Appendix J: AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces

Table of Contents **Page**

This document contains technical guidelines for the data collection requirements for microbiology methods. The document also contains specific programmatic requirements and information related to the AOAC *Official Methods*^{SM} and *Performance Tested Methods*^{SM} programs. Actual programmatic requirements and information may differ from the information stipulated in this guidance document. For the most current programmatic requirements, contact AOAC INTERNATIONAL staff at aoac@aoac.org.

The guidelines were approved by the AOAC Methods Committee on Microbiology and Official Methods Board in September 2011.

1 Scope

The purpose of this document is to provide comprehensive AOAC INTERNATIONAL (AOAC) technical guidelines for conducting microbiological validation studies of food and environmental analysis methods submitted for AOAC® *Official Methods of Analysis*℠ (OMA) status and/or *Performance Tested Methods*℠ (PTM) certification.

2 Applicability

These guidelines are applicable to the validation of any candidate method, whether proprietary or nonproprietary, that is submitted to AOAC for OMA status or PTM certification. Circumstances, unforeseen by AOAC, may necessitate divergence from the guidelines in certain cases. The PTM Program requires a Method Developer Study and an Independent Laboratory Study. The OMA Program requires a Single-Laboratory Validation (SLV) Study (also known as the Precollaborative Study), an Independent Validation Study, and a Collaborative Study. A harmonized PTM-OMA program can be followed in which PTM certification is sought and, if successful, serves as the SLV and Independent Validation phase of the OMA program. This approach provides a certification while working toward OMA status. *See* Table 1 for more detail.

3 Terms and Definitions

3.1 Analyte

Microorganism or associated biochemicals (e.g., DNA, proteins, or lipopolysaccharides) measured or detected by the method of analysis.

3.2 Candidate Method

The method submitted for validation.

3.3 Candidate Method Result

The final result of the qualitative or quantitative analysis for the candidate method. For methods with a confirmation phase, only presumptive positive results that confirm positive are considered as positive for the candidate method. All other results are considered as negative for the candidate method.

Table 1

AOAC Program	Study Requirements	Relevant Guideline Sections		
		Qualitative	Quantitative	Confirmatory Identification
PTM	Method Developer Validation Study	4.1	5.1	6.1
OMA	SLV (Precollaborative Validation) Study	4.1.2 and 4.1.3	5.1.2 and 5.1.3	6.1.2
	Independent Validation Study	4.2	5.2	6.2
	Collaborative Validation Study	4.3	5.3	6.3
Harmonized PTM-OMA	Method Developer Validation Study	4.1	5.1	6.1
	Independent Validation Study	4.2	5.2	6.2
	Collaborative Validation Study	4.3	5.3	6.3

3.4 Collaborative Study (CS)

A validation study performed by multiple laboratories to estimate critical candidate method performance parameters.

3.5 Composite Test Portion

Test portions taken from multiple samples of the same matrix combined together.

3.6 Confirmatory Identification Method

Method of analysis whose purpose is to determine the identity of an analyte. (Biological Threat Agent Method; BTAM)

3.7 Confirmatory Phase

A procedure specified in some qualitative assays whereby a preliminary presumptive result is confirmed by a subsequent and different method.

3.8 Confirmed Result

The qualitative response from the confirmatory phase of a candidate method.

3.9 Enrichment Pool

A pool comprised of aliquots from multiple test portion enrichments.

3.10 Exclusivity

The nontarget strains, which are potentially cross-reactive, that are not detected by the method.

3.11 Fractional Recovery

Validation criterion that is satisfied when an unknown sample yields both positive and negative responses within a set of replicate analyses. The proportion of positive responses should fall within 25 and 75% and should ideally approximate 50% of the total number of replicates in the set. A set of replicate analyses are those replicates analyzed by one method (either candidate or reference). Only one set of replicates per matrix is required to satisfy this criterion.

An alternate plan acceptable to the Statistics Committee can be used.

3.12 Inclusivity

The strains or isolates of the target analyte(s) that the method can detect. (BTAM)

3.13 Limit of Detection$_{50}$ (LOD_{50})

The analyte concentration at which the probability of detection (POD) is equal to 50%.

3.14 Matched Analyses

Two or more analyses or analytical results on the same unknown sample, which can be traced to the same test portion.

3.15 Matrix

The food, beverage, or environmental surface material to be included in the validation as per the intended use of the method.

3.16 Method Developer Validation Study or Single-Laboratory Validation (SLV or Precollaborative) Study

A validation study performed by a single laboratory in order to systematically estimate critical candidate method performance parameters. The method developer study is usually performed by the organizing laboratory or Study Director.

3.17 Precision

The closeness of agreement between independent test results under stipulated conditions. (ISO 5725-1)

3.18 Presumptive Phase

The initial qualitative determination of the analyte in a test portion. In some qualitative microbiological assays, confirmation of results is required as specified in the method.

3.19 Presumptive Result

The qualitative response from the presumptive phase of a candidate method that includes a confirmatory phase.

3.20 Probability of Detection (POD)

The proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level or concentration. POD is concentration dependent. Several POD measures can be calculated, e.g., POD_R (reference method POD), POD_C (confirmed candidate method POD), POD_{CP} (candidate method presumptive result POD) and POD_{CC} (candidate method confirmation result POD). Other POD estimates include:

dPOD – the difference between any two POD values

LPOD – the POD value obtained from combining all valid collaborator data sets for a method for a given matrix at a given analyte level or concentration

dLPOD – the difference between any two LPOD values

3.21 Qualitative Method

Method of analysis whose response is either the presence or absence of the analyte detected either directly or indirectly in a specified test portion.

3.22 Quantitative Method

Method of analysis whose response is the amount (count or mass) of the analyte measured either directly (e.g., enumeration in a mass or a volume), or indirectly (e.g., color absorbance, impedance, etc.) in a specified test portion.

3.23 Reference Method

Preexisting recognized analytical method against which the candidate method will be compared. (BTAM)

3.24 Repeatability

Precision under repeatability conditions. (ISO 5725-1)

3.25 Repeatability Conditions

Conditions where independent test results are obtained with the same method on equivalent test items in the same laboratory by the same operator using the same equipment within short intervals of time.

3.26 Reproducibility

Precision under reproducibility conditions. (ISO 5725-1)

3.27 Reproducibility Conditions

Conditions where independent test results are obtained with the same methods on equivalent test items in different laboratories with different operators using separate instruments.

3.28 Robustness Study

A study which tests the capacity of a method to remain unaffected by small but deliberate variations in method parameters and which provides an indication of its reliability during normal usage. (USP 31)

3.29 Sample

The batch of matrix from which replicate test portions are removed for analysis. The sample (naturally contaminated, uncontaminated, or inoculated) contains analyte, if present, at one homogeneous concentration.

3.30 Test Portion

A specified quantity of the sample that is taken for analysis by the method.

3.31 Unmatched Analyses

Two or more analyses or analytical results on the same unknown sample, which cannot be traced to the same test portion.

4 Qualitative Methods—Technical Protocol for Validation

4.1 Method Developer Validation Study or Single-Laboratory Validation (SLV or Precollaborative) Study

4.1.1 Scope

The Method Developer Validation Study is intended to determine the performance characteristics of the candidate method. The study is designed to evaluate performance parameters including inclusivity, exclusivity, and probability of detection (POD). For PTM submissions, robustness is also included. The Method Developer Study is normally conducted in a single laboratory, usually the method developer's laboratory. Alternatively, the method developer can contract the work to an independent site.

The SLV or Precollaborative Study is a formal submission requirement for OMA microbiology methods and is normally conducted in the method developer laboratory. It precedes the Collaborative Study. The purpose of an SLV Study is to define the applicability claims of a proposed OMA method by demonstrating the applicability of the method to various foods and/or environmental samples. For OMA methods, the applicability statement immediately follows the method title. The applicability statement for microbiological methods is generally concerned with target analyte and matrix coverage.

4.1.2 Inclusivity/Exclusivity Study

4.1.2.1 Species/Strain Selection

The choice of inclusivity strains should reflect the genetic and/or serological and/or biochemical diversity of the organisms involved, as well as other factors such as virulence, frequency of occurrence and availability. Select at least 50 pure strains of the target organism(s) to be analyzed as pure culture preparations. For *Salmonella* methods, the number of target organisms is increased to at least 100 serovars that are selected to represent the majority of known somatic groups and subtypes of *Salmonella*.

The choice of exclusivity strains should reflect closely related, potentially cross-reactive organisms. Other factors such as virulence, frequency of occurrence and availability should be considered. Select at least 30 strains of potentially competitive organisms.

Species/strains specified for use must be traceable to the source. The source and origin of each species/strain should be documented.

4.1.2.2 Study Design

Inclusivity strains are cultured by the candidate method enrichment procedure. The target concentration for testing is 100 times the LOD_{50} of the candidate method. Test one replicate per strain. Exclusivity strains are cultured in nonselective media. The target level is the growth limit of the organism. Test one replicate per strain. If the cross reactive strain is detected repeat the analysis using the enrichment conditions prescribed in the candidate method. Report all results.

Inclusivity and exclusivity evaluations shall be performed together as one study. Inclusivity and exclusivity test samples must be blind coded, randomized and intermingled so the analysts cannot know the identity, sequence or concentration of the test samples.

4.1.2.3 Data Reporting

Report inclusivity data as determined in *4.1.2.2* as number of strains detected. For example, "Of the 50 specific inclusivity strains tested, 47 were detected and 3 were not detected. Those strains not detected were the following: …"

Report exclusivity data as determined in *4.1.2.2* as number of strains not detected. For example, "Of the 30 specific exclusivity strains tested, 28 were not detected and 2 were detected. Those detected were the following: …"

The study report should include a table titled "Inclusivity/Exclusivity Panel Results," which lists all strains tested, their source, origin and essential characteristics plus testing outcome. Any unexpected results must be discussed.

