Appendix []: AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Cannabis and Cannabis Products

Table of Contents

1 Scope

The purpose of this document is to provide comprehensive AOAC INTERNATIONAL (AOAC) technical guidelines for conducting microbiological validation studies for analysis methods of cannabis and cannabis products submitted for AOAC® Official Methods of Analysis (OMA) status, Performance Tested Methods (PTM), and/or Reviewed and Recognized (R^2) 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, PTM, or R^2 certification. Circumstances, unforeseen by AOAC, may necessitate divergence from the guidelines in certain cases. The PTM and R^2 Programs require 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 or R^2-OMA program can be followed in which PTM or R^2 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.

Table 1

AOAC Program	Study Requirements	Relevant Guideline Sections		
		Qualitative	Quantitative	Confirmatory Identification
PTM/R^2	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 or R^2-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 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.

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 Decontamination

The process of removing pathogenic microorganisms from products to allow for safe handling and consumption

3.10 Enrichment Pool

A pool comprised of aliquots from multiple test portion enrichments.

3.11 Exclusivity

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

3.12 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.

3.13 Inclusivity

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

3.14 Limit of Detection50 (LOD50)

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

3.15 Limit of Quantitation (LOQ)

The lowest amount of analyte in a laboratory sample which can be quantitatively determined with a defined confidence.

3.16 Matched Analyses

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

3.17 Material

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

3.18 Matrix

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

3.19 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.

3.20 Precision

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

3.21 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.

3.22 Presumptive Result

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

3.23 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., PODR (reference method POD), PODC (confirmed candidate method POD), PODCP (candidate method presumptive result POD) and PODCC (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

3.24 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.

3.25 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.

3.26 Reference Method

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

3.27 Remediation

The process of removing or reducing the level of microbial contamination in a product to a level of compliance.

3.28 Repeatability

Precision under repeatability conditions. (ISO 5725-1)

3.29 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.

3.30 Reproducibility

Precision under reproducibility conditions. (ISO 5725-1)

3.31 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.

3.32 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)

3.33 Sterilization

The process of complete elimination or destruction of all form of microbial life (both vegetative cells and spores) which is carried out by various physical or chemical methods.

3.34 Test Portion

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

3.35 Unmatched Analyses

Two or more analyses or analytical results on the same unknown material, 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 and R^2 submissions, robustness, product consistency, and stability are 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 LOD50 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.

4.1.2.4 In-Silico Analysis

For molecular methods, see section 10 for in-silico analysis guidelines that can serve as supplemental information for the inclusivity study.

4.1.3 Matrix Study

4.1.3.1 Reference Method

Candidate methods are compared to a cultural reference method, where applicable. The following are examples of sources 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, USP, Dairy Standard Methods.

Recognizing that there may be a lack of reference methods available for the cannabis matrix, guidance in AOAC Standard Method Performance Requirements should be followed in conjunction with reputable reference method sources. When appropriate, Method developers should coordinate with the study director and/or consultant for best practices to be followed.

4.1.3.2 Matrix Categories

AOAC INTERNATIONAL recognizes claims for the range of specific cannabis 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. See section 7 for guidance on matrix categorization.

4.1.3.3 Levels of Contamination

Each matrix 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 (PODR) or candidate method POD (PODC) in the range of 0.25–0.75. Finally, one sample should be contaminated at such a level to assure a POD 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 cannabis and cannabis products, depending on the matrix.
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.

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.

4.1.3.4 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. If naturally contaminated material is used, 2 lots/batches of 20 replicates should be analyzed, and one lot must produce fractionally positive results.

4.1.3.5 Test Portion Size, Compositing and Pooling

Sample sizes required are as written in each method and/or SMPR.

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.

4.1.3.6 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.

