off also defines a positive result of the assay.
- If the assay has an equivocal zone, cut-off values (limits) for the equivocal zone.

- If your interpretation of the initial equivocal results requires re-testing, (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 must be developed before the pivotal clinical study that evaluates the clinical performance of the assay).

- If one of the reported outputs of your assay can be an equivocal result, the interpretation and recommendation for how the user should follow up the equivocal results.

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

IX(D)(4). Analytical Sensitivity (Limit of Detection)

Determine the limit of detection (LoD) of your assay at the preclinical stage in each appropriate specimen type using your device. The study needs to include testing serial dilutions of viable (live) *B. anthracis* in replicates of 3-5. Each dilution is to be made using *B. anthracis* negative pooled human samples such as blood or sputum or an equivalent matrix. Report the LoD as the level of *B. anthracis* that gives a 95% detection rate. Based on the titration results, the LoD must be further confirmed by preparing at least 20 additional replicates at the LoD concentration and demonstrating that *B. anthracis* was detected ≥ 95% of the time. The LoD must be established using organism preparations quantified in units of CFU/ml and/or DNA copy numbers

IX(D)(5). Carry-Over and Cross-Contamination Studies (For Multi-sample Assays and Devices That Require Instrumentation)

Demonstrate that carry-over and cross-contamination do not occur with your device. In a carry-over and cross-contamination study, high positive samples are to be used in series alternating with high negative samples in patterns dependent on the operational function of the device. Perform at least 5 runs with alternating high positive and negative samples. The high positive samples in the study need to 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 need to 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 95%.

IX(D)(6). Interference/Inhibitory Substances

Test the effects of potentially endogenous interfering substances encountered in blood, human specimens, colony cultures, or liquid culture derived from clinical specimens and exogenous interfering substances that could be introduced during sample purification or reaction set-up. These interfering substances may interfere with assay performance. Include the tabulated data for the evaluated endogenous and exogenous interfering substances for your device in the submission.

IX(D)(7). Precision/Reproducibility/Repeatability

Conduct within-laboratory precision studies for devices that include instruments or automated components. Characterize within-day, day-to-day, intra-laboratory, and inter-laboratory precision. Follow the protocol for a nucleic acid amplification assay below:

- Perform reproducibility studies at three sites (two external and one in-house site).
- Use 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.
- Have at least two operators each day at each facility perform the test.

Prepare reproducibility panels by spiking each matrix (e.g., blood, human specimens, colony cultures, or liquid culture derived from clinical specimens) with *B. anthracis* at a low (near LoD), medium, or high level. Negative sample panels must be unspiked specimens for each matrix.

Each panel is to consist of 6-9 samples that include three levels of analyte as described below:

- A “high negative” sample with a concentration of analyte below the clinical cut-off so that re-testing of this sample is negative approximately 95% 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.

You may refer to the CLSI document EP15-A2 [Reference 6], EP5-A2 [Reference 3], and EP12-A2 [Reference 7] for guidance on reproducibility study design.

X. References

1. Clinical and Laboratory Standards Institute (CLSI). Procedures for the Handling and Processing of Blood Specimens for Common Laboratory Tests; Approved Guideline,

Fourth Edition. CLSI document GP44-A4. Wayne, PA: Clinical and Laboratory Standards Institute; 2010.

2. Clinical and Laboratory Standards Institute (CLSI). Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline, Third Edition. CLSI document MM03. Wayne, Pennsylvania: Clinical and Laboratory Standards Institute; 2015.

3. Clinical and Laboratory Standards Institute (CLSI). Evaluation of Precision of Quantitative Measurement Procedures; Approved Guideline, Third Edition. CLSI document EP05-A3. Wayne, Pennsylvania: Clinical and Laboratory Standards Institute; 2014.

4. Clinical and Laboratory Standards Institute (CLSI). Assessment of the Diagnostic Accuracy of Laboratory Tests Using Receiver Operating Characteristics Curves; Approved Guideline, Second Edition. CLSI document EP24-02. Wayne, Pennsylvania: Clinical and Laboratory Standards Institute; 2011.

5. Clinical and Laboratory Standards Institute (CLSI). Interference Testing in Clinical Chemistry; Approved Guideline, Third Edition. CLSI document EP07. Wayne, Pennsylvania: Clinical and Laboratory Standards Institute; 2018.

6. Clinical and Laboratory Standards Institute (CLSI). User Verification of Precision and Estimation of Bias; Approved Guideline, Third Edition. CLSI document EP15-A3. Wayne, Pennsylvania: Clinical and Laboratory Standards Institute; 2014.

7. Clinical and Laboratory Standards Institute (CLSI). User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline, Second Edition. CLSI document EP12-A2. Wayne, Pennsylvania: Clinical and Laboratory Standards Institute; 2008.