4.1.3 Matrix Study

4.1.3.1 Reference Method

Candidate methods are compared to a cultural reference method where applicable. The following methods are examples of acceptable reference methods: AOAC OMA, U.S. Food and Drug Administration *Bacteriological Analytical Manual* (BAM), U.S. Department of Agriculture–Food Safety and Inspection Service *Microbiology Laboratory Guidebook* (MLG) (for meat and poultry products), International Organization for Standardization (ISO) and Health Canada *Compendium of Analytical Methods*.

4.1.3.2 Food Categories

AOAC INTERNATIONAL recognizes claims for the range of specific food matrices successfully validated in the Method Developer Study, or the PCS and CS. The number of different matrices required for testing depends on the applicability of the method. All claimed matrices must be included in the Method Developer Study and the PCS.

4.1.3.3 Environmental Surfaces

The number of different surface types required for testing depends on the applicability of the method. The Study Director may choose from the following surfaces: stainless steel, plastic (polyethylene, polypropylene, or polycarbonate), ceramic (glazed earthen material or glass), rubber, sealed concrete (a commercially available product that "seals concrete pores"), cast iron (coated to prevent rusting), and air filter material. Alternatively, the method claim may be limited to one or more specific surfaces. All claimed surface types must be included in the Method Developer Study or the PCS.

For surfaces to be sampled with a swab, each test area should measure 1″ × 1″. For surfaces to be sampled with a sponge, each test area should measure 4″ × 4″.

4.1.3.4 Levels of Contamination

Each matrix (food, beverage, or surface material) is divided into at least three samples. One sample serves as the uncontaminated level (for naturally contaminated matrices, an uncontaminated level is not required), one or more samples are contaminated at levels that will produce at least one reference method POD (POD_R) or candidate method POD (POD_C) in the range of 0.25–0.75. Finally, one sample should be contaminated at such a level to assure a POD_C of nearly 1.00, with as high a degree of confidence as possible. Depending on the laboratory's confidence in satisfying this validation criterion, it may be advisable to prepare a fourth sample targeting the fractional POD range. All outcomes for each contamination level tested, whether fulfilling the POD requirement or not must be reported.

The target concentration for the fractional POD range is typically 0.2–2 CFU/test portion for foods and beverages, depending on the matrix. The target concentration for POD = 1.00 is approximately 5 CFU/test portion for foods and beverages. Target concentrations for fractional PODs on environmental surfaces can be in the range 10^4–10^6 CFU/surface area, depending on the surface, organism, and environmental conditions of the testing area.

A 5-tube 3-level Most Probable Number (MPN) estimation of contamination levels (1) must be conducted on the day that the analysis of test samples is initiated. The MPN analysis scheme may also make use of the reference method replicates. *See Annex A* for details.

For environmental surface studies, an MPN analysis is not applicable.

If the method is intended to detect more than one target organism simultaneously from the same test portion, the validation study should be designed so that target organisms are inoculated into a common sample and the validation tests are performed in a simultaneous manner.

4.1.3.5 Number of Test Portions

The number of replicate test portions method per level is 5 for the high inoculation level, 20 for the fractional positive level and 5 for the uncontaminated level.

4.1.3.6 Test Portion Size, Compositing and Pooling

Sample sizes required are as written in each method.

Test portion compositing is the combining of test portions prior to enrichment and can be validated alongside the standard test portion size if desired. The standard test portion size is utilized for the reference method and the standard test portion size is mixed with X uncontaminated test portions to create composite test portions for validation by the candidate method. For example, if a candidate method is to be validated for 375 g composites (15 × 25 g analytical units), then, for each level, one set of 20 composited test portions are made by combining twenty single 25 g inoculated test portions with twenty 350 g uninoculated test portions to form the twenty 375 g composited test portions. These 375 g candidate method composites are then compared to the 25 g reference method test portions. MPNs are performed only on the batch samples from which the reference method test portions are taken. Acceptance criteria for composited test portions are the same as for the standard test portion size.

Pooling is the post-enrichment combining of aliquots from more than one enriched test portion. This is validated by preparing replicate test portions for the candidate method and replicate test portions for the reference method, either as matched or unmatched test portions. At the conclusion of the enrichment procedure, test each enriched test portion by the candidate and/or reference method as appropriate. In addition, pool (dilute) an aliquot of each test portion with X aliquots, as specified by the candidate method, of known negative enriched test portions. Acceptance criteria for pooled enriched test portions are the same as for the standard test portion analyses.

4.1.3.7 Source of Contamination

Naturally contaminated matrix is preferred as a source of inoculum, if available. An effort should be made to obtain naturally contaminated matrix as it is most representative of the method usage environment. If naturally contaminated matrix cannot be found, then pure culture preparations may be used for artificial inoculation.

Numerous strains representing different serotypes or genotypes are required, if applicable. Typically a different isolate, strain, biovar or species is used for each matrix. The product inoculation should be conducted with a pure culture of one strain per target analyte. Mixed cultures are used only for multianalyte methods.

4.1.3.8 Preparation of Artificially Contaminated Samples

4.1.3.8.1 Food

Microorganisms in processed foods are typically stressed, thus the contaminating microorganisms are also stressed for these types

of foods. Microorganism stress may occur at the time of inoculation or during preparation of the food. Raw and cold-processed foods should be inoculated with unstressed organisms, heat-processed foods with heat-stressed organisms (e.g., heat culture at 50°C for 10 min), and dry foods with lyophilized culture. Mix well by kneading, stirring or shaking as appropriate. Frozen foods should be thawed, inoculated, mixed and refrozen.

The degree of injury caused by heat stressing should be demonstrated, for nonspore-formers, by plating the inoculum in triplicate on selective and nonselective agars. The degree of injury is calculated as follows:

$$(1 - \frac{n_{select}}{n_{nonselect}}) \times 100$$

where n_{select} = mean number of colonies on selective agar and $n_{nonselect}$ = mean number of colonies on nonselective agar. The heat stress must achieve 50–80% injury of the inoculum. The inoculum should be added to the sample, mixed well and allowed to equilibrate in the matrix for 48–72 h at 4°C for refrigerated foods, for a minimum of 2 weeks at –20°C for frozen foods or for a minimum of 2 weeks at room temperature for dried foods prior to analysis.

4.1.3.8.2 Environmental Surfaces

Strains should be grown in conditions suitable for target organism to achieve stationary phase cells. The selected surface types will receive an inoculum of cells sufficient to provide fractional recovery by either the candidate method or reference method, if applicable. Inoculation levels may need to be adjusted depending on the strain/surface being used to achieve fractional recovery. The initial culture should be diluted into an appropriate stabilizing medium for inoculation onto test surface. The stock culture should also be diluted to a volume that will allow for even distribution of inoculum over entire test surface area, but without producing excessive accumulation of liquid that may dry unevenly. The surface is allowed to dry for 16–24 h at room temperature (20–25°C). The surface must be visually dry at the time of test portion collection.

4.1.3.9 Preparation of Naturally Contaminated Samples

Naturally contaminated matrix may be mixed with uncontaminated matrix of the same food or incubated to achieve a level yielding fractionally positive results. Naturally contaminated surface materials may be used as is, as long as the requirement for yielding fractionally positive results is achieved.

4.1.3.10 Need for Competitive Microflora

It is more realistic and challenging to include microorganisms that act as competitors to the analyte microorganisms. The purpose of including these organisms is to more closely simulate conditions found in nature. It is sufficient to demonstrate this recovery in one matrix. This requirement may be satisfied in the SLV (Precollaborative) Study. The competitor contamination levels, which may be naturally occurring or artificially introduced, should be 10 times higher than the target microorganism.

4.1.3.11 Environmental Surface Sampling

The candidate method submitter will determine which surface will be sampled by sponge or swab. An environmental sampling sponge is a porous moisture absorbing matrix, approximately 2″ (5 cm) × 3″ (7.5 cm) often contained in a presterilized sample bag. An environmental swab is a sampling device comprised of synthetic (e.g., dacron) or cotton tips affixed to a wood or polymeric stick, delivered in a presterilized package.

Sponges and swabs are premoistened with a neutralizing broth, such as Dey-Engley (2), prior to sampling. The entire sampling area is sponged or swabbed in both a horizontal and vertical motion. Use the sponges to sample a 100 cm^2 (4″ × 4″) area and swabs to sample a 5 cm^2 (1″ × 1″) area. Sponges/swabs containing samples are placed back into their individual respective bag or tube and held at room temperature for 2 hours prior to initiation of testing.

4.1.3.12 Confirmation of Test Portions

Follow the reference method as written for isolation and confirmation of typical colonies from all candidate method test portions regardless of presumptive result. The method developer can perform their own confirmation procedure in addition to the reference method confirmation procedure.

4.1.3.13 Data Analysis and Reporting

Each level of each matrix must be analyzed and reported separately. The following section describes the data analysis to be performed according to the POD model. It is acceptable to analyze data according to the Chi Square statistical methodology for paired studies, and the Relative Limit of Detection (RLOD) for unpaired studies, as defined in the current revision of ISO 16140. Refer to ISO 16140 for detailed descriptions of Chi Square and RLOD.

4.1.3.13.1 Raw Data Tables

For each matrix and level, report each result from each test portion separately. *See Annex B* for raw data table format.

4.1.3.13.2 Probability of Detection (POD)

POD is the proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level or concentration. POD is concentration dependent.

The POD estimate is calculated as the number of positive outcomes divided by the total number of trials.

Estimate the POD with a 95% confidence interval for the candidate method, the reference method and, if included, the presumptive and confirmed results. *See Annex C* for details.

4.1.3.13.3 Difference of Probabilities of Detection (dPOD)

Difference of probabilities of detection is the difference between any two POD values.

Estimate the $dPOD_C$ as the difference between the candidate method and reference method POD values. Calculate the 95% confidence interval on the $dPOD_C$.

$$dPOD_C = POD_C - POD_R$$

Estimate the $dPOD_{CP}$ as the difference between the candidate presumptive result POD (POD_{CP}) and the candidate confirmed result POD (POD_{CC}) values. Calculate the 95% confidence interval on the $dPOD_{CP}$. *See Annex C* for details.

$$dPOD_{CP} = POD_{CP} - POD_{CC}$$

If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level.

4.1.3.13.4 Summary Data Tables

For all matrices and levels, use the summary table from *Annex D*.

4.1.3.13.5 Graph of Data

For each matrix, graph POD_R, POD_C, and dPOD by level with 95% confidence intervals. *See* example in *Annex E*.

4.1.3.13.6 Data Analysis and Reporting in the Absence of a Reference Method

If no appropriate reference method is available for the target analyte, indicate "Not Applicable" (NA) where appropriate in the summary tables.

4.1.4 Robustness Study [*Performance Tested Methods*℠ (PTM) submissions only]

4.1.4.1 Strain Selection

Robustness strains are prepared and analyzed as vegetative cells, spores or components thereof as applicable to the candidate method. One material is tested at a level that yields fractional recovery and one nontarget material is analyzed at the growth level achieved in a nonselective broth or at a high inoculation level.

4.1.4.2 Study Design

Minor, reasonable variations in a method of a magnitude that might well be expected to occur when the method is used are deliberately introduced and tested. Variations in method parameters that can be influenced by the end user should be tested. Use a screening factorial experimental design.

The method developer is expected to make a good faith effort to choose parameters that are most likely to affect the analytical performance and determine the range of variations that can occur without adversely affecting analytical results.

Ten replicates of each material are tested for each treatment combination.

4.1.4.3 Data Analysis and Reporting

The results are analyzed for variable detection due to changes in parameter settings. Report the appropriate statistical measures of the measured variable(s) (e.g., Ct, absorbance, POD value, etc.) for each set of replicates for each treatment combination. This should include at least means, standard deviations, and confidence intervals where appropriate.

4.2 Independent Validation Study

4.2.1 Scope

A validation study to corroborate the analytical results obtained by the method developer and to provide additional single laboratory data. The independent validation study traditionally verifies POD in the hands of an independent trained user and is required for PTM certification and OMA approval.

4.2.2 Reference Method

If there is a reference method, then the candidate method is compared to a reference method. The reference method should be the same as that used in the Method Developer Study.

4.2.3 Matrices

The independent laboratory must test at least one matrix that was tested in the Method Developer Study. The total number of matrices to be evaluated by the independent laboratory is dependent on the claim of the candidate method. For every five foods claimed, one food matrix shall be included in the independent study and for every five environmental surfaces claimed, one surface shall be included in the independent study. The choice of matrices for the Independent Study is made by the appropriate method volunteer(s) in consultation with the Study Director.

4.2.4 Study Design

The study design for validation of qualitative methods in the independent study follows the Method Developer Validation Study design. Contamination levels, number of test portions, test portion size, source of contamination, preparation of samples, confirmation of test portions, and data analysis and reporting are found in Section *4.1.3*. If composite test portions or pooling was validated in the Method Developer Validation Study, include it also in the Independent Validation Study.

4.3 Collaborative Study (CS)

4.3.1 Scope

The Collaborative Study (CS) report is a formal submission requirement for OMA methods only. The purpose of the Collaborative Study is to estimate the reproducibility and determine the performance of the candidate method among collaborators.

4.3.2 Number of Laboratories

At least 12 laboratories per matrix should be included due to potential failure to follow protocol. A minimum of 10 valid laboratory data sets per matrix are required.

4.3.3 Reference Method

The reference method used in the Collaborative Study must be the same as that used in the Method Developer Study or SLV (PCS). The reference method should be carried out by the organizing laboratory.

4.3.4 Matrix Selection

At least one matrix from those studied in the PTM or PCS shall be chosen by the appropriate method volunteer(s) in consultation with the Study Director for collaborative study. For methods with more than one sample preparation/enrichment, one matrix per procedure may be required in the collaborative study. The determination if the procedures differ significantly to warrant expanding the collaborative study is made by the appropriate method volunteer(s) in consultation with the Study Director. The Statistical Advisor and reviewers can be consulted during this determination. Examples of what constitutes a different sample preparation procedure would include different test portion size, different enrichment media or conditions, different dilution volume and different homogenization equipment. The AOAC appropriate method volunteer, Statistical Advisor and collaborative study protocol reviewers shall make the final selection of the matrix(es) with consideration of the PTM or PCS data and the relative importance of the matrices to food safety. The data from both the PCS and CS studies form the basis for defining the method applicability statement.

4.3.5 Analyte Level Estimation

Refer to Section *4.1.3.4*. Use the reference method (or candidate method if there is no reference method) test portions with additional levels to estimate the MPN using the formula in *Annex A*. The levels of contamination are one high level, one level where fractional recovery is expected, and one uninoculated level.

4.3.6 Number of Test Portions

The number of test portions is 12 at the high level, 12 at the fractional level, and 12 uncontaminated per method per laboratory. Test portions are to be randomized and blind-coded when sent to participating laboratories for analysis.

4.3.7 Test Portion Size, Compositing and Pooling

Sample sizes required are as written in each method.

Test portion compositing is the combining of test portions prior to enrichment and can be validated alongside the standard test portion size if desired. The standard test portion size is utilized for the reference method and the standard test portion size is mixed with X uncontaminated test portions to create composite test portions for validation by the candidate method. For example, if a candidate method is to be validated for 375 g composites (15 × 25 g analytical units), then, for each level, one set of 20 composited test portions are made by combining twenty single 25 g inoculated test portions with twenty 350 g uninoculated test portions to form the twenty 375 g composited test portions. These 375 g candidate method composites are then compared to the 25 g reference method test portions. MPNs are performed only on the batch samples from which the reference method test portions are taken. Acceptance criteria for composited test portions are the same as for the standard test portion size.

Pooling is the post-enrichment combining of aliquots from more than one enriched test portion. This is validated by preparing replicate test portions for the candidate method and replicate test portions for the reference method, either as matched or unmatched test portions. At the conclusion of the enrichment procedure, test each enriched test portion by the candidate and/or reference method as appropriate. In addition, pool (dilute) an aliquot of each test portion with X aliquots, as specified by the candidate method, of known negative enriched test portions. Acceptance criteria for pooled enriched test portions are the same as for the standard test portion analyses.

4.3.8 Source of Contamination

Refer to *4.1.3.7*.

4.3.9 Preparation of Artificially Contaminated Samples

Refer to *4.1.3.8*.

4.3.10 Preparation of Naturally Contaminated Samples

Refer to *4.1.3.9*.

4.3.11 Confirmation of Test Portions

Follow the reference method as written for isolation and confirmation of typical colonies from all candidate method test portions regardless of presumptive result.

4.3.12 Data Analysis and Reporting

Each concentration level of each matrix must be analyzed and reported separately. Data may be excluded due to an assignable cause if sufficient justification is provided. Excluded data must be reported, but should not be included in the statistical analysis. The following section describes the data analysis to be performed according to the POD model. It is acceptable to analyze data according to the Chi Square statistical methodology for paired studies, and the RLOD for unpaired studies, as defined in the current revision of ISO 16140. Refer to ISO 16140 for detailed descriptions of Chi Square and RLOD.

4.3.12.1 Raw Data Tables

For each matrix and concentration level, report each result from each test portion separately. *See Annex B* for raw data table format.

4.3.12.2 Estimate of Repeatability

Estimate the repeatability standard deviation (s_r) for qualitative methods according to *Annex F*.

4.3.12.3 Estimate of Reproducibility

Cross-laboratory estimates of probabilities of detection and their differences depend upon an assumption that the same performance is achieved in each laboratory. This assumption must be tested and the laboratory effect estimated. If the effect is large, method performance cannot be expected to be the same in two different laboratories.

For each matrix and level, calculate the standard deviation of the laboratory POD values (s_{POD}) and associated 95% confidence interval to estimate the reproducibility. *See Annex F* for details.

4.3.12.4 Cross-Laboratory Probability of Detection (LPOD)

Report the LPOD estimates by matrix and concentration with 95% confidence intervals for the candidate method and, if included, the presumptive and confirmed results. *See Annex F* for details.

4.3.12.5 Difference of Cross-Laboratory Probability of Detection (dLPOD)

Difference probability of detection is the difference between any two LPOD values.

Estimate the $dLPOD_C$ as the difference between the candidate and reference LPOD values. Calculate the 95% confidence interval on the $dLPOD_C$.

Estimate the $dLPOD_{CP}$ as the difference between the presumptive and confirmed LPOD values. Calculate the 95% confidence interval on the $dLPOD_{CP}$. *See Annex F* for details.

If the confidence interval of a dLPOD does not contain zero, then the difference is statistically significant.

4.3.12.6 Summary Data Tables

For all matrices and levels, use the summary table from *Annex G*.

4.3.12.7 Graph of Data

For each matrix, graph POD_R, $LPOD_C$, and $dLPOD_C$ by level with 95% confidence intervals. *See* example in *Annex E*.

4.3.12.8 Data Analysis and Reporting in the Absence of a Reference Method

If no appropriate reference method is available for the target analyte, indicate "Not Applicable" where appropriate in the summary tables.

5 Quantitative Methods—Technical Protocol for Validation

5.1 Method Developer Validation Study or SLV (Precollaborative) Study

5.1.1 Scope

The Method Developer Validation Study is intended to determine the performance of the candidate method. The study is designed to

evaluate performance parameters including inclusivity, exclusivity, repeatability, bias, and robustness. The Method Developer Study is normally conducted in a single laboratory, usually the method developer's laboratory. Alternatively, the method developer can contract the work to an independent site.

The SLV (Precollaborative) Study is a formal submission requirement for OMA microbiology methods and is normally conducted in the method developer laboratory. It precedes the Collaborative Study. The purpose of an SLV (Precollaborative) Study is to define the applicability claims of a proposed OMA microbiology method by demonstrating the applicability of the method to various food categories. For OMA methods, the applicability statement immediately follows the method title. The applicability statement for microbiological methods is generally concerned with target analyte and food type coverage.

5.1.2 Inclusivity/ Exclusivity

This requirement is not applicable to total viable count, yeast & mold count, or similar total enumeration methods that are not directed at specific microorganisms. The requirement applies to selective or differential quantitative methods.

5.1.2.1 Strain Selection

The choice of inclusivity strains should reflect the genetic and/or serological and/or biochemical diversity of the target organism(s). Select at least 50 pure strains of the target organism(s) to be analyzed as pure culture preparations. For *Salmonella* methods, the number of target organisms is increased to at least 100 serovars that are selected to represent the majority of known somatic groups and subtypes of *Salmonella*.

The choice of exclusivity strains should reflect closely related, potentially cross-reactive organisms. Other factors such as virulence, frequency of occurrence and availability should be considered. Select at least 30 pure strains of potentially competitive organisms.

Species/strains specified for use must be traceable to the source. The source and origin of each species/strain should be documented.

5.1.2.3 Study Design

Inclusivity strains are cultured in nonselective media. The target concentration for testing is 100 times the LOD_{50} of the method. Test one replicate per strain.

Exclusivity strains are cultured in nonselective media. The target level is the growth limit of the organism. Test one replicate per strain.

Inclusivity and exclusivity evaluations shall be performed together as one study. Inclusivity and exclusivity test samples must be blind coded and intermingled so the analysts cannot know the identity or concentration of the test samples.

5.1.2.4 Data Reporting

Report inclusivity data as number of strains detected. For example, "Of the 50 specific inclusivity strains tested, 47 were detected and 3 were not detected. Those strains not detected were the following: …"

Report exclusivity data as number of strains not detected. For example, "Of the 30 specific exclusivity strains tested, 28 were not detected and 2 were detected. Those detected were the following: …"

The study report should include a table titled "Inclusivity/ Exclusivity Panel Results," which lists all strains tested, their source, origin and essential characteristics plus testing outcome.

5.1.3 Matrix Study

5.1.3.1 Reference Method

Candidate methods are compared to a reference method where applicable. The following methods are examples of acceptable reference methods: AOAC OMA, FDA BAM, FSIS MLG (for meat and poultry products), ISO and Health Canada *Compendium of Analytical Methods*.

5.1.3.2 Food Categories

AOAC INTERNATIONAL recognizes claims for only the range of food categories or specific food types successfully validated in the Method Developer Study or the PCS and CS. The number of different matrices depends on the applicability of the method. All claimed matrices must be included in the Method Developer Study and the PCS.

5.1.3.3 Levels of Contamination

For the artificially contaminated food types, three inoculated levels (high, medium, and low) and one uninoculated level are required. For naturally contaminated food, three contamination levels (high, medium, and low) are required, and no uninoculated level. The low level should be near the limit of detection, and the medium and high levels should cover the analytical range of the candidate method. If the claimed range of the method is greater than 4 logs, intermediate levels may be required at the discretion of the appropriate method volunteer(s) in consultation with the Study Director.

If the method is intended to detect more than one target organism simultaneously from the same test portion, the validation study should be designed so that target organisms are inoculated into a common sample and the validation tests are performed in a simultaneous manner.

5.1.3.4 Number of Test Portions

For each level, analyze five test portions by the candidate method and five test portions by the reference method.

5.1.3.5 Source of Contamination

Naturally contaminated matrix is preferred as a source of inoculum, if available. Inoculating cultures are used only if the method is for a specific target analyte which may not routinely be found in all food types (e.g., enumeration of *Listeria* spp.) or a certain type has been referenced and the subject flora (e.g., yeast) has not been found in measurable levels.

5.1.3.6 Preparation of Artificially Contaminated Samples

Microorganisms in processed foods are typically stressed, thus the contaminating microorganisms are also stressed for these types of foods. Microorganism stress may occur at the time of inoculation or during preparation of the food. Raw and cold-processed foods should be inoculated with unstressed organisms, heat-processed foods with heat-stressed organisms (e.g., heat culture at 50°C for 10 min), and dry foods with lyophilized culture. Mix well by kneading, stirring or shaking as appropriate. Frozen foods should be thawed, inoculated, mixed and refrozen.

The degree of injury caused by heat stressing should be demonstrated, for nonspore-formers, by plating the inoculum in triplicate on selective and nonselective agars. The degree of injury is calculated as follows:

$$(1 - \frac{n_{select}}{n_{nonselect}}) \times 100$$

where n_{select} = mean number of colonies on selective agar and $n_{nonselect}$ = mean number of colonies on nonselective agar. The heat stress must achieve 50–80% injury of the inoculum. The inoculum should be added to the sample, mixed well and allowed to equilibrate in the matrix for 48–72 h at 4°C for refrigerated foods, for a minimum of 2 weeks at –20°C for frozen foods or for a minimum of 2 weeks at room temperature for dried foods prior to analysis.

5.1.3.7 Use of Artificially and Naturally Contaminated Test Samples

Approximately 50% of the food types should be naturally contaminated unless the method is for a specific microorganism that may not be naturally occurring in that number of food types. For the food types that are naturally contaminated, three different lots are required per food type. There are no uncontaminated levels required for the food types that are naturally contaminated.

The balance of the food types may be either naturally contaminated or artificially contaminated.

5.1.3.8 Need for Competitive Flora

For those candidate methods that are specific for target organisms, it is more realistic and challenging to include microorganisms that act as competitors to the analyte microorganisms. The purpose of including these organisms is to more closely simulate conditions found in nature. It is sufficient to demonstrate this recovery in one food type. This requirement may be satisfied in the Matrix Study. The competitor contamination levels, which may be naturally occurring or artificially introduced, should be at least 10 times higher than the target microorganism.

5.1.3.9 Confirmation of Test Portions

Follow the reference method as written for isolation and confirmation of typical colonies from all candidate method test portions.

5.1.3.10 Data Analysis and Reporting

5.1.3.10.1 General Considerations

Data often do not show a statistically normal distribution. In order to normalize the data, perform a logarithmic transformation on the reported CFU/unit (including any zero results) as follows:

$$\text{Log}_{10}\,[\text{CFU/unit} + (0.1)\text{f}]$$

where f is the reported CFU/unit corresponding to the smallest reportable result, and unit is the reported unit of measure (e.g., g, mL, filter). For details, *see Annex H*.

5.1.3.10.2 Initial Review of Data

If there is a reference method, plot the candidate method result versus the reference method result. The vertical *y*-axis (dependent variable) is used for the candidate method and the horizontal *x*-axis (independent variable) for the reference method. This independent variable *x* is considered to be accurate and have known values. Usually major discrepancies will be apparent.

5.1.3.10.3 Outliers

It is often difficult to make reliable estimations (average, standard deviation, etc.) with a small bias in presence of outliers. Data should be examined to determine whether there exists an occasional result that differs from the rest of the data by a greater amount than could be reasonably expected or found by chance alone. Perform outlier tests (Cochran and Grubbs) in order to discard significantly outlying values (3). There must be an explanation for every excluded result; no results can be excluded on a statistical basis only. To view the data adequately, construct a stem-leaf display, a letter-value display, and a boxplot (4).

Results excluded for justifiable cause must be reported, but should not be included in the statistical analysis.

5.1.3.10.4 Repeatability (s_r)

Calculate repeatability as the standard deviation of replicates at each concentration of each matrix for each method.

5.1.3.10.5 Mean Difference Between Candidate and Reference Where Applicable

Report the mean difference between the candidate and reference method transformed results and its 95% confidence interval. In addition, report the reverse transformed mean difference and confidence interval in CFU/unit or spores/mL.

5.1.4 Robustness Study (PTM submissions only)

5.1.4.1 Strain Selection

Robustness strains are prepared and analyzed as vegetative cells, spores or components thereof as applicable to the candidate method. One target strain is tested using the candidate method enrichment at a high and low level within the quantitative range of the candidate method. One nontarget strain is enriched in a nonselective broth and tested at the high level.

5.1.4.2 Study Design

Minor, reasonable variations in a method of a magnitude that might well be expected to occur when the method is used are deliberately introduced and tested. Variations in method parameters that can be influenced by the end user should be tested. Use a screening factorial experimental design.

The method developer is expected to make a good faith effort to choose parameters that are most likely to affect the analytical performance and determine the range of variations that can occur without adversely affecting analytical results.

Five replicates at each target concentration and five replicates of the nontarget are tested for each factorial pattern.

5.1.4.3 Data Analysis and Reporting

The results are analyzed for effects on bias and repeatability. Standard deviations (s_r) at each concentration are compared to determine if any robustness parameter value causes more than a 3-fold increase in s_r.

5.2 Independent Validation Study

5.2.1 Scope

A validation study to corroborate the analytical results obtained by the method developer and to provide additional single laboratory

data. The independent validation study traditionally verifies repeatability in the hands of an independent trained user.

5.2.2 Reference Method

If there is a reference method, then the candidate method is compared to a reference method. The reference method should be the same as that used in the method developer study.

5.2.3 Matrices

The independent laboratory must test at least one matrix that was tested in the Method Developer Study. The total number of matrices to be evaluated by the independent laboratory is dependent on the claim of the candidate method. For every five foods claimed, one food matrix shall be included in the independent study and for every five environmental surfaces claimed, one surface shall be included in the Independent Study. The choice of matrices for the Independent Study is made by the appropriate method volunteer(s) in consultation with the Study Director.

5.2.4 Study Design

The study design for validation of quantitative methods in the independent study follows the Method Developer Validation Study design. Contamination levels, number of test portions, source of contamination, preparation of samples, confirmation of test portions, and data analysis and reporting are found in Section *5.1.3*.

5.3 Collaborative Study (CS)

5.3.1 Scope

The Collaborative Study (CS) is a formal submission requirement for OMA methods and succeeds the SLV (Precollaborative) Study. The purpose of the Collaborative Study is to estimate the reproducibility and determine the performance of the candidate method among collaborators.

5.3.2 Number of Laboratories

A minimum of eight laboratories reporting valid data for each food type is required. It is suggested that at least 10–12 laboratories begin the analysis.

5.3.3 Reference Method

Candidate methods are compared to a reference method where applicable. The reference method(s) used in the collaborative study must be the same as those used in the SLV (Precollaborative) Study.

5.3.4 Matrix Selection

At least one matrix from those studied in the PTM or PCS shall be chosen by the appropriate method volunteer(s) in consultation with the Study Director for collaborative study. For methods with more than one sample preparation/enrichment, one matrix per procedure may be required in the collaborative study. The determination if the procedures differ significantly to warrant expanding the collaborative study is made by the appropriate method volunteer(s) in consultation with the Study Director. The Statistical Advisor and reviewers can be consulted during this determination. Examples of what constitutes a different sample preparation procedure would include different test portion size, different enrichment media or conditions, different dilution volume and different homogenization equipment. The appropriate AOAC method volunteer(s) shall make the final selection of the matrix(es) with consideration of the PTM or PCS data and the relative importance of the matrices to food safety. The data from both the PCS and CS studies form the basis for defining the method applicability statement.

5.3.5 Levels of Contamination

For the artificially contaminated food types, three inoculated levels (high, medium, and low) and one uninoculated level are required. For naturally contaminated food, three contamination levels (high, medium, and low) are required, and no uninoculated level. The low level should be near the limit of detection, and the medium and high levels should cover the analytical range of the candidate method. If the claimed range of the method is greater than 4 logs, intermediate levels may be required at the discretion of the appropriate method volunteer(s) in consultation with the Study Director.

If the method is intended to detect more than one target organism simultaneously from the same test portion, the validation study should be designed so that target organisms are inoculated into a common sample and the validation tests are performed in a simultaneous manner.

5.3.6 Number of Test Portions

For each contamination level, two test portions are analyzed by the candidate method and two test portions are analyzed by the reference method in each laboratory.

5.3.7 Enumeration of Specific Microorganisms

If the candidate method is for quantitation of a specific microorganism, it may be necessary to include certain food types known to support the growth of such analytes. The inoculating microorganisms must represent different genera, species and/or toxin-producing microorganisms that are intended to be included in the method applicability statement. The choice of strains should be broad enough to represent the inherent variation in the microorganisms of interest.

5.3.8 Source of Contamination

Refer to section *5.1.3.5*.

5.3.9 Preparation of Artificially Contaminated Samples

Refer to section *5.1.3.6*.

5.3.10 Use of Artificially and Naturally Contaminated Test Samples

The use of both naturally and artificially contaminated test samples is strongly encouraged. Because naturally contaminated foods are not always available particularly for methods applicable to specific microorganisms, artificially contaminated test samples may be used.

5.3.11 Confirmation of Test Portions

Follow the reference method as written for isolation and confirmation of typical colonies from all candidate method test portions.

5.3.12 Data Analysis and Reporting

For a detailed explanation of the quantitative method calculations to be performed, refer to *Appendix D* (3).

5.3.12.1 General Considerations

Data often do not show a statistically normal distribution. In order to normalize the data, perform a logarithmic transformation on the reported CFU/unit (including any zero results) as follows:

$$Log_{10} [CFU/unit + (0.1)f]$$

where f is the reported CFU/unit corresponding to the smallest reportable result, and unit is the reported unit of measure (e.g., g, mL, 25 g). For details, *see Annex H*.

5.3.12.2 Initial Review of Data

Plot the candidate method result versus the reference method result. The vertical *y*-axis (dependent variable) is used for the candidate method and the horizontal *x*-axis (independent variable) for the reference method. This independent variable *x* is considered to be accurate and have known values. Usually major discrepancies will be apparent.

Construct a Youden plot. For a given matrix–level combination, plot replicate pairs as first replicate versus second replicate. Usually major discrepancies will be apparent: displaced means, unduly spread replicates, outlying values, differences between methods, consistently high or low laboratory rankings, etc.

Only valid data should be included in the statistical analysis.

5.3.12.3 Outliers

It is often difficult to make reliable estimations (average, standard deviation, etc.) with a small bias and in presence of outliers. Data should be examined to determine whether any laboratory shows consistently high or low values or an occasional result that differs from the rest of the data by a greater amount than could be reasonably expected or found by chance alone. Perform outlier tests (Cochran and Grubbs) in order to discard the outlying values and to obtain a better estimate (3). There must be an explanation for every excluded data set; no data sets can be excluded on a statistical basis only. To view the data adequately, construct a stem-leaf display, a letter-value display, and a boxplot (4).

5.3.12.4 Performance Indicators

Performance indicators for quantitative methods include repeatability and reproducibility standard deviations of the transformed data.

5.3.12.4.1 Repeatability (s_r)

Calculate repeatability as the standard deviation of replicates at each concentration of each matrix for each laboratory.

5.3.12.4.2 Reproducibility (s_R)

Calculate reproducibility as the standard deviation of replicates at each concentration for each matrix across all laboratories.

5.3.12.5 Mean Difference between Candidate and Reference Methods Where Applicable

Report the mean difference between the candidate and reference method transformed results and its 95% confidence interval. In addition, report the reverse transformed mean difference and confidence interval in CFU/unit.

5.3.12.6 Calculations

For details, refer to *Appendix D* (3).

6 Confirmatory Identification Methods

6.1 Method Developer Validation Study or SLV (Precollaborative) Study

6.1.1 Scope

The Method Developer Study is intended to determine the performance of a microbiological confirmatory identification method. The study is designed to evaluate performance parameters including inclusivity, exclusivity, and robustness. The Method Developer Study is normally conducted in a single laboratory, usually the method developer's laboratory. Alternatively, the method developer can contract the work to an independent site.

The SLV (Precollaborative) Study is a formal submission requirement for OMA microbiology methods and is normally conducted in the method developer laboratory. It precedes the Collaborative Study. The purpose of an SLV (Precollaborative) Study is to define the applicability claims of a proposed OMA microbiology method. For OMA methods, the applicability statement immediately follows the method title.

6.1.2 Inclusivity/Exclusivity Study

6.1.2.1 Species/Strain Selection

The choice of inclusivity strains should cover the genetic, serological, biochemical or physical diversity of the target agent group(s) as appropriate for the method. The number of organisms required for validation will be determined by the diversity of the target agent group(s) and the intended use claim. The number of strains tested should be no less than 50 for each target species claimed, if available. For *Salmonella* methods, the number of target organisms is increased to at least 100 serovars that are selected to represent the majority of known somatic groups of *Salmonella*.

The choice of exclusivity strains should include organisms not claimed by the confirmatory identification method. The choice of exclusivity strains should reflect closely related, potentially competitive organisms. Other factors such as virulence, frequency of occurrence and availability should be considered. The number of species/strains tested should be no less than 30.

Species/strains selected for testing must be different than those used to develop the method if possible. Species/strains specified for use must be traceable to the source. The source and origin of each species/strain should be reported. Species/strains must have Certificate of Analysis from the source documenting the identity and method(s) used to determine the identity or be well characterized before use with documentation on file.

The study designs presented are intended to be a suggested guideline. Specific study designs and numbers of strains will be determined by the Methods Committee on Microbiology on a case by case basis.

6.1.2.2 Study Design

Inclusivity strains are prepared and analyzed as vegetative cells on the media designated in the candidate method. All media recommended for use with the candidate method must be validated. Test one replicate per strain per medium using the candidate method.

Exclusivity strains are prepared and analyzed as vegetative cells on the media designated in the candidate method. All media recommended for use with the candidate method must be validated. Test one replicate per strain per medium using the candidate method.

Inclusivity and exclusivity evaluations shall be performed together as one study. Inclusivity and exclusivity test samples must be blind coded and intermingled so the analysts cannot know the identity of the test samples.

6.1.2.3 Data Analysis and Reporting

Analyze the data for correct identification, misidentification or unidentified organism. The data is reported as number of species/strains correctly identified.

The data is reported as number of species/strains correctly identified. For example, "Of the 50 specific inclusivity strains tested, 48 were correctly identified and 2 were misidentified. Those strains misidentified were the following: …" or "Of the 30 specific exclusivity strains tested, 27 were correctly unidentified and 3 were misidentified. Those misidentified by the method were the following: …"

The study report should include a table titled "Inclusivity/Exclusivity Panel Results," which lists all species/strains tested their source, origin and essential characteristics plus testing outcome.

6.1.3 Robustness Study (PTM submissions only)

6.1.3.1 Strain Selection

Robustness strains are prepared and analyzed as vegetative cells on agar(s) recommended by the candidate method. Prepare 10 inclusivity strains and five exclusivity strains for testing.

6.1.3.2 Study Design

Minor, reasonable variations in a method of a magnitude that might well be expected to occur when the method is used are deliberately introduced and tested. Variations in method parameters that can be influenced by the end user should be tested. Use a screening factorial experimental design.

The method developer is expected to make a good faith effort to choose parameters that are most likely to affect the analytical performance and determine the range of variations that can occur without adversely affecting analytical results.

Test one replicate of each inclusivity and exclusivity organism for each factorial pattern.

6.1.3.3 Data Analysis and Reporting

The results are analyzed for the number of misidentifications when method parameters are altered. Report the identification results for each factorial pattern.

6.2 Independent Validation Study

6.2.1 Scope

A validation study to corroborate the analytical results obtained by the method developer and to provide additional single laboratory data. The independent validation study verifies the inclusivity and exclusivity in the hands of an independent trained user.

6.2.2 Study Design

Inclusivity and exclusivity strains are prepared and analyzed as vegetative cells on the media designated in the candidate method. All media recommended for use with the candidate method must be tested by the Independent laboratory. Test one replicate per strain per medium using the candidate method. For inclusivity, the independent laboratory must test at least 10 strains randomly selected from the ≥30 selected earlier per pathogenic species claimed and at least one strain per nonpathogenic species claimed. For exclusivity, the independent laboratory must test at least 10 strains not claimed by the method. The strains selected should be different from those used to develop the method where possible.

Inclusivity and exclusivity evaluations shall be performed together as one study. Inclusivity and exclusivity test samples must be blind coded and intermingled so the analysts cannot know the identity of the test samples.

The study designs presented are intended to be a suggested guideline. Specific study designs and numbers of strains will be determined by the Methods Committee on Microbiology on a case by case basis.

Species/strains selected for testing must be different than those used to develop the method if possible. Species/strains specified for use must be traceable to the source. The source and origin of each species/strain should be reported. Species/strains must have Certificate of Analysis from the source documenting the identity and method(s) used to determine the identity or be well characterized before use with documentation on file.

6.2.3 Data Analysis and Reporting

Analyze the inclusivity data for correct identification, misidentification and unidentified organisms.

Species/strains selected for testing must be different than those used to develop the method if possible. Species/strains specified for use must be traceable to the source. The source and origin of each species/strain should be reported. Species/strains must have Certificate of Analysis from the source documenting the identity and method(s) used to determine the identity or be well characterized before use with documentation on file.

The data are reported as number of species/strains correctly identified. For example, "Of the 10 specific inclusivity strains tested, 9 were correctly identified and 1 was misidentified. The strain misidentified was the following: …"

The study report should include a table titled "Inclusivity Panel Results," which lists all species/strains tested, their source, origin and essential characteristics plus testing outcome.

Analyze the exclusivity data for misidentifications and unidentified organisms. The data is reported as number of strains correctly unidentified. For example, "Of the 10 specific exclusivity strains tested, 7 were correctly unidentified and 3 were misidentified. Those misidentified by the method were the following: …"

The study report should include a table titled "Exclusivity Panel Results," which lists all strains tested, their source, origin and essential characteristics plus testing outcome.

6.3 Collaborative Study

6.3.1 Scope

The Collaborative Study is a requirement for OMA methods and succeeds the SLV (Precollaborative) Study. The purpose of the Collaborative Study is to estimate the reproducibility and determine the performance of the candidate method among collaborators.

6.3.2 Number of Collaborators

A minimum of 10 laboratories reporting valid data are required. The Study Director should plan on including additional laboratories due to potential invalid data sets, so it is recommended that at least 12 collaborators be included in the collaborative study.

6.3.3 Number of Tests

Each collaborator receives a minimum of 12 organisms recommended by the Methods Committee on Microbiology. Data collection at all test sites must begin on the same day to control for the age of the cultures.

Species/strains selected for testing must be different than those used to develop the method if possible. Species/strains specified for use must be traceable to the source. The source and origin of each species/strain should be reported. Species/strains must have Certificate of Analysis from the source documenting the identity and method(s) used to determine the identity or be well characterized before use with documentation on file.

6.3.4 Data Analysis and Reporting

Analyze the inclusivity data for correct identification, misidentification and unidentified organisms by laboratory. The data are reported as number of species/strains correctly identified by laboratory. For example, "Of the N specific inclusivity strains tested, N-2 were correctly identified and 2 were misidentified in Laboratory 1. Those strains misidentified were the following: …"

The study report should include a table titled "Inclusivity Panel Results," which lists all species/strains tested, their source, origin and essential characteristics plus testing outcome by laboratory.

Analyze the exclusivity data for misidentifications and unidentified organisms. The data are reported as number of strains correctly unidentified. For example, "Of the M specific exclusivity strains tested, M-3 were correctly unidentified and 3 were misidentified in Laboratory 1. Those misidentified by the method were the following: …"

The study report should include a table titled "Exclusivity Panel Results," which lists all strains tested, their source, origin and essential characteristics plus testing outcome by each laboratory.

7 Safety

Personnel should be aware of safety issues in the laboratory and have the appropriate training to carry out microbiological procedures dealing with the growth and safe disposal of microorganisms and biochemicals, particularly where pathogens are under test. The appropriate biohazard containment facilities and protective clothing should be available.

8 References

(1) U.S. Food and Drug Administration (FDA) (2010) *Bacteriological Analytical Manual*, http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm109656.htm

(2) U.S. Food and Drug Administration (FDA) (2005) *Bacteriological Analytical Manual*, http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/UCM064244

(3) *Official Methods of Analysis* (2012) 19th Ed., *Appendix D*, AOAC INTERNATIONAL, Gaithersburg, MD

(4) Tukey, J.W. (1977) *Exploratory Data Analysis*, Addison-Wesley, pp 8 ff, 33, and 39 ff

(5) AOAC INTERNATIONAL *Official Methods of Analysis Online*, http://www.eoma.aoac.org/

(6) U.S. Food and Drug Administration (FDA) (2011) *Bacteriological Analytical Manual Online*, http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/default.htm

(7) U.S. Department of Agriculture–Food Safety and Inspection Service (FSIS) *Microbiology Laboratory Guidebook Online*, http://www.fsis.usda.gov/Science/Microbiological_Lab_Guidebook/index.asp

(8) International Organization for Standardization (ISO) http://www.iso.org/iso/prods-services/ISOstore/store.html

(9) Health Canada *Compendium of Analytical Methods–Microbiological Methods*, http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/index-eng.php

(10) Feldsine, P., Abeyta, C, & Andrews, W.H. (2002) *J. AOAC Int.* **85**, 1187–1200

(11) International Organization for Standardization (ISO) Standard 16140, *Microbiology of Food and Animal Feeding Stuffs — Protocol for the Validation of Alternative Methods*, http://www.iso.org/iso/search.htm?qt=16140&searchSubmit=Search&sort=rel&type=simple&published=true

(12) LaBudde, R.A. (2008) *Statistical Analysis of Interlaboratory Studies*, *XX. Measuring the Performance of a Qualitative Test Method*, TR290, Least Cost Formulations, Ltd, Virginia Beach, VA

(13) LaBudde, R.A. (2009) *Coverage Accuracy for Binomial Proportion 95% Confidence Intervals for 12 to 100 Replicates*, TR297, Least Cost Formulations, Ltd, Virginia Beach, VA

(14) LaBudde, R.A. (2009) *Statistical Analysis of Interlaboratory Studies, XXII. Statistical Analysis of a Qualitative Multicollaborator Study as a Quantitative Study Under the Large Sample Approximation*, TR296, Least Cost Formulations, Ltd, Virginia Beach, VA

ANNEX A
MPN Analysis of Contaminated Matrix

The most probable number ("MPN"), also known as the maximum likelihood estimate, is obtained as the root of the following equation:

$$\sum_{k=1}^{K}\left[\frac{d_k m_k}{\exp(d_k\,\mathrm{MPN})-1}-d_k\left(n_k-m_k\right)\right]=0$$

where the summation over k = 1, 2, … , K ranges over the serial dilution sets, and d_k = the amount of sample used in the k-th dilution set; m_k = the number of replicates in the k-th dilution set; n_k = the number of positive results in the k-th dilution set; MPN = the most probable number estimate.

A 95% confidence interval for the MPN estimate can be obtained as the 2.5 and 97.5% quantiles of sampling distribution of MPN generated by bootstrap resampling with 10 000 realizations. For bootstrap resampling to be acceptable, at least one dilution set with fractional response must have five replicates or more.

Approximate confidence intervals may also be found from one of the following formulas:

$$I=\sum_{k=1}^{K}\left[\frac{d_k^2 m_k \exp(d_k\,\mathrm{MPN})}{\left(\exp(d_k\,\mathrm{MPN})-1\right)^2}\right]$$

$$\mathrm{UCL,\ LCL}\ =\mathrm{MPN}\pm\frac{1.9600}{\sqrt{I}}$$

directly on MPN.

$$\mathrm{UCL,\ LCL}\ =\exp\left[\ln(\mathrm{MPN})\pm\frac{1.9600}{\mathrm{MPN}\sqrt{I}}\right]$$

for intervals on ln(MPN).

When an equal number of replicates in each set and a constant dilution ratio between sets are used, tables, such as those in the FDA *Bacteriological Analytical Manual* Appendix 2, may be used to supply estimates of MPN with 95% confidence intervals.

It is strongly recommended that no less than five replicates be used in each dilution set, and that the replicates tested in the reference laboratory be included as one of the dilutions for each concentration level. Dilution sets with fewer replicates supply unreliable estimates. For fractional detection concentration levels, a dilution ratio of 1/2 or 1/3 is recommended instead of the customary 1/10.

Example: A candidate test method is evaluated at an expected 50% fractional detection concentration level. Twenty replicates are analyzed in the reference laboratory. During test portion preparation, an additional five replicates are made each of 3 and 1/3 times the desired concentration level. All 30 test portions are tested by the reference method in the reference laboratory, with presence or absence results (*see* Table A1).

"The MPN estimate is 0.053 MPN/g (1.3 MPN/25 g) with a 95% confidence interval from bootstrap resampling of 0.034 MPN/g (0.85 MPN/25 g) to 0.086 MPN/g (2.2 MPN/25 g)."

Table A1

	Initial	Estimate	Bootstrap LCL	Bootstrap UCL
MPN	0.055	0.053	0.034	0.086
Direct			0.027	0.079
ln based			0.032	0.087
Series	Dilution factor	No. tubes	No. positive	Dilution estimate
1	3.00000	5	5	0.333
2	1.00000	20	15	0.024
3	0.33333	5	1	0.012

ANNEX B
Raw Format Data Table Template and Example for Qualitative Method Single Laboratory and Collaborative Studies

The purpose of the Raw Format Data Table is to document in a software-friendly format all of the factors, variables, and measurements in the experiment. By matrix and concentration level, report each result from each method for each test portion separately.

Each row (record) in the Raw Format Data Table should contain the following columns (fields):

(*1*) *Matrix type*.—An identifier indicating the matrix involved, such as "EGGS." The same exact identifier must be used for the same matrix.

(*2*) *Concentration level*.—The MPN/test portion for the level. (The MPN/test portion, and not MPN/g or MPN/mL, is the relevant measure for statistical analysis of the data.)

(*3*) *Laboratory*.—An identifier indicating the laboratory involved, such as "01."

(*4*) *Method*.—An identifier indicating the test method used, such as "REF" for the reference method, "C-P" for the candidate presumptive method, or "C-C" for the candidate confirmation method.

(*5*) *Replicate*.—A unique identifier for the test portion involved. If this identifier is common to two rows in the table, this implies the results are matched by test portion. Example identifiers might be "01," "001," or "A1."

(*6*) *Result*.—"0" for absence or "1" for presence (detection).

In computer format, the Raw Format Data Table should be given either as: (*1*) a "fixed-format" file with fixed column widths and blanks or tabs as separators and a file extension of ".txt" or ".xls"; or (*2*) a "comma-separated value" file with commas as separators between columns and identifiers within quotes, and a file extension of ".csv".

It is desirable to include a "header" record as the first record in the file with identifiers for each column.

An example file named "ecoli.csv" might be:

"matrix", "level", "lab", "method", "replicate", "result"
"spinach", "2.20", "01", "cpres", "001", 0
"spinach", "2.20", "01", "cconf", "002", 1
"spinach", "2.20", "01", "ref", "003", 1
"spinach", "2.20", "01", "cpres", "004", 1
"spinach", "2.20", "01", "cconf", "005", 1
"spinach", "2.20", "01", "ref", "006", 1
etc.

ANNEX C
Calculation of POD and dPOD Values from Qualitative Method Single Laboratory Data

In general, four different probabilities detected (PODs) are to be calculated: POD_R (for the reference method), POD_C (for the confirmed candidate method), POD_{CP} (for the candidate presumptive method), and POD_{CC} (for the candidate confirmation method).

For each of these four cases, calculate the POD as the ratio of the number positive (x) to total number tested (N):

$$\mathrm{POD} = \frac{x}{N}$$

where POD is POD_C, POD_R, etc.

The POD estimates and 95% confidence interval (LCL, UCL) estimates are given by:

(*1*) *For the case where x = 0.*

$$\mathrm{POD} = 0$$

$$\mathrm{LCL} = 0$$

$$\mathrm{UCL} = 3.8415/(N + 3.8415)$$

(*2*) *For the case where x = N.*

$$\mathrm{POD} = 1$$

$$\mathrm{LCL} = N/(N + 3.8415)$$

$$\mathrm{UCL} = 1$$

(*3*) *For the case where 0 < x < N.*

$$\mathrm{POD} = x/N$$

$$\mathrm{LCL} = \frac{x + 1.9207 - 1.9600\sqrt{x - \frac{x^2}{N} + 0.9604}}{N + 3.8415}$$

$$\mathrm{UCL} = \frac{x + 1.9207 + 1.9600\sqrt{x - \frac{x^2}{N} + 0.9604}}{N + 3.8415}$$

where 1.9600 = z, the Gaussian quantile for probability 0.975, $1.9207 = z^2/2$, $0.9604 = z^2/4$ and $3.8415 = z^2$.

Finally, if $x \leq 1$, set LCL = 0. If $x \geq N\text{-}1$, set UCL = 1.

The confidence interval corresponds to the uncorrected Wilson-score method, modified for x = 1 and x = N–1 to improve coverage accuracy on the boundary.

dPOD for Unpaired Studies

The differences in proportions detected are estimated by:

$$\mathrm{dPOD_C} = \mathrm{POD_C} - \mathrm{POD_R}$$

$$\mathrm{dPOD_{CP}} = \mathrm{POD_{CP}} - \mathrm{POD_{CC}}$$

If the replicates tested by the candidate and reference methods are unpaired (i.e., the enrichment conditions differ between the methods, thus the methods require analysis of distinct test portions), the associated 95% confidence interval (LCL, UCL) for the expected value of dPOD = $POD_1 - POD_2$ is estimated by:

$$\mathrm{LCL} = \mathrm{dPOD} - \sqrt{(\mathrm{POD_1} - \mathrm{LCL_1})^2 + (\mathrm{POD_2} - \mathrm{UCL_2})^2}$$

$$\mathrm{UCL} = \mathrm{dPOD} + \sqrt{(\mathrm{POD_1} - \mathrm{UCL_1})^2 + (\mathrm{POD_2} - \mathrm{LCL_2})^2}$$

where (LCL_1, UCL_1) is a 95% confidence interval for POD_1 and (LCL_2, UCL_2) is a 95% confidence interval for POD_2, as determined above.

dPOD for Paired Studies

If the replicates tested by the candidate and reference methods are paired (i.e., the enrichment conditions are the same, thus common test portions are analyzed by both methods), the associated 95% confidence interval (LCL, UCL) for the expected value of dPOD = $POD_1 - POD_2$ is estimated by the following:

Let

$$d_i = x_{1i} - x_{2i}$$

denote the numerical difference of the two method results on test portion i. Note that d_i must take on only the values –1, 0, or +1.

The recommended method for estimating dPOD is the mean of differences d_i:

$$d\mathrm{POD} = \frac{\sum_{i=1}^{N} d_i}{N}$$

where N is the number of test portions.

The recommended approximate 95% confidence interval is the usual Student-*t* based interval, with the standard error of dPOD computed in the usual manner from the replicate differences:

$$s_d = \sqrt{\frac{\sum_{i=1}^{N} (d_i - d\mathrm{POD})^2}{N - 1}}$$

$$\mathrm{SE}_{d\mathrm{POD}} = \frac{s_d}{\sqrt{N}}$$

and

$$LCL = d\mathrm{POD} - t_c \cdot \mathrm{SE}_{d\mathrm{POD}}$$

$$UCL = d\mathrm{POD} + t_c \cdot \mathrm{SE}_{d\mathrm{POD}}$$

where t_c is the 97.5% quantile of the Student-*t* distribution for N-1 degrees of freedom, and the 95% confidence interval is (LCL, UCL).

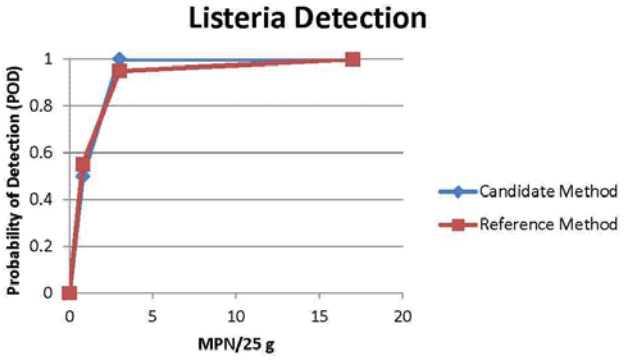
The degree of coverage accuracy for this approximate confidence interval will improve as N increases and the Central Limit Theorem forces the distribution of dPOD to become normal. Given the finite range of the d_i's, this will happen quickly, even for small N.

ANNEX D
Summary Data Table for Qualitative Method Single Laboratory Studies

Table D1. Comparative results for the detection of *Listeria innocua* in raw shrimp

Statistic	Concentration	Candidate presumptive (CP)			Candidate confirmed (CC)			Candidate method (C)			Reference method (R)			C vs R	CP vs CC
	MPN/25 g	N	x	POD(CP)	N	x	POD(CC)	N	x	POD(C)	N	x	POD(R)	dPOD(C,R)	dPOD(CP,CC)
Estimate	0.00	20	0	0.00	20	0	0.00	20	0	0.00	20	0	0.00	0.00	0.00
LCL	0.00			0.00			0.00			0.00			0.00	–0.16	–0.16
UCL	1.50			0.16			0.16			0.16			0.16	0.16	0.16
Estimate	0.80	20	12	0.60	20	10	0.50	20	10	0.50	20	11	0.55	–0.05	0.10
LCL	0.43			0.39			0.30			0.30			0.34	–0.33	–0.19
UCL	1.39			0.78			0.70			0.70			0.74	0.24	0.37
Estimate	3.00	20	20	1.00	20	20	1.00	20	20	1.00	20	19	0.95	0.05	0.00
LCL	1.58			0.84			0.84			0.84			0.76	–0.12	–0.16
UCL	5.68			1.00			1.00			1.00			1.00	0.24	0.16
Estimate	17.00	20	20	1.00	20	20	1.00	20	20	1.00	20	20	1.00	0.00	0.00
LCL	0.27			0.84			0.84			0.84			0.84	–0.16	–0.16
UCL	1060			1.00			1.00			1.00			1.00	0.16	0.16

ANNEX E
Example of Graph of POD Values from Qualitative Method Single Laboratory Data



Notes:
(*1*) The concentration plotted should be MPN/test portion.
(*2*) Confidence intervals may also be plotted.
(*3*) Collaborative data should be plotted analogously.

ANNEX F
Calculation of LPOD and dLPOD Values from Qualitative Method Collaborative Study Data

For a multilaboratory trial where L = number of laboratories, R = replicates per laboratory, N = LR = total replicates, LPOD estimate is given by

$$\text{LPOD} = \frac{x}{N}$$

where x is the number of positive results.

Method for estimating LPOD 95% confidence intervals:

Step 1.—Enter data into AOAC spreadsheet with 1 for positive response and 0 for negative response. Record the mean LPOD, s(R), and s(r).

Step 2.—Calculate s(L), standard deviation due to laboratory effect as:

$$s(L) = \sqrt{s(R)^2 - s(r)^2}$$

Step 3.—Calculate s(POD) as the standard deviation of the individual laboratory POD estimates.

$$s(POD) = \sqrt{\frac{\sum (POD_i - LPOD)^2}{L-1}}$$

Step 4.—Calculate degrees of freedom, df for s(POD) as follows:

$$df = \frac{\left[\frac{s(L)^2}{L} + \frac{s(r)^2}{N}\right]^2}{\frac{\left[\frac{s(L)^2}{L}\right]^2}{L-1} + \frac{\left[\frac{s(r)^2}{N}\right]^2}{N-L}}$$

Step 5.—Calculate 95% confidence limits on LPOD:

If $0.15 \le \text{LPOD} \le 0.85$:

$$LCL = \max\left\{0, LPOD - \frac{t_{0.975,df}\ s(POD)}{\sqrt{L}}\right\}$$

$$UCL = \min\left\{1, LPOD + \frac{t_{0.975,df}\ s(POD)}{\sqrt{L}}\right\}$$

If LPOD <0.15 or LPOD > 0.85:

$$LCL = \frac{x + 1.9207 - 1.9600\sqrt{x - \frac{x^2}{N} + 0.9604}}{N + 3.8415}$$

$$UCL = \frac{x + 1.9207 + 1.9600\sqrt{x - \frac{x^2}{N} + 0.9604}}{N + 3.8415}$$

where x is the number of observed positive outcomes and N is the total number of trials.

If LPOD = 0:

$$LCL = 0$$

$$UCL = 3.8415/(N + 3.8415)$$

If LPOD = 1:

$$LCL = N/(N + 3.8415)$$

$$UCL = 1$$

Step 6.—Calculate 95% confidence intervals for dLPOD:

dLPOD is the difference between any two LPOD estimates, for example to compare a candidate method to a reference method:

$$dLPOD_C = LPOD_C - LPOD_R$$

The associated 95% confidence interval (LCL, UCL) for the expected value of dLPOD = $LPOD_1 - LPOD_2$ is estimated by:

$$LCL = dLPOD - \sqrt{(LPOD_1 - LCL_1)^2 + (LPOD_2 - UCL_2)^2}$$

$$UCL = dL\,POD + \sqrt{(LPOD_1 - UCL_1)^2 + (LPOD_2 - LCL_2)^2}$$

Example

Suppose the reference method in an interlaboratory study gave the following results when 12 replicate test portions were tested in each of 10 laboratories: *see* Table F1.

Here, x = 76, N =120, and LPOD = 0.6333 (= 76/120).

The repeatability standard deviation

$$s_r^2 = \frac{\sum_{i=1}^{L}(n_i - 1)s_i^2}{\sum_{i=1}^{L}(n_i - 1)} = \frac{\sum\left[x_i - \left(x_i^2/n_i\right)\right]}{N - L} = \frac{\left[\left(7 - 49/120\right) + \left(9 - 81/120\right) + \ldots + \left(9 - 81/120\right)\right]}{120 - 10}$$

$$= 0.2242$$

$$s_r = \sqrt{s_r^2} = \sqrt{0.2242} = 0.4735$$

where s_i^2 is the variance of the results from laboratory i, x_i is the number of positive detections from laboratory i, n_i is the number of observations from laboratory i, N is the total number of data, and L is the number of laboratories.

Table F1

	Method R			R
Lab	Positive	Negative	Total	POD
1	7	5	12	0.5833
2	9	3	12	0.7500
3	6	6	12	0.5000
4	10	2	12	0.8333
5	5	7	12	0.4167
6	7	5	12	0.5833
7	5	7	12	0.4167
8	7	5	12	0.5833
9	11	1	12	0.9167
10	9	3	12	0.7500
All	76	44	120	

And $\sqrt{LPOD(1 - LPOD)} = 0.4819$, suggesting s_L will be small compared to s_r.

The among-laboratory standard deviation is

$$s_L^2 = \max\left\{0, \frac{\sum(POD_i - LPOD)^2}{L - 1} - \frac{s_r^2}{n}\right\}$$

$$= \max\left\{0, \frac{\left[(0.5833 - 0.6333)^2 + \ldots + (0.75 - 0.6333)^2\right]}{10 - 1} - \frac{0.2242}{12}\right\}$$

$$= \max\{0, 0.02963 - 0.0187\}$$

$$= 0.01093$$

and $s_L = \sqrt{0.01093} = 0.1045$, which is noticeably less than s_r, as expected.

The reproducibility standard deviation is

$$s_R^2 = s_r^2 + s_L^2$$
$$= 0.01093 + 0.2242$$
$$= 0.2351$$

so $s_R = \sqrt{0.2351} = 0.4849 \approx s_r$

The results are summarized in Table F2.

The "homogeneity test" reported above is the T statistic based on the χ^2 distribution, so the *p*-value of 0.1703 should be compared to 0.10. The test indicates the observed value of s_L = 0.1046 is not statistically significant, so the study was not large enough to reliably detect an interlaboratory effect of this size.

Table F2

Parameter	Value
LPOD	0.6333
s_r	0.4735
s_L	0.1046
s_R	0.4850
p-Value for *T*-test	0.1703

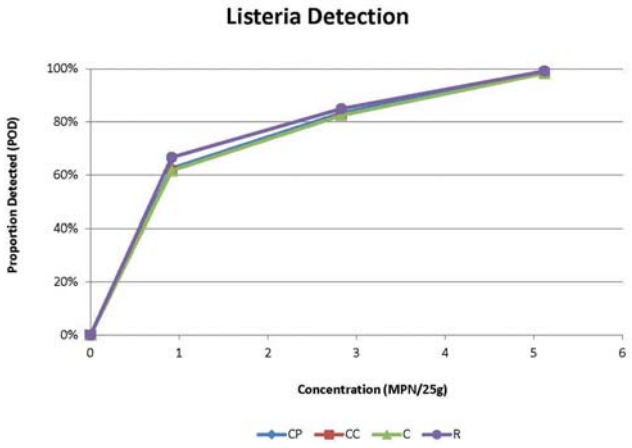
ANNEX G
Data Summary Table Template and Example for Qualitative Method Collaborative Studies

Table G1. Comparative results for the detection of *Listeria innocua* in raw shrimp by the candidate and reference methods in an interlaboratory study

			Candidate presumptive (CP)			Candidate confirmed (CC)			Candidate result (C)			Reference method (R)			C vs R	CP vs CC
Statistic	Concn, MPN/25 g	Lab	N	x	POD(CP)	N	x	POD(CC)	N	x	POD(C)	N	x	POD (R)	dLPOD (C,R)	dLPOD (CP,CC)
		01	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		02	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		03	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		04	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		05	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		06	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		07	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		08	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		09	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
		10	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00
Estimate	0.00	All	120	0	0.00	120	0	0.00	120	0	0.00	120	0	0.00	0.00	0.00
LCL	0.00				0.00			0.00			0.00			0.00	–1.00	–1.00
UCL	0.02				0.17			0.03			0.03			0.03	0.03	0.03
s_r					0.00			0.00			0.00			0.00		
LCL					0.00			0.00			0.00			0.00		
UCL					0.17			17			0.17			0.17		
s_L					0.00			0.00			0.00			0.00		
LCL					0.00			0.00			0.00			0.00		
UCL					0.03			0.03			3.00			0.03		
s_R					0.00			0.00			0.00			0.00		
LCL					0.00			0.00			0.00			0.00		
UCL					0.24			0.24			0.24			0.24		
P_T					1.0000			1.0000			1.0000			1.0000		
		01	12	8	0.67	12	8	0.67	12	8	0.67	12	7	0.58	0.08	0.00
		02	12	9	0.75	12	8	0.67	12	8	0.67	12	7	0.58	0.08	0.08
		03	12	8	0.67	12	8	0.67	12	8	0.67	12	6	0.50	0.17	0.00
		04	12	6	0.50	12	6	0.50	12	6	0.50	12	10	0.83	–0.33	0.00
		05	12	7	0.58	12	7	0.58	12	7	0.58	12	7	0.58	0.00	0.00
		06	12	6	0.50	12	6	0.50	12	6	0.50	12	8	0.67	–0.17	0.00
		07	12	8	0.67	12	8	0.67	12	8	0.67	12	6	0.50	0.17	0.00

Table G1. (*continued*)

			Candidate presumptive (CP)			Candidate confirmed (CC)			Candidate result (C)			Reference method (R)			C vs R	CP vs CC
Statistic	Concn, MPN/25 g	Lab	N	x	POD(CP)	N	x	POD(CC)	N	x	POD(C)	N	x	POD (R)	dLPOD (C,R)	dLPOD (CP,CC)
		08	12	7	0.58	12	7	0.58	12	7	0.58	12	11	0.92	–0.33	0.00
		09	12	8	0.67	12	8	0.67	12	8	0.67	12	9	0.75	–0.08	0.00
		10	12	8	0.67	12	8	0.67	12	8	0.67	12	9	0.75	–0.08	0.00
Estimate	0.92	All	120	75	0.63	120	74	0.62	120	74	0.62	120	80	0.67	–0.05	0.01
LCL	0.73				0.53			0.53			0.53			0.58	–0.36	–0.37
UCL					0.72			0.71			0.71			0.76	–0.04	0.12
s_r					0.50			0.50			0.50			0.47		
LCL					0.44			0.44			0.44			0.42		
UCL					0.52			0.52			0.52			0.52		
s_L					0.00			0.00			0.00			0.04		
LCL					0.00			0.00			0.00			000		
UCL					0.13			0.11			0.11			0.22		
s_R					0.50			0.50			0.50			0.47		
LCL					0.45			0.45			0.45			0.42		
UCL					0.52			0.52			0.52			0.52		
P_T					0.9634			0.9867			0.9867			0.3711		
etc.																



ANNEX H
Logarithmic Transformation of Data from Quantitative Method Single Laboratory and Collaborative Data

Quantitative microbiological count data from experiments spanning multiple dilutions often do not show a Poisson nor a Gaussian statistical distribution. When the underlying physical mechanism allows for "clustering," typically a logarithmic transformation will normalize the data.

Perform a logarithmic transformation on the reported CFU/unit (including any zero results) as follows:

$$Y = \log_{10} [CFU/unit + (0.1)f]$$

where f is the reported CFU/unit corresponding to the smallest reportable result, and "unit" is the reported unit of measure (e.g., g, mL, 25 g).

Examples

(*1*) For the control concentration, the CFU/g is reported as "<0.003." So CFU/unit = 0.0, and $Y = \log_{10} [0.0 + (0.1)(0.003)] = -3.52$.

(*2*) For the low concentration, the CFU/g is 0.042. So $Y = \log_{10} [0.042 + (0.1)(0.003)] = -1.37$.

(*3*) For the high concentration, the CFU/g is 0.231. So $Y = \log_{10} [0.231 + (0.1)(0.003)] = -0.64$.