Class II Special Controls Guideline Document: Toxin Gene Amplification Assays for the Detection of *Clostridium difficile*

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

Office of *In Vitro* Diagnostics and Radiological Health
Division of Microbiology Devices

Preface

Public Comment

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I. Introduction

This special controls guideline was developed to support the classification of a *Clostridium difficile* toxin gene amplification assay into class II (special controls).

This guideline identifies measures that FDA considers as mitigations to the risks to health associated with these devices and that provide a reasonable assurance of safety and effectiveness. Firms submitting a 510(k) premarket notification for a *Clostridium difficile* toxin gene amplification assay will need either to (1) comply with the particular mitigation measures set forth in the special controls guideline or (2) use alternative mitigation measures, but demonstrate to the Agency's satisfaction that those alternative measures identified by the firm will provide at least equivalent assurance of safety and effectiveness.

II. *Clostridium difficile* – Background

Clostridium difficile (*C. difficile*) is a gram-positive, spore-forming anaerobic bacillus consisting of toxigenic and non-toxigenic strains. When a colonized individual has been treated with certain antibiotics, disruption of the colonic flora may cause toxigenic *Clostridium difficile* to establish and produce toxin in the colon. Typically two types of toxin are produced: toxin A (enterotoxin, *TcdA*), and toxin B (cytotoxin, *TcdB*); toxin B is the primary virulence factor. The genes that encode toxin A and toxin B are part of the pathogenicity locus (PaLoc) that is found in toxigenic strains. Most pathogenic strains are toxin A and B positive (A+B+) but some pathogenic variant isolates may be toxin A negative, B positive (A-B+). Some strains of toxigenic *Clostridium difficile* also produce a toxin called *C. difficile* transferase (CDT), or binary toxin. The binary toxin locus contains two genes, *cdtA* and *cdtB,* that are not part of the PaLoc. The clinical presentation of *Clostridium difficile* infection (CDI) varies from mild self-limiting diarrhea to severe, bloody antibiotic associated diarrhea to the development of pseudo membranous colitis and toxic

megacolon. The mode of infection is the oral-fecal route. The populations at risk for infection are primarily institutionalized patients (e.g., patients in hospitals and nursing homes) who have received antibiotic treatment. In the last several years, there have been outbreaks of CDI caused by hypervirulent and fluoroquinolone-resistant strains belonging to PCR ribotype 027, PFGE type NAP1, and REA type B1. CDI is also being recognized as a cause of diarrhea among people in community settings without recent in-patient hospital exposure.

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 a *Clostridium difficile* toxin gene amplification assay. 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,[1] (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 the following device described in 21 CFR 866.3130:

21 CFR 866.3130. *Clostridium difficile* toxin gene amplification assay

> *(a) Identification.* A *Clostridium difficile* toxin gene amplification assay is a device that consists of reagents for the amplification and detection of target sequences in *Clostridium difficile* toxin genes in fecal specimens from patients suspected of having *Clostridium difficile* infection (CDI). The detection of clostridial toxin genes, in conjunction with other laboratory tests, aids in the clinical laboratory diagnosis of CDI caused by *Clostridium difficile.*

V.Risks to Health

FDA has identified four risks to health associated with the use of the device. The risks are false negative test results, false positive test results, failure of the test to be used or perform properly, and failure to properly interpret the test results. The measures to mitigate these identified risks are summarized in the table below.

FDA has identified the risks of false negative test and false positive test results, both of which can lead to individual and public health consequences, as issues of safety and

[1] For additional information regarding 510(k) submissions, refer to 21 CFR 807.87 and the Center for Devices and Radiological Health (CDRH) Device Advice (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/default.htm).

effectiveness associated with this device. Device failure leading to no result (e.g., due to failure of reagents, instrumentation, data management, or software) or an invalid result could delay diagnosis and could require additional specimen collection. Failure of devices for detection of *C. difficile* toxin genes to perform as expected or failure to interpret results correctly may lead to incorrect patient management decisions. In the context of individual patient management, a false negative report could lead to delays in providing (or failure to provide) a definitive diagnosis, appropriate treatment, infection control and prevention measures. A false positive report could lead to unnecessary or inappropriate treatment or unnecessary control and prevention actions.

Under this guideline, manufacturers who intend to market a device of this type must conduct a risk analysis prior to submitting a premarket notification to identify any other risks specific to their device. The premarket notification must describe the risk analysis method used. If you elect to use an alternative approach to mitigate a particular risk identified in this guideline, or if you or others identify additional potential risks from use of a device of this type, you must provide sufficient detail regarding the approaches used to mitigate these risks and a justification for your approach.

