Class II Special Controls Guideline: Gastrointestinal Microorganism Multiplex Nucleic Acid-Based Assays for Detection and Identification of Microorganisms and Toxin Genes from Human Stool Specimens

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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

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Preface

Public Comment

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Guideline for Industry and Food and Drug Administration Staff

I. Introduction

This special controls guideline was developed to support the classification of a gastrointestinal microorganism multiplex nucleic acid-based assay into class II (special controls).

This guideline identifies measures that FDA believes will mitigate the risks to health associated with these devices and provide a reasonable assurance of safety and effectiveness. Firms submitting a 510(k) for a gastrointestinal microorganism multiplex nucleic acid-based 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 an equivalent assurance of safety and effectiveness.

II. Gastrointestinal Microorganisms – Background

Infectious gastroenteritis is an inflammation of the stomach and intestines caused by certain viruses, bacteria, or parasites. Common symptoms include vomiting and diarrhea, which can be more severe in infants, the elderly, and people with suppressed immune systems. Gastroenteritis can be spread easily through person-to-person contact and contaminated food, water, and surfaces.

The Centers for Disease Control and Prevention reports that between 1999 and 2007 gastroenteritis-associated deaths in the United States increased from nearly 7,000 to more than 17,000 per year. Norovirus and *Clostridium difficile* accounted for two-thirds of the deaths.[1]

III. Premarket Notifications – 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 gastrointestinal microorganism multiplex nucleic acid-based 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,[2] (2) comply with the special controls identified in this guideline, and (3) obtain a substantial equivalence determination from FDA prior to marketing the device.

This guideline identifies the classification regulation for gastrointestinal microorganism multiplex nucleic acid-based assay. In addition, other sections of this guideline list the risks to health and describe mitigation measures that, if followed by manufacturers and combined with the general controls, will address the risks associated with these devices and will generally lead to a timely premarket notification (510(k)) review.

This document will supplement other FDA documents regarding the specific content requirements of a premarket notification submission for a gastrointestinal microorganism multiplex nucleic acid-based assay. You must also comply with the documents entitled "Class II Special Controls Guidance Document: Instrumentation for Clinical Multiplex Test Systems" (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm077819.htm) and "Class II Special Controls Guidance Document: Norovirus Serological Reagents" (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm295088.htm).

IV. Scope

The scope of this document is limited to devices identified and classified under 21 CFR 866.3990.

[1] http://www.cdc.gov/media/releases/2012/p0314_gastroenteritis.html

[2] For additional information regarding 510(k) submissions, refer to 21 CFR 807.87, the Center for Devices and Radiological Health (CDRH) Device Advice (http://www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/default.htm), FDA's guidance entitled "Guidance for the Content of Premarket Submissions for Software Contained in Medical Devices" (http://www.fda.gov/downloads/MedicalDevices/.../ucm089593.pdf), and FDA's guidance entitled "Format for Traditional and Abbreviated 510(k)s" (http://www.fda.gov/ MedicalDevices/DeviceRegulationandGuidance/ GuidanceDocuments/ucm084365.htm).

21 CFR 866.3990. Gastrointestinal microorganism multiplex nucleic acid-based assay

> (a) *Identification.* A gastrointestinal microorganism multiplex nucleic acid-based assay is a qualitative *in vitro* diagnostic device intended to simultaneously detect and identify multiple gastrointestinal microbial nucleic acids extracted from human stool specimens. The device detects specific nucleic acid sequences for organism identification as well as for determining the presence of toxin genes. The detection and identification of a specific gastrointestinal microbial nucleic acid from individuals exhibiting signs and symptoms of gastrointestinal infection aids in the diagnosis of gastrointestinal infection when used in conjunction with clinical evaluation and other laboratory findings. A gastrointestinal microorganism multiplex nucleic acid-based assay also aids in the detection and identification of acute gastroenteritis in the context of outbreaks.

The microorganisms detected and identified by devices of this type include the following microorganism types, subtypes and toxin genes:

Campylobacter (*C. jejuni*, *C. coli* and *C. lari* only)	Norovirus GI/GII
Clostridium difficile (*C. difficile*) toxin A/B	Rotavirus A
Cryptosporidium (*C. parvum* and *C. hominis* only)	*Salmonella*
Escherichia coli (*E. coli*) O157	Shiga-like Toxin producing *E. coli* (STEC) stx 1/stx 2
Enterotoxigenic *Escherichia coli* (ETEC) LT/ST	*Shigella* (*S. boydii*, *S. sonnei*, *S. Flexneri*, and *S. dysenteriae*)
Giardia (*G. lamblia* only – also known as *G. intestinalis* and *G. duodenalis*)	

This document is not intended to address non-nucleic acid-based methodologies or devices that target microorganisms, toxin genes, or some combination of microorganisms and toxin genes other than those listed in the table above.

V. Risks to Health

FDA has identified the risks of failure of (1) the device to detect and identify a targeted organism when such organism is present in the specimen (i.e., false negative test result for presence of organism); (2) detection of the targeted microorganism when such organism is not present in the specimen (i.e., false positive test result for presence of organism), both of which can lead to individual and/or public health consequences; and (3) failure to correctly interpret test results as the risks to health associated with this device that require special controls. The measures to mitigate these identified risks are summarized in the table below.

Failure of the device to detect and identify a targeted organism when such organism is present in the specimen (i.e., false negative result) may lead to a delay in finding the true cause of the gastrointestinal infection, additional diagnostic tests, and unnecessary treatment or inappropriate

antibiotic use. For certain microorganisms targeted by the device, failure of detection may contribute to incorrect patient management to prevent transmission of infection, or delay recognition of an outbreak. An incorrect positive test result (i.e., false positive result) also may lead to unnecessary or ineffective antibiotic therapy and a delay in determining the true cause of the patient's illness, which for some microorganisms may lead to a more serious infection. Additionally, in the context of public health, a false positive test result may lead to misallocation of resources used for disease surveillance and prevention.

Failure to correctly interpret test results in the context of other clinical and laboratory findings may lead to inappropriate or delayed treatment. For example, a microorganism present as a colonizer may be correctly detected but not be the true cause of illness. Although this identical risk would be present from use of any microbiological assay in this setting, simultaneous testing of multiple analytes in a multiplex assay may be more likely to detect an unanticipated colonizer that might not be tested for individually.

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 Risks	Mitigation Measures
Failure of the device to detect and identify a targeted organism when such organism is present in the specimen (i.e., false negative result for presence of organism)	• Section VI (Specific Device Description Requirements) • Section VII (Performance Studies) • Section VIII (Labeling)
Detection of the targeted microorganism when such organism is not present in the specimen (i.e., false positive result for presence of organism)	• Section VI (Specific Device Description Requirements) • Section VII (Performance Studies) • Section VIII (Labeling)
Failure to correctly interpret test results	• Section VI (Specific Device Description Requirements) • 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, test methodology, specimen storage

conditions, ancillary reagents, controls, interpreting test results and reporting, and instrumentation and software.

VI(A). Intended Use

Your 510(k) must include labeling that describes the intended use of your product. The intended use must specify the specimen type (e.g., human stool specimens), the microorganisms that the device detects and identifies, the nature of the analyte and target (e.g., RNA, DNA, or both RNA and DNA), the clinical indication(s) for which the test is to be used, and the specific population(s) for which the test is intended (including disclaiming age groups if there is not adequate data to support the use of the device in patients in that age group). The intended use must also state whether the test is qualitative or quantitative, whether analyte detection is presumptive, and any specific conditions of use (e.g., whether the test is intended to be used in conjunction with other laboratory tests). The intended use must clearly state that the device is to serve only as an aid in diagnosis of gastrointestinal infections. Additional qualifications may be appropriate based on the results of the clinical studies.