4.1.3.7 Preparation of Artificially Contaminated Samples

4.1.3.7.1 Cannabis and Cannabis Products

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

$(1-(n_{select}/n_{nonselect})*100$

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 4C for refrigerated cannabis products, for a minimum of 2 weeks at –20C for frozen cannabis products, or for a minimum of 2 weeks at room temperature for dried cannabis and cannabis products prior to analysis.

4.1.3.8 Preparation of Naturally Contaminated Samples

Naturally contaminated matrix may be mixed with uncontaminated matrix of the same cannabis or cannabis product or incubated to achieve a level yielding fractionally positive results.

4.1.3.9 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 suffi cient 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.

4.1.3.10 Confirmation of Test Portions

Follow the reference method (or confirmatory 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.

4.1.3.11 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.

4.1.3.11.1 Raw Data Tables

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

4.1.3.11.2 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.

4.1.3.11.3 Difference of Probabilities of Detection (dPOD)

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

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

dPODC = PODC – PODR

Estimate the dPODCP as the difference between the candidate presumptive result POD (PODCP) and the candidate confirmed result POD (PODCC) values. Calculate the 95% confidence interval on the dPODCP. See Annex C for details.

dPODCP = PODCP – PODCC

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

4.1.3.11.4 Summary Data Tables

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

4.1.3.11.5 Graph of Data

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

4.1.4 Robustness Study [Performance Tested Methods (PTM) or R^2 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 or R^2 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 PTM or R^2 Validation Study, one Independent Study is made by the appropriate method volunteer(s) in consultation with the Study Director and relevant SMPRs.

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 collaborators 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 and collaborators.

4.3.4 Matrix Selection

At least one matrix from those studied in the PTM or PCS shall be chosen by the appropriate volunteer expert(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 expert(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 4.1.3.3. 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 collaborator. Test portions are to be randomized and blind-coded when sent to participating collaborators 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 4.1.3.6.

4.3.9 Preparation of Artificially Contaminated Samples

Refer to 4.1.3.7.

4.3.10 Preparation of Naturally Contaminated Samples

Refer to 4.1.3.8.

4.3.11 Confirmation of Test Portions

Follow the reference method or confirmation procedure 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 (sr) 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 (sPOD) 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 dLPODC as the difference between the candidate and reference LPOD values. Calculate the 95% confidence interval on the dLPODC.
Estimate the dLPODCP as the difference between the presumptive and confirmed LPOD values. Calculate the 95% confidence interval
on the dLPODCP. 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 PODR, LPODC, and dLPODC by level with 95% confidence intervals. See example in Annex E.

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 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.

5.1.2.2 Study Design

Inclusivity strains are cultured in nonselective media. The target concentration for testing is 100 times the LOD50 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.

5.1.2.3 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.

5.1.2.4 In-Silico Analysis

For molecular methods, see section 10 for in-silico analysis guidelines that can serve as supplemental information for the inclusivity study.

5.1.3 Matrix Study

5.1.3.1 Reference Method

Candidate methods are compared to a cultural reference method, where applicable. The following are examples of sources 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, USP, Dairy Standard Methods.

Recognizing that there may be a lack of reference methods available for the cannabis matrix, guidance in AOAC Standard Method Performance Requirements should be followed in conjunction with reputable reference method sources. When appropriate, Method developers should coordinate with the study director and/or consultant for best practices to be followed.

5.1.3.2 Matrix Categories

AOAC INTERNATIONAL recognizes claims for the range of specific cannabis 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. See section 7 for guidance on matrix categorization.

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

$(1-(n_{select}/n_{nonselect})*100$

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 4C for refrigerated cannabis products, for a minimum of 2 weeks at –20C for frozen cannabis products, or for a minimum of 2 weeks at room temperature for dried cannabis and cannabis products prior to analysis.

5.1.3.7 Use of Artificially and Naturally Contaminated Test Samples

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

The balance of the cannabis or cannabis product 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 cannabis or cannabis product 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 or confirmation procedure 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:

Log10 [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, filter). For details, see Annex H.