Table 1 – Identified Risks and Mitigation Measures

Identified Risk	Mitigation Measures
A false positive test result for an individual may lead to inappropriate use of antibiotics for treatment	• Section VI (Specific Device Description Requirements) • Section VII (Performance Studies) • Section VIII (Labeling)
A false negative test result for an individual may lead to a potential delay in antibiotic treatment	• Section VI (Specific Device Description Requirements) • Section VII (Performance Studies) • Section VIII (Labeling)
Failure of the test to be used or perform properly	• Section VIII (Labeling)
Failure to properly interpret test results	• Section VIII (Labeling)

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, the test results your device may yield, and how the user of the device should interpret the test results.

VI(A). Intended Use

Your 510(k) must include labeling that describes the intended use of your product. You must clearly state the clinical indications for which the test is to be used, the nature of the analyte, the target to be detected and if there are possible limitations to device. The intended use must include the patient population to be tested, specimen type for which testing will be indicated, whether the test is qualitative and any specific conditions of use. You must specify whether your device is to be used as an adjunct to other laboratory tests and clinical findings.

VI(B). Reagents and Other Device Components

When describing reagents and other device components in your 510(k), you must describe in detail your design requirements for the device that address or mitigate risks associated with reagents and instruments used in a toxin gene amplification test procedure to detect target sequences in *Clostridium difficile* toxin genes. The performance data in the 510(k) must support the conclusion that design requirements have been met. Examples include:

- Designing your reagent for use in a closed tube test system (e.g., self-containing cartridge) to minimize false positives due to amplicon or carryover contamination.
- Developing methods for extraction and purification that yield suitable quality and quantity of nucleic acid from patient samples for use in the test system with your reagents.
- Optimizing your reagents and test procedure for recommended instruments.

You must include illustrations or photographs of any non-standard equipment or methods, if applicable.

VI(B)(1). Instruments – Hardware and Software

For instruments and systems that measure multiple signals, and for other complex laboratory instrumentation that has not been previously cleared, refer to FDA's guidance document entitled "Class II Special Controls Guidance Document: Instrumentation for Clinical Multiplex Test Systems" (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm077819.htm) for details on the types of instrument-related data you must provide to support clearance.

If your system includes software, you must submit software information detailed in accordance with the level of concern associated with your software. The level of concern must be driven by a hazard analysis in the absence of mitigations (i.e., the hazard analysis must be performed as though none of the individual hazard mitigations were present). The level of concern of *in vitro* diagnostic devices of this type is expected to typically be moderate because software flaws could indirectly affect the patient and potentially result in

injury when the healthcare provider and patient do not get accurate information. The level of concern is based on how the operation of the software associated with the functioning of the device could affect the patient or operator and is defined below.

- Major - The level of concern is Major if (1) a failure or latent flaw could directly result in death or serious injury to the patient or operator and/or (2) if a failure or latent flaw could indirectly result in death or serious injury of the patient or operator through incorrect or delayed information or through the action of a care provider.
- Moderate – The level of concern is Moderate if (1) a failure or latent design flaw could directly result in minor injury to the patient or operator and/or (2) if a failure or latent flaw could indirectly result in minor injury to the patient or operator through incorrect or delayed information or through the action of a care provider.
- Minor - The level of concern is Minor if failures or latent design flaws are unlikely to cause any injury to the patient or operator.

See Table 2 below for the software documentation required in the 510k submission dependent on the level of concern associated with the subject device.

Table 2 - Required Documentation Based on Level of Concern

SOFTWARE DOCUMENTATION	MINOR CONCERN	MODERATE CONCERN	MAJOR CONCERN
Level of Concern	A statement indicating the Level of Concern and a description of the rationale for that level.		
Software Description	A summary overview of the features and software operating environment.		
Device Hazard Analysis	Tabular description of identified hardware and software hazards, including severity assessment and mitigations.		
Software Requirements Specification (SRS)	Summary of functional requirements from SRS.	The complete SRS document.	
Architecture Design Chart	No documentation is necessary in the submission.	Detailed depiction of functional units and software modules. May include state diagrams as well as flow charts.	
Software Design Specification (SDS)	No documentation is necessary in the submission.	Software design specification document.	
Traceability Analysis	Traceability among requirements, specifications, identified hazards and mitigations, and Verification and Validation (V&V) testing.		
Software Development	No documentation	Summary of software life	Summary of software life cycle

Environment Description	is necessary in the submission.	cycle development plan, including a summary of the configuration management and maintenance activities.	development plan. Annotated list of control documents generated during development process. Include the configuration management and maintenance plan documents.
Verification and Validation Documentation	Software functional test plan, pass / fail criteria, and results.	Description of V&V activities at the unit, integration, and system level. System level test protocol, including pass/fail criteria, and tests results.	Description of V&V activities at the unit, integration, and system level. Unit, integration and system level test protocols, including pass/fail criteria, test report, summary, and tests results.
Revision Level History	Revision history log, including release version number and date.		
Unresolved Anomalies (Bugs or Defects)	No documentation is necessary in the submission.	List of remaining software anomalies, annotated with an explanation of the impact on safety or effectiveness, including operator usage and human factors.	