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

- The identity of the microorganisms that your device is designed to detect.
- How the device test results will be used to aid in laboratory identification of gastrointestinal microorganisms.

VI(B). Test Methodology

You must describe in detail the methodology used by your device. This must include describing the following elements as applicable to your device:

- Test platform (e.g., real-time PCR, hybridization assay, bead arrays).
- Specificity of the microorganism sequences being detected (i.e., methodologies used in addition to the evaluation of clinical specificity to demonstrate that the target sequence detected only the microorganism of interest).
- Information regarding the rationale for the selection of specific target sequences and the methods used to design detection elements.
- Limiting factors of the assay (e.g., saturation level of hybridization, maximum cycle number).
- Device design to minimize false positives due to contamination or carryover.
- Sample types (e.g., stool or stool in preservative holding media).
- Specimen collection and handling methods, including methods and instrumentation for collection, stabilization, and concentration of specimens.
- Reagent components provided or recommended for use, and their function within the system (e.g., buffers, enzymes, fluorescent dyes, chemiluminescent reagents, other signaling/amplification reagents).
- The potential for specific and non-specific probe cross-hybridization.

- Description of any internal controls and external controls specific to use with the device.
- Instrumentation and software involved in the use of your device, including the components and their function within the system.
- The computational path from raw data to the reported result (e.g., how raw signals are converted into a value), if appropriate. This would include sufficient software controls for identifying and dealing with obvious problems in the dataset. It would also include adjustment for background and normalization, if applicable.
- Illustrations or photographs of non-standard equipment or methods, as appropriate, and provide a detailed description.

When applicable, you must describe design control specifications for your device that address or mitigate risks associated with primers, probes and controls used in nucleic-acid based multiplex test procedures to detect organism nucleic acid, such as the following:

- Minimization of false positives due to contamination or carryover of a sample.
- Prevention of probe cross-contamination for multiplexed tests in which many probes are handled during the manufacturing process.
- Correct placement and identity of assay features (e.g., probes).
- Use of multiple probes to enable detection of variants caused by mutations within the target nucleic acid segment(s), or variants within a designated viral, bacterial, or parasitic strain (or lineage).
- Development of, or recommendations regarding, validated methods for nucleic acid extraction and purification that yield suitable quality and quantity of microorganism nucleic acids for use in the test system with your reagents. You must address suitable validated extraction method(s) for the different specimen types claimed in the device intended use.
- Optimization of your reagents and test procedure for the recommended instrument(s).

In your 510(k), you must provide performance information that supports the conclusion that your design control specifications have been met. You must also provide information to verify the design of your reagents (e.g., rationale for selection of specific primers/probes). See Section VII – Performance Studies.

VI(C). Specimen Storage Conditions

If you recommend 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 period. Each recommended storage temperature must be evaluated, and for wide temperature ranges, both ends of the range must be evaluated (e.g., test both 15ºC and 30ºC for a room temperature claim of 15-30ºC).

VI(D). Ancillary Reagents

Ancillary reagents are reagents specified in device labeling as "required but not provided" in order to carry out the assay as indicated in its instructions for use and to achieve the test performance claimed in labeling for the assay. For example, for purposes of this document, if your device labeling specifies the use of a specific reagent (e.g., 'Brand X Extraction Buffer' or other buffers shown to be equivalent), and use of any other extraction buffer may alter the performance characteristics of your device from that reported in your labeling, then Brand X Extraction Buffer or other buffers shown to be equivalent are ancillary reagents of concern. 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. You may consult with the Division of Microbiology Devices in the Office of *In Vitro* Diagnostics and Radiological Health (OIR) at FDA if you are unsure whether this aspect of the special controls applies to your device.

By contrast, if your device relies on 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 include the elements described below. FDA will evaluate whether the information submitted is sufficient to support a demonstration that your device is at least as safe and effective, that 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, 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 in your 510(k) how you intend to mitigate risks through implementation of any necessary controls over ancillary reagents. These may include, where applicable:
    - User labeling to assure appropriate use of ancillary reagents (see "Labeling" for further discussion).
    - Plans for assessing user compliance with labeling instructions regarding ancillary reagents.
    - Material specifications for ancillary reagents.
    - Identification of reagent lots that will allow appropriate performance of your device.

- Stability testing.
- Complaint handling.
- Corrective and preventive actions.
- Plans for alerting users in the event of an issue involving ancillary reagents that would impact the performance of the assay.
- Any other issues that must be addressed in order to assure safe and effective use of your test in combination with identified ancillary reagents, in accordance with your device's instructions for use.

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

If you have questions regarding identification, use, or control of ancillary reagents, you may contact the Division of Microbiology Devices in OIR to obtain advice.

VI(E). Controls

You must describe with particularity the following concerning quality control and calibration in your premarket submission:

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

Controls must provide information about (1) sample quality, (2) nucleic acid quality, and (3) process quality. You may contact the Division of Microbiology Devices in OIR when designing specific controls for your device. Generally you must include the following types of controls:

VI(E)(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 applicable for assays performed in single-test, disposable cartridges or tubes.

Negative sample control
The negative sample control contains non-target nucleic acid or, if used to evaluate extraction procedures, it contains whole microorganism(s) not targeted by the assay. It reveals non-specific priming or detection and indicates that signals are not obtained in the absence of target sequences. This control must be tested daily in the analytical and clinical studies. Examples of acceptable negative sample control materials include:

- Patient specimen from a non-infected individual
- Samples containing a non-target microorganism (e.g., stool specimen spiked with non-target microorganism)
- Surrogate negative control (e.g., extracted DNA or RNA)

VI(E)(2). Positive Controls

External positive control for complete assay
The positive control is designed to mimic a patient specimen, contains target nucleic acids, and is used to control the entire assay process, including nucleic acid extraction, amplification, and detection. Acceptable positive assay control materials include negative stool samples or other appropriate specimen matrix mimicking the assayed specimen type, spiked with whole microorganism(s) targeted by the assay. This positive control must be run daily during the analytical and clinical studies. When applicable, representative analytes may be tested in a rotating manner using a pre-defined schedule.

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

VI(E)(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), equipment function (e.g., thermal cycler), and the presence of inhibitors in the samples. The need for this control is determined on a device case-by-case basis through agency feedback. An example of acceptable internal control is human housekeeping genes nucleic acid co-extracted with the microorganism nucleic acids targeted by the assay. Refer to Clinical and Laboratory Standards Institute (CLSI) document MM3-A2, *Molecular Diagnostic Methods for Infectious Disease* [REF. 1], for additional information.

VI(E)(4). Interpreting Test Results/Reporting

In your 510(k), you must describe with particularity how positive, negative, equivocal (if applicable), or invalid results are determined and how they are to be interpreted. Interpretative algorithms must be clearly explained. In your 510(k) submission, 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., negative/positive), this cut-off also defines a positive result of the assay.

If the assay has an equivocal zone, you must provide cut-off values (limits) for the equivocal zone. If your interpretation of the initial equivocal results requires re-testing, you must provide

- A recommendation whether re-testing should be repeated from the same nucleic acid preparation, a new extraction, or a new patient specimen, and
- An algorithm for defining a final result by combining the initial equivocal result and the results after re-testing. 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, you must provide the interpretation and recommendation for how the user should follow-up the equivocal results for each pathogen on your panel.

If the assay has an invalid result, you 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 re-testing is recommended). If re-testing is recommended, you must provide information similar to that for the 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).

VI(E)(5). Instrumentation and Software

For instruments and systems that measure and sort multiple signals, and other complex laboratory instrumentation that has not been previously cleared, refer to the document "Class II Special Controls Guidance Document: Instrumentation for Clinical Multiplex Test Systems" (http://www.fda.gov/RegulatoryInformation/Guidances/ucm077819.htm), for details on the types of data that must be provided to support instrument 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) 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) 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 510(k) 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 Environment Description	No documentation is necessary in the submission.	Summary of software life cycle development plan, including a summary of the configuration management and maintenance activities.	Summary of software life cycle development plan. Annotated list of control documents generated during development process. Include the configuration management and maintenance plan documents.
Verification and	Software	Description of	Description of

Validation Documentation	functional test plan, pass/fail criteria, and results.	V&V activities at the unit, integration, and system level. System level test protocol, including pass/fail criteria, and test results.	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.	

Configuration of the hardware and software components must be very similar or identical to that anticipated for the final version of the device before beginning clinical studies. If any significant changes are made to the hardware or software after the completion of the clinical studies but before the clearance and distribution of the device, you must perform a risk assessment and include it in your 510(k) submission.

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 consider in developing and maintaining your device under good software life cycle practices consistent with FDA regulations.

- The guidance entitled "General Principles of Software Validation" found at http://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/UCM085371.pdf.
- The guidance entitled "Off-the-Shelf Software Use in Medical Devices" found at http://www.fda.gov/ MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/ucm073778.htm.
- 21 CFR 820.30 – Design Controls of the Quality System Regulation.
- ISO 14971:2007; Medical devices – Application of risk management to medical devices.
- AAMI SW68:2001; Medical device software – Software life cycle processes.

VII. Performance Studies

VII(A). General Study Requirements

Your 510(k) submission must include detailed descriptive information regarding the studies that you conducted to establish each of the performance characteristics outlined below. Controls must approximate the composition and nucleic acid concentration of a sample in order to adequately challenge the system, as well as address reproducibility around the cut-off. You must run appropriate 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.

Prospective clinical studies are necessary to determine the performance of your device in conditions similar to the intended use. In general, for both clinical studies and reproducibility studies, you must conduct testing at three (3) sites representative of where you intend to market the device (e.g., clinical laboratory sites, one of which may be in-house). If your product labeling calls for the use of ancillary reagents, the premarket performance testing submitted to support your 510(k) must use the ancillary reagents that your instructions for use reference.

You must provide appropriate specific information in your 510(k) submission describing the protocols used during your assay development in order for FDA to accurately interpret acceptance criteria and data summaries contained in your application. When referring to CLSI protocols or guidelines, you must indicate whether all aspects of the guideline were followed or if not, which specific aspects of the protocols or guidelines were followed.

For analytes with specific FDA guideline or guidance documents, you must indicate whether all aspects of the guideline or guidance were followed and provide justification if not. 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 to obtain advice before initiating the study.

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

VII(B). Preanalytical Factors

Consideration of preanalytical factors is critical for multiplexed nucleic acid tests. In your 510(k), you must address the following issues regarding preanalytical factors:

VII(B)(1). Specimen Collection and Handling

You must specify the specimen type(s) your assay is intended to measure. Appropriate specimen types depend on a variety of factors, including the time of collection and the preservative (or none) into which the sample is to be collected. Specifically, a stool specimen has to be collected in its native form or in preservative at the time in the clinical progression of the disease state during which the organism will be present. Specimen types for gastrointestinal microorganism nucleic acid-based assay devices could include native stool, stool collected in transport/preservative media, and rectal swabs.

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

If you intend to claim multiple stool transport media, you must demonstrate that your device generates equivalent results. If you recommend specimen storage and/or shipping conditions, you must demonstrate that your device generates equivalent results for the stored specimens at several time points throughout the duration of the recommended storage period. Each recommend storage temperature must be evaluated, and for wide temperature ranges, both ends of the range must be evaluated (e.g., test both 15ºC and 30ºC for a room temperature claim of 15-30ºC). You must state your acceptance criteria for all specimen stability parameters.

CLSI document MM13-A, *Collection, Transport, Preparation and Storage of Specimens for Molecular Methods* [REF. 2], CLSI document GP44-A4, *Procedures for Handling and Processing of Blood Specimens for Common Laboratory Tests* [REF. 3], and CLSI document M29-A4, *Protection of Laboratory Workers from Occupationally Acquired Infections* [REF. 4], contain additional information regarding this topic.

VII(B)(2). Fresh versus Frozen Samples (Stability)

Performance for detection of viruses, bacteria, and parasites may differ for fresh versus frozen samples. If you use any frozen samples in your studies, or are claiming the use of frozen samples for testing with your device, you must assess the effect of freezing samples prior to testing and the effect of multiple freeze/thaw cycles on the performance of your device. You must test representative analytes with a minimum of 60 samples per analyte, with most of the samples containing levels close to the LoD, and the rest of the samples containing concentrations throughout the clinically relevant analyte concentration range. The study must demonstrate positive agreement of at least 95% with a lower bound of a 95% (two-sided) confidence interval exceeding 90%. You must assess the effect of repeated freeze/thaw cycles on the yield of the nucleic acid and its influence on the assay performance.

VII(B)(3). Nucleic Acid Extraction

Different extraction methods may yield nucleic acids of varying quantity and quality, and therefore the extraction method can be crucial to a successful result. Therefore, you must evaluate your assay's analytical and clinical performance characteristics for each of the targeted organisms using the entire pre-analytical process (including the extraction procedure) that you recommend for use with your device. This must include demonstrating reproducibility at 3 sites and confirmation of the LoD of your assay with each extraction procedure recommended in your labeling.

If you recommend multiple extraction methods, the LoD of your device must be determined separately for each method. Additionally, the reproducibility study design must allow for evaluation of each extraction procedure. Provided the LoD results are equivalent for the different extraction methods, it is acceptable for different methods to be used at different testing sites in the reproducibility study.

If LoD and reproducibility study results demonstrate equivalent performance using the different extraction procedures, it may be acceptable for clinical study testing sites to use different extraction procedures. Furthermore, data may be pooled in the final analysis if clinical performance of the device is equivalent for each extraction method.

You must provide information on how your device controls for extraction efficiency (e.g., the presence of an internal control added to each tested sample).

VII(B)(4). Well-to-Well Cross-Contamination with Automated Extraction Systems

If automated systems are used or recommended for nucleic acid extraction, you must include a check of potential well-to-well cross-contamination as part of the performance qualification of the extraction instrument. You must provide a software hazard analysis for automated extraction systems as part of your 510(k). A validation study of the extraction process can be designed in a grid such that a nucleic acid-containing sample with a concentration at the highest anticipated clinical level is surrounded on all sides by a "no template control" or a "high negative" sample. The results must demonstrate that well-to-well cross-contamination does not occur. A representative analyte from each of the analyte types represented in the assay must be tested in the study design (e.g., a representative virus, a representative bacterium, and a representative parasite).

VII(B)(5). Cut-off Determination

You must explain how the cut-off for each target or analyte was initially determined as well as how it was validated. The cut-off must be determined using appropriate statistical methods. The performance of your device using the pre-determined cut-off must then be validated in an independent set of positive stool samples. 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 specimens. For details about ROC analysis, refer to CLSI document EP24-A2, *Assessment of the Diagnostic Accuracy of Laboratory Tests Using Receiver Operating Characteristic Curves*) [REF. 5].

VII(C). Analytical Performance

VII(C)(1). Limit of Detection (LoD)

LoD is defined as the lowest concentration (e.g., CFU/ml for bacteria) of analyte that can be consistently detected in a stool sample approximately 95% of the time. The LoD must be

determined for each targeted organism and toxin gene marker in the most challenging stool matrix (e.g., raw stool or stool in transport media, etc.) claimed for use with your device. The number of strains tested may vary by analyte, but in general, you must determine the LoD for a minimum of two strains for each organism and for each toxin gene targeted by your device.

LoD determination can be accomplished by preparing serial dilutions of culture isolates or clinical specimens into an appropriate pooled negative human stool sample matrix. The method for LoD determination includes the re-growth and re-titering of stocks. You must confirm the titer of the microorganisms prior to use in the study using: tissue culture infectious dose 50 ($TCID_{50}$) units, colony forming units/mL (CFU/mL), genome equivalent copies/mL (copies/mL), plaque forming units/mL (PFU/mL), or infected cells/mL. Initially the LoD can be estimated by testing a small number of replicates at each dilution. Confirmation of the LoD must then be performed by testing a minimum of 20 replicates at the lowest concentration that produces a positive result greater than or equal to 95% of the time. You must apply the entire process of the test system from sample preparation to amplicon detection when evaluating assay LoD.

Refer to CLSI document EP17-A2, *Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures* [REF. 6], when designing your LoD studies.

VII(C)(2). Analytical Reactivity (Inclusivity)

You must demonstrate reactivity for the diversity of gastrointestinal microorganism subtypes, strains, genotypes, serotypes, or species that the assay has been designed to detect. For each microorganism targeted by your device, you must include multiple well-characterized strains that are clinically relevant and represent temporal and geographical diversity. For each toxin gene marker that your device targets, you must include multiple strains of each microorganism targeted by your device and known to carry the specific marker. For example, if your assay targets and identifies *Salmonella spp.,* you must test multiple strains representing the various *Salmonella enterica* subspecies and the common contemporary serotypes of *Salmonella enterica* subsp. enterica that infect humans in the U.S., as well as other *Salmonella* species.

Strains tested in the inclusivity studies will vary depending on the device's targeted analytes. Concentrations of the microorganisms tested must be no higher than two to three times the respective LoD. All microorganism identities and concentrations must be confirmed. Each strain must be tested in extraction triplicate. Examples of strains for LoD and analytical reactivity studies are shown in Table 3 below.

Table 3: Example Strains for LoD and Reactivity

Reactivity	
Adenovirus 40	*Salmonella enterica* subsp. *enterica*, Serotype Paratyphi B
Adenovirus 41	*Salmonella enterica* subsp. *enterica*, 4:i:-
Campylobacter jejuni	*Salmonella enterica* subsp. *enterica*, Agona
Campylobacter jejuni subsp. *jejuni*	*Salmonella enterica* subsp. *enterica*, Braenderup
Campylobacter lari	*Salmonella enterica* subsp. *enterica*, Brandenburg
Campylobacter lari subsp. *lari*	*Salmonella enterica* subsp. *enterica*, Choleraesuis var. Decatur
Campylobacter coli	*Salmonella enterica* subsp. *enterica*, Choleraesuis var. Kunzendorf

Reactivity	
Campylobacter	*Salmonella enterica* subsp. *enterica*, Choleraesuis var. sensu stricto
Clostridium difficile toxin A/B	*Salmonella enterica* subsp. *enterica*, Corvallis
Cryptosporidium parvum	*Salmonella enterica* subsp. *enterica*, Derby
Cryptosporidium hominis	*Salmonella enterica* subsp. *enterica*, Dublin
Entamoeba histolytica	*Salmonella enterica* subsp. *enterica*, Enteritidis
Escherichia coli O157	*Salmonella enterica* subsp. *enterica*, Hadar
Escherichia coli O91 (Produces shiga-like toxin II)	*Salmonella enterica* subsp. *enterica*, Heidelberg
Escherichia coli O113 (Produces shiga toxin 2)	*Salmonella enterica* subsp. *enterica*, Infantis
Escherichia coli O113 (Produces shiga toxin 1 and 2)	*Salmonella enterica* subsp. *enterica*, Javiana
Escherichia coli O111 (Produces shiga toxin 1 and 2*)*	*Salmonella enterica* subsp. *enterica*, Kentucky
Escherichia coli O104 (Produces shiga toxin 2)	*Salmonella enterica* subsp. *enterica*, Mississippi
Escherichia coli O26	*Salmonella enterica* subsp. *enterica*, Montevideo
Escherichia coli O78:H11 (Produces LT and ST)	*Salmonella enterica* subsp. *enterica*, Muenchen
Escherichia coli O25:K98:NM (Produces LT)	*Salmonella enterica* subsp. *enterica*, Newport
Escherichia coli	*Salmonella enterica* subsp. *enterica*, Oranienburg
Escherichia coli O78:K80:H12 (Produces ST)	*Salmonella enterica* subsp. *enterica*, Panama
Giardia intestinalis	*Salmonella enterica* subsp. *enterica*, Paratyphi A
Giardia lamblia	*Salmonella enterica* subsp. *enterica*, Paratyphi B
Norovirus GI	*Salmonella enterica* subsp. *enterica*, Paratyphi C
Norovirus GII	*Salmonella enterica* subsp. *enterica*, Saintpaul
Rotavirus Group A	*Salmonella enterica* subsp. *enterica*, Stanley
Salmonella enterica subsp. *enterica*, Serotype Choleraesuis	*Salmonella enterica* subsp. *enterica*, Tennessee
Salmonella enterica subsp. *enterica*, Serotype Javiana	*Salmonella enterica* subsp. *enterica*, Thompson
Salmonella enterica subsp. *enterica*, Serotype Tennessee	*Salmonella enterica* subsp. *enterica*, Typhimurium
Salmonella enterica subsp. *enterica*, Serotype Paratyphi A	*Salmonella enterica* subsp. *enterica*, Virchow
Salmonella enterica subsp. *enterica*, Serotype Enteritidis	*Salmonella enterica* subsp. *arizonae*, 53:g,z51:-
Salmonella enterica subsp. *enterica*, Serotype Typhimurium	*Salmonella enterica* subsp. *diarizonae*, 17:z10:e,n,z15
Salmonella enterica subsp. *arizonae*	*Salmonella enterica* subsp. *salamae*, 11:l,z28:e,n,x
Salmonella enterica subsp. *enterica*, Serotype Paratyphi C	*Salmonella enterica* subsp. *houtenae*, 6,7:z4,z24:-
Salmonella enterica subsp. *enterica*, Serovar Typhimurium	*Salmonella enterica* subsp. *indica*, 11:b:1,7
Salmonella enterica subsp. *enterica*, Serotype Dublin	*Salmonella bongori*, 66:z35:-
Salmonella enterica subsp. *enterica*, serotype Typhi	*Shigella dysenteriae* (Subgroup A)
Salmonella bongori type strain	*Shigella dysenteriae* (Subgroup A, serotype 8)
Salmonella enterica subsp. *enterica*, Serotype Virchow	*Shigella flexneri* (Subgroup B, serotype 3)
Salmonella enterica subsp. *enterica*, Serotype Hadar	*Shigella flexneri* (Subgroup B, serotype 4a)
Salmonella enterica subsp. *enterica*, Serotype Agona	*Shigella flexneri* (Subgroup B, serotype 6)
Salmonella enterica subsp. *enterica*, Serotype Paratyphi B var. Java	*Shigella boydii* (Subgroup C, serotype 8)
Salmonella enterica subsp. *enterica*, Serotype Derby	*Shigella boydii* (Subgroup C, serotype 10)
Salmonella enterica subsp. *enterica*, Serotype Newport	*Shigella boydii* (Subgroup C, serotype 11)
Salmonella enterica subsp. *enterica*, Serotype Braenderup	*Shigella dysenteriae* (Subgroup A, serotype 9)
Salmonella enterica subsp. *enterica*, Serotype Choleraesuis	*Shigella dysenteriae* (Type strain, Subgroup A, serotype 1)
Salmonella enterica subsp. *enterica*, Serotype Stanley	*Shigella dysenteriae* (Subgroup A, serotype 11)
Salmonella enterica subsp. *enterica*, Serotype Panama	*Shigella dysenteriae* (Subgroup A, serotype 1)
Salmonella enterica subsp. *enterica*, Serotype Heidelberg	*Shigella sonnei*
Salmonella enterica subsp. *enterica*, Serotype Montevideo	*Shigella sonnei*, Subgroup D
Salmonella enterica subsp. *enterica*, Serotype Muenchen	*Shigella flexneri*
Salmonella enterica subsp. *enterica*, Serotype Thompson	*Vibrio cholerae* pacini
Salmonella enterica subsp. *enterica*, Serotype Paratyphi B var. L(+) tartrate+	*Vibrio cholerae asiaticae* (Trevisan) Pfeiffer
Salmonella enterica subsp. *enterica*, Serotype Bareilly	*Vibrio cholerae*
Salmonella enterica subsp. *enterica*, Serotype Oranienburg	*Yersinia enterocolitica* subsp. *enterocolitica*, biotype 1 (serotype O:8)

Reactivity	
Salmonella enterica subsp. *enterica*, Serotype Kentucky	*Yersinia enterocolitica* subsp. *enterocolitica*, biotype 1 (serotype 8)
Salmonella enterica subsp. *enterica*, Serotype Anatum	*Yersinia enterocolitica* subsp. *enterocolitica* (serotype O:9)
Salmonella enterica subsp. *enterica*, Serotype Saintpaul	*Yersinia enterocolitica* subsp. *enterocolitica*, biotype 4 (serotype 3)
Salmonella enterica subsp. *enterica*, Serotype Infantis	*Yersinia enterocolitica* subsp. *enterocolitica*, biotype 2 (serotype 9)
Salmonella enterica subsp. *enterica*	*Yersinia enterocolitica*

If appropriate, *in silico* results based on target sequence alignment identity can be used to guide the selection of strains that should be "wet tested." *In silico* analyses must include clinically relevant strains and represent temporal, geographical, and phylogenetic diversity for each claimed target. With this approach, increasing numbers of representative microorganisms selected from groups with decreasing levels of identity to the target region can be selected for further laboratory testing. You must provide a clear rationale for the inclusion of the selected strains, the metrics used to assess inclusivity, and a clear presentation of the sequence alignment in the specific regions of interest for each microorganism evaluated. In addition, you must provide information regarding primer and probe sequences and any sequence differences in the target region for each species tested along with calculated % homology. You may contact the Division of Microbiology Devices in OIR for guidance in study design.

VII(C)(3). Analytical Specificity (Cross-reactivity)

You must determine analytical specificity for your gastrointestinal microorganism multiplex nucleic acid-based assay for a comprehensive number of bacteria, viruses, and parasites that are not targeted by your device. You must include microorganisms that are phylogenetically related to the analytes detected by your device as well as other microorganisms that may be present in human stool specimens but not targeted by your assay. When your assay detects a specific species of an analyte, you must evaluate other species in the genus for potential cross reactivity. This testing must be performed using potential cross-reacting microorganisms at high concentrations (e.g., 10^6 - 10^9 CFU/mL or viral particles/mL).

To supplement exclusivity wet testing, *in silico* evidence can also be included in your submission to support the specificity of the nucleic acid targets for your assay and as an aid in determining the microorganisms to be tested in your cross-reactivity study. You may contact the Division of Microbiology Devices in OIR for guidance in study design.

The following tables contain examples of the types of microorganisms (i.e., gastroenteritis causing microorganisms that are not targeted by your assay and commensal flora) that might be appropriate to include in an exclusivity study for a gastrointestinal microorganism multiplex nucleic acid-based assay:

Table 4: Pathogenic Flora

Acinetobacter baumannii	*Escherichia coli* (Migula) Castellani and Chalmers serotype O111:H8 strain CDC 1999-3249) (Produces Shiga toxin 1 and 2)

Adenovirus serotype 1	*Escherichia fergusonii*
Adenovirus serotype 3	*Escherichia hermanii*
Adenovirus serotype 4	*Escherichia vulneris*
Adenovirus serotype 5	*Gardnerella vaginalis*
Adenovirus serotype 8	*Helicobacter felis*
Adenovirus serotype 14	*Helicobacter pylori*
Adenovirus serotype 18	Hepatitis A virus
Adenovirus serotype 31	*Klebsiella oxytoca*
Aeromonas hydrophila	*Klebsiella ozaenae (K. pneumonia* subsp. *ozaenae)*
Aichi virus	*Listeria grayi*
Arcobacter butzleri	*Listeria monocytogenes*
Arcobacter cryaerophilus	Norovirus GIV
Astrovirus Type 1	*Plesiomonas shigelloides*
Astrovirus Type 2	*Porphyromonas asaccharolytica*
Bacillus cereus	*Providencia alcalifaciens*
Campylobacter fetus subsp. *fetus*	*Providencia rettgeri*
Campylobacter fetus subsp. *venerealis*	*Providencia stuartii*
Campylobacter hyointestinalis	Rotavirus A (strain WA)
Campylobacter jejuni subsp. *jejuni*	Rotavirus Group B
Campylobacter upsaliensis	Rotavirus Group C
Chlamydia trachomatis	*Salmonella enterica* subsp. *enterica* serovar Choleraesuis
Clostridium perfringens	*Salmonella enterica* subsp. *enterica* serovar Typhimurium (formerly *Salmonella choleraesuis* subsp. *choleraesuis* serotype Typhimurium)
Clostridium septicum	Sapovirus GI
Clostridium sordellii	Sapovirus GII
Clostridium tertium	Sapovirus GIII (porcine)
Clostridium tetani	Sapovirus GIV
Coxsackie virus	*Serratia liquefaciens*
Cronobacter sakazakii	*Serratia marcescens* subsp. *marcescens*
Cryptosporidium meleagridis	*Shigella boydii*
Cryptosporidium muris	*Shigella dysenteriae* serotype 1 strain AMC 43-A-14
Cytomegalovirus	*Shigella sonnei*
Echovirus	*Stenotrophomonas maltophilia*
Edwardsiella tarda	*Streptococcus dysgalactiae* subsp. *dysgalactiae*
Enterovirus (Human enterovirus D (Enterovirus Type 70)), strain J670/71	*Streptococcus pyogenes*
Enterovirus (Sabin 3)	*Vibrio parahaemoliticus*
Escherichia blattae	*Yersinia bercovieri*
Escherichia coli (Migula) Castellani and Chalmers strain CDC EDL 1284 [929-78] (serotype O124:NM) (enteroinvasive)	*Yersinia pseudotuberculosis*

Escherichia coli (Migula) Castellani and Chalmers strain CFT073 (uropathogenic strain)	*Yersinia rohdei*
Escherichia coli (Migula) Castellani and Chalmers (serotype O16:K1(L):NM)	

Table 5: Commensal Flora

Abiotrophia defectiva	*Clostridium difficile* (non-toxigenic)	*Eubacterium rectale*
Acinetobacter haemolyticus	*Clostridium fallax*	*Faecalibacterium prausnitzii* (formerly *Fusobacterium prausnitzii*)
Acinetobacter lwoffii	*Clostridium haemolyticum*	*Fusobacterium varium*
Actinomyces naeslundii	*Clostridium histolyticum*	*Gemella morbillorum*
Akkermansia muciniphila	*Clostridium innocuum*	*Hafnia alvei*
Alcaligenes faecalis subsp. *faecalis*	*Clostridium methylpentosum*	*Helicobacter fennelliae*
Anaerococcus tetradius	*Clostridium nexile*	
Atopobium vaginae	*Clostridium novyi*	*Klebsiella pneumoniae* subsp. *pneumonia*
Bacillus subtilis subsp. *spizizenii*	*Clostridium paraputrificum*	*Lactobacillus acidophilus*
Bacillus subtilis subsp. *subtilis*	*Clostridium ramosum*	*Lactobacillus casei*
Bacteroides caccae	*Clostridium scindens*	*Lactobacillus reuteri*
Bacteroides fragilis	*Clostridium sphenoides*	*Lactococcus lactis* subsp. *lactis*
Bacteroides stercoris	*Clostridium sporogenes*	*Leminorella grimontii*
Bacteroides thetaiotaomicron	*Clostridium symbiosum*	*Listeria innocua*
Bacteroides vulgatus	*Corynebacterium genitalium*	*Mycoplasma fermentans*
Bifidobacterium adolescentis	*Corynebacterium glutamicum*	*Peptoniphilus asaccharolyticus*
Bifidobacterium bifidum	*Desulfovibrio piger*	*Peptostreptococcus anaerobius*
Bifidobacterium longum subsp. *longum*	*E. coli* (strain: (Migula) Castellani and Chalmers) strain Crooks	*Porphyromonas levii*
Blastocystis hominis	*E. coli* (strain: (Migula) Castellani and Chalmers) serotype O26:K60(B6)	*Prevotella melaninogenica*
Campylobacter concisus	*E. coli* (strain: (Migula) Castellani and Chalmers) O Group 26	*Proteus mirabilis*
Campylobacter curvus	*E. coli* (strain: (Migula) Castellani and Chalmers) serotype O103:K:H8	*Proteus penneri*
Campylobacter gracilis	*E. coli* (strain: (Migula) Castellani and Chalmers) serotype O111:NM	*Proteus vulgaris*
Campylobacter helveticus	*E. coli* (strain: (Migula) Castellani and Chalmers) – feces, human (feces from a healthy human),	*Pseudomonas aeruginosa*

	strain HGH21	
Campylobacter hominis	*E. coli* (strain: (Migula) Castellani and Chalmers) – adult, human NewYork, strain ECOR2	*Pseudomonas putida*
Campylobacter rectus	*E. coli* (strain: (Migula) Castellani and Chalmers) – adult, human Sweden, ECOR 9 (reference strain)	*Ruminococcus bromii*
Campylobacter showae	*E. coli* (strain: (Migula) Castellani and Chalmers) – adult, human Tonga, ECOR 41 (reference strain)	*Salmonella subterranea*
Campylobacter sputorum biovar *sputorum*	*Eggerthella lenta*	*Staphylococcus aureus* subsp. *aureus* strain FDA 209
Candida albicans	*Entamoeba dispar*	*Staphylococcus aureus* subsp. *aureus,* Cowan's serotype 1 (contains a protein A)
Candida catenulata	*Entamoeba moshkovskii*	*Staphylococcus epidermidis*
Capnocytophaga gingivalis	*Enterobacter aerogenes*	*Streptococus intermedius*
Cedecea davisae	*Enterobacter cloacae* subsp. *cloacae*	*Streptococcus salivarius*
Chryseobacterium gleum	*Enterococcus casseliflavus*	*Streptococcus sp.*
Citrobacter amalonaticus	*Enterococcus cecorum*	*Streptococcus uberis*
Citrobacter freundii	*Enterococcus dispar*	*Trabulsiella guamensis*
Citrobacter koseri	*Enterococcus faecalis*	*Veillonella atypical*
Citrobacter sedlakii	*Enterococcus faecalis* vanB	*Veillonella parvula*
Clostridium beijerinckii	*Enterococcus faecium*	
Clostridium bifermentans	*Enterococcus faecium* vanA	
Clostridium bolteae	*Enterococcus gallinarum*	
Clostridium butyricum	*Enterococcus hirae*	
Clostridium chauvoei	*Enterococcus raffinosus*	

VII(C)(4). Competitive Inhibition Studies

These studies must challenge your assay with combinations of analytes that are commonly found in stool samples in patients with mixed infections of microorganisms that are targeted by your assay. In these studies, you must demonstrate that a high concentration of one targeted microorganism does not inhibit detection of another targeted organism that is present at concentrations near the LoD and vice versa. You may reference current published literature to support the most appropriate organism combinations to be included in these studies.

VII(C)(5). Interference Testing

Interfering substances
In order to assess the inhibitory effects of substances encountered in human stool, you must conduct an interference study using relevant concentrations of potential interferents. Potentially

interfering substances to test include those that may pre-exist in the specimen (e.g., blood, mucin, triglycerides, cholesterol, laxatives, anti-diarrheal medications, anti-acids, anti-fungals, antibiotics, anti-inflammatories, and others that may be used in the intended use patient population), as well as those that may be introduced during specimen collection and sample preparation. Examples of potentially interfering substances are presented in Table 6 below. You must test the effect of each interfering substance on detection of analytes in your gastrointestinal microorganism multiplex nucleic acid-based assay. The amount of microorganism in the sample must be at the specific cutoff concentration. You must evaluate each interfering substance at its potentially highest concentration ("the worst case"). If no significant clinical effect is observed, no further testing is necessary.

You must also assess other commonly prescribed or over-the-counter medications and their metabolites, as appropriate. Since spiking experiments may not necessarily be an accurate model of the *in vivo* scenario, alternative experimental designs such as assessing the effect of medications in patients included in the clinical studies received may need to be considered, as appropriate.

Refer to CLSI document EP07-A2, *Interference Testing in Clinical Chemistry* [REF. 7], when designing your Interference studies.

Table 6: Non-microbial Agents for Cross-reactivity and Interference Studies

Non-microbial agents
Whole blood
Mucin
Fecal fat – triglcerides
Fecal fat – cholesterol
Hemoglobin (tarry stool)
Pepto-Bismol (Bismuth subsalicylate)
Kaopectate (Attapulgite)
Imodium (Loperamide hydrochloride)
Nystatin (antifungal)
Hydrocortisone
Calcium Carbonate (antacids)
Magnesium Hydroxide, Aluminum Hydroxide (antacids)
Mineral Oil
Sennosides (laxative)
Naproxen Sodium (non-steroid anti-inflammatory)
Benzalkonium Chloride, Ethanol (moist towellets)
Ampicillin sodium salt (152 µmol/L) (antibiotic)
Polymyxin B sulfate, bacitracin zinc (antibiotic, topical)

Microbial interference

You must evaluate your assay for interference by microorganisms that are not targeted by your assay using clinically relevant concentrations of potentially interfering microorganisms (usually 10^6 CFU/ml or higher for bacteria and 10^5 PFU/ml or higher for viruses). Potentially interfering microorganisms to be considered for testing are the same as those mentioned in regards to the

cross-reactivity study. You must evaluate potential microbial interference at the device cut-off determined for each analyte.

VII(C)(6). Precision (In-house/Within-laboratory Repeatability)

The need for an in-house precision study will be determined for each individual device. Contact the Division of Microbiology Devices in OIR for guidance specific to your device.

If performing a within-laboratory precision study is deemed necessary for your device, you must test sources of variability such as operators, days, and assay runs. Testing must be performed over a minimum of 12 days (not necessarily consecutive) with replicates of each blood culture sample every run.

Samples must be prepared by spiking an appropriate pooled negative human stool sample matrix with low concentrations of each targeted microorganism and toxin gene. For each targeted microorganism/toxin gene, you must test a minimum of two different concentrations. In addition, a negative sample must also be included in the panel. At a minimum, the following panel members must be included in the precision study:

- A negative sample: a sample with no, or an undetectable amount of targeted 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).

Your precision study report must include the following information: number of days and runs, number of operators, and the acceptance criteria applied to your study. In general for qualitative tests, components of variation must be assessed individually as well as in total. For qualitative tests that have an underlying quantitative output, components of variation must be numerically analyzed (e.g., mean, standard deviation, and % coefficient of variation).

CLSI documents EP05-A2, *Evaluation of Precision Performance of Quantitative Measurement Methods* [REF. 8], and EP12-A2, *User Protocol for Evaluation of Qualitative Test Performance* [REF. 9], contain further information about designing and performing repeatability studies.

VII(C)(7). Multi-Site Reproducibility Study

The protocol for the reproducibility study may vary slightly depending on the assay format although the panel of samples must be prepared in the same manner and consist of the same panel members described for in-house precision studies above.

If your device targets a large number of analytes, it may be reasonable for the reproducibility study to include a subset of representative targeted organisms. Contact the Division of Microbiology Devices in OIR for guidance specific to your device.

Generally you must use the following approach when performing your reproducibility studies:

- Evaluate the reproducibility of your test at three testing sites (one of which may be in-house).
- Use a five day testing protocol, including a minimum of two runs per day (unless the assay design precludes multiple runs per day), three replicates of each panel member per run, with at least two operators at each facility performing the test each day. A minimum of 90 extraction replicates must be tested for each analyte and each concentration.
- You must provide training only to the same extent that you intend to train users after marketing your device.

Your reproducibility study report must include the following information: number of days and runs, number of operators, and acceptance criteria applied to the study. In general for qualitative tests, components of variation must be assessed individually as well in total. For qualitative tests that have an underlying quantitative output, components of variation must be numerically analyzed as well (e.g., mean, standard deviation, and % coefficient of variation). In addition, you must provide the percentage of invalid results for each site separately and for all sites combined.

CLSI document EP15-A2, *User Verification of Performance for Precision and Trueness* [REF. 10], contains additional information on reproducibility study design.

VII(C)(8). Carryover Studies and Cross-contamination Studies (for Multi-sample Assays and Devices that Require Instrumentation)

For multi-sample assays and devices that require instrumentation, you must demonstrate that carryover and cross-contamination do not occur when using your device. In a carryover and cross-contamination study, high positive samples must be tested in a series alternating with negative samples in patterns dependent on the operational function of the device. The study must include a minimum of five runs in which alternating positive and negative samples are performed. The high positive samples in the study must have a concentration where 95% or more of the results obtained from specimens of diseased patients in the intended use population are positive.

VII(D). Clinical Performance

Clinical study protocols must be completed and reviewed by the investigators prior to the study's initiation. Copies of the original study protocols, protocol modifications, and any other relevant study information must be included in your 510(k) submission.

You may contact the Division of Microbiology Devices in OIR to request a review of your proposed studies as part of the pre-submission review process. You must conduct prospective clinical studies to determine the performance of your device for the specific intended use. Enrolled patients must be representative of the entire intended use population for all assay analytes (i.e., one population must not be overrepresented (*C. difficile*-infected)). You must develop a detailed study protocol that includes the specific patient inclusion and exclusion criteria, the type and number of specimens needed, the directions for use, and a statistical analysis plan that accounts for variances to prevent data bias. You must include a description of how the studies support the proposed intended use. . You may contact the Division of Microbiology Devices in OIR to request a review of proposed studies prior to study initiation.

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

VII(D)(1). Reference Assays

You must compare the performance of your device to established reference methods of culture or a predetermined algorithm that uses composite comparator methods. Examples of the reference methods include:

1. Comparison to FDA-cleared device, if available.
2. Two well-characterized and validated nucleic acid amplification tests (NAAT) followed by bi-directional sequencing analysis. You may contact the Division of Microbiology Devices in OIR for guidance with the validation study design.
3. Culture or FDA-cleared EIA-based test method and one well-characterized and validated NAAT followed by bi-directional sequencing analysis.

If you use NAAT followed by bi-directional sequencing as part of a composite comparator method, then the primers you utilize must be well-characterized and validated, including evaluation of the limit of detection for the primers and demonstration of analytical reactivity. The NAAT assays must utilize primers designed to amplify regions of the genomic sequence that are not covered by your gastrointestinal microorganism multiplex nucleic acid-based assay. You may provide published literature or laboratory data in your submission in support of the primers used for NAAT or sequencing. You must perform the sequencing reaction on both strands of the amplicon (bi-directional sequencing) and demonstrate that the generated sequence (both forward and reverse sequences of the produced amplicon) met sequence acceptability criteria as follows:

- The generated sequences, from bi-directional sequencing, must be at least 200 bases of an acceptable quality, defined as a minimum of 90% of the total bases with PHRED quality score of 20 or higher (equivalent to an error rate of 1% or lower).
- For sequences containing ambiguous nucleotides, the total number of ambiguous nucleotides in the acceptable quality sequences generated using bi-directional sequencing must not exceed 5% of total bases (or 10 bases per 200 bp read).
- Blast analysis of the acceptable quality sequences generated by bi-directional sequencing must have at least 95% query coverage compare to reference and at least 95% identity to reference.

- Sequence matches the reference or consensus sequence with an Expected Value (E-Value) < 10^{-30} for the specific target (for a BLAST search in GenBank, http://www.ncbi.nlm.nih.gov/Genbank/).

CLSI documents MM18-A, *Interpretive Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing* [REF. 11], and MM9-A, *Nucleic Acid Sequencing Methods in Diagnostic Laboratory Medicine* [REF. 12], contain additional information on microbial sequencing.

VII(D)(2). Study Sites

You must conduct your studies at a minimum of three different geographical sites representing testing environments where the device will ultimately be used (e.g., clinical laboratories) and by laboratory personnel likely to perform the testing in clinical practice. At least two of the study sites must be in the United States. Testing sites can be independent from specimen collection sites. However, a minimum of three geographically diverse specimen collection sites must be chosen. If some of the studies are conducted outside the U.S., you must document the relevance of your studies to U.S. clinical practice and demographics.

VII(D)(3). Study Population

You must conduct your studies using samples from your proposed target population. Patient enrollment in the study must be based on signs and symptoms and meet any additional inclusion criteria for the study. In order to preserve the true prevalence of your target analytes in your clinical study patient population, patients previously enrolled in the clinical study must not be allowed to re-enter the same clinical study. You must collect all relevant clinical and laboratory information available for your clinical study patients. This includes:

- Age (children, adults, geriatric population)
- Sex
- Patient population (e.g., outpatient, ER, hospitalized, immunocompromised)
- The type of clinical sign(s) and symptoms(s)
- The duration and severity of the symptom(s) prior to enrolment
- The method of transmission
- Any prior and concomitant medications (including the dose, type, frequency and duration)
- Any additional laboratory test results
- A final diagnosis if available

The clinical information appropriate for consideration may vary with the study group of interest. You must include patients from each age group in your clinical studies (e.g., less than 5, 6-21, 22-59, and greater than 60 years old) that you don't disclaim in your intended use that your device can detect.

VII(D)(4). Study Design

Sample size for a prospective study for estimating sensitivity or positive percent agreement is determined by the estimated prevalence of clinical disease and the expected prevalence for each analyte. You must present a detailed summary substantiated by literature references of the expected prevalence for each analyte in your study.

Clinical studies must primarily involve testing of fresh prospectively-collected specimens, each from a unique patient. However, a portion of the total tested specimens may consist of prospectively collected and archived frozen stool specimens. Prospectively collected archived specimens must be collected sequentially from all patients meeting study inclusion criteria and representing the assay intended use population and must be collected between two predetermined dates, so there is no bias and the prevalence of the analyte is preserved. Generally 1,500 prospective samples must be collected and analyzed by your assay in order to obtain sufficient statistical power for FDA to make a substantial equivalence determination.

In the event that the prospective clinical specimen testing does not yield a sufficient number of positive results for some targeted analytes, it is acceptable to supplement with retrospective specimens that are known to be positive for specific analytes.

Results from testing of frozen (prospective or retrospective archived) specimens are acceptable for inclusion in support of test performance provided that studies are performed to demonstrate that freezing specimens does not alter the performance of the device in comparison to testing of fresh specimens.

In addition, inclusion of contrived specimens may also be an acceptable enrichment approach. Microorganisms used for contrived sample testing must represent different clinical isolates and not multiple isolates of the same organisms from the same patient.

Retrospective archived and contrived specimens must be tested in a blinded manner along with some negative samples and randomly distributed with testing performed at a minimum of three clinical testing sites.

VII(D)(5). Presentation of Clinical Study Results

You must present sensitivity and specificity (or positive and negative agreement) with 95% confidence intervals separately for each targeted microorganism and toxin gene that your device identifies. Also, you must present (1) the results of your test for the specimens that have more than one analyte as determined by the reference method, and (2) the results for the reference method for the specimens that have more than one microorganism as determined by your device.

All samples tested in your clinical study must be tested as described in the instructions for use for your device. For example, if the samples with initial invalid results are to be re-tested according to the assay instructions, then these samples must be re-tested in the clinical study and the final results for these samples must be used in your statistical analysis. You must provide

the percent of re-tested samples due to equivocal results (if applicable) and due to invalid results for each individual analyte and for all combined.

Performance for prospectively collected specimens (fresh and archived) must be presented separately in your 510(k) submission. Additionally, separate analyses must be presented for retrospective and contrived specimens.

You must present the study data demonstrating the performance of your assay 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.

VIII. Device Specific Labeling

Your labeling for devices for a gastrointestinal microorganism multiplex nucleic acid-based assay must include the information described below to help ensure that users understand the appropriate uses of the device.

VIII(A). Intended Use

The intended use must specify the specimen type (e.g., human stool specimens), the microorganisms that the device detects and identifies, the nature of the analyte and target (e.g., RNA, DNA, or both RNA and DNA), the clinical indication(s) for which the test is to be used, and the specific population(s) for which the test is intended (including disclaiming age groups if there is not adequate data to support the use of the device in patients in that age group). The intended use must also state whether the test is qualitative or quantitative, whether analyte detection is presumptive, and any specific conditions of use (e.g., whether the test is intended to be used in conjunction with other laboratory tests). The intended use must clearly state that the device is to serve only as an aid in diagnosis of gastrointestinal infections. Additional qualifications may be appropriate based on the results of the clinical studies.

VIII(B). Device Description

The device description must clearly describe the test methodology used by the device.

VIII(C). Procedure

This section must include a detailed description of the entire testing process from patient sampling to result reporting.

VIII(D). Directions for Use

The Directions for Use must provide clear and concise instructions that systematically describe the procedures for using the device and the types of control measures that will minimize risks of

inaccurate results. Instructions must encourage use of additional control measures and testing of control materials to ensure use in a safe and effective manner.

Detailed device handling and storage instructions must be included as well as a clear description of the expiration dating for both open and closed storage conditions for the device and any reagents or other components.

VIII(E). Quality Control

Quality control recommendations in the package insert must include a clear explanation of what controls should be used with the assay and the expected results for the control material.

If controls are included with the device, the 510(k) submission must include the specifications for the control materials.

VIII(F). Warnings, Contraindications, Precautions, and Limitations

All warnings, contraindications, precautions, and limitations relevant to the specific device must be included in the device labeling. In doing so, at a minimum, you must include a discussion of certain populations where device performance may differ or where the device has not been studied (e.g., immunocompromised patients). In addition, the following statements must be included as limitations:

- All assay results should be used and interpreted in the context of a full clinical evaluation as an aid in the diagnosis of gastrointestinal infection.
- There is a risk of false positive values resulting from cross-contamination by target organisms, their nucleic acids, or the amplified product.
- There is a risk of false positive values resulting from non-specific signals in the assay.
- Analyte targets (virus, bacteria, or parasite nucleic acid sequences) may persist in vivo, independent of virus, bacteria, or parasite viability. Detection of analyte target(s) does not guarantee that the corresponding live organism(s) is present, or that the corresponding organism(s) is the causative agent for clinical symptoms.
- The detection of viral, bacterial, or parasitic sequences is dependent upon proper specimen collection, handling, transportation, storage, and preparation (including extraction). Failure to observe proper procedures in any one of these steps can lead to incorrect results.
- Underlying polymorphisms in primer-binding regions can affect the targets being detected and subsequently the test results returned.
- *Salmonella*: not all *Salmonella* serotypes were tested in validation studies.
- There is a risk of false negative values resulting from improperly collected, transported, or handled specimens.
- There is a risk of false negative values due to the presence of strain/species sequence variability in the targets of the assay, procedural errors, amplification inhibitors in specimens, or inadequate numbers of organisms for amplification.

- The performance of this test has not been established for monitoring treatment of infection with any of the targeted microorganisms.
- Positive and negative predictive values are highly dependent on prevalence. False negative test results are more likely when prevalence of disease is high. False positive test results are more likely when prevalence is low.
- The effect of interfering substances has only been evaluated for those listed in the labeling at its indicated amount or concentration. Interference by substances other than those described in the "Interference" section of the package insert can lead to erroneous results.
- Cross-reactivity with gastrointestinal tract organisms other than those listed in the "Analytical Specificity" section of the package insert may lead to erroneous results.
- This test is a qualitative test and does not provide the quantitative value of detected organism present.

VIII(G). Specimen Collection

You must provide instructions for specimen collection. If you recommend that raw stool, preserved stool, or processed stool specimens can be stored for later testing, you must provide information for validated storage conditions.

VIII(H). Interpretation and Reporting of Assay Results

You must describe how the operator should interpret each of the possible device results (e.g., positive, equivocal, and negative). You must also describe the recommendations for retesting or reporting of specimens that are equivocal (if this is a possible device output) or when specimen processing fails (e.g., whether another aliquot of the same specimen or a fresh specimen is necessary). See also Section VI(E)(4) of this document entitled "Interpreting Test Results/Reporting." If appropriate, you must include photographs and/or diagrams to indicate how to interpret results for tests with a qualitative result.

VIII(I). Performance Characteristics

Labeling must include a summary of the study designs and study results described in Section VII of this document that would aid the user in interpreting test results and understanding device performance; this must include descriptions of both clinical and analytical study results.

IX. References

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

2. Clinical and Laboratory Standards Institute. 2006. Collection, Transport, Preparation, and Storage of Specimens for Molecular Methods; Approved Guideline. MM13-A. Clinical and Laboratory Standards Institute, Wayne PA.

3. Clinical and Laboratory Standards Institute. 2010. Procedures for Handling and Processing of Blood Specimens for Common Laboratory Tests; Approved Guideline. GP44-A4. Clinical and Laboratory Standards Institute, Wayne, PA.

4. Clinical and Laboratory Standards Institute. 2005. Protection of Laboratory Workers from Occupationally Acquired Infections; Approved Guideline. M29-A4. Clinical and Laboratory Standards Institute, Wayne PA.

5. Clinical and Laboratory Standards Institute. 2011. Assessment of the Diagnostic Accuracy of Laboratory Tests Using Receiver Operating Characteristic Curves; Approved Guideline. EP24-A2. Clinical and Laboratory Standards Institute, Wayne PA.

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

7. Clinical and Laboratory Standards Institute. 2005. Interference Testing in Clinical Chemistry; Approved Guideline. EP07-A2. Clinical and Laboratory Standards Institute, Wayne PA.

8. Clinical and Laboratory Standards Institute. 2004. Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline. EP05-A2. Clinical and Laboratory Standards Institute, Wayne PA.

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

10. Clinical and Laboratory Standards Institute. 2006. User Verification of Performance for Precision and Trueness; Approved Guideline. EP15-A2. Clinical and Laboratory Standards Institute, Wayne PA.

11. Clinical and Laboratory Standards Institute. 2008. Interpretive Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing; Approved Guideline. MM18-A. Clinical and Laboratory Standards Institute, Wayne PA.

12. Clinical and Laboratory Standards Institute. 2004. Nucleic Acid Sequencing Methods in Diagnostic Laboratory Medicine; Approved Guideline; CLSI document MM9-A. Clinical and Laboratory Standards Institute, Wayne PA.