5.1.3.10.2 Initial Review of Data

If there is a reference method (or confirmatory 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 (sr)

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.

5.1.4.3Data Analysis and Reporting

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

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 (or confirmatory 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 cannabis or cannabis product type claimed, one cannabis or cannabis product matrix 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 collaborators reporting valid data for each cannabis or cannabis product type is required. It is suggested that at least 10–12 collaborators begin the analysis.

5.3.3 Reference Method

Candidate methods are compared to a reference method (or confirmatory 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 cannabis or cannabis product types, three inoculated levels (high, medium, and low) and one uninoculated level are required. For naturally contaminated cannabis or cannabis product, 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 by each collaborator.

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 cannabis or cannabis product 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 cannabis and cannabis products 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 (or confirmatory 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:

$$\text{Log10 [CFU/unit + (0.1)f]}$$

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 (or confirmatory 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 (sr)

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

5.3.12.4.2 Reproducibility (sR)

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 Methods

6.1 Reference ISO 16140-6:2019

7 Matrix Categorization

7.1 Scope

The following matrix categories and subcategories are intended to provide a guidance for method validation study design. The list is non-exhaustive, and per SMPR guidance and method specific requirements, matrices and subcategories that are included in the study design may need to be extended or modified.

7.2 Matrix Categories and Subcategories

7.2.1 Plant/Flower

- Buds
- Kief*
- Joints (pre-rolls)
- Fresh Frozen
- Shake/Trim

7.2.2 Concentrates

- Isolate
- Shatter/Wax
- Vape oil/cartridges
- Distillate
- Hash/Rosin
- Kief*

7.2.3 Infused Edibles

- Chocolate bars
- Baked Goods
- Gummies
- Tinctures
- Capsules/pills
- Beverages

7.2.4 Infused Non-Edibles

- Lotion
- Balm/salve
- Bath bombs/bath salts
- Transdermal Patches
- Oils (topical)

*Matrix classification varies depending on regional designation. Matrix should be classified under the category appropriate to the study and can only qualify as a required matrix for a single matrix category.

Inclusion of matrices outside of those listed above may be used for validation purposes, however, these matrices will be considered supplemental to the minimum number of matrices required by method-specific SMPRs.

8 Decontamination of Matrix

8.1 Scope

The conformity assessment programs of AOAC INTERNATIONAL require candidate methods to be evaluated for all matrices claimed in the method's scope. The decontamination and sterilization techniques described in this section are most applicable to cannabis plant material. Testing can be performed using materials naturally contaminated or artificially inoculated with the target analyte(s). Matrix study designs are based on requirements outlined in official validation guidelines (e.g., Official Methods of Analysis SM Appendix J: AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces) and/or Standard Method Performance Requirements (SMPRSM). These validation studies are designed to challenge the methods at the Limit of Detection (LOD50) for qualitative methods or Limit of Quantitation (LOQ) for quantitative methods.

Qualitative method analysis requires the evaluation of multiple lots of a single matrix when naturally contaminated or multiple contamination levels (control, low/fractional, high) of a single lot when artificially contaminated. In both instances, test portions producing fractional positive results, those in which the low level (or 1 of the 2 naturally contaminated lots) produces 25-75% positive results, is required. For quantitative methods, testing should challenge the method at the LOQ and data should be obtained that spans the range of 2 logs (e.g., ~10 CFU/g, ~100 CFU/g, and ~1,000 CFU/g). Obtaining these levels can be difficult to achieve and may require matrix manipulation, including reducing the presence of naturally occurring microbial bioburden to achieve fractional results (qualitative) or low levels (quantitative).

Decontamination processes need to be thorough enough to reduce the bioburden to the levels required for validation/verification but should not impact the overall integrity of the matrix. For cannabis flower, this means ensuring that key components of the plant, potency, moisture content, terpene profiles are not drastically altered. This document discusses various approaches to decontaminating cannabis matrices for the purposes of microbial method validation/verification and provides

recommendations to ensure the integrity of the matrix is maintained after the decontamination step.

