U.S. FOOD & DRUG ADMINISTRATION

Memorandum

To: Foods Program Governance Board

From: FDA Foods Program Regulatory Science Steering Committee (RSSC)
Date: Sep 5th, 2019
Subject: Guidelines for the Validation of Analytical Methods for Nucleic Acid Sequence-Based Analysis for the FDA Foods Program, 1st Edition

The FDA Foods Program Regulatory Science Steering Committee (RSSC), made up of representatives from Center for Food Safety and Applied Nutrition (CFSAN), the Center for Veterinary Medicine (CVM), the Office of Regulatory Affairs (ORA), the National Center for Toxicological Research (NCTR), and the Office of the Chief Scientist of the FDA, is charged with the task of prioritizing, coordinating and integrating human food- and animal food-related science and research activities across the operating units of FDA's Foods Program.

As a regulatory agency tasked with ensuring the safety of the nation's food supply, it is imperative that the laboratory methods needed to support regulatory compliance, investigations and enforcement actions meet the highest analytical performance standards appropriate for their intended purposes. Development of standardized validation requirements for all regulatory methods used in our laboratories to detect chemical and radiological contaminants, as well as microbial pathogens, is a critical step in ensuring that we continue to meet the highest standards possible.

The attached document, now formally adopted by the RSSC, establishes the requirements that must be fulfilled in the evaluation of nucleic acid sequence-based analysis methods to be used in our testing laboratories. These guidelines are posted on FDA's Foods Program Methods website. Please share these methods validation guidelines with anyone who may be conducting or supervising methods validation projects or otherwise needs to be aware of these updated requirements.

Because nucleic acid sequence based-methods are used in both chemistry and microbiology programs within the FDA, the Molecular Methods Technical Advisory Group, one of the hierarchical committees under the RSSC and consisting of members from both the chemistry and microbiology programs, was charged with the development of these guidelines. They were developed with input and ultimate approval from both the Microbiological Methods Validation Subcommittee (MMVS) and Chemistry Methods Validation Subcommittee (CMVS), which are charged with providing guidance and oversight to all validation studies and are principally responsible for the content of these Guidelines. The Chemistry Research Coordination Group (CRCG) and the Microbiology Research Coordination Group (MRCG), and associated Technical Advisory Groups, also provided input. Additional questions and comments about the Guidelines may be directed to the MMVS, MRCG, CMVS or CRCG.

Thank you,

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Guidelines for the Validation of Analytical Methods for Nucleic Acid Sequence-Based Analysis of Food, Feed, Cosmetics and Veterinary Products

Edition 1.2

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December 2023

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The first edition of these guidelines was published in 2019 at the request of the US FDA Foods Program. In cooperation with members of the Foods Program Regulatory Science Steering Committee, direct input, review, and consent were provided by the following FDA research and regulatory offices:

Center for Food Safety and Applied Nutrition
Office of Regulatory Science
Office of Food Safety
Office of Applied Research and Safety Assessment

Center for Veterinary Medicine
Office of Research
Office of New Animal Drug Evaluation

Office of Regulatory Affairs
Office of Regulatory Science

APPROVAL PAGE

This document is approved by the FDA Foods and Veterinary Medicine (FVM) Regulatory Science Steering Committee (RSSC). The **FVM RSSC Project Manager is responsible for updating the document as change requirements are met, and disseminating updates to the RSSC and other stakeholders, as required.**

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RSSC Chair

Guidelines for the Validation of Analytical Methods Using Nucleic Acid Sequenced-Based Technologies

Table of Contents

1.0 INTRODUCTION

1.1 Purpose

The U.S. Food and Drug Administration (FDA) is responsible for ensuring the safety of much of the nation's food supply. The FDA Foods Program (FFP), which includes the Center for Food Safety and Applied Nutrition (CFSAN), and the Center for Veterinary Medicine (CVM), as well as related activities under the Office of Regulatory Affairs (ORA), supports the FDA mission as related to food, feed, cosmetics, veterinary products, and environmental samples. The mission of these offices includes preventing foodborne illness, fostering good nutrition, and improving the safety and efficacy of animal and veterinary products. Foods Program laboratories, in conjunction with ORA, contribute to this mission through routine surveillance programs, targeted regulatory analysis, emergency response when contaminated food or feed is detected or suspected in a public health or animal health incident, and in the prevention of fraudulent or misbranded products being brought to market. The effectiveness of these activities is highly dependent on the quality and performance of the laboratory methods needed to support regulatory compliance, investigations, and enforcement actions. To ensure that the methods employed in Foods Program laboratories meet the highest analytical performance standards, the Foods Program Regulatory Science Steering Committee (RSSC) here establishes criteria by which all analytical methods for targeted nucleic acid sequence-based analyses in food, feed, cosmetics, veterinary products, and environmental samples to be used in FDA laboratories shall be evaluated and validated.

In this document, the following terms are used:

- "shall" indicates a requirement;
- "should" indicates a recommendation;
- "may" indicates a permission;
- "can" indicates a possibility or capability.

This document defines four standard levels of performance for use in the validation/verification of analytical methods for the detection, identification, and/or quantification of specific nucleic acid sequences in food, feed, cosmetics, veterinary products, and environmental samples. **This document further defines three validation levels and a verification level for Next Generation Sequencing (NGS) for bacterial genomic DNA.**

These criteria are consistent with several related guidelines produced by international food standards setting organizations including, but not limited to, Codex Alimentarius, the International Organization for Standardization (ISO), the European Union Reference Laboratory for Genetically Modified Food and Feed, and also with the FDA Foods Program Methods Validation Processes and Guidelines.*Guidelines for the Validation of Microbiological Methods for the FDA Foods Program* and *Guidelines for the Validation of Chemical Methods for the FDA Foods Program*.

1.2 Scope

These criteria apply to all Foods Program laboratories that develop and participate in the validation of targeted nucleic acid sequence or next generation sequencing-based analytical methods for food, feed, cosmetics, veterinary products, and environmental samples for Agency-wide implementation in a regulatory capacity. These criteria do not apply to methods developed by or submitted to FDA under a codified process or official guidance (e.g., in the Code of Federal Regulations, CPGs, etc.), such as methods developed or submitted as part of the veterinary drug approval process, which includes animals with intentionally altered genomic DNA. For such studies, the appropriate CVM or other Program guidance documents should be followed. At the time of final approval by the Foods Program RSSC, this document will supersede all other intra-agency documents pertaining to human and animal food-related method validation criteria for targeted nucleic acid-sequence based analysis or whole genome sequencing (WGS) of bacterial strains. In addition, this guidance is a forward-looking document; the requirements described here will only apply to *newly* developed methods and those for which significant modifications have been made to an existing method. Once a method has been validated, it can be implemented by other laboratories following an appropriate method verification process.

1.3 Administrative Authority and Responsibilities

All criteria established in this document for analytical method validation have been adopted and approved by the Foods Program RSSC. The document, *FFP Method Development, Validation and Implementation Program*, establishes the standard operating procedure for the approval and tracking of method development and validation activities within the Foods Program. Single laboratory validation (SLV) studies can be managed wholly by the respective Center and Office management structure. Oversight and coordination of multi-laboratory validation (MLV) studies are the responsibility of the Methods Validation Subcommittees (MVS).

1.4 The Method Validation Subcommittees

Under the authority of the RSSC, targeted nucleic acid sequence-based analyses will be overseen by either the Chemistry Methods Validation Subcommittee (CMVS) or the Microbiology Methods Validation Subcommittee (MMVS), based on the analytical target of the assay. These committees are governed by the organizational structure, roles and responsibilities as detailed in their charters. Briefly, the validation subcommittees will oversee and coordinate, in collaboration with the originating laboratory, all collaborative laboratory validation studies for methods for the detection, identification, and/or quantification of specific nucleic acid sequences in food or feed developed within the FDA. This includes the evaluation of Single Laboratory Validation (SLV) results and the evaluation of any subsequent collaborative validation study plan. Correspondence between the method developer(s) and the MMVS or CMVS will be by email using the following addresses: Microbiology.mvs@fda.hhs.gov; Chemistry.mvs@fda.hhs.gov

Where possible, MLVs should be discussed with the appropriate Technical Advisory Group (TAG) for feedback and guidance as well as with the Chemistry Research Coordinating Group (CRCG) or the Microbiology Research Coordinating Group (MRCG) to ensure the broadest possible consideration of factors before committing resources to an MLV.

1.5 General Responsibility of the Originating Laboratory

It is the responsibility of the originating laboratory to ensure proper adherence to all criteria described in this document. The originating laboratory should work in consultation with the appropriate MVS and/or its designated TAG throughout the MLV process. It will be the responsibility of the originating laboratory to include their respective QA/QC manager in all aspects of the validation process.

1.6 Method Validation Definition

Method validation is the process of demonstrating or confirming that a method is suitable for its intended purpose. Intended purposes may include, but are not limited to:

- qualitative or quantitative analyses
- screening or confirmatory analyses
- subtyping analyses
- matrix or platform extensions
- method or reagent modifications
- emergency operations analyses

Method validation criteria may include:

- sensitivity
- accuracy
- trueness
- reproducibility and robustness/ruggedness
- precision

Method validation is achieved by conducting experiments to determine performance characteristics and quantify method performance and is performed after the initial method development and optimization.

1.7 Applicability

This document establishes evaluation criteria for methods to detect and/or identify the presence of specific nucleic acid sequences derived from: microbes (including viruses, fungi, and parasites), insects, plants, or animals, including organisms with intentionally altered genomic DNA, either used as or present as contaminants in foods, dietary supplements, and cosmetics. Methods commonly in use include, but are not limited to:

- qualitative assays
- quantitative assays
- sequencing assays

Templates used for analysis may include, but are not limited to, genomic DNA, chloroplast and/or mitochondrial DNA, and reverse-transcribed RNA (cDNA).

This document also establishes performance criteria elements for validating and verifying an NGS method. Specifically, guidance is provided for short read technology on non-targeted whole genome sequencing of pure bacterial isolates.

1.8 Requirements

Method validation is required for:

- Submission of a new or original method.
- Platform, matrix, and analyte extensions.
- Modifications to a method that may alter its performance specifications.

2.0 CRITERIA AND GUIDANCE FOR THE VALIDATION OF FDA DEVELOPED METHODS

This section provides validation criteria and guidance for all newly developed methods or any existing validated method(s) that has been significantly modified. Moreover, the NGS section provides criteria for verification of a validated method.

2.1 Validation Definitions

2.1.1 General Validation Tools and Protocol Guidance

Depending on the type of method being evaluated, the analysis of method blanks, matrix blanks, reference materials (standards) and spikes will be used for the calculations of accuracy, and precision, as well as determining ruggedness/robustness. The following general validation tools should be used to generate method performance characteristics as described below. Note: Some of these items are not applicable to all of the method types covered in this document.

Extraction Blank: This type of blank incorporates all the reagents and steps of the nucleic acid extraction and is processed simultaneously with the samples. Extraction controls are used to demonstrate that the extraction reagents are free of contamination. Additionally, these controls are used to demonstrate that no cross-contamination between samples has occurred.

Matrix Blank: This type of blank is a substance that closely matches the samples being analyzed with regard to matrix components. Matrix blanks are used to demonstrate that sample matrix and equipment used does not interfere with or affect the analytical signal.

Positive Control: DNA/RNA known to contain the target sequence and give a strong positive signal.

Internal Amplification Control: Internal amplification controls should be included in the PCR assays design to ensure that PCR inhibitors are not present. Internal controls are amplified using different primer and probe sets from those used to amplify assay targets and may be based on exogenous DNA/RNA or endogenous DNA/RNA. This may not apply to all amplification technologies (e.g., LAMP).

Matrix Spikes: Matrix effects can be assessed by spiking known amounts of analyte into a matrix of interest. Accuracy and precision are calculated from these results. The data can also be used to evaluate robustness/ruggedness of the method resulting from changes in the sample matrix.

No Template Control (for PCR): This type of blank incorporates all reagents used in the PCR except the template DNA but including the internal control. It serves to demonstrate that reagents are analyte-free, and the equipment used does not interfere with or affect the analytical signal.

Reference Materials and Certified Reference Materials: The use of known reference materials (when available and applicable) should be incorporated to assess the accuracy of the method, as well as for obtaining information on interferences.

Replicate Analyses: The precision of the analytical process can be evaluated using replicate analyses. The originating laboratory should assure that adequate sample replicates are performed and that results from replicate measurements of each analyte are compared.

Statistics: Statistical techniques are employed to evaluate accuracy, trueness, precision, linear range, limits of detection and quantitation, and measurement uncertainty.

For additional definitions, please see Appendix 4, Glossary of Terms.

2.1.2 Reference Method

A reference method is a method by which the performance of an alternate or new method may be measured or evaluated. In some cases, an appropriate reference method may not be available. However, there are some instances in which the use of a reference method is appropriate such as when replacing a method specified for use in a compliance program. Consultation between the originating laboratory, the appropriate MVS, and the Program Office is suggested when deciding if the use of a reference method will be necessary. Use of a reference method is required for microbiological methods when available (see Section 2.1.1 of *Guidelines for the Validation of Microbiological Methods for the FDA Foods Program*).

2.2 Criteria to be Evaluated by Validation Type

Performance characteristics that may be evaluated to validate a method will vary depending on the intended use of the method, the type of method, and the degree to

which it has been previously validated. Criteria listed below are meant to serve as guidance for validation protocols.

New Qualitative Methods: These methods are developed to detect analytes (DNA/RNA) from a matrix of interest in a strictly qualitative way. At a minimum, the following performance characteristics should be assessed: sensitivity, specificity, false positive rate, false negative rate, limit of detection, and ruggedness/robustness. For microbiological methods, see Section 2.4.2 of *Guidelines for the Validation of Microbiological Methods for the FDA Foods Program* for guidance.

New Quantitative Methods: These methods are developed to detect analytes (DNA/RNA) from a matrix of interest in a quantitative manner. At a minimum, the following performance characteristics should be assessed: accuracy, precision, specificity, limit of detection, limit of quantitation, linearity (efficiency), range, measurement uncertainty, robustness/ruggedness, confirmation of identity and extraction efficiency. For microbiological methods, see Section 2.4.2 of *Guidelines for the Validation of Microbiological Methods for the FDA Foods Program* for guidance.

New Identification Methods: These methods are developed to unequivocally identify specific analytes (based on their nucleic acid sequences). At a minimum, the following performance characteristics should be assessed: accuracy, precision, specificity, ruggedness/robustness, confirmation of identity. See Section 2.4.4. for guidance. For microbiological methods, see Table 3 (section 2.3.2.2) of the *Guidelines for the Validation of Microbiological Methods for the FDA Foods Program*

Method, Matrix and Platform Extensions: Validating the extension of methods that have previously been validated requires a careful evaluation of the intended purpose of the extension. To implement the modified method, generally the standard or existing method is first performed. The modified method performance then is verified by comparison with that of the original method. Method, Matrix and Platform extension validations should generally be performed using Level 2 guidelines and should compare the proposed new changes to the existing reference method. For microbiological methods, see the *Guidelines for the Validation of Microbiological Methods for the FDA Foods Program*

2.3 The Method Validation Process

Method validation exercises confirm by examination (and the provision of objective evidence) that the requirements for a method have been fulfilled. All methods used by the FDA in support of its regulatory and compliance roles must be validated according to the guidelines established.

This approach is based on the Food Emergency Response Network (FERN), SOP No: FERN-ADM.0008.01, FERN Validation Guidelines for FERN Chemical, Microbiological, and Radiological Methods, as well as AOAC guidelines for single-laboratory validation and collaborative studies. Key validation parameters for each level are summarized in Table 1. It is the responsibility of the originating (developing) laboratory to perform

validations up to and through the single laboratory validation levels. It is highly recommended that originating laboratories work with the appropriate Technical Advisory Group when determining the appropriate level of validation beyond the SLV level.

Note: *The level of validation required will depend on the intended use of the method. Not all methods will or should be validated to the highest level.*

2.3.1 Validation Levels for Microbiological Analytes

2.3.1.1 Single-laboratory Validation The originating lab has done a more comprehensive initial study with defined inclusivity/exclusivity levels as shown in Table 1. If available, a comparison has been done to an existing reference method. Results of the SLV have been evaluated and approved by the MMVS. This is the first step in the validation process for methods designed for routine regulatory applications. All FDA SLV protocols should be reviewed by MMVS before research is initiated.

Intended Use: Methods validated to this level of scrutiny can be used immediately for emergencies. Slightly higher false-positive rates may be acceptable as all samples analyzed will require confirmatory testing.

2.3.1.2 Independent Laboratory Validation The purpose of an ILV is to determine if a method can be successfully performed by a laboratory other than the originating laboratory. An ILV study may be required for methods extensions and/or method modifications that do not require a collaborative study. An ILV is required under 2 circumstances: 1) for entirely new methods or modified methods that have not been fully validated through collaborative study; or, 2) for methods extensions of fully validated methods (through collaborative study), where the sample preparation procedure has been changed for a particular matrix or set of matrices. It is not required for methods extensions where the method remains unchanged and where the scope of the method is being extended to include additional matrices. Determination of the need for an ILV is at the discretion of the MMVS. **(See Table 1A)**

Intended Use: Methods validated to this level of scrutiny can be used immediately for emergencies only and not for regulatory purposes unless the purpose of the ILV is for the extension of a fully validated method. Slightly higher false-positive rates may be acceptable as all samples analyzed will require confirmatory testing.

2.3.1.3 Multi-Laboratory Validation Study A multi-laboratory study is an inter-laboratory study in which collaborators in multiple laboratories use a defined method of analysis to analyze identical portions of homogeneous materials to assess the performance characteristics obtained for that method of analysis (W. Horwitz, IUPAC, 1987). It is designed to measure **reproducibility**, so that it can be determined if the method can be successfully performed by laboratories other than the originating laboratory. For methods having more than one sample preparation or enrichment scheme, it is necessary to test one matrix per sample preparation or enrichment scheme. The criteria defined for this

level of scrutiny (to be performed by the originating and collaborating labs) are closely aligned with other recognized and established validation criteria for collaborative studies, *e.g.* AOAC and ISO. This includes criteria for inclusivity/exclusivity, analyte contamination levels, competitor strains, aging, and a comparison to an existing, recognized reference method when available.

Intended Use: All methods validated to this level of scrutiny are acceptable for use in all regulatory circumstances, *e.g.,* confirmatory analyses, regulatory sampling, outbreak investigations, and surveillance and compliance support.

Table 1A- General Guidelines for the Validation of Qualitative Detection Methods for Microbial Analytes*

Criteria	Single Laboratory Validation Study	Independent Laboratory Validation Study	Multi-Laboratory Validation Study
Participating Laboratory	Originating Laboratory	Collaborator	Collaborators
# of Target Organism (Inclusivity)[a]	50 (unless 50 aren't available)[b,c]	≠NA	≠NA
# of Non-Target Organism (Exclusivity)[a]	30 strains[d]	≠NA	≠NA
# of Collaborators Providing Usable Data	NA	1	10
# of Foods	1 or more[e]	1 or more[e]	1 or more[e]
# of Analyte Levels/Food Matrix	3 levels: Minimum of two inoculated levels (one fractional[f] and one 1 log higher) and one uninoculated level	3 levels: Minimum of two inoculated levels (one fractional[f] and one 1 log higher) and one uninoculated level	3 levels: Minimum of two inoculated levels (one fractional[f] and one 1 log higher) and one uninoculated level
Replicates per Food at Each Level Tested	20 for the fractional level (5 each for the uninoculated and high levels)	20 for the fractional level (5 each for the uninoculated and high levels)[g]	8 per level
Aging of Inoculated Samples Prior to Testing	Yes[h]	Yes[h]	Yes[h]
Addition of Competitor Strain[i]	In 1 food at +1 log>analyte at fractional positive[f] analyte level	In 1 food at +1 log>analyte at fractional positive[f] analyte level	In 1 food at +1 log>analyte at fractional positive[f] analyte level
BAM Reference Method Comparison Requirement[j]	Yes, if available	Yes, if available	Yes, if available

*Analysts should consult with MMVS to determine appropriate statistics before initiating study.
[a] Using pure cultures without a food matrix.
[b] The target concentration for testing is 10 to 100 times the LOD_{50} of the candidate method. Inclusivity testing is only necessary for new methods and where deemed necessary by MMVS. Inclusivity testing unnecessary for methods extensions to new matrices.
[c] 100 serotypes for *Salmonella* testing.

[d] At growth limit, i.e., 10^9 CFU/ml for target organisms. Exclusivity testing is only necessary for new methods and where deemed necessary by MMVS. Exclusivity testing unnecessary for methods extensions to new matrices. Exclusivity non-target organisms are grown in a non-selective rich medium.
[e] For FDA regulatory use, methods are only valid for foods that have been tested; the MMVS may require that a new method be validated for 3 foods within a food category.
[f] Must be adjusted to achieve fractional positive results (one or both methods *i.e.* the reference and alternate methods must yield 50%±25% of tests positive) at this level; the high-level inoculum should be approximately1 log greater than that used to achieve fractional results. All 5 replicates at the high inoculum should yield positive results.
[g] Independent lab test portions are blind coded.
[h] Period of aging depends on food being tested. Perishable foods should be aged under refrigeration for 48 – 72 h. Frozen and shelf stable foods should be aged for a minimum of 2 weeks at -20°C or at room temperature, respectively. MMVS can change aging requirements if circumstances warrant.
[i] An appropriate competitor is one that gives similar reactions in enrichment and detection systems. Natural background microflora can fulfill this requirement if it present in the matrix at a level 1 log greater than the target analyte. Perform aerobic plate counts on all foods tested to determine levels of background microflora. This requirement may be waived or modified by consent of the FDA MMVS should the pathogen be unstable in the matrix.
[j] Independent Laboratory and Collaborative Validation Studies should use the BAM reference method if available. Other reference methods can be used per MMVS approval.
≠ Not Applicable

2.3.2 Validation Levels for All Other Analytes

2.3.2.1 Level One This is a single laboratory validation level with the fewest validation requirements and is suggested for emergency or limited use only. Method performance at the single laboratory validation level may determine if further validation is warranted.

Intended use: Emergency or limited use, and emergency matrix, analyte, or platform extensions. Examples of where Level One validation would be acceptable include isolated consumer complaints, single-occurrence samples, and application of a method developed for a specific analyte(s) to a matrix not previously validated in response to a real or perceived threat to food safety or public health.

It would be expected that a more rigorous validation (Level Two or above) would be undertaken for a method that would have widespread usage.

2.3.2.2 Level Two This is a single laboratory validation level. The originating laboratory has conducted a Level One validation and compared the new method with an existing reference method if available and appropriate.

Intended Use: Level Two validations include: episodic regulatory testing, minor method modifications, analyte and matrix extensions of screening methods. If a method validated at this level is expected to have use that is widespread, long term, or of high public visibility its validation should be extended to at least Level Three.

2.3.2.3 Level Three This is a multi-laboratory validation level. Level Three validation employs a minimum of one additional collaborating laboratory and the originating laboratory. The acceptance criteria are similar to the AOAC full collaborative study level with comparison to an existing reference method when available and appropriate. Level Three validations may employ fewer spike levels and or replicates when compared to a full AOAC collaborative study.

Intended Use: Methods validated to this level of scrutiny are acceptable for use in many regulatory circumstances including screening analyses, confirmatory analyses, outbreak investigations, short term regulatory surveys, and limited compliance support. If the method is expected to have use that is widespread, long term, of high public visibility or involved in international trade conflicts, it would be appropriate to have its validation extended to Level Four.

2.3.2.4 Level Four This validation level has criteria equivalent to a full AOAC or ISO Collaborative Study. It is designed to measure inter-laboratory reproducibility, so that it can be determined if the method can be successfully performed by multiple laboratories other than the originating laboratory.

Intended Use: All methods validated to this level of scrutiny are acceptable for use in any and all regulatory circumstances including confirmatory analyses, routine regulatory sampling, outbreak and/or recall investigations, and routine surveillance and compliance support.

2.4 Acceptability Criteria

There are various acceptability ranges for method validation performance criteria that may be appropriate depending on the application or intended use of the methodology. For nucleic acid-based methodologies, there are several quality parameters and acceptance criteria, which vary depending on the methodology being employed. These features will be defined under the general headings of qualitative polymerase chain reaction (PCR) analysis, quantitative PCR analysis, detection of specific nucleic acid sequences by Sanger sequencing and NGS validation criteria.

Table 1B provides guidance for the design of validation studies at all levels. If the assay to be validated is designed to detect a prokaryotic microorganism, please also refer to Table 1A above or *Guidelines for the Validation of Microbiological Methods for the FDA Foods Program* for additional study design parameters.

Table 1B- Validation Parameters for Qualitative and Quantitative PCR-Based Methods for Non-Microbiological Methods Validation Studies

	L1-Emergency	L2 –SLV	L3- MLV	L4-MLV
Number of labs	1	1	≥2	8-10
Matrix sources per matrix	≥1	≥3*	≥3* if food = 1; ≥1 if food >1	≥3* if food = 1; ≥ 1 if food >1
No. of spike levels	≥2	≥3	≥3	≥3
Replicates if matrix source = 1	≥4 (Quantitative) ≥6 (Qualitative)	≥6 (Quantitative) ≥9 (Qualitative)	≥3 (Quantitative) ≥6 (Qualitative)	≥2 (Quantitative) ≥6 (Qualitative)

Replicates if matrix source > 1	≥2 (Quantitative) ≥2 (Qualitative)	≥2 (Quantitative) ≥3 (Qualitative)	≥2 (Quantitative) ≥3 (Qualitative)	≥2 (Quantitative) ≥3 (Qualitative)

*Recommended where available

2.4.1 Qualitative PCR Methods

Nucleic acid-based methods that are used for the detection of a specific DNA/RNA sequence which could be part of a mixture of related targets should allow for the unequivocal detection of a nucleic acid sequence that is specific to the target organism, group or sub-set of organisms (family, genus, pathogenic strain, etc.), or transformation event in the case of genetically altered organisms. For instance, target-specific methods that are used for detection of a single transformation event should allow for unequivocal detection, identification and/or confirmation of a nucleic acid sequence that is unique or specific to that transformation event. For food authentication, the specific target sequence/s should uniquely define the target as required. For qualitative PCR methods, the basic performance characteristics are:

- Extraction Efficiency
- Sensitivity (Limit of Detection-LOD)
- Specificity (Selectivity)
- False-Negative and Positive Rates
- Robustness/Ruggedness

Extraction Efficiency: Empirical results from testing the extraction method for its efficiency should be provided for each matrix being validated; this is necessary to demonstrate the extraction is sufficient and reproducible. Extraction efficiency for a given matrix can be determined by spiking known amounts of the target analyte into that matrix prior to extraction. Extraction blanks will be included to ensure that cross-contamination does not occur during the extraction protocol.

Sensitivity: Data obtained from testing the method at different concentrations of the target sequence in order to determine the sensitivity of the method should be provided. Limits of detection (LOD) should be defined using samples comprised of single ingredients only. The LOD is usually understood as the concentration of the target DNA/RNA at which an amplification product is detected with a probability of at least 0.95 ($LOD_{95\%}$). The LOD should be determined by means of a dilution series of the target DNA/RNA. For each dilution level, 12 PCR replicate measurements are performed. The dilution level with the lowest number of copies for which all 12 replicates are positive is considered to be an approximate value for $LOD_{95\%}$. This data may be represented as DNA/RNA weight/reaction (ng or pg, etc.) or the target copy number/reaction. After the LOD of the assay is determined using a dilution series of the target DNA/RNA, the originating lab should perform experiments to estimate the LOD of the assay in various food matrices.

A real-time PCR method may employ a Ct cutoff value above which a result is considered negative. It is the responsibility of the originating laboratory to determine if a cutoff value should be established and if so, what cutoff value should be used. The decision shall be based upon assay optimization, validation data and, if available, results of testing naturally incurred/infected and epidemiologically linked products. If Ct cutoff values are employed, they will likely vary among different real-time PCR methods.

Specificity: The method should be tested with DNA/RNA from closely related or potentially co-occurring non-target species/varieties and DNA/RNA from the reference species/variety material. Demonstration of the specificity of a novel assay can be accomplished in several phases.

- *Theoretical test for specificity*: Carry out a computer-aided ("*in-silico*") test, examining the oligonucleotide sequences (primer, probe) as well as the amplicon sequence for similarities to other sequences by searching suitable databases (e.g BLASTn).

- *Experimental test for specificity*: The method must be tested with DNA/RNA from non-target species/varieties (exclusivity) and DNA/RNA from the reference species/variety (inclusivity) material. This testing should include closely related materials and cases where the limits of the sensitivity are truly tested.

Note: The number of species used for inclusivity/exclusivity testing will vary with the analysis being conducted and the target organism/s. Samples for inclusivity assessment should be chosen to reflect the genetic diversity of species on which the assay will be used; samples for exclusivity testing should be chosen to reflect related and potentially cross-reactive organisms and species, as well as those likely to co-occur in food products. Both inclusivity and exclusivity testing should be performed on purified samples and amounts of DNA/RNA should be equal between inclusivity samples and exclusivity samples. Samples used in specificity testing should be traceable to the source. A method developer who has concerns regarding samples for specificity testing can work with the MVS to determine the best course of action. For methods which may be used as confirmatory testing on a purified target (e.g., bacterial isolates), please refer to *Guidelines for the Validation of Microbiological Methods for the FDA Foods Program*, section 2.3.2 "Validation Criteria for Identification Methods", for additional study design parameters.

False Negative and Positive Rates: A false negative (FN) occurs if the test result is negative and the actual condition is positive (present at a concentration ≥ LOD); a false positive (FP) occurs if the test result is classified positive when the actual condition is negative. Both FN and FP rates should be determined as <5% using a valid statistical approach. An example approach for determining FN and FP rates as <5% is provided in Appendix 3.

Robustness/Ruggedness: The robustness/ruggedness of a method is determined by measuring its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure. The method should provide the expected results when small deviations are introduced from the experimental conditions described in the procedure. For qualitative analyses, all replicates should give positive results. Depending on the use of the intended method, the target amount/concentration to be tested can be either at the limit of detection or level of concern/interest.

Note: The following factors are potential examples of robustness/ruggedness testing:

- thermal cycler (brands and models)
- master mix (e.g., final concentrations of salts, dNTPs, or adjuvants such as BSA or glycerol)
- reaction volume
- probe and primer concentrations
- thermal cycling parameters

The following table provides guidance for a multifactorial robustness test that may be used to validate PCR assays.

Table 2. Example Robustness/Ruggedness Testing Matrix* (n≥3)

Factor	*Combination*							
	1	2	3	4	5	6	7	8
PCR Equipment	A	A	A	A	B	B	B	B
PCR kit or reagent provider	X	X	Y	Y	X	X	Y	Y
Primer concentration	NC	-30%	NC	+30%	NC	-30%	NC	+30%
Probe concentration	NC	-30%	+30%	NC	-30%	NC	NC	+30%
MM Volume	-5%	-5%	+5%	+5%	+5%	+5%	-5%	-5%
Annealing Temp.	+1ºC	-1ºC	+1ºC	-1ºC	-1ºC	+1ºC	-1ºC	+1ºC

NC: No change from optimized conditions
MM: Master mix

**From: Guidelines for the single-laboratory validation of qualitative real-time PCR methods-Bundesamt fur Verbraucherschutz und Lebensmittelsicherheit-March 2016*

2.4.2 Quantitative PCR Methods

The analysis of nucleic acid, especially in processed foods, requires the detection of very small amounts of target-specific DNA/RNA. The result of a quantitative PCR analysis is often expressed in % as the amount of target nucleic acid relative to an endogenous control or a taxon-specific PCR product, therefore, this measurement involves two PCR-

based determinations – that of the target-specific DNA/RNA sequence and that of the comparator. Each of these determinations has its own uncertainties, and the two are likely to have different measurement characteristics. It is thus important that both measurements are properly validated, and that the assay is fit for purpose. For quantitative PCR methods, the basic performance characteristics are:

- Extraction Efficiency: See Section 2.4.1
- PCR Efficiency
- Dynamic Range (Range of quantification)
- Sensitivity (Limit of Detection-LOD): See Section 2.4.1
- Sensitivity (Limit of Quantification-LOQ)
- Specificity (Selectivity): See Section 2.4.1
- Precision (Repeatability and Reproducibility): Standard Deviations
- Robustness/Ruggedness: See Section 2.4.1
- Trueness

Standard Curves: The generation of standard curves is required for the optimization of quantitative PCR analyses. At the initial stages of method development, standard curves should be derived from pure samples of target nucleic acid. Assays used for the analysis of food should also include standard curves generated using a relevant food matrix or matrices (see section 3.1 below for selection of relevant matrices). These standard curves will be used to determine the PCR efficiency, dynamic range, and limit of quantification, which are discussed below.

PCR Efficiency: PCR efficiency is a measure of how close the observed reaction is to a true statistical doubling of amplified product over successive cycles and is based on C_T values. The efficiency is tested by preparing a standard curve of the template DNA/RNA and determining the C_T-value for each dilution. If the amplification efficiency is 100%, a two-fold reduction in template DNA/RNA should result in an increase in the C_T value of one cycle. Therefore, if DNA/RNA is diluted 10-fold, the theoretical difference in C_T values between the two concentrations of template nucleic acid should be approximately 3.32 cycles. The average slope of the standard curve should be between -3.1 and -3.6, which corresponds to an efficiency of 90%-110%. Most current real-time PCR analysis software packages will calculate the slope and PCR efficiency of an assay based on the C_T values of the standard curve. In rare cases, quantification can still be performed using assays with efficiency outside the optimal range. The originating laboratory should contact the appropriate MVS with questions regarding validating such assays. The slope of the standard curve is calculated using the equation $y = mx + b$ (where y = C_T value and x = log target amount). Subsequently, the PCR efficiency is calculated as $E = 10^{-1/m} - 1$, where E = efficiency and m = slope of the linear standard curve. Significant deviations in PCR efficiency may indicate the presence of PCR inhibitors or that the assay needs to be further optimized. Reaction efficiencies determined using known amounts of target spiked into a food matrix will also indicate extraction efficiency for that matrix.

Dynamic Range-:: The dynamic range is the concentration range over which the target nucleic acid sequence will be reliably quantified. This desired concentration range defines the standard curves which will be used for quantification. Generally, a minimum five-log concentration range with three replicates each is necessary to determine the dynamic range of the assay; a well-designed and well-optimized real time PCR assay will have a linear range of 6-8 orders of magnitude. However, each method should be validated for a dynamic range that is relevant to the application. If a method is validated for a given range of values, the range may not be extended without further validation. The R^2 value of the standard curves used to determine the dynamic range should be ≥0.98.

Limit of Quantification (LOQ): The limit of quantification is the lowest amount of analyte in a sample that can be reliably quantified. There are multiple experimental approaches to determine the LOQ, such as assaying spiked samples that have a known amount of analyte, or analyzing a number of samples that contain known amounts of analyte. The limit of quantification is the minimum nucleic acid concentration for which all 12 replicates give a positive result with a C_T coefficient of variability (CV) of no more than 0.5 C_T. The quantification should be determined by spiking the target organism into a relevant food matrix prior to sample preparation and DNA/RNA extraction. Quantification should be expressed in units which are relevant to the intended purpose of the method, for example as mg/kg, parts per million, or percentage in a food matrix.

Repeatability and Reproducibility Standard Deviations ($RSD_{repeatability}$ and $RSD_{reproducibility}$): $RSD_{repeatability}$ is the relative standard deviation of results obtained with the same method, by the same analyst, in the same laboratory, with the same equipment, on the same samples (repeatability conditions). The $RSD_{repeatability}$ should not exceed 25% over the whole dynamic range of the assay. The relative standard deviation between laboratories ($RSD_{reproducibility}$) should be less than 35%. These values account for the inherent variability of biological systems. RSD values may be higher at lower target levels due to stochastic effects which occur during both sampling and the PCR process itself.

Trueness: Trueness compares the obtained value from a series of samples to the actual or reference value. Trueness should be within ± 25% of the accepted reference value across the whole dynamic range of the assay.

2.4.3 Qualitative and Quantitative Multiplex Assays

For multiplex assays, all method validation must be carried out in multiplex and performance metrics described above must be reported for each individual target as it performed under multiplex conditions. For probe-based assays, the signals from the fluorophores on different targets must not interfere with each other. Multiplex intercalating dye-based assays will not be considered quantitative because intercalating dyes do not distinguish between different targets in a multiplex assay.

2.4.4 Detection of Specific Nucleic Acid Sequences by Sanger Sequencing

In a Sanger sequencing-based method, dideoxy-nucleotide (ddNTP) chain terminators are used to determine the specific nucleotide sequence of the target nucleic acid. The

guidelines set forth in this document are focused on validation of the laboratory procedures used to generate a specific, target DNA/cDNA sequence. The validation of laboratory procedures detailed here is distinguished from a fit-for-purpose determination that a specific gene or target region can be used to effectively and reliably differentiate the target organism or group. That work should be performed during method development and information on the appropriateness of the specific gene or target region should be provided or referenced as background information in the validation report.

Current Sanger sequencing-based methods are most commonly carried out via a multi-step process which includes not only appropriate sampling and nucleic acid extraction but also: 1) conventional PCR amplification of the target region, 2) PCR cleanup for removal of unincorporated primers and nucleotides, 3) a sequencing reaction in which the PCR product is used as template for the incorporation of fluorescently labelled dideoxy chain terminators, 4) sequencing reaction cleanup for removal of unincorporated fluorescent dideoxy chain terminators, and 5) simultaneous size-dependent separation and nucleic acid sequence determination via capillary electrophoresis. The entire workflow, from sampling and DNA/cDNA extraction through analysis of sequencing data, must be validated as a single method. Changes to any step in the workflow will require method extension; significant changes may require revalidation. For procedures based on Sanger sequencing, the basic performance characteristics are:

- Extraction Efficiency: See Section 2.4.1
- Quality of Sequencing Template
- Quality of Sequencing Data
- Robustness/Ruggedness
- Precision
- Specificity
- Accuracy
- Confirmation of Identity

Quality of Sequencing Template: PCR products used as sequencing templates should consist of specific amplicons from the intended target region in an amount sufficient for the sequencing reaction. The minimum amount of template required or the range of template over which the sequencing reaction will be successful should be determined during the method development and SLV processes and that information should be provided in the MLV proposal package. PCR optimization parameters are the same as those described for robustness/ruggedness in Section 2.4.1. PCR products to be used as sequencing templates should produce a single, distinct band of the expected size on a gel (i.e. agarose, acrylamide etc.).

Quality of Sequencing Data: Important parameters in determining quality of sequencing data are base quality scores, visual inspection of chromatograms, and length of sequence obtained. The primary parameter used for evaluating sequencing data is the base quality score. It should be noted that the way in which these scores are generated can vary depending on the specific software package being used and this should be considered if

multiple platforms are used for an MLV. For most platforms, a quality score of 20 or higher indicates accuracy of 99% or above for a given base call in a sequence and is considered "high quality." Generally, at least 90% of the bases in a sequence should have high quality scores. Bidirectional sequencing, in which both forward and reverse strands of the DNA/RNA target region are sequenced, should be employed to generate higher quality data and greater confidence in results. When data are obtained from single reads, in which only one strand is sequenced, higher quality value cutoffs, for example 98% instead of 90%, are recommended. On visual examination, chromatograms should show level baselines, high signal-to-noise ratios, and clear, single peaks for base positions. Signal intensities should be consistent, and chromatograms should not contain broad, high-intensity peaks indicative of the presence of unincorporated fluorescently labeled ddNTPs. The length of the sequence obtained should be reported, and a minimum acceptable length should be established based on the length of the target region. The procedure and parameters used for analysis of raw sequence data, including any reference sequences, should be clearly stated and should be objective. Methods intended for routine regulatory use should not include manual trimming or manual editing of sequences, if possible, as these may introduce unwanted operator bias. Peaks not able to be assigned by the automated software should be scored as sequencing ambiguities and cut-offs should be established for acceptable levels of sequencing ambiguities.

Robustness/Ruggedness: For sequencing assays in which a PCR product serves as the sequencing template, robust, specific amplification of the target region is a major determinant of method robustness and sequencing success. In addition, robustness may be affected by conditions used for the sequencing reaction. Guidelines for robustness testing detailed in section 2.4.1 apply to both the PCR and sequencing reactions in a Sanger sequencing method.

Precision: Sequencing methods must demonstrate precision in terms of both repeatability and reproducibility. Repeatability, or within-run precision, is demonstrated when the same sequence is obtained from the same sample by the same analyst in the same laboratory under the same conditions. Reproducibility, or between-run precision, is demonstrated when the same sequence is obtained from samples run using the same method in different laboratories.

Specificity: For sequencing-based methods, specificity testing determines that the sequence generated is unique to the species or group being tested (e.g. sequence variation within the species inclusion group must be sufficiently small compared to its variation with the exclusion group). This ensures that the target group can be effectively differentiated. A maximum acceptable level of variation within a target group should be established during method development and provided in the validation report. A given sequencing method must be validated with a sufficient number and variety of organisms that are closely related to the target or likely to co-occur (Section 2.4.1), and acceptable levels of within-species vs. between-species variability must be demonstrated.

Accuracy: For a sequencing method, accuracy is the extent to which the sequence obtained for a given sample matches that of a known reference. Reference sequences used to determine accuracy are from organisms/species whose identity has been independently determined. Quality cut-offs for reference sequences should be equal to or greater than those established for the target (unknown) sequence.

Confirmation of Identity: Identity of the target sequence should be confirmed by comparing the sequences obtained to a library of reference sequences obtained from specimens of known origin.

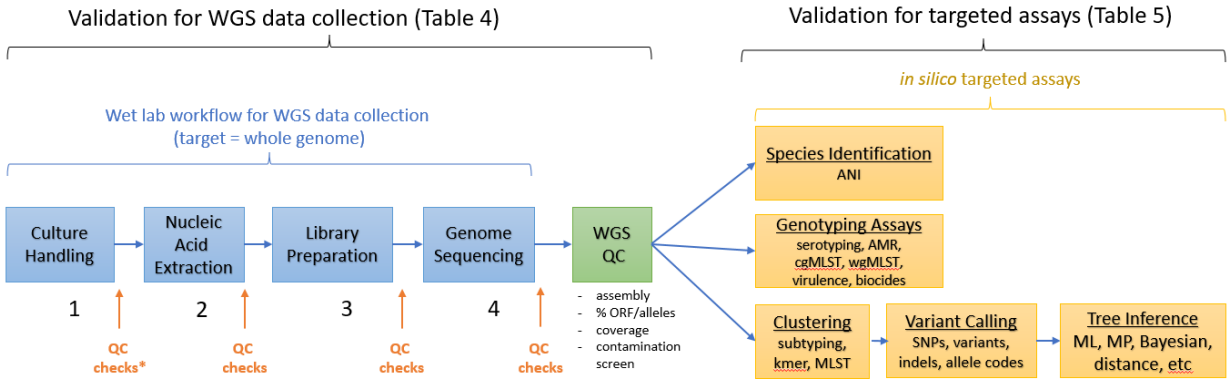
2.4.5 Next Generation Sequencing of Pure Bacterial Isolates

Next Generation Sequencing (NGS) has revolutionized how bacterial genomes are sequenced and analyzed for species identification, serotyping, virulence typing, evaluating phylogeny as well as many other genotypic characterizations. For over two decades, Pulsed-Field Gel Electrophoresis (PFGE) was the gold standard for sub-typing and the primary laboratory tool for investigating foodborne outbreaks to identify the source of contamination during traceback analysis. NGS methods have replaced PFGE as the gold standard. Many different types of laboratories (regulatory, public health, animal, environmental, veterinary, agriculture) routinely use short read genome sequencing methods for identifying and characterizing bacterial isolates.

The goal of this section is to provide validation criteria for the standardization of short read next generation sequencing analytic (primary sequence generation) and bioinformatic (sequence evaluation and analysis of) processes for bacterial isolates in FDA laboratories. These performance metrics provide guidance for the development of NGS methods that can generate high quality and reliable data for regulatory and research purposes.

The overall NGS workflow, including primary genomic sequence data acquisition and bioinformatic data analysis pipeline components are illustrated below in figure 1. Workflow components can be validated in a modular approach.

Figure 1. NGS Validation Workflow for Pure Bacterial Isolates



*More information on QC checks for wet lab workflow can be found in the draft reference document titled CDC NGS QC Guidance for Illumina Workflows.

The following three tables (3-5) outline the performance criteria for establishing NGS method validation for short read sequencing. This section is intended for the analysis of pure bacterial cultures and does not include genomic sequencing from whole genome sequencing/genome skimming of eukaryotes, chloroplasts, mitochondria, parasites, viruses or metagenomic applications (Contact MMVS for guidance on the validation of non-bacterial sequencing). Please refer to the glossary (Appendix 4) for detailed explanations of performance characteristics, other validation criteria and NGS terms.

Tables 3a-d provide descriptive details for designing different types of validation (SLV, ILV, and MLV) and verification studies. This table also provides further performance criteria for generating and collecting primary genomic sequence data and for bioinformatic pipelines for targeted gene prediction typing methods and phylogenetic clustering workflows.

Table 3. Study Design & Primary Data Collection

Performance Criteria	Single Laboratory Validation Study (SLV)	Independent Laboratory Validation (ILV) Study	Multi-Laboratory Validation (MLV) Study	Verification Study
Participating Laboratory	Originating Laboratory	Collaborator	Collaborators	Single Lab
# of Collaborators Providing Usable Data	NA	1	10[a]	NA
Reference Method Requirement[j]	Yes, if available	Yes, if available	Yes, if available	Yes, if available
Table 3b. Genomic Primary Data Collection and Targeted Confirmation and Typing – Pure Culture[b]				
Genomic Primary Data Collection and Targeted Confirmation and Typing – Pure Culture[b]				
Isolate Panel for Primary NGS/WGS Data Collection	"CFSAN WGS strain validation set" or modification (data set should represent variability of the organism(s)[c,d]	Same Panel[c]	Recommend relevant strains for study outcome (at least 50% of original panel)	Recommend relevant strains for study outcome (at least 50% of original panel)[e]
Repeatability (Precision within run)[f]	Isolate DNA run 3X at the same time on the same instrument (30% Strain Panel)	Isolate DNA run 3X at the same time on the same instrument (30% Strain Panel)	Isolate DNA run 2X (50% Strain Panel)	Isolate DNA run 1X (50% Strain Panel)
Reproducibility (Precision between runs)[f]	Isolate DNA run 3X on different runs	Isolate DNA run 3X on different	Isolate DNA run 1X (50% Strain Panel)	NA

	(100% Strain Panel)	runs (100% Strain Panel)		
Table 3c. Dataset for Targeted Workflow Assays				
Dataset for Targeted Workflow Assays[g,h]	200 Isolates for Inclusivity 100 Isolates for Exclusivity	Same Panel	At least 50% of original panel	At least 50% of original panel
Repeatability (Precision within run)	Dataset run 2X (Method developers should confirm no variation)[i]	NA	NA	NA
Reproducibility (Precision between runs)	NA	Run dataset 1X	Run dataset 1X	Run dataset 1X
Table 3d. Phylogenomic Clustering and Tree Inferences				
Phylogenomic Clustering and Tree Inferences[j] (refer to Table 5)	Assemble dataset that approximates the complexity and diversity for each use case (e.g., species, serovars, outbreak clusters)[k]	Replicate originating laboratory results with defined benchmark datasets	Replicate originating laboratory results with defined benchmark datasets	Replicate originating laboratory results with defined benchmark datasets
Repeatability (Precision within run)	Build in enough replicates to capture expected variation (i.e., 10X, 100X, 1000X)	NA	NA	NA
Reproducibility (Precision between users/laboratories)	NA	Run validation dataset 1X	Run validation dataset run 1X	Run validation dataset run 1X

[a] Recommendation for at least one non-FDA lab.
[b] Using pure microbial isolates.

[c] Recommended representative panel of microbial isolates that are commercially available (strain validation set will be available through Microbiologics, Inc.). May use other representative panels depending on the application. Contact MMVS for strain validation set.
[d] For bioinformatic pipelines, number of required isolates may be greater. Consult with MMVS or CMVS prior to initiating study.
[e] Participation in ILV or MLV qualifies for verification.
[f] Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Food and Feed, October 2019. 3rd edition, USFDA
[g] Some examples: speciation, genotyping, serotyping, antimicrobial resistance, virulence markers, phylogenomic clustering, etc.
[h] For *Salmonella*, inclusivity and exclusivity may be higher numbers. Consult with MMVS.
[i] For algorithms where stochastic variation is expected, method developers should build in enough replicates to capture expected variation (i.e., 10X, 100X, 1000X). Consultation with statistician is advised.
[j] Method developers should use benchmark datasets using the link below or develop and submit their own benchmark datasets
https://github.com/globalmicrobialidentifier-WG3/datasets
[k] Consult with MMVS/CMVS (SME or TAG) when designing this type of validation study.

NA=Not Applicable

Table 4 provides descriptive details for assessing the various NGS primary sequence generation components (modules) which includes a). Performance Criteria Elements, b). Parameters Measured and c). Minimum Acceptance Criteria Requirements.

Table 4. Performance Criteria for Assessing NGS Methods

NGS Method Component (Module)	Performance Criteria Element	Parameter Measured	Minimum Acceptance Criteria
Sample Preparation/ Handling	Reproducibility/ Repeatability	• Identity and purity of pure cultures confirmed • Stored and cultured to minimize genetic change • Metadata associated with sample retained	Minimum Requirement, see Appendix 2 and 5
Nucleic Acid Extraction	Reproducibility/ Repeatability	• Quality and quantity of nucleic acid meets or exceeds downstream requirements • No introduction of impurities/contamination	Minimum Requirement, see Appendix 2 and 5
Library Preparation	Reproducibility/ Repeatability	• Quality/Quantity of sample library meets or exceeds downstream requirements	Minimum Requirement, see Appendix 2 and 5
Genome Sequencing[a] (WGS QC)	Accuracy Specificity Sensitivity Reproducibility Repeatability	• Raw Sequence QC • Quantity and Quality • Cluster Density • Clusters Passing Filter • Overall % • Avg. Depth of Genome Coverage • Mean Read Length • Sequence Length Distribution • Assembly Length (Mbp) • # Contigs	Minimum Requirement[a], see Appendix 2 and 5

		• N50 • Contamination • % ORF/ % Core Present	

[a] For validation submissions in which a novel or updated QC workflow is utilized (i.e., MicroRunQC, FastQC, etc.), we recommend that method developers provide a detailed description for review to the Bioinformatic Technical Advisory Group. The Bioinformatics TAG will determine if a review by the MMVS/CMVS is required.

Table 5 provides descriptive details for the performance criteria to be used in validating bioinformatic workflows (*in silico* gene(s) prediction assays) which includes a). Performance Criteria Elements, b). Parameters Measured and c). Minimum Acceptance Criteria Requirements.

Table 5. Validation Criteria for *in silico* Assays

NGS Bioinformatic Module (*in silico* assays)[a]	**Performance Criteria Element**	**Parameter Measured**	**Minimum Acceptance Criteria[b]**
Species identification	Accuracy Specificity Sensitivity	Correct species name identified	Precision at ≥ 95% Level of agreement at ≥ 95% Repeatability and reproducibility 100%
Genotyping method (serotyping, AMR, virulence, toxin, biocides, cgMLST, wgMLST, etc)	Specificity Sensitivity Accuracy/Level of Agreement	Correct identification of a known set of genes or alleles.	Precision at ≥ 95% Level of agreement at ≥ 95% Repeatability and reproducibility 100%
Phylogenomic clustering methods[c]	Specificity and Sensitivity for cluster assignment.	Comparison to empirical benchmark datasets and/or simulated datasets, for trees and for variants.	Unique to each study.

	Specificity and Sensitivity for calling variants (SNPS and/or indels). Accuracy of entire tree OR specific splits (e.g., leading to an outbreak lineage).		

[a] A bioinformatic method that approximates an existing validated wet bench method (species ID, serotyping, AMR, etc). Results of these methods are reported in FDA's regulatory workflow. The Bioinformatics TAG will determine if a review by the MMVS/CMVS is required.
[b] Repeatability should be 100% for species ID and genotypic assays (variation is not expected for these assays). Repeatability should be nearly 100% for phylogenomic clustering (e.g., tree inference should uncover significant splits accounting for the stochastic nature of these algorithms). Level of agreement in comparing new NGS method to gold standard method should be 95% or better.
[c] Consult MMVS/CMVS before designing this type of validation study.

3.0 Additional Procedural Guidance

3.1 Food Matrix and Sample Selection

Food matrix and sample source selection should be based on the types of foods most likely to be used in the analysis or based on historical cases of contamination. A nucleic acid-based method intended for use in processed foods should be tested on samples subjected to similar processing. Special attention should be paid to sample purity or subsampling requirements, as well as processing conditions and matrix characteristics which are known to have particularly adverse effects on nucleic acid-based assays; these include high-temperature and high-pressure treatments (e.g., canning) and low pH (e.g., tomato-based products). The number of food categories used during the validation process depends on the intended use of the method. See Appendix 1 for a listing of food categories and examples of processing methods for each. It is recommended that validations attempt to ascertain the effects of sample age, handling, and storage conditions, when applicable, as these can affect the stability of DNA/RNA.

If the assay to be validated is designed to detect a microorganism, please refer to *Guidelines for the Validation of Microbiological Methods for the FDA Foods Program* for additional information on food matrix and sample source selection.

3.2 Matrix Extensions

The validation of method performance with a new matrix is intended to assure that the method will continue to produce accurate and reliable results. Emergency matrix

extensions (Level 1 in Table 1B) are intended for those instances in which a validated method is used with a matrix not previously validated in response to a real or perceived threat to food safety or public health. Matrix extensions of validated methods that are intended to increase the regulatory scope and applicability, such as running the method on a recurring basis, would minimally fall under Level 2 validation in Table 1B. It is generally assumed that the more closely related a new food matrix is to a previously validated matrix for a defined analyte, the greater the probability that the new matrix will behave similarly. Appendix 1 provides guidance on commodity categories. The number of different food categories to be validated depends on the applicability and intended use of the method. Depending on how many categories will be validated, a minimum of 1 – 3 representative matrices from each category should be selected, depending on the level of validation required and the number of food categories being tested (refer to Tables 1A and 1B).

3.3 Platform Extensions

Expanding the use of a validated method to include another significantly different instrument or platform requires further validation. Such instances include the use of an instrument or platform similar in scope and function to that currently validated for approved use. However, it may have differences in throughput, configuration, chemistry, or detection methodology. Platform extension validation should be performed as described in Table 1B, Level 2. For NGS methods, platform extension validation should be performed as described in Tables 3a-d. Depending on the complexity of the platform extension, it is recommended to consult with the appropriate Method Validation Subcommittee or TAG for guidance. In planning platform extensions, acceptance criteria for these comparisons must be established by the validating laboratory. Care must be taken to ensure that the new platform produces equivalent results to the originally validated method.

3.4 *In silico* Workflow Updates

Bioinformatic workflows are constantly being updated to include new information or more efficient scripts and new ways of displaying the output data. New versions of validated workflows should be validated using at least 30% of the original dataset that represents the complexity and diversity of that dataset. Alternatively, other appropriate datasets may be used; however, it is recommended to consult with the Bioinformatic Technical Advisory Group for advice and guidance. Repeatability should be 100% for species ID and genotypic assays (variation is not expected for new versions). Repeatability should be nearly 100% for phylogenomic clustering. Level of agreement in comparing new version workflow to original validated workflow should be 100%. Care must be taken to ensure that the new version produces equivalent results to the originally validated workflow.

3.5 Method Modifications of Existing Validated Methods

Validated methods may be modified due to a number of different circumstances. The level of modification to an existing method will impact the specific criteria necessary to support

the validated status of the method. At a minimum, performance characteristics of the modified method should be compared to those of the original method. It is recommended to consult with the appropriate Method Validation Subcommittee or TAG for guidance.

3.6 Reference Materials

All reference specimens used for confirmation of identity should be identified by an authoritative taxonomic expert using established identification techniques. Sequences included in a reference library should be collected from a wide variety of organisms or groups likely to be encountered based on the intended purpose of the method. These reference libraries must be curated for quality and accuracy. Optimally, reference sequences should be generated under a defined set of conditions to assure quality. If reference sequences are derived from public databases, it is incumbent on the originating laboratory to verify the validity and quality of the sequence used. In addition, accession numbers for all sequences used for the development of molecular assays (and any supporting documentation) should be included in the validation package.

3.7 Reporting of Results of Laboratory Validations

See Appendix 2 for a listing of information that should be provided from the originating laboratory when the results for a laboratory validation study are prepared for review. This list of information is in addition to the results obtained for the validation criteria.

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Appendix 1. Example Food Categories for Validation Studies (Not Inclusive)

Food Categories

Meat: Raw, Heat processed, Frozen, Fermented, Cured

Poultry: Raw, Heat processed, Frozen, Other

Seafood: Raw, Heat processed, Frozen, Smoked

Fruits and vegetables: Raw, Heat processed, Frozen, Dry, Fermented, Cured/Salted, Juice/Concentrate, Low Moisture

Dairy: Raw, Heat processed, Frozen, Fermented, Dry

Chocolate/Bakery: Low Moisture, Dry, Milk Chocolate

Animal Feed: Low Moisture, Dry Pet

Pasta: Un-cooked, Pre-cooked

Miscellaneous: Dressings, Spices, Mayonnaise, Egg/Derivatives, Cereal/Rice

Representative Food Products within Categories (Not Inclusive)

Meats: Ground beef, ground pork, meat by-products, glandular products, frog legs, rabbit carcasses, lamb, sausage, frankfurters, lunch meat, beef jerky, meat substitutes

Poultry: Ground chicken, ground turkey, cooked chicken, raw chicken parts

Seafood: Raw shrimp, fish sticks, surimi, raw fish filet, raw oysters, raw mussels, raw clams, cooked crawfish, smoked fish, crabmeat (fresh or pasteurized)

Fruits, Vegetables, and Nuts: Fresh / frozen fruits or dried fruits, orange juice, apple juice, apple cider, tomato juice, melon cubes, berries, pecans, walnuts, peanut butter, coconut, almonds, lettuce, spinach, kale, collard greens, cabbage, bean sprouts, seed sprouts, spent water from sprouts, peas, mushroom, green beans

Dairy: Yogurt, cottage cheese, hard and soft cheeses, raw or pasteurized liquid milk (skim, 2% fat, whole, buttermilk), infant formula, coffee creamer, ice cream, nonfat dry milk / dry whole milk, dried buttermilk, dried cheese spray

Chocolate / bakery: Frosting and topping mixes, candy and candy coating, milk chocolate

Animal feed: Dry food, meat and bone meal, chicken and feather meal

Pet food: Dry pet food, wet pet food, canned pet food, raw pet food, ingredients/supplements, pet treats

Uncooked Pasta: Uncooked noodles, macaroni, spaghetti

Miscellaneous: Shell eggs, liquid whole eggs, oral or tube feedings containing egg, dried whole egg or dried egg yolk, dried egg whites, Oregano, pepper, paprika, black pepper, white pepper, celery seed or flakes, chili powder, cumin, parsley flakes, rosemary, sesame seed, thyme, vegetable flakes, onion flakes, onion powder, garlic flakes, allspice, Wheat flour, casein, cake mixes, whey, nonfat dry milk/dry whole milk, corn meal, dried whole egg or dried egg yolk, dried egg whites, soy flour, dried yeast, cereals, dried buttermilk, dry cheese spray

Appendix 2. Information to be Provided with Method Validation Documents

For Qualitative and Quantitative PCR-Based Assays:

Assay Design

- Type of assay: oligonucleotide probe-based or double stranded DNA dye-based
- Name of target gene or region
- Internal control/amplification control type: exogenous or endogenous
- Exact oligonucleotide sequences for all primers and probe(s)
- Length of PCR product (amplicon)
- Dye and probe characteristics. For probe-based assays, provide a brief description of the probe chemistry and the identities and locations of fluorophores and quenchers, including internal quenchers. For dye-based assays, state which dye is being used.
- Any other reporter molecules.

Sample Preparation and Nucleic Acid Extraction

- Form and quantity of sample required. Include information on subsampling or sample compositing as well as relevant aspects of handling and storage.
- Method or kit used for DNA extraction. Include any relevant modifications as well as information on RNAse treatment. Independent assessments of DNA/RNA quality and quantity are not required as long as the method is shown to yield acceptable/reliable PCR results. For quantification of targets in complex food matrices, normalization of total DNA/RNA amounts prior to PCR may result in higher quality data.

PCR Conditions

- Reaction: reaction volume; identities and concentrations of all reaction components, including buffer or master mix, all primers, all probes and/or dyes, template DNA/RNA, Mg^{2+}, and additives (e.g., BSA, DMSO, or glycerol).
- Platform: State make and model of real-time PCR platform as well as name and version of accompanying software. Include brief descriptions of physical format (e.g., 96 well thermal block or other) and optical system.
- Thermal cycling conditions. Include PCR cycling conditions for both dye-and probe-based assays; also include melt conditions for dye-based assays. Optimal cycling conditions should be determined empirically and not through software-based calculations of primer or probe annealing temperature, as annealing temperatures can be significantly affected by specific reaction conditions.

Data Analysis

- Specify which software program and version was used for data analysis.
- Report and explain any adjustments made to baseline and threshold determination, or other software default analysis parameters.

- For dsDNA dye-based assays, analysis of melt curves must be performed to confirm the presence of a single, sharp melting peak optimally with a melting temperature (T_m) of approximately 80-90°C in all samples and standards.

For Sanger Sequencing Assays:

Assay Design

- Name of target gene or region
- Length of target gene or region
- Exact oligonucleotide sequences for PCR primers
- Exact oligonucleotide sequences for sequencing primers

Sample Preparation and Nucleic Acid Extraction

- Form and quantity of sample required. Include information on subsampling as well as relevant aspects of handling and storage.
- Method or kit used for DNA/RNA extraction. Independent assessments of DNA quality and quantity are not required as long as the method is shown to yield acceptable, reliable PCR and sequencing results.
- Range over which the quantity of extracted DNA has been tested and shown to provide acceptable results.

PCR and Sequencing Reaction Conditions

- Reaction: reaction volume; identities and concentrations of all reaction components, including buffer or master mix, all primers, all probes and/or dyes, template DNA/RNA, Mg^{2+}, and additives (e.g., BSA, DMSO, or glycerol)
- Thermal cyclers used for both PCR and sequencing reactions
- Thermal cycling conditions for both PCR and sequencing reactions. Optimal cycling conditions should be determined empirically and not through software-based calculations of primer annealing temperature, as annealing temperatures can be significantly affected by specific reaction conditions.

Sequencing Platform and Sequencing Data Quality

- Make and model of capillary electrophoresis platform
- Length and percentage of high quality bases for all species used in specificity and confirmation of identity
- Specify whether validation used bidirectional or single reads

Data Analysis

- Parameters used for processing of raw data on capillary electrophoresis platform
- Software program and procedure used for downstream data analysis.
- Details/location of reference sequence database used for confirmation of identity

For Next Generation Sequencing Assays: Refer to Appendix 5

Assay Design

- List study design: (SLV, ILV, MLV or Verification)
- Instrument Platform (Make/Model):

- Instrument Control Software Name/Version:
- Sample Type:
- Library Preparation:
- Cartridge Type:

Sample Preparation and Nucleic Acid Extraction

- Form and quantity of sample required. Include information on subsampling as well as relevant aspects of handling and storage
- Method or kit used for nucleic acid extraction

Sequencing Conditions

- Library prep kits and protocols including relevant details for handling and storage
- Sequencing cartridges, flow cells and sequencing protocols

Sequencing Platform and Sequencing Data Quality

- Make and model of NGS platform.
- Describe all expected minimum sequence quality metrics (Appendix 5)

For Next Generation Sequencing Data Analysis Assays: Refer to Appendix 8

Assay Design

- Workflow name, version, developer and location
- Study Category: (SLV, ILV, MLV or Verification)
- Assay Category: (Species Identification, Genotyping (Molecular Typing) or Phylogenomic Clustering)
- Input Dataset Collection
- Benchmark Dataset (if applicable)

Data Analysis

- Parameters used for processing of raw data on capillary electrophoresis platform
- Software program and procedure used for downstream data analysis
- Details/location of reference sequence database used for confirmation of identity
- Species Identification: Performance metrics including Accuracy/Level of Agreement, Sensitivity and Specificality
- Genotyping (Molecular Typing): Performance metrics including Accuracy/Level of Agreement, Sensitivity and Specificality
- Phylogenomic Clustering: Performance metrics including sensitivity and specificity for cluster assignment, and sensitivity for variant determination (SNPs, MLST etc)

Appendix 3. Example statistical approach to confirm false negative (FN) and false positive (FP) rates as <5%

Zero acceptance number sampling is a statistical approach commonly used to test a hypothesis (or criteria) for the frequency of defective items in a population (e.g., such as FN or FP rates with repeated testing). For this approach, all tested samples must have the correct response in order to accept the hypothesis (i.e., accept only when zero "defective" responses observed). The minimum number of samples that must be tested depends on the criteria for the defect rate and the level of statistical confidence:

$$n = \log(\alpha) \Big/ \log(1 - p)$$

where 1-α is the confidence level and *p* is the maximum acceptable defect rate per sample (e.g., FN or FP rate). Sample sizes to assess selected criteria for FN or FP rates with varying levels of confidence are provided in the following table.

	Confidence Level			
FN or FP rate	80%	90%	95%	99%
<1%	161	230	299	459
<2%	80	114	149	228
<5%	***32***	***45***	***59***	***90***
<10%	16	22	29	44

For example, if the goal is to have 95% confidence that the FN rate is <5% then test 59 samples with the nucleic acid target present at the concentration of interest, typically the LOD or a relevant level of concern, in a range of matrices. The criteria are satisfied if all 59 test results are positive for the target.

This sample size formula is related to the Clopper-Pearson confidence interval for Binomial proportions and frequently used for zero defect acceptance sampling plans for commodity lots. The rationale for the sample size is that when the probability of a defective (incorrect) test response is *p* for each sample then $(1 - p)^n$ is the probability that *n* samples will have the correct response. The minimum sample size required for a specified level of confidence follows from setting the probability of that outcome equal to the type I error rate *α* and solving for *n*.

Appendix 4. Additional Glossary of Terms

Adapted from: ISO 16577: 2016, Molecular biomarker analysis – Terms and definitions. NGS terms were adapted from ISO documents and other references.

Accuracy Closeness of agreement between a measured quantity value and a true quantity value of a measurand**.**

Adapters Short sequence-specific oligos ligated to the 5' and 3′ends of each DNA fragment in a sequencing library as part of NGS library preparation. The adapters are complementary to the short sequences present on the surface of Illumina flow cells.

Alignment The process of mapping sequencing reads to a reference sequence.

Amplicon DNA sequence produced by a DNA-amplification technology, such as PCR.

Amplicon Sequencing Sequencing of DNA amplicons in which PCR is used to amplify the target.

Analyte Component of a system to be analyzed.

Annealing Pairing of complementary single strands of nucleic acids to form a double-stranded molecule.

Assembly Reconstructing fragment sequences into higher order structures based on their overlap and reference sequence, where appropriate.

BAM File Binary version of SAM file, a typical output of the secondary phase of data analysis. BAM is a binary sequence file format that uses BZGF compression and indexing. BAM is the binary compressed version of the SAM (Sequence Alignment/Map) format, which contains information about each sequence read in an NGS data set with respect to its alignment position on a reference genome, variants in the read versus the reference genome, mapping quality, and the sequence quality string in an ASCII string that represents PHRED quality scores.

Benchmark Dataset a collection of data used as a standard or point of reference against which things may be compared or assessed.

Calibration Operation that establishes a relation between the quantity values with measurement uncertainties provided by measurement standards and corresponding indications with associated measurement uncertainties and uses this information to establish a relation for obtaining a measurement result from an indication.

Certified Reference Material (CRM) Reference material, accompanied by documentation issued by an authoritative body and providing one or more specified property values with associated uncertainties and traceability, using valid procedures.

Cluster, Epidemiological An aggregation of cases grouped in place and time that are suspected to be greater than the number expected, even though the expected number may not be known.

Cluster, Flow Cell A clonal grouping of template DNA bound to the surface of a flow cell. Each cluster is seeded by a single template DNA strand and is clonally amplified through bridge amplification until the cluster has ~1000 copies. Each cluster on the flow cell produces a single sequencing read. For example, 10,000 clusters on the flow cell would produce 10,000 single reads and 20,000 paired-end reads.

Cluster Generation Amplification of single molecules resulting in millions of unique, clonal clusters across a flow cell. (For more information refer to Illumina website.)

Combinatorial Dual Indexes Pairs of indexes such that every i5 index would be paired with each i7 index in a matrix to create unique index pairs, but not unique single-sided indexes.

Consensus Sequence When two or more DNA sequences are aligned, the overlapping portions can be combined to create a single consensus sequence. In positions where all overlapping sequences have the same base (a single column of the multiple alignment), that base becomes the consensus. Any position where there is disagreement among aligned bases can be written as the letter N to designate “unknown.”

Contigs A stretch of continuous sequence, *in silico*, generated by aligning overlapping sequencing reads.

Coverage Distribution A metric describing the percentage of bases sequenced across the genome or target region at a given depth (eg, 95% of bases covered with a minimum 10× coverage). Average or mean sequencing depth by itself (eg, 30× mean coverage) does not take into account the percentage of bases sequenced below acceptable threshold limits or bases that were not sequenced at all. For example, when a data set has a reported "coverage distribution of 95% with a minimum 10× coverage," this also indicates that 5% of bases were covered below the 10× threshold or not covered at all. For this reason, coverage distribution is commonly used along with mean coverage to describe sequencing results.

Coverage Level (or, depth) The average number of sequenced bases that align to, or “cover,” known reference bases. For example, a whole genome sequenced at 30×

coverage means that, on average, each base in the genome was sequenced 30 times. At higher levels of coverage, base calls can be made with a higher degree of confidence.

Cross-Reactivity Degree to which binding occurs between an antibody and antigenic determinants, or primers and a target sequence, which are not the analyte of primary interest.

Cycle threshold (C_T) In real-time quantitative PCR, the cycle at which the fluorescence from the reaction crosses a specified threshold level at which the signal can be distinguished from background levels.

de Bruijn graph This is a graph theory method for assembling a long sequence (like a genome) from overlapping fragments (like sequence reads). The de Bruijn graph is a set of unique substrings (words) of a fixed length (a k-mer) that contain all possible words in the data set exactly once. For genome assembly, the sequence reads are split into all possible k-mers and overlapping k-mers are linked by edges in the graph. Reads are then mapped onto the graph of overlapping k-mers in a single pass, greatly reducing the computational complexity of genome assembly.

Denaturation Process of partial or total alteration of the native structure of a macromolecule resulting from the loss of tertiary and/or secondary structure that is a consequence of the disruption of stabilizing weak bonds. DNA that has been converted from double-stranded to a single-stranded form by a denaturation process such as heating.

De-Novo Assembly The piecing together of genetic material without the use of a reference sequence.

Deoxyribonuclease/Ribonuclease (DNase/RNase) Enzyme that catalyzes the hydrolytic cleavage of deoxyribonucleic acid/ribonucleic acid that may produce a single nucleotide residue by cleavage at the end of the chain or a polynucleotide by cleavage at a position within the chain

Deoxyribonuclease/Ribonuclease Inhibitor Substance that either fully or partially blocks deoxyribonuclease/ribonuclease activity.

Deoxyribonucleic acid (DNA) Polymer of deoxyribonucleotides occurring in double strand (dsDNA) or single strand (ssDNA) form that is the carrier of genetic information, encoded in the sequence of bases (nitrogen containing ring compounds that are either purines or pyrimidines), and is present in chromosomes and chromosomal material of cell organelles as well as in plasmids and in viruses.

Deoxyribonucleotide Triphosphate (dNTP) Generic term referring to a deoxyribonucleotide that includes: deoxyadenosine nucleotide triphosphate (dATP), deoxycytidine nucleotide triphosphate (dCTP), deoxyguanosine nucleotide triphosphate (dGTP), deoxythymidine nucleotide triphosphate (dTTP) and deoxyuridine nucleotide triphosphate (dUTP).

Detection Limit/Limit of Detection measured quantity value, obtained by a given measurement procedure, for which the probability of falsely claiming the absence of a component in a material is β, given a probability α of falsely claiming its presence.

DNA Extraction Sample treatment for the liberation and separation of DNA from other cellular components.

DNA Polymerase Enzyme that synthesizes DNA by catalyzing the addition of deoxyribonucleotide residues to the free 3'-hydroxyl end of a DNA molecular chain, starting from a mixture of the appropriate triphosphorylated bases.

DNA Probe Short sequence of DNA labelled isotopically or chemically that is used for the detection of a complementary nucleotide sequence.

Electrophoresis Technique used for separating, identifying, and purifying molecules (e.g. plasmid DNA, DNA fragments resulting from digestion, RNA, protein, and PCR products) based upon the differential movement of charged particles through a matrix when subjected to an electric field.

Endogenous DNA Sequence Defined reference DNA sequence native to a corresponding taxon.

End-Point PCR Method where the amplicons are detected at the end of the PCR reaction, typically by gel electrophoresis and the amplified product is visualized with a fluorescent dye.

Error/Error of Measurement/Measurement Error Measured quantity value minus a reference quantity value.

Event Transgene construct and its unique site of insertion into a genome.

Event-Specific Method Detection method that targets DNA sequences at the integration site unique to a specific transformation event.

Exonuclease Enzyme that hydrolyses (cleaves) terminal phosphodiester bonds of a nucleic acid.

False Negative Error of failing to reject a null hypothesis when it is in fact not true.

False Negative Rate Probability that a known positive test sample has been classified as negative by the method**.** The false negative rate is the number of misclassified known positives divided by the total number of positive test samples.

$$\%\ \text{false negative results} = \left(\frac{\#\ \text{of misclassified known positive samples total}}{\#\ \text{of positive test results (including misclassified)}}\right) \times 100$$

False Positive Error of rejecting a null hypothesis when it is actually true.

False Positive Rate Probability that a known negative test sample has been classified as positive by the method. The false positive rate is the number of misclassified known negatives divided by the total number of negative test samples.

$$\%\ \text{false positive results} = \left(\frac{\#\ \text{of misclassified known negative samples total}}{\#\ \text{of negative test results (including misclassified)}}\right) \times 100$$

FASTQ A file of sequences of individual reads with corresponding quality indicators for each base. A typical result of primary analysis. Each sequence read is represented as a header line with a unique identifier for each sequence read and a line of DNA bases represented as text (GATC), which is very similar to the FASTA format. A second pair of lines is also present for each read, another header line and then a line with a string of ASCII symbols, equal in length to the number of bases in the read, which encode the PHRED quality score for each base.

Fitness for Purpose Applicability of a prescribed method or the degree to which data produced by a measurement process enables a user to make technically and administratively correct decisions for a stated purpose.

Flow Cell A glass slide or other solid surface with one, two, or eight physically separated lanes used as a consumable on NGS instruments. Sequencing templates are immobilized on the flow cell surface, which is designed to present the DNA in a manner that facilitates access to enzymes while ensuring high stability of surface-bound template and low nonspecific binding of fluorescently labeled nucleotides. Solid-phase amplification (cluster generation) creates up to 1000 identical copies of each single template molecule in close proximity. Densities on the order of ten million clusters per square centimeter are achieved.

Fluorescence Resonance Energy Transfer/FRET Distance dependent energy transfer from a donor molecule to an acceptor molecule resulting in enhanced fluorescence of the acceptor molecule after excitation with electromagnetic radiation of a defined wave length.

Fluorescent Probe Oligonucleotide or oligonucleotide analogue of defined sequence coupled with one or more fluorescent molecules emitting a fluorescent signal after specific hybridization to the target nucleic acid sequence which can be detected by the specific equipment.

Fluorophore Molecule with a functional group that absorbs energy of a specific wavelength and re-emits energy at a different (but equally specific) wavelength dependent on both the fluorophore and the chemical environment.

Fragment A short stretch of nucleic acid resulting from the fragmentation of longer stretches and sequenced. The required size of a fragment is specific to the type of experiment and sequencer possibilities.

Fragmentation Splitting of genetic material into fragments of desired sizes: mechanically (nebulisation, sonication) or enzymatically.

Genetic Engineering/Genetic Modification Selective, deliberate alteration of genes (genetic material) by means of recombinant DNA technology.

Genetically Engineered Content/GE Content Measured value that identifies and quantifies levels of genetically engineered (GE) traits or GE-derived material in a product.

Genetically Engineered Organism (GEO) Organism in which the genetic material has been changed through modern biotechnology in a way that does not occur naturally by multiplication and/or natural recombination.

Hot-Start PCR Method that uses a thermostable DNA polymerase enzyme which becomes activated at a specific temperature through an initial heating step to reduce non-specific amplification.

Hybridization Non-covalent sequence-specific interaction of two complementary nucleic acid sequences (either RNA and/or DNA) under an appropriate set of reaction conditions to give a double-stranded molecule.

Hybridization Probe Fragment of DNA/RNA of variable length which is used to detect the presence of nucleotide sequences (the target) that are complementary to the nucleotide sequence in the probe.

Identification Assay Procedure or method that is used to identify a single organism, trait, analyte, or pest at a specified taxonomic level.

InDel (Shortened term for Insertion/Deletion) insertions or deletions of one or more nucleotides that can cause a reading frame shift. Indels may be a product of errors in DNA sequencing, the result of alignment errors, or true mutations in one sequence with respect to another. In the context of NGS, indels are detected in sequence reads after alignment to a reference genome. Indels are called in a sample after variant detection has established a high probability that the indel is present in multiple reads with adequate coverage and quality, and not the result of errors in sequencing or alignment.

Indexes (Barcodes or Tags) A unique short DNA sequence added to each DNA fragment during library preparation. The unique sequences allow many libraries to be pooled together and sequenced simultaneously. Sequencing reads from pooled libraries are identified and sorted computationally, based on their barcodes, before final data analysis. Library multiplexing is a useful technique when working with small genomes or targeting genomic regions of interest. Multiplexing with barcodes can exponentially increase the number of samples analyzed in a single run, without drastically increasing run cost or run time.

Indexing Strategy Illumina and other commercial vendors support several indexing methods, including single and dual indexing. With single indexing, up to 48 unique 6-base indexes can be used to generate up to 48 uniquely tagged libraries. With dual indexing, up to 24 unique 8-base Index 1 sequences and up to 16 unique 8-base Index 2 sequences can be used in combination to generate up to 384 uniquely tagged libraries.

Inhibition Control Sample that enables the analyst to check that there has been no inhibition affecting the results of a DNA amplification assay. This amplicon may or may not be different from the target fragment. An inhibition control makes it possible to unambiguously interpret a negative result (highlighting the false negatives obtained in the presence of inhibitors).

Insert During library preparation, sample DNA is fragmented, and the fragments are ligated or “inserted” in between two oligo adapters. The original sample DNA fragments are also referred to as “inserts.”

Integration-Border Region Junction region where one element originates from the host organism and the other originates from the DNA introduced during transformation.

Endogenous Amplification Control Gene sequence naturally present in template DNA (e.g., housekeeping gene with known copy #/genome) that is amplified to check the quality and yield of a DNA extract.

Junction Region DNA sequence encompassing two consecutive sequences EXAMPLE A promoter and the coding region of a gene.

k-mer (also referred to as k-tup, k-tuple) A short word composed of a defined number (k) of nucleotides in a DNA sequence (GATC) that is used as an element of an algorithm. A sequence read can be broken down into shorter (length of k) segments of text (either overlapping or non-overlapping words). The length of the word is called the k-tup size. Very fast exact matching methods can be used to find words that are shared by multiple sequence reads or between sequence reads and a reference genome.

Lane A physical path on a flow cell where fluidics pass through allowing next generation sequencing to occur. On Illumina platforms, flow cells can have one or multiple lanes (MiSeq and HiSeq, respectively)

Library Preparation A molecular biology protocol that converts a genomic DNA sample (or cDNA sample) into a sequencing library (fragmentation and tagmentation with adaptors and indexes), which can then be sequenced on an NGS instrument.

Library A pool of nucleic acid fragments which has undergone all processing steps and is ready for actual sequencing.

Ligation The enzymatic process of joining two nucleic acid fragments.

Limit of Quantification/LOQ/Determination Limit Lowest concentration or content of the analyte of interest per defined amount of matrix that can be measured with reasonable statistical certainty consistently under the experimental conditions specified in the method. Generally expressed in terms of the signal or measurement (true) value that will produce estimates having a specified relative standard deviation (RSD).

Linearity Ability of a method of analysis, within a certain range, to provide an instrumental response or results proportional to the quantity of analyte to be determined in the laboratory sample.

Long Reads The output of current and emerging third generation sequencing technologies, such as PacBio and Oxford Nanopore, where single reads can reach 10,000 to 100,000 bp or longer.

Loop-Mediated Isothermal Amplification (LAMP) Method for achieving isothermal DNA amplification by utilizing a set of four to six specially designed primers and a polymerase with high strand displacement activity.

Massively Parallel Sequencing Another name for next-generation sequencing.

Mate Pair-End-Read Strategy for sample preparation where the longer fragment (thousands of bases) is circularized using labelled adapters, the molecule is subsequently fragmented, but only the fragments containing the labelled adapters are sequenced.

Matrix All relevant components of a sample inclusive of analyte.

Measurand Quantity intended to be measured.

Measurement Precision Closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions. Measurement precision is usually expressed numerically by measures of imprecision, such as standard deviation, variance, or coefficient of variation under the specified conditions of measurement. Measurement precision is used to define measurement repeatability, intermediate measurement precision and measurement reproducibility.

Measurement Repeatability Measurement precision under a set of repeatability conditions of measurement.

Measurement Reproducibility Measurement precision under reproducibility conditions of measurement.

Measurement Trueness Closeness of agreement between the average value of an infinite number of replicates measured quantity values and a reference quantity value.

Measurement uncertainty Non-negative parameter characterizing the dispersion of the quantity values attributed to a measurand based on the information used. Measurement uncertainty includes components arising from systematic effects, the assigned quantity values of measurement standards, as well as the definitional uncertainty. It is understood that the measurement uncertainty is associated with a stated quality value attributed to the measurand. A modification of this value results in a modification of the associated uncertainty.

Melting Curve Analysis describing the dissociation characteristics of double-stranded DNA observed during heating. The information gathered can be used to infer the presence and identity of single-nucleotide polymorphisms.

Melting Temperature (T_m) Temperature at which 50 % of double-stranded DNA helices are dissociated since a DNA helix melts in a temperature range rather than at one very specific temperature.

Metagenomics The study of nucleotide sequences isolated from all organisms in a given sample, often conducted as a taxonomic survey using direct PCR (with universal 16S

primers) of DNA extracted from environmental samples. Shotgun metagenomics is the identification of entire nucleotide sequences in a given sample, then attempts both taxonomic and functional identification of genes encoded by an organism's DNA.

Microsatellite Repetitive DNA elements, also known as simple sequence repeats (SSR), consisting of short in tandem repeat motifs of one to a few nucleotides that tend to occur in non-coding DNA of eukaryotic genomes and that are sometimes referred to as variable number of tandem repeats (VNTRs).

Molecular Beacon Fluorescent probe that consists of three different parts: the central component is complementary to the target nucleic acid sequence, whereas the 5´- and the 3´- components are complementary to each other; the reporter is attached to one arm of the molecule; and the end of the other arm carries a quencher.

Multiplex PCR PCR technique that employs multiple pairs of primers combined simultaneously within a single reaction mixture to produce multiple amplicons.

Multiplexing A process by which unique short DNA sequences, or "indexes," are added to each DNA fragment during NGS library preparation. The unique sequences allow many libraries to be pooled together and sequenced simultaneously. Sequencing reads from pooled libraries are identified and sorted computationally before final data analysis. Library multiplexing is a useful technique when working with small genomes or targeting genomic regions of interest. Multiplexing can exponentially increase the number of samples analyzed in a single run, without drastically increasing run cost or run time.

N50 The sequence length of the shortest contig at 50% of the total genome length. It can be thought of as the point of half of the mass of the distribution. This term is useful to assess the quality of genome assemblies, where the higher the N50 value is indicative of a more complete draft genome.

Negative DNA Target Control Well-characterized DNA preparation material that does not contain target nucleic acid.

Nested PCR Specific PCR technique in which a second PCR is used to amplify a DNA sequence within an amplicon produced during a first PCR run.

Next-Generation Sequencing (NGS) Term commonly used for massively parallel sequencing technology, or sequencing of millions of small fragments of DNA in parallel.

Nicking Enzyme Amplification Reaction (NEAR) Isothermal method for exponential *in vitro* DNA amplification.

Nucleic Acid Extraction Sample treatment for liberation of target nucleic acid. Nucleic acid extraction could be DNA or RNA extraction.

Nucleic Acid Purification Procedure or process involving sequential steps used to separate DNA and/or RNA from other components in a sample

Nucleic Acid Sequence Based Amplification (NASBA) Isothermal transcription-based *in-vitro* nucleic acid amplification process that involves the concomitant action of an RNA-directed DNA polymerase, a ribonuclease, and a DNA-directed RNA polymerase to synthesize large quantities of sequence-specific RNA and DNA molecules.

Nucleotide Diversity (also known as "color balance") refers to the relative proportion of nucleotides A, C, G and T present in every cycle of an NGS sequencing run. Well-balanced or high diversity libraries have roughly equal proportions of all four nucleotides in each cycle throughout the sequencing run. Low diversity libraries have a high proportion of certain nucleotides and a low proportion of other nucleotides in a cycle. "Color balance" is required for effective image analysis on Illumina sequencing systems.

Oligonucleotide (also known as "oligo") A short DNA or RNA sequence.

Outlier Member of a set of values which is inconsistent with other members of that set as determined by statistical analysis.

Output Capacity A number of read bases in sequencing, typically measured in thousands to trillions of bases (kb, Mb, Gb, Tb), can be related to an experiment, chip, instrument, etc.

Paired-End Sequencing A process of sequencing from both ends of a DNA fragment in the same run.

Paired-End Read A method of reading a fragment where the fragment is first read from one end and then from the other.

Passive Reference Dye Fluorescent molecules present in the reaction mix used to normalize the signal and may be coupled with nucleic acid sequences or other molecules not taking part in the reaction.

Pathogen/Pathogenic Organisms Sub-category of the definition of pest with specific focus on a micro-organism causing disease which is detected or identified predominantly by using molecular diagnostic procedure development.

PCR Master Mix Mixture of the reagents required for PCR but excluding DNA template and controls.

PCR Reagent Control Containing all the amplification reagents except the extracted test sample template DNA. This control is used to demonstrate the absence of contaminating nucleic acids in the reagents. Instead of the template DNA, for example, a corresponding volume of nucleic acid free water is added to the reaction.

PCR Target Sequence Specific region of DNA that becomes selectively amplified during PCR-based detection, identification and/or quantification. The PCR target sequence is characterized by being located between the primers, and in the case of real-time PCR, may include the probe hybridization site.

Percent Error Relative error expressed as a percentage.

Phred Score Phred assigns a quality score to each base, which is equivalent to the probability of error for that base. The Phred score is the negative log (base 10) of the error probability; thus a base with an accuracy of 99% receives a Phred score of 20.

Polymerase Chain Reaction (PCR) *In vitro* enzymatic technique to increase the number of copies of a specific DNA fragment by several orders of magnitude.

Positive DNA Target Control/Positive PCR Control Any reliable source of well-characterized positive sample material, containing intact target nucleic acid sequences for PCR. Reference DNA or DNA extracted from a certified reference material is generally used to demonstrate that PCR reagents are working as intended.

Precision The relationship of how close two or more measurements are to each other. In statistical terms, this is the reciprocal of the variance.

Primary Analysis Conversion of raw data to sequences (ACGT), assessment of the quality of base reading, chip occupation and overall success of sequencing.

Primer Strand of nucleic acid sequence that serves as a starting point for DNA synthesis.

Primer Extension Enzymatic reaction that synthesizes a new DNA strand by adding a deoxyribonucleotide to the 3’ end of the primer sequence.

Qualitative Method Method of analysis that yields a binary result.

Quality Assurance Planned and systematic actions necessary to provide adequate confidence that analytical results will satisfy given requirements for quality.

Quality Score (also known as "Q-score") A metric in DNA sequencing that predicts or estimates the probability of an error in base calling. A quality score (Q-score) serves as a compact way to communicate very small error probabilities. A high Q-score implies that a base call is more reliable and less likely to be incorrect. See also Phred score.

Quantitative Analysis Analyses in which the amount or concentration of an analyte may be determined and expressed as a numerical value in appropriate units.

Quantitative Method Analytical method by which an amount or concentration of an analyte may be determined and expressed as a numerical value in appropriate units.

Quencher Molecule which acts as an energy acceptor and quenches the fluorescence signal of the reporter molecule.

Range of Linearity/Dynamic Range Upper and lower limits of quantification as expressed by a set of reference materials (or dilutions) with a suitable level of precision and accuracy.

Read Accuracy Indicates the occurrence of errors (in %) after primary analysis.

Read Depth A metric used to assess coverage of DNA sequencing. For DNA, depth is defined as the number of times a nucleotide is read or covered. For RNA, depth is indicated by the total number of reads per sample.

Read Length The number of nucleotides in a given fragment, or the maximum length of a fragment which can be sequenced at a time (indicated in bases).

Real-Time PCR Enzymatic procedure which combines the *in vitro* amplification of specific DNA segments with the detection of specific PCR products during the amplification process. While the PCR reaction is producing copies of the relevant DNA sequence, the fluorescent marker becomes un-quenched at the same time, so it fluoresces in direct proportion to the amount of DNA present (which can theoretically be back-calculated to infer the original amount of that particular DNA present in a sample prior to initiation of PCR).

Recovery Proportion of the amount of analyte, present in, added to, or present in and added to the analytical portion of the test material, which is presented for measurement following extraction from the matrix.

Reference Genome A draft or complete genome against which new sequence reads, or assemblies, are aligned and compared.

Reference Material/Reference Sample Material or substance, one or more of whose property values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials.

Relative Error Absolute error divided by the magnitude of the true (best accepted) value.

Repeatability The degree of agreement between results obtained by the same user at the same laboratory using the same instrument under the same conditions.

Repeatability Standard Deviation Standard deviation of test results or measurement results obtained under repeatability conditions**.** It is a measure of the dispersion of the distribution of test or measurement results under repeatability conditions. Similarly, “repeatability variance” and “repeatability coefficient of variation” can be defined and used as measures of the dispersion of test or measurement results under repeatability conditions.

Repeatability Relative Standard Deviation RSD_r is calculated by dividing the repeatability standard deviation by the mean. Relative standard deviation (RSD) is a useful measure of precision in quantitative studies and reflects precision under repeatability conditions. The RSD is also known as coefficient of variation.

Reporter Molecule Fluorescent molecule used to detect the hybridization of specific probes by excitation with electromagnetic radiation of an appropriate wavelength.

Reproducibility (or between-lab reproducibility) The degree of agreement between results obtained by different users at different laboratories using different instruments.

Reproducibility Condition of Measurement/Reproducibility Condition Condition of measurement, out of a set of conditions that includes different locations, operators, measuring systems, and replicate measurements on the same or similar objects.

Reproducibility Standard Deviation Standard deviation of test results or measurement results obtained under reproducibility conditions**.** It is a measure of the dispersion of the distribution of test or measurement results under reproducibility conditions. Similarly, “reproducibility variance” and “reproducibility coefficient of variation” can be defined and used as measures of the dispersion of test or measurement results under reproducibility conditions.

Restriction Endonucleases Class of enzymes that cleave (i.e. restrict) DNA at specific and unique internal location(s) along its length.

Restriction Fragment Length Polymorphism (RFLP) Nucleotide difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples with specific restriction endonucleases.

Reverse Transcriptase Class of RNA-directed DNA polymerase enzymes, which allow for the synthesis of DNA (complementary to the RNA) using suitable primers and reaction conditions.

Reverse Transcription Process of making DNA from an RNA template, using the enzymatic activity of a reverse transcriptase associated with one or more oligonucleotide primers under a suitable set of conditions.

Reverse Transcription PCR (RT-PCR) Process by which an RNA strand is first reverse transcribed into its DNA complement (complementary DNA or cDNA) using reverse transcriptase and the resulting cDNA is amplified using traditional or real-time PCR.

Ribonucleic Acid (RNA) Polymer of nucleotides that consist of a nucleobase (adenine, guanine, thymine, or uracil), a ribose sugar, and a phosphate group. Synthesis of proteins in cells is directed by genetic information carried in the sequence of nucleotides in a class of RNA known as messenger RNA (mRNA).

RNA Extraction Separation of RNA from the other cellular components in a test sample.

Robustness is the stability of the method against small variations of the internal factors (intrinsic) method parameters, such as sample preparation and other internal factors, and variability of sample matrix (within-lab variation).

Ruggedness is the stability of the method against external influencing factors, such as analyst, laboratory, instrument, reagents and days.

Robustness/Ruggedness Measure of the capacity of an analytical procedure to remain unaffected by small variations in method parameters and provides an indication of the method's reliability during normal usage.

SAM File File containing alignment of fragments together with quality indicators and possibly other information.

Sample Small portion or quantity, taken from a population or lot that is ideally a representative selection of the whole.

Sample Enrichment Techniques used to concentrate genetic material in the preparation of a sample so that it contains the maximum amount of the intended targeted.

Scaffold The second level of the association of fragment sequences to higher structures (Sequencing Read -> Contig -> Scaffold).

Screening Method Broad-spectrum procedure that rapidly and reliably identifies a large number of negative (or positive) test samples and restricts the number of test samples requiring the application of a more rigorous and specific method.

Secondary Analysis Filtration of reads on the basis of quality, alignment and assembly of reads, determination of the alignment quality indicators and assembly, administration of sequence variants.

Selectivity Property of a measuring system, used with a specified measurement procedure, whereby it provides measured quantity values for one or more measurands such that the values of each measurand are independent of other measurands or other quantities in the phenomenon, body, or substance being investigated.

Sensitivity (True Positive Rate) Measures the proportion of positives that are correctly identified by a diagnostic test. Quotient of the change in an indication of a measuring system and the corresponding change in a value of a quantity being measured. Sensitivity of a measuring system can depend on the value of the quantity being measured.

Sensitivity (NGS Assay) Refers to the ability to detect a particular variant in a given sample. The lower the allele frequency, the higher the sensitivity needed to detect it. Next-generation sequencing provides higher sensitivity than capillary electrophoresis, offering the ability to detect rare mutations.

Sequencing By Synthesis (SBS) Technology that uses four fluorescently labeled nucleotides (or other labels or signals) to sequence the tens of millions of clusters on the flow cell surface in parallel. During each sequencing cycle, a single labeled deoxynucleotide triphosphate (dNTP) is added to the nucleic acid chain. The nucleotide label serves as a “reversible terminator” for polymerization: after dNTP incorporation, the fluorescent dye is identified through laser excitation and imaging, then enzymatically cleaved to allow the next round of incorporation. Base calls are made directly from signal intensity measurements during each cycle. Because all four reversible terminator-bound

dNTPs (A, C, T, G) are present as single, separate molecules, natural competition minimizes incorporation bias.

Sequencing Primer A PCR primer adjacent to an adapter sequence that indicates the starting point of the sequencing read. During the sequencing process, the primer anneals to a portion of the sequencing adapter on the template strand. The DNA polymerase enzyme binds to this site and incorporates complementary nucleotides base by base into the growing opposite strand.

Sequencing Reads The data strings of A, T, C, and G bases corresponding to each DNA fragment in a sequencing library. In Illumina technology, when a library is sequenced, each DNA fragment produces a cluster on the surface of a flow cell and each cluster generates a single sequencing read. (For example, 1 million clusters on a flow cell would produce 1 million single reads and 2 million paired-end reads.) Read lengths can range from 25 bp to 300 bp or higher depending on application needs.

Sequencing Run A single process consisting of multiple cycles on the sequencing platform that generate raw sequence reads.

Short-Reads The output of next generation sequencing using Single-End and Pair-End sample preparation methods.

Signal Intensity The brightness of the fluorophores that are attached to a substrate. In sequencing by synthesis, base calls are made directly from signal intensity measurements during each cycle.

Simple Sequence Repeat Marker (SSR Marker) Region of DNA consisting of a short (1 bp to 6 bp) sequence (repeat unit) that is tandemly repeated many (typically 5 to 50) times. SSRs are commonly known as microsatellites. The number of repeat units present at a specified SSR, and thus the overall length of the SSR, often vary among individuals.

Single-Laboratory Validation A validation process that evaluates functional characteristics of a method, including repeatability, when applying a specific method within a specific laboratory, by (a) specific operator(s) using specific equipment.

Single Nucleotide Polymorphism (SNP) DNA sequence variation that occurs when a single nucleotide, A, T, C, or G, in the genome (or other shared sequence) differs between members of a species (or between paired chromosomes in an individual).

Single Nucleotide Polymorphism Calling (SNP Calling) A Process of detecting single nucleotide polymorphisms (SNPs) in a given sequence.

Single-Read A method of reading a fragment where the fragment is read from one end only during sequencing.

SMRT Cell A type of sequencing chip used in Pacific Biosciences instruments (Single Molecule Real Time technology).

Species Group of organisms that have a high level of genetic (DNA) similarity and are capable of interbreeding (often containing subspecies, varieties or races).

Specificity (True Negative Rate) The proportion of negative test results of a diagnostic test that correctly identify true negatives. Property of a method to respond exclusively to the characteristic or analyte under investigation.

Stacked Gene Insertion of two or more (synthetic) genes into the genome of an organism.

Stacked Event Accumulation of two or more transformation events as a result of traditional breeding and/or successive transformation steps.

Surrogate Pure compound or element added to the test material, the chemical and physical behavior of which is taken to be representative of the native analyte.

Systematic Measurement Error/Systematic Error of Measurement/Systematic Error Component of measurement error that, in replicate measurements, is consistent with a magnitude that remains constant or varies in a predictable manner.

Tagmentation A rapid enzymatic reaction where double-stranded DNA is simultaneously fragmented and tagged by ligation with Illumina adapter sequences and PCR primer binding sites. The combined reaction eliminates the need for a separate mechanical shearing step during library preparation.

Taq DNA Polymerase Thermostable DNA polymerase derived from the thermophilic bacterium *Thermus aquaticus* commonly utilized to catalyze PCR reactions (needed for thermal cycles utilized in the PCR technique).

Target Fragment Nucleic acid fragment targeted for detection, e.g. by PCR.

Target Sequence Specific DNA sequence targeted for detection, e.g. by PCR.

Target Size The total size of all the target regions in a targeted resequencing experiment. Target sizes will vary among different predesigned panels or custom panels. (For example, a panel of 22 target regions, where each target region is 100 kb, will have a total target size of 2200 kb.)

Taxon Particular group or category into which related organisms are classified.

Taxon-Specific (Endogenous) Target Sequence DNA sequence that is known to be specific for a target taxon and is consistently present in the target taxon but consistently absent in other taxa.

Template Strand of DNA or RNA that specifies the base sequence of a newly synthesized strand of DNA or RNA, the two strands being complementary.

Test Sample Sample prepared for testing or analysis, the whole quantity or part of it being used for testing or analysis at one time.

Thermocycler/Thermal Cycler Automated laboratory apparatus used to raise and lower the temperature of a sample in discrete, preprogramed steps.

Threshold Cycle (C_t) Point of the amplification curve at which the fluorescence signal rises above the baseline or crosses a pre-defined threshold setting.

Throughput The amount of data produced by a next-generation sequencing instrument. Usually defined in terms of megabases (Mb) or gigabases (Gb). 1 megabase = 1,000,000 bases. 1 gigabase = 1,000,000,000 bases.

Transcriptome Total mRNA present in a cell.

Transgene Gene or genetic material (i.e., DNA) that is inserted into the genome of a cell via recombinant DNA techniques. This genetic material may include promoters, leader sequence, termination codons, etc.

Transgenic Organism Organism that contains genetic material, originally derived from different species, that has been inserted into the genome using recombinant DNA techniques.

Uracil N-Glycosylase (UNG) Enzyme which removes uracil from deoxyuridine-containing nucleic acid sequences of either double or single stranded DNA, leaving apyrimidinic sites.

Validated Range Range that is part of the concentration range of an analytical method which has been subjected to validation.

Validated Test Method Accepted test method for which validation studies have been completed to determine the accuracy and reliability of this method for a specific purpose.

Validation Process that determines the fitness of an assay/method for intended purpose. All new or significantly modified methods should go through a validation process.

Variant Calling A process of detecting sequence variants (SNPs, indels, etc.) in a given sample compared to a reference sequence.

VCF File A file containing information about the sequence variants identified, a typical output of the secondary phase of data analysis.

Vector Agent used to carry new genes into cells. A vector can also be an organism that spreads pathogens.

Verification Provision of objective evidence that a given item fulfils specified requirements. Only a previously validated assay/method can be verified. A newly developed or significantly modified assay/method should be validated.

Whole Genome Sequencing A comprehensive method that provides a base-by-base assessment of a draft or complete genome.

Whole Genome Skimming: a next generation sequencing approach that employs shallow sequencing of an entire genome to uncover conserved ortholog sequences for phylogenomic studies, often used in eukaryotic groups with larger genomes.

Appendix 5. Sequencing Method Validation Submission Form (Library Preparation, Platform, and Associated Chemistries)

1. Instrument Platform (Make/Model):
2. Instrument Control Software Name/Version:
3. Sample Type:
4. Library Preparation:
5. Cartridge Type:

Notes:

a. This table was developed for short-read sequencing, specifically Illumina platforms. For other platforms and technologies, such as long-read sequencing, submitter should develop appropriate table of sequencing performance criteria.
b. If any criterion is not reported, please provide an explanation or indicate "not applicable" if appropriate.
c. If any study result is not within the range of expected results, please provide an explanation.
d. Additional relevant criteria not listed on this form should be included to effectively evaluate validation study.
e. Please submit additional worksheets that includes all criteria metrics for each individual run (reference method versus experimental method)

Criteria Performance (Illumina based)	Expected Results	Validation Study Results (Run 1)
Library Loading Concentration		
Cluster Density		
Clusters Passing Filter		
Total % Reads $\geq$ Q 30		
% PhiX Aligned		

PhiX Error Rate		
Error Rate (if applicable)		
Contamination		
N50		
Average Depth of Coverage		
de novo Assembly: Sequence Length (Mbp)		
Contigs		
Mean Read Lengths for Read 1 and Read 2		
Mean Q Scores for Read 1 and Read 2		
Yield Total (# of bases sequenced)		
Sequence Length Distribution		
% Core Present or % ORFs		
Other(s):		

Appendix 6. iSeq Example for Sequencing Method Validation Submission Form (Library Preparation, Platform, and Associated Chemistries)

1. Instrument Platform (Make/Model): **iSeq 100**
2. Instrument Control Software Name/Version: **iSeq 100 Software System Suite v2.0**
3. Sample Type: **16S Amplicon**
4. Library Preparation: **Illumina DNA Prep**
5. Cartridge Type: **Illumina 300 cycle**

Notes:

a. This table was developed for short-read sequencing, specifically Illumina platforms. For other platforms and technologies, such as long-read sequencing, submitter should develop appropriate table of sequencing performance criteria.
b. If any criterion is not reported, please provide an explanation or indicate "not applicable" if appropriate.
c. If any study result is not within the range of expected results, please provide an explanation.
d. Additional relevant criteria not listed on this form should be included to effectively evaluate validation study.
e. Please submit additional worksheets that includes all criteria metrics for each individual run (reference method versus experimental method)

Criteria Performance (Illumina based)	Expected Results	Validation Study Results (Run 1)
Library Loading Concentration	75-100 pM	80 pM
Cluster Density	N/A iSeq 100 does not report out cluster density	N/A iSeq 100 does not report out cluster density
Clusters Passing Filter	55-95 %	80%
Total % Reads $\geq$ Q 30	$\geq$ 80	82
% PhiX Aligned	1-5	1%

PhiX Error Rate	0-2	< 1
Error Rate (if applicable)	0-2	< 1
Contamination	< 1%	0
N50	Appropriate size ranges	493699 (*Salmonella*)
Average Depth of Coverage	> 30X (*Salmonella*) > 20X (*Listeria*) > 40X (*E. coli*) > 20X (*Campylobacter*) > 40X (*Vibrio*)	64.7X (*Salmonella*)
De novo Assembly: Sequence Length (Mbp)	4.4 to 5.6 (*Salmonella*) 2.8 to 3.1 (*Listeria*) 4.2 to 5.9 (*E. coli/Shigella*) 1.4 to 2.2 (*Campylobacter*) 3.8 to 5.5 (*Vibrio* spp)	4993163 (*Salmonella*)
Contigs	≤ 400 (*Salmonella*) ≤ 100 (*Listeria*) ≤ 600 (*E. coli*) ≤ 200 (*Campylobacter*) ≤ 200 (*Vibrio* spp)	33 (*Salmonella*)
Mean Read Lengths for Read 1 and Read 2	2 x 150 chemistry	R1: 135 R2: 133
Mean Q Scores for Read 1 and Read 2	≥ 30	R1: 34 R2: 32
Yield Total (# of bases sequenced)	4 M (Appropriate yield for flow cell and # of libraries)	3.9 M
Sequence Length Distribution	< 5% of reads are < 120 bp when raw input reads are 300 bp long	3% < 120 bp 75% > 150 bp

	> 50% of the reads are > 150 bp when raw input reads are 300 bp long	
% Core Present or % ORFs	≥ 95% (*Listeria mono.*) ≥ 85 (*E. coli, Shigella, Salmonella, Campylobacter jejuni*)	92% (*Salmonella*)
Other(s):		

Appendix 7. MiSeq Example for Sequencing Method Validation Submission Form (Library Preparation, Platform, and Associated Chemistries)

1. Instrument Platform (Make/Model): **Illumina MiSeq**
2. Instrument Control Software Name/Version: **MiSeq Control Software 2.5.0.5**
3. Sample Type: **Bacterial Whole Genome**
4. Library Preparation: **Illumina DNA Prep**
5. Cartridge Type: **Illumina 500 cycle**

Notes:

a. This table was developed for short-read sequencing, specifically Illumina platforms. For other platforms and technologies, such as long-read sequencing, submitter should develop appropriate table of sequencing performance criteria.
b. If any criterion is not reported, please provide an explanation or indicate "not applicable" if appropriate.
c. If any study result is not within the range of expected results, please provide an explanation.
d. Additional relevant criteria not listed on this form should be included to effectively evaluate validation study.
e. Please submit additional worksheets that include all criteria metrics for each individual run (reference method versus experimental method).

Criteria Performance (Illumina based)	Expected Results	Validation Study Results (Run 1)
Library Loading Concentration	10 pM	10 pM
Cluster Density	600-1200 K/mm^2	1050 K/mm^2
Clusters Passing Filter	$\geq$ 80 %	84%
Total % Reads $\geq$ Q 30	> 75	82
% PhiX Aligned	1-5 %	1%

PhiX Error Rate	0-2	< 1
Error Rate (if applicable)	0-2	< 1
Contamination	< 1%	0
N50	Appropriate size ranges	493699 (*Salmonella*)
Average Depth of Coverage	> 30X (*Salmonella*) > 20X (*Listeria*) > 40X (*E. coli*) > 20X (*Campylobacter*) > 40X (*Vibrio*)	64.7X (*Salmonella*)
De novo Assembly: Sequence Length (Mbp)	4.4 to 5.6 (*Salmonella*) 2.8 to 3.1 (*Listeria*) 4.2 to 5.9 (*E. coli/Shigella*) 1.4 to 2.2 (*Campylobacter*) 3.8 to 5.5 (*Vibrio* spp)	4993163 (*Salmonella*)
Contigs	≤ 400 ((*Salmonella*) ≤ 100 (*Listeria*) ≤ 600 (*E. coli*) ≤ 200 (*Campylobacter*) ≤ 200 (*Vibrio)*	33 (*Salmonella*)
Mean Read Lengths for Read 1 and Read 2	2 x 250 chemistry ≥ 230	R1: 238 R2: 237
Mean Q Scores for Read 1 and Read 2	≥ 30	R1: 34 R2: 32
Yield Total (# of bases sequenced)	8-10 Mbp (Appropriate yield for flow cell and # of libraries)	8.7 Mbp (20 *Salmonella*)
Sequence Length Distribution	< 5% of reads are < 120 bp when raw input reads are 300 bp long > 50% of the reads are > 150 bp when raw input reads are 300 bp long	3% < 120 bp 75% > 150 bp

% Core Present or % ORFs	≥ 95% (*Listeria mono.*) ≥ 85 (*E. coli, Shigella, Salmonella, Campylobacter jejuni*)	92% (*Salmonella*)
Other(s):		

Appendix 8. Validation Submission Form for *in silico* Assays

1. Assay Name:
2. Version:
3. Location:
4. Developer:
5. Study Category: (SLV, ILV, MLV or Verification)
6. Assay Category: (Species Identification, Genotyping (Molecular Typing) or Phylogenomic Clustering)
7. Input Dataset Collection:
8. Output:
9. Benchmark Dataset (if applicable):

Notes:

a. If any criterion is not reported, please provide an explanation or indicate "not applicable" if appropriate.
b. If any study result is not within the range of expected results, please provide an explanation.
c. Additional relevant criteria not listed on this form should be included to effectively evaluate validation study.
d. Please submit additional worksheets that include all criteria metrics for each individual run (reference method versus experimental method).

Performance Criteria	Expected Results	Validation Study Results (Run 1)
Species Identification		
Accuracy/Level of Agreement		
Sensitivity		
Specificity		
Genotyping (Molecular Typing)		

Accuracy/Level of Agreement		
Sensitivity		
Specificity		
Phylogenomic Clustering		
Sensitivity for Cluster Assignment		
Specificity for Cluster Assignment		
Sensitivity for variant determination (SNPs, MLST, etc)		
Specificity for variant determination (SNPs, MLST, etc)		
Accuracy of entire tree or specific split		
Other(s):		

Appendix 9. SeqSero Example for Validation Submission Form for *in silico* Assays

1. Assay Name: **SeqSero2**
2. Version: **1.1.1**
3. Location: **www.galaxytrakr.org tool "SeqSero2 v1.1.1 collection workflow" and Raven2**
4. Developer: **Zhang et al., 2019, AEM; https://doi.org/10.1128/AEM.01746-19. and Charles Strittmatter (GalaxyTrakr Team)**
5. Study Category: **_X_ SLV ___ ILV ___ MLV ___ Verification**
6. Assay Category: **___ Species Identification**
   _X_ Genotyping (Molecular Typing)
   ___ Phylogenetic Clustering
7. Input Dataset Collection: **See Appendix A of submission package (Full dataset)**
8. Output: **See Appendix A of submission package (Full dataset)**
9. Benchmark Dataset (if applicable): **Not applicable**

Notes:

a. If any criterion is not reported, please provide an explanation or indicate "not applicable" if appropriate.
b. If any study result is not within the range of expected results, please provide an explanation.
c. Additional relevant criteria not listed on this form should be included to effectively evaluate validation study.
d. Please submit additional worksheets that include all criteria metrics for each individual run (reference method versus experimental method).

Performance Criteria	Expected Results	Validation Study Results (Run 1)
Species Identification		
Accuracy/Level of Agreement	> 95%	N/A
Sensitivity	> 95%	N/A

Specificity	> 95%	N/A
Genotyping (Molecular Typing)		
Accuracy/Level of Agreement	≥ 95%	97.3%
Sensitivity	≥ 95%	94.74% (Serovar Name Match) 97.66% (Antigen Match)
Specificity	≥ 95%	100%
Phylogenomic Clustering		
Sensitivity for Cluster Assignment	Unique to each study	N/A
Specificity for Cluster Assignment	Unique to each study	N/A
Sensitivity for variant determination (SNPs, MLST, etc)	Unique to each study	N/A
Specificity for variant determination (SNPs, MLST, etc)	Unique to each study	N/A
Accuracy of entire tree or specific split	Unique to each study	N/A
Other(s):		