You must clearly describe how raw signals are converted into a result including adjustment to the background signal for normalization, if applicable. You must also include the following information for software development and implementation in the submission:

- System and Software Requirements
- Hazard Analysis
- Architecture Design Chart
- Software Design Specification
- Software Development Environment Description
- Verification and Validation
- Traceability Analysis
- Unresolved Anomalies

Before beginning clinical studies, the configuration of the hardware and software components must be very similar or identical to the final version of the device. A risk assessment must be performed if any significant changes are made to the hardware or software after the completion of the clinical studies and before the clearance and distribution of the device.

For additional information on how FDA believes the level of concern is to be determined as well as additional descriptions of software documentation, see FDA's guidance entitled "The Content of Premarket Submissions for Software Contained in Medical Devices" (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm089543.htm).

Below are additional references to help you develop and maintain your device under good software life cycle practices consistent with FDA regulations:

- The guidance entitled "General Principles of Software Validation" (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm085281.htm).
- The guidance entitled "Off-the-Shelf Software Use in Medical Devices; Final Guidance for Industry and FDA Staff" (http://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm073779.pdf).
- The guidance entitled "Guidance for the Content of Premarket Submissions for Software Contained in Medical Devices" (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm089543.htm).
- 21 CFR 820.30 Subpart C – Design Controls of the Quality System Regulation.
- ISO 14971 -1; Medical devices - Risk management - Part 1: Application of Risk Analysis.
- AAMI 62304:2006; Medical device software - Software life cycle processes.

VI(B)(2). Ancillary Reagents

Ancillary reagents are those reagents that a 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 only, 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.[2]

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

If the instructions for use of your device specify one or more ancillary reagents of concern, you must 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 must address the elements described below. FDA will evaluate whether the information submitted is sufficient to support a demonstration that your device is substantially equivalent to a legally marketed device.

- You must include in your 510(k) a risk assessment addressing the use of ancillary reagents, including risks associated with management of reagent quality and variability. This assessment must include risks associated with inconsistency between instructions for use provided directly with the ancillary reagent and those supplied by you with your assay, and any other issues that could present a risk of obtaining incorrect results with your assay.
- Using your risk assessment as a basis for applicability, you must describe with particularity in your 510(k) how you intend to mitigate risks through implementation of any necessary controls over ancillary reagents. These include, where applicable:
    - User labeling to assure appropriate use of ancillary reagents.
    - 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 affect the performance of the assay.

[2] Even if you establish that one or more alternative ancillary reagents may be used in your assay, each of those named alternatives may still be an ancillary reagent of concern. If you are unsure whether this aspect of the special controls applies to your device, contact the Division of Microbiology Devices in the Office of *In Vitro* Diagnostics and Radiological Health (OIR).

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

If you have questions regarding identification, use, or control of ancillary reagents, contact the Division of Microbiology Devices in the Office of *In Vitro* Diagnostics and Radiological Health (OIR) regarding your planned study.

VI(C). Testing Procedures Using your Device

In your 510(k), you must provide a detailed description of the principles of operation for your device, including those for detecting target sequences in toxin genes from *Clostridium difficile*. You must specifically describe testing conditions, procedures, and controls designed to safeguard against conditions that can cause false positive and false negative results. These include, but are not limited to:

- Overall design of the testing procedure, including control elements incorporated into the recommended testing procedures. Control materials must approximate the lower range of clinically relevant nucleic acid levels and must be extracted as a clinical sample.
  - Description of, or recommendations for, any internal controls (e.g., internal controls that monitor contamination, extraction efficiency, and amplification inhibition).
  - Features and additional controls that monitor procedural errors or factors (e.g., degradation of the master mix) that adversely affect amplification and detection.

You must include a description of all additional procedures, methods, and practices incorporated into your directions for use (See Section VIII - Labeling) that mitigate risks associated with testing for *Clostridium difficile* toxin genes.

VI(D). Interpreting Test Results/Reporting

In your 510(k), you must describe in detail how positive, negative, equivocal, or invalid results are determined and how they are to be interpreted by the intended user. You must indicate the cut-off values for all outputs of the assay.

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

If the assay has an equivocal zone, you must provide ranges (limits) for the equivocal zone and recommendations for how the user should follow up the equivocal results. If your interpretation of the initial equivocal results requires retesting, your 510(k) must address:

- Whether retesting should be done by the same assay or a different method.
- Whether retesting should be repeated from the same preparation, a new extraction, or a new patient specimen.
- An algorithm for defining a final result by combining the initial equivocal result and the results after retesting if retesting is done by the same assay as the initial testing. This algorithm must be developed before the pivotal clinical studies that evaluate the clinical performance of the assay.

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

VII. Performance Studies

VII(A). General Study Requirements

In your 510(k), you must provide detailed descriptive information on the studies you conducted to establish each of the performance characteristics outlined below. In general, for the analytical studies to establish precision and for the clinical studies, you must conduct testing at three sites, representative of where you intend to market the device (i.e., clinical laboratory sites).

You must evaluate performance of your assay for each specimen type recommended.

In order to accurately interpret acceptance criteria or data summaries during the review, you must provide appropriate specific information concerning protocols. When referring to Clinical and Laboratory Standards Institute (CLSI) guidelines or standards, you must indicate which specific aspects of the guidelines or standards were followed. If you have further questions regarding your planned study and the clinical claims you intend to support, contact the Division of Microbiology Devices in OIR before initiating the study.

VII(B). Controls

When conducting the performance studies described below, you must run appropriate external controls every day of testing for the duration of the analytical and clinical studies.

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. For devices based on gene amplification technology, include the following types of controls:

VII(B)(1). Negative Controls

Blanks or no template control
The blank, or "no-template" control, contains buffer or specimen 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. This control 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, it contains the whole organism. It reveals non-specific priming or detection and indicates that signals are not obtained in the absence of target sequences. An example of an acceptable negative sample control material is a patient specimen from a non-*Clostridium difficile* infected individual.

VII(B)(2). Positive Controls

Positive control for complete assay
The positive assay control contains target toxin gene sequences 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. Acceptable positive assay controls include whole organisms containing target sequences detected by the device.

Positive control for amplification and detection
The positive control for amplification and detection contains purified target nucleic acid at or near the limit of detection for a qualitative assay and is not usually taken through the nucleic acid extraction process. It verifies the integrity of the instrument and the reaction components when negative results are obtained. It indicates that the target can be detected if it is present in the extracted sample. Examples of this type of control include DNA plasmids containing the target sequence, or purified full length double stranded genomic DNA from a toxigenic *C. difficile* isolate.

In some cases where a positive control for the complete assay is included, a separate positive control for amplification and detection would not be needed.

VII(B)(3). Internal Control

The internal control is a non-target nucleic acid sequence that is co-extracted and/or co-amplified with the target nucleic acid. It controls for integrity of the reagents (polymerase,

primers, etc.), equipment function (thermal cycler), and the presence of inhibitors in the samples. Examples of acceptable internal control materials include human nucleic acid co-extracted with the *Clostridium difficile* 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. Refer to Clinical and Laboratory Standards Institute (CLSI) document MM3-A2, *Molecular Diagnostic Methods for Infectious Disease* [Ref. 1], for additional information.

VII(C). Analytical Performance Studies

You must establish the following performance characteristics for your *Clostridium difficile* toxin gene assay in your 510(k):

VII(C)(1). Analytical Sensitivity

Limit of Detection

You must determine the limit of detection (LoD) for your assay. Your study must include serial dilutions of at least two strains of toxigenic *Clostridium difficile* and three to five replicates for each dilution tested in negative stool matrix. One strain tested must be NAP 1, Ribotype 027, Toxinotype III (Tox A+/B+). You must report the LoD as the level of analyte that gives a 95% detection rate. The LoD must be confirmed by preparing at least 20 additional replicates at the LoD concentration and demonstrating that the strains were detected 95% of the time. Portions of the serial dilution can be cultured and quantified in units of CFU/mL and used to determine CFU/reaction. All analytical studies must be conducted using whole organisms and must be based on multiples of the LoD expressed in CFU/mL. You must determine the LoD for each analyte in the matrix tested by the device. Refer to CLSI document EP17-A2 [Ref. 2], when designing your studies. Some examples of strains for inclusion in your LoD studies are shown in Table 3.

Table 3 - Toxigenic strains of *C. difficile* for analytical sensitivity (reactivity and LoD) studies

Strain	Toxinotype and/or Toxin
ATCC 43255 (CCUG19126, VPI 10463)	0, A+B+
ATCC 9689 (90556-M6S)	0, A+B+
ATCC 700792 (14797-2)	A+B+
ATCC 17858 (1253)	A+B+
ATCC BAA-1805	III, A+B+
ATCC BAA-1382 (630)	A+B+
ATCC 51695 (BDMS 18 AN)	A+B+

ATCC 43600 (2149)	A+B+
ATCC 43599 (2022)	A+B+
ATCC 43596 (545)	A+B+
ATCC 43594 (W1194)	A+B+
ATCC 17857 (870)	A+B+
ATCC 43598 (1470)	VIII, A-B+
CCUG 8864	X, A-B+

Assay Cut-off and Equivocal Zone
In your submission, you must explain how the assay cut-off was determined and how the cut-off values were validated (see also the section VI(D)). The cut-off must be determined using appropriate statistical methods. For example, you may provide a result distribution, 95th and 99th percentiles, percent of the non-negative (positive or equivocal) results, and other statistics, for the clinical samples without any toxigenic *C. difficile* 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. If the assay has an equivocal zone, you must explain how you determined the limits of the equivocal zone. The performance of your device using the pre-determined cut-off (and equivocal zone, if applicable) must be validated in an independent population consistent with the defined intended use of your device.

Analytical Reactivity (Inclusivity)
You must demonstrate that the test can detect at least twenty strains of toxigenic *C. difficile* in addition to the strains used in the LoD study. These must be well characterized clinically relevant *C. difficile* isolates of varying strains and toxinotypes representing temporal and geographical diversity. The diversity of the strains tested for reactivity must be supported by inclusion of at least five different toxinotypes in the study. The dilutions must be made in pooled *C. difficile* negative human stool sample at concentrations that are two to three times the LoD. Relevant strains reactivity studies include, but are not limited to, the strains shown in Table 3.

VII(C)(2). Analytical Specificity

Cross-reactivity
You must test for potential cross-reactivity with medically relevant levels of viruses, fungi, and bacteria (usually 10^6 CFU/mL or higher for bacteria and fungi, and 10^5 PFU/ml or higher for viruses). Bacteria and viruses' identities and titers must be confirmed by actual counts rather than a theoretical calculation deduced from estimation. Relevant microorganisms for cross reactivity testing include, but are not limited to, the microorganisms listed in Table 4.

Table 4 - Microorganisms for analytical specificity (cross-reactivity) studies.

Genera and Species	Strain
Abiotrophia defective	ATCC 49176
Acinetobacter baumannii	ATCC 19606
Aeromonas hydrophila	ATCC 7966
Alcaligenes faecalis subsp. Faecalis	ATCC 15554
Bacillus cereus	ATCC 13472
Bacteroides fragilis	ATCC 25285
Campylobacter coli	ATCC 43479
Campylobacter jejuni sub sp .jejuni	ATCC 33292
Candida albicans	ATCC 10231
Citrobacter freundii	ATCC 8090
Clostridium bifermentans	ATCC 638
Clostridium butyricum	CCRI-11128
Clostridium haemolyticum	ATCC 19398
Clostridium novyi	ATCC 19402
Clostridium orbiscindens	ATCC 49531
Clostridium perfringens	ATCC 13124
Clostridium scindens	ATCC 35704
Clostridium septicum	ATCC 12464
Clostridium sordellii	ATCC 9714
Clostridium difficile (non-toxigenic)	ATCC 43593
Clostridium difficile (non-toxigenic)	ATCC 43601
Clostridium sporogenes	ATCC 15579
Edwardsiella tarda	ATCC 15947
Enterobacter aerogenes	ATCC 13048
Enterobacter cloacae	ATCC 13047
Enterococcus faecalis vanB	ATCC 51299
Escherichia coli	ATCC 23511
Escherichia coli O157:H7	ATCC 700927
Helicobacter pylori	ATCC 43504
Klebsiella oxytoca	ATCC 33497
Lactobacillus acidophilus	ATCC 4356
Listeria monocytogenes	ATCC BAA-389
Peptostreptococcus anaerobius	ATCC 27337
Plesiomonas shigelloides	ATCC 14029
Porphyromonas asaccharolytica	ATCC 25260
Prevotella melaninogenica	ATCC 25845
Proteus mirabilis	ATCC 25933
Providencia alcalifaciens	ATCC 9886
Pseudomonas aeruginosa	ATCC 35554
Salmonella choleraesuis (typhimurium)	ATCC 14028
Salmonella enterica subsp. Arizonae	ATCC 13314
Salmonella enterica subsp. Enterica	ATCC 7001
Serratia liquefaciens	ATCC 27592

Genera and Species	Strain
Serratia marcescens	ATCC 13880
Shigella boydii	ATCC 9207
Shigella dysenteriae	ATCC 11835
Shigella sonnei	ATCC 29930
Staphylococcus aureus	ATCC 43300
Staphylococcus epidermidis	ATCC 14990
Streptococcus agalactiae	ATCC 12973
Vibrio parahaemolyticus	ATCC 17802
Adenovirus	
Rotavirus	
Norovirus	
Enterovirus	
Echovirus	
Coxsackie virus	
Cytomegalovirus	
Human DNA	

Interference

You must conduct a comprehensive interference study using medically relevant concentrations of the interferent and more than one strain of toxigenic *C. difficile* to assess the potentially inhibitory effects of substances encountered in blood and stool specimens. Potentially interfering substances include, but are not limited to, biological and chemical substances occasionally used or found in peri-anal, rectal and/or stool specimens, blood, and mucus. Additional examples of potentially interfering substances are presented in Table 5. You must test interference at the assay cut-off determined for each *C. difficile* strain and for each of the interfering substances. You must evaluate each interfering substance at its potentially highest concentration ("worst case"). If no significant clinical effect is observed, no further testing is necessary. Refer to the CLSI document EP7-A2 [Ref. 3] for additional information.

Table 5 - Substances for interference studies

Substance	Active Ingredient
Anti-Fungal /Anti-Itch Vaginal	Nystatin
Creams/Ointments/Suppositories	Hydrocortisone
Anti-Hemorrhoid Creams/Ointments	Phenylephrine
Antacids	Calcium Carbonate/ Aluminum Hydroxide/ Magnesium Hydroxide
Enemas	Mesalazine/Mineral Oil
Condoms with Spermicidal Lubricant	Nonoxynol-9
Anti-Diarrheal Medication	Loperamide Hydrochloride/ Bismuth Subsalicylate
Laxatives	Sennosides
Antibiotics (Oral and Topical)	Antibiotic
Non-Steroidal Anti-Inflammatory	Naproxen Sodium

Moist Towelettes	Benzalkonium Chloride, Ethanol
Fecal Fat	Lipids etc.
Blood	Glucose, Hormones, Enzymes, Ions, Iron, etc.
Mucus	Immunoglobulins, Lysozyme, Polymers, etc.

VII(C)(3). Precision

Within-Laboratory Precision/Repeatability
Depending on the device methodology, within-laboratory precision studies may or may not be required; contact the Division of Microbiology Devices regarding the need for within-laboratory precision studies for your device. If precision studies are required, you must conduct these studies using the instruments and/or automated components anticipated for use in your clinical study. You may perform these studies in-house.

You must test sources of variability (e.g., operators, days, assay runs) for a minimum of 12 days (not necessarily consecutive), with two operators, each performing two runs per day, and two replicates of each sample per run. These test days must span at least two calibration cycles if the calibration cycle is shorter than two months. The test panel must consist of 3-6 positive samples with organisms at two levels in addition to a negative sample as follows:

- A negative sample: a sample with no analyte such that results of repeated tests of this sample are negative 100% of the time.
- 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.
- A "moderate positive" sample: a sample with a concentration at which 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 cutoff, 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%.[3] For details, see CLSI EP17-A2 [Ref. 2],. CLSI documents EP5-A2 [Ref. 4], and EP12-A [Ref. 5], which contain further information about designing and performing precision studies.

Between Laboratory Reproducibility
The protocol for the reproducibility study may vary slightly depending on the device format. In general, the protocol must:

- Use the same sample panel as described in the repeatability study above.
- Evaluate the reproducibility of your test at three testing sites (e.g., at least two external sites and one in-house site).

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

- Use a five-day testing protocol, including a minimum of two runs per day, (unless the assay design precludes multiple runs per day) and three replicates of each panel member per run, with at least two operators at each facility perform the test each day. A minimum of 90 replicates must be tested for each analyte and each concentration.

If you are considering clearance for rapid test devices that are likely to be used in point-of-care settings, you may contact the Division of Microbiology Devices.

The CLSI document, EP15-A2 [Ref. 6], contains additional information on reproducibility study design.

VII(C)(4). Carry-Over/Cross-contamination Studies

Multi-sample assays and devices that require instrumentation
For multi-sample assays and devices that require instrumentation, you must demonstrate that carry-over and cross-contamination do not occur with your device (including the nucleic acid extraction method). In a carry-over study, high positive samples must be used in series alternating with negative samples in patterns dependent on the operational function of the device. You must include at least five runs with alternating high positive and negative samples during the carry-over study. The high positive sample in the carry-over study must be high enough to exceed 95% or more of the results obtained from specimens of positive patients from the intended use population. Negative samples must be samples with no analyte such that results of repeated tests are negative 100% of the time. The carry-over and cross-contamination effect can then be estimated by the percent of negative results for the negative samples that are adjacent to high positive samples in the carry-over study compared to the percent of negative results in the absence of adjacent high positive samples. For details, see Haeckel [Ref. 7].

VII(C)(5). Specimen Collection, Handling, Storage, and Shipping Conditions

If you recommend specimen collection, transport, and storage conditions, you must demonstrate that your device generates equivalent results using specimens that are handled in the same manner as recommended in the device package insert. For the specimen storage conditions, you must demonstrate that your device generates equivalent results for the stored specimens at several time points throughout the duration of the recommended storage and at both ends of your recommended temperature range. If transport media is recommended for storage or shipping, you must conduct appropriate studies to demonstrate that the device will perform as described when the specimen is preserved in transport media.

VII(C)(6). Nucleic Acid Extraction Methods

You must conduct analytical and clinical studies to demonstrate the efficiency and reproducibility of your recommended nucleic acid extraction procedure(s) for use for claimed specimen types. These analytical studies must include determination of the Limit of

Detection (LoD) for each claimed specimen type as well as reproducibility studies for each claimed specimen type (see Sections VII(C)(1) and VII(C)(3)). The reproducibility evaluation for the nucleic acid extraction must be conducted at three sites (e.g., two external and one in-house site) in the matrix specified in your labeling, at analyte concentrations near the clinical cut-off.

If you choose to obtain clearance for multiple extraction methods, you must demonstrate the LoD and reproducibility for each method. If the extraction method introduces minimum variability to the overall assay performance, combining the extraction method variable with each site performance variable may be an acceptable alternative to evaluating every extraction method at every site. 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 described above do not show significant differences, no further reproducibility studies are needed. However, if the initial extraction equivalency studies from the three sites indicate statistically significant differences in assay performance, the reproducibility study must be expanded to include testing each extraction method at three study sites (e.g., site 1 extraction method A, site 2 extraction method A, and site 3 extraction method A).

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

VII(C)(7). Expected Values

You must establish the range of expected values as obtained with your device in a population with signs and symptoms consistent with CDI. You must assay a statistically relevant number of specimens that are representative of the intended use including the specified sample matrix. You must provide these results based on your new device performance, and summarize the distribution of the population according to specimen type, age and geographic location.

VII(D). Clinical Performance Studies

You must conduct prospective clinical studies to determine the performance of your device for all the specimen types you claim in your labeling. You must prospectively collect specimens from individuals with signs and symptoms consistent with clinical suspicion of CDI. All results must include 95% two-sided confidence intervals. For methods of

calculation of confidence intervals (CI), see CLSI EP12-A2 [Ref. 5]. You must compare your device to toxigenic culture which is comprised of culture followed by a cytotoxin assay performed on cultured isolates. Toxigenic culture is a widely recognized reference method for evaluating the performance of devices that aid in the diagnosis of *C. difficile* infection. .

If you have questions about the use of alternative comparator methods in the clinical performance studies, contact the Division of Microbiology Devices.

VII(D)(1). Study Protocol

You must develop a detailed study protocol that includes patient inclusion and exclusion criteria, type and number of specimens needed, description of the reference method, directions for use, and a statistical analysis plan that accounts for variances to prevent data bias. The study inclusion and exclusion criteria must match the intended use population of your device. A minimum set of demographic characteristics, including age, sex, and signs and symptoms must be captured. You must include this and any other relevant protocol information in your 510(k).

Contact the Division of Microbiology Devices to request a review of your proposed studies and selection of appropriate specimen types as part of the pre-submission review process, particularly if you plan to submit a premarket notification for the first time. For more information regarding the pre-submission process, see FDA's guidance entitled "Requests for Feedback on Medical Device Submissions: The Pre-Submission Program and Meetings with Food and Drug Administration Staff" (http://www.fda.gov/downloads/medicaldevices/deviceregulationandguidance/guidancedocuments/ucm311176.pdf).

VII(D)(2). Study Sites

You must conduct your studies at a minimum of three different geographical sites (one of which may be in-house) representing the testing environment where the device will ultimately be used (e.g., clinical laboratory) and by individuals likely to perform the test in clinical practice. At least two of the study sites must be US sites.

VII(D)(3). Study Population

You must conduct your studies on individuals who meet the study inclusion and exclusion criteria for suspected CDI. You must provide all relevant clinical, laboratory, and demographic information when available including age, sex, and signs and symptoms of CDI.

You must include a meaningful number of samples from each age group. You must 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.

VII(D)(4). Presentation of Results

In your 510(k), you must describe in detail how the samples were selected, and include reasons that samples were excluded. You must include a data analysis plan to account for all subjects enrolled and for all specimens collected.

You must initially analyze and present data from each study site separately to evaluate any inter-site variation and include results of the analysis in the 510(k). It may be possible to pool clinical study results from the individual sites in the package insert if you can demonstrate that there are no significant statistical or clinical differences in the results or populations among sites.

Consult with the Division of Microbiology Devices prior to study initiation regarding proposed studies that may include retrospective or banked specimens to supplement specimens from prospectively enrolled subjects.

You must provide line listings of the study data, including appropriate daily external control testing data during all analytical and clinical studies in an acceptable electronic format that supports independent data analyses (e.g., Microsoft Excel, delimited text files, or SAS files).

VIII. Labeling

Your labeling for devices for *C. difficile* toxin gene detection must include the information described below to help ensure users understand the appropriate uses of the device and how to use the device properly.

VIII(A). Intended Use

The intended use statement must specify the analyte detected by your assay, the clinical indications for which the test is to be used, and the specific population for which the test is intended. The intended use statement must specify whether the test is qualitative or quantitative and must state that it serves only as an aid in diagnosis.

VIII(B). Device Description

You must provide a detailed description of the test methodology used in your device.

VIII(C). Directions for Use

You must provide clear and concise instructions that delineate the technological features of the specific device and how it is to be used on patients. You must include a general description of the analysis procedure, from sample collection up to, and including result reporting. Instructions must encourage users to familiarize themselves with the features of the device and to adhere to these instructions to use the device safely and effectively. You

must include handling and storage instructions. You must describe stability (i.e. expiration dating) under the open and closed storage conditions that you recommend to users.

VIII(D). Limitations

You must clearly describe any assay limitations including those that a health care provider needs to know prior to ordering the test. In addition to any warnings and precautions relevant to your assay, you must also include the applicable limiting statements listed below:

- False negative test results may occur from improper specimen collection, handling or storage, presence of inhibitors, technical errors, or because the number of organisms in the specimen is below the analytical sensitivity of the test.
- A negative result should not be used as the sole basis for treatment or other patient management decisions.
- The positive and negative predictive values of the test may vary depending on the prevalence of infection.
- This test does not distinguish between viable and non-viable toxigenic *C. difficile* organisms.
- Mutations or polymorphisms in primer or probe binding regions may affect detection of new or unknown *C. difficile* variants resulting in a false negative result with the assay. A negative test result does not preclude infection with toxigenic *C. difficile*.
- This test is for use with liquid or soft human stool specimens. Performance characteristics of other specimen types have not been established.
- This test is not for determining susceptibility of *C. difficile* to antimicrobial agents.
- This test does not differentiate the *C. difficile* 027/NAP1/BI strains from other toxigenic genotypes.
- Results are to be interpreted in conjunction with other clinical and laboratory findings.

VIII(E). Expected Values

You must include the range of expected values (e.g., frequency of positive results) obtained with your device with an explanation of the results. You must also summarize the study used to determine the range of expected values, including the number of samples, age, sex, and demographics of the population.

VIII(F). Performance Characteristics

You must include in the package insert a summary of the study designs and the results of the studies described in Section VII that would aid users in interpreting test results. This includes clinical and analytical performance characteristics.

IX. References

1. Clinical and Laboratory Standards Institute. 2006. Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline-Second Edition. MM3-A2. Clinical and Laboratory Standards Institute, Wayne PA

2. Clinical and Laboratory Standards Institute. 2012. Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures; Approved Guideline - Second Edition. EP17-A2. Clinical and Laboratory Standards Institute, Wayne PA.

3. Clinical and Laboratory Standards Institute. 2005. Interference Testing in Clinical Chemistry; Approved Guideline. EP7-A2. Clinical and Laboratory Standards Institute, Wayne PA.

4. Clinical and Laboratory Standards Institute. 2004. Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline - Second Edition. EP5-A2. Clinical and Laboratory Standards Institute, Wayne PA.

5. Clinical and Laboratory Standards Institute. 2008. User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline - Second Edition. EP12-A2. Clinical and Laboratory Standards Institute, Wayne PA.

6. Clinical and Laboratory Standards Institute. 2008. User Verification of Performance for Precision and Trueness; Approved Guideline - Second Edition. EP15-A2. Clinical and Laboratory Standards Institute, Wayne PA.

7. Haeckel R. Proposals for the Description and Measurement of Carry-over Effects in Clinical Chemistry. Pure Appl. Chem. 1991; 63:302-306.