The decontamination techniques presented here are well-established in other industries, have some baseline data to support their use in the cannabis industry, and can be performed without significantly altering the plant matrix.

8.2 Decontamination Approach

8.2.1 Irradiation

8.2.1.1 Gamma irradiation

i. Uses gamma rays (high energy photons) produced during the decay of the radioisotope Cobalt 60 radiation.
ii. Treatment conditions vary from 0–20 kGy/min (kiloGray), and treatment times also vary.
iii. Pros: Effective kill rates, can penetrate organic matter for surface and internal decontamination, high sterility assurance, fewer variables to control, no significant impact on CBD and THC; no residue left on product
iv. Cons: Terpene losses up to 38% (Hazekamp 2016), expensive

8.2.1.2 Electron-beam (E-beam)

i. Product is bombarded with high-energy electrons produced by an electrical current; beta emitter
ii. Treatment times are usually shorter than gamma or X-ray; treatment conditions around 15 kW (KWs) and an energy
iii. Capacity of 5.25 megaelectronvolt (MeV)
iv. Pros: Effective kill rates, generally less harsh on/damaging, fast, high sterility assurance level, no residue left on product
v. Cons: Limited penetration depth, expensive

8.2.1.3 X-ray irradiation

i. Uses an electron beam produced by a current and focus that beam on a specific metal, which creates X-rays (photons) through a process called Bremsstrahlung.
ii. Treatment conditions vary: Radsource suggests 3-7 hour treatment time with a total 2000 Gy dose
iii. Pros: Internal and external decontamination; CBD and THC preserved; no residue left on product, high sterility assurance
iv. Cons: Terpene content may be altered, expensive

8.2.1.4 UV light:

i. UV-C light is typically used for air sterilization as a preventative measure; however some DIY decontamination methods are to use UV-C light bulbs set up in the dry/cure room or in a UV-C disinfection chamber for 360 degree exposure.

ii. Pros: Easy to use, inexpensive, no residue left on produce,
iii. Cons: Varied microbial sensitivity, surface level only

8.2.2 Electromagnetic radiation (non-ionizing)

8.2.2.1 Microwave

i. Used infrequently but some processors combine microwave, vacuum and agitation to accelerate the dry/cure process
ii. Treatments vary in time and range from 3-30 GHz
iii. Pros: Good microbial reduction of external and internal microbial contaminants, no residue left on product
iv. Cons: Process creates heat, which can alter cannabinoid, terpene, and moisture content

8.2.2.2 Radio frequency (RF)

i. Uses RF to create dipoles (molecules with separated positive and negative charges) and align in an electric field, causing rotation and heat
ii. Treatments conditions range from 100 MHz to <10 GHz (up to 10) and times vary
iii. Pros: Good microbial reduction of external and internal microbial contaminants, no residue left on product, longer wavelengths and penetration depth than microwaves
iv. Cons: Process creates heat, which can alter cannabinoid, terpene, and moisture content, process is dependent bound water

8.2.3 Reactive oxygen species and ionized gases (surface decontamination methods)

8.2.3.1 Ozone

i. Created by reacting oxygen through an electric current creating O3 (ozone)
ii. Treatment times vary and concentrations range from 10-1000 ppm ozone. Generally 10-30 minute exposure.
iii. Pros: External microbial decontamination, does not alter cannabinoid or terpene profile, no residue left on product, fast
iv. Cons: Surface level decontamination, moisture loss I the flower matrix

8.2.3.2 Hydrogen peroxide

i. Some cultivators submerge flower after harvest and allow to dry
ii. Vaporized H2O2 like TheBOX flash vaporizes aqueous hydrogen peroxide and distributes inside an enclosed chamber; treatment conditions and times vary
iii. Pros: surface contamination, no residual chemicals left on the plant matter, no significant impact on CBD, THC, or terpenes

iv. Cons: May increase water activity/moisture content to the plant matter, may discolor product, mostly surface level decontamination

8.2.3.3 Cold plasma

i. Generated by high voltage current passed through air, creating a mix of electrons, ions, photons, and free radicals; does not exceed tens of degrees Celsius, making it “cold”

ii. Treatment times and conditions vary

iii. Pros: External microbial decontamination, no residue left on product

iv. Cons: Surface level decontamination

8.2.4 Heat treatment

8.3.4.1 Pasteurization

8.3.4.2 Steam treatment

8.3.4.3 Autoclaving

8.3.4.4 Heating/baking: can decarboxylate flower and change appearance and terpene profile

8.2.5 Cold temperature treatment

8.3.5.1 Freeze drying

8.3.5.2 Fresh frozen

8.2.6 Carbon dioxide, ethanol, butane, propane, etc. (extraction/remediation)

i. Pros: These organic solvents will remove microbial contaminants internally and externally

ii. Cons: Extraction removes the majority of cannabinoids and terpenes from the plant material, rendering mostly the insoluble matter afterwards, which does not represent the original material

8.3 Methods to Confirm Integrity of Product Maintained following Decontamination

To evaluate the effectiveness of decontamination procedures, decontaminated product should be evaluated using methods described below. It is recommended that decontaminated product be analyzed with a minimum of the Microbial Burden, Cannabinoid Profile and the Moisture Content/Water Activity in order to establish the efficacy of the product decontamination and verification of the integrity of the product post treatment. The Microbial Burden in the treated product should have a reduction to a level that meets the requirements of the method validation study and/or is below the regulatory limit of the governing body.

8.3.1 Microbial Burden

Analysis of treated product for Microbial contamination should be undertaken based upon the requirements that have been defined by the governing entity and/or legislation.

8.3.1.1 Classical Determination of Microbiological Parameters in Cannabis Products

For enumeration of defined groups of microorganisms such as Yeast and Mold, Total Coliform or Aerobic Plate Count, the use of established protocols is highly recommended. These methods utilized media or substrate enriched in metabolites and nutrients that enhance the growth of the target microbiological groups. In some procedures, catalysis of the metabolites by the target microbiological groups results in some change in the media associated with the colony growth. A simple example, Lactose is often included in media used to enumerate Total Coliform since these organisms ferment Lactose and therefore produce gas. In addition, incubation times and temperatures are specific to the procedure further enhancing growth or target microbiological groups.

8.3.1.2 Genetic Based Determination of Detection of species and/or groups known to be pathogenic

i. Real Time PCR Analysis has been established as a very useful tool in the determination of microbiological contamination of Cannabis products. Amplification of target DNA in the sample produces an increase of fluorescence during thermocycle sequence which indicates the presence or absence of the targeted organism(s).
ii. Microarray analysis generally does not require enrichment. DNA from the target organism(s) is amplified from lysed and labelled with fluorescent probes. The labelled DNA is hybridized to complementary DNA immobilized in wells of the microarray and analysis completed by the analysis of the fluorescence in the wells of the microarray.
iii. Indirect metabolite readings (e.g. Sol)
iv. Sequencing

8.3.1.3 Cannabinoid profile

For quantification of the major cannabinoids (THCA, CBDA, THC, CBD, and others) established chromatography methods should be used.

8.3.1.6 Water Activity/Moisture Content

The level of water, or moisture, in cannabis products is recognized as a key factor in its safety. Low levels of water help control microbial growth and it is considered a critical control point for preventing microbial contamination. Cannabis flower that is not dried to water activity levels below 0.6 (USP recommendations; Sarma 2020) can support mold growth during storage and transportation which may lead to the production of mold spores and/or mycotoxins. Water concentrations are typically measured in one of two ways: water activity or moisture content.

Water activity - Water activity or aw is the ratio of the vapor pressure of water in a material or substance to the vapor pressure of pure water. Water activity measurements are determined from a calculation of relative humidity. Relative humidity is the percentage of water in the air (vapor pressure) compared with the total amount of water that the air could hold (saturation vapor pressure) at a given temperature. A water activity test works by placing a sample in a sealed measuring container. When the vapor pressure of the water in the substance and the water in the air reaches equilibrium, the relative humidity of the air surrounding the sample is equal to the water activity of the sample. Water activity meters typically use electrical capacitance or resistance hygrometer to measure this humidity, although some will measure the dew point of the air.

Moisture Analyzers - Moisture analyzers, sometimes referred to as moisture balances, measure the moisture content through the Loss On Drying (LOD) method. This is a three step process where an initial weight is obtained on a moisture analyzer, the instrument then heats the sample to dry it, followed by a final weight of the sample. The weight after drying is subtracted from the weight before, so the loss of moisture is determined using the loss of mass. The heat generating system can vary in moisture analyzers. Some moisture analyzers use halogen and some use infrared or glass-free metal heaters positioned above a precision balance. The heat generating system can vary in moisture analyzers. Some moisture analyzers use halogen and some use infrared or glass-free metal heaters positioned above a precision balance.

8.3.1.7 Qualitative Analysis

i. Color
ii. Deformities
iii. Texture

iv. Sensory evaluation
v. Trichome integrity

9 Suitability of Testing Media

9.1 Scope

The present document is intended to give adequate information concerning the suitability of testing methods for cannabis-related products. The suitability test should demonstrate that tested products have no inhibitory effects on the growth of selected control microorganisms under aerobic conditions. In other terms, obtained results in recovery test media have to be completely representative: the designed suitability testing method should be able to neutralize all possible antimicrobial effects related to the tested product. In numerical terms, the growth of control microorganisms has to be predictable and verifiable.

Should any antimicrobial feature be present in the cannabis-related product under examination, one of the below-mentioned options would be carried out on the product before suitability testing:

a) Dilution
b) Filtration
c) Neutralization
d) Inactivation.

It has to be taken into account that tested products may have antimicrobial features and related effects because of the presence of antimicrobial agents (preservatives, fungistatic substances, etc.). Consequently, should the suitability test give unsatisfactory results, all analytical evaluations based on the used method would be questioned, and a new suitability test would be required with the aim of neutralizing the inhibitory agent(s).

In addition, should any modification in the testing method and/or in the cannabis-related product be introduced with potential influence on analytical performance, the suitability test would be obligatorily repeated (confirmation needed).

It has to be noted that growth promotion testing procedures must be conducted in order to then conduct suitability testing, taking into account that:

1) Growth promotion testing should demonstrate that culture media can effectively support the growth of selected microorganisms, while
2) Suitability testing should demonstrate the ability of the test to detect or enumerate selected microorganisms in the presence of the tested product.

This document has been written taking into account:

a) The United States Pharmacopeia (USP <51>, <60>, <61>, <62>, and <1111>) with reference to general concepts concerning suitability of testing methods;

b) The United States Pharmacopeia (USP <2021>, <2022>, and <2023>) with reference to microbiological features of cannabis and cannabis-derived products as non-sterile nutritional and dietary supplements;

c) The Bacteriological Analytical Manual and Pharmaceutical Microbiology Manual of the Food and Drug Administration;

d) The International Pharmacopoeia;

e) The European Pharmacopoeia;

f) The "Guidelines for Assuring Quality of Medical Microbiological Culture Media" (the Australian Society of Microbiology, Inc., 2nd Edition, July 2012)

g) The recent AOAC SMPR 2021.009; Version 9; June 17, 2021 (Method Name: Standard Method Performance Requirements for Viable Yeast and Mold).

h) Current Good Manufacturing Practice (cGMP).

All operations have to be performed with the aim of avoiding microbial contamination of the product under examination, and also assuring that control microorganisms are not negatively influenced.

9.2 Preparation of Test Strains

The following Table 1 shows standard microorganisms for suitability testing, and correlated preparation procedures (1-8). The listed microorganisms are provided as examples, but may not be required for all methods or applications. Method developers should coordinate with the study director and/or consultant for best practices to be followed.

Table 1: standard microorganisms for suitability testing, and correlated preparation procedures (1-8)

Microorganism	Test strain (examples)	Preparation
Staphylococcus aureus	ATCC 6538 NCIMB 9518 CIP 4.83 NBRC 13276	Casein soya bean digest agar or Casein soya bean digest broth Temperature: 30–35 °C

		Incubation time: 18–24 h
Pseudomonas aeruginosa	ATCC 9027 NCIMB 8626 CIP 82.118 NBRC 13275	Casein soya bean digest agar or casein soya bean digest broth Temperature: 30–35 °C Incubation time: 18–24 h
Bacillus subtilis	ATCC 6633 NCIMB 8054 CIP 52.62 NBRC 3134	Casein soya bean digest agar or Casein soya bean digest broth Temperature: 30–35 °C Incubation time: 18–24 h
Candida albicans	ATCC 10231 NCPF 3179 IP 48.72 NBRC 1594	Sabouraud-dextrose agar or Sabouraud dextrose broth Temperature: 20–25 °C Incubation time: 2–3 days

Aspergillus brasiliensis	ATCC 16404 IMI 149007 IP 1431.83 NBRC 9455	Sabouraud dextrose agar or Potato dextrose agar Temperature: 20–25 °C Incubation time: 5–7 days (or until good sporulation is obtained)

Viable microorganisms used for inoculation should be not more than 5 passages removed from the original master seed-lot. With relation to test suspensions, the following solutions are recommended:

1) Buffered sodium chloride-peptone solution at pH 7.0, or
2) Phosphate buffer at pH 7.2.

An exception has to be mentioned with concern to *A. brasiliensis*: in this ambit, a 0.05 % of polysorbate 80 addition may be performed. Anyway, test suspensions have to be used within 120 minutes or within 24 hours if stored at 2-8 °C.

9.3 Suitability of the Method in the Presence of the Tested Product

Suitability testing methods are performed in two ways, depending on the control microorganism (1-8):

a) The determination of total aerobic microbial count (TAMC), and
b) The determination of total yeast and mold count (TYMC).

The enumeration method can be one of the below-mentioned systems, taking into account the nature of the tested products (1-8):

1) Membrane Filtration
2) Plate-Count methods
3) The Most- Probable-Number (MPN) method.

The following Table 2 shows standard microorganisms for suitability testing, and correlated suitability conditions The listed microorganisms are provided as examples, but may not be required for all methods or applications. Method developers should coordinate with the study director and/or consultant for best practices to be followed.

Table 2: standard microorganisms for suitability testing, and correlated suitability conditions

Microorganism	Test strain (examples)	Suitability testing - TAMC	Suitability testing - TYMC
Staphylococcus aureus	ATCC 6538 NCIMB 9518 CIP 4.83 NBRC 13276	Casein soya bean digest Agar MPN casein soya bean digest broth Inoculum: ≤ 100 CFU Temperature: 30–35 °C Incubation time: ≤ 3 days	-
Pseudomonas aeruginosa	ATCC 9027 NCIMB 8626 CIP 82.118 NBRC 13275	Casein soya bean digest agar/MPN casein soya bean digest broth Inoculum: ≤ 100 CFU Temperature: 30–35 °C Incubation time: ≤ 3 days	-

Bacillus subtilis	ATCC 6633 NCIMB 8054 CIP 52.62 NBRC 3134	Casein soya bean digest agar/MPN casein soya bean digest broth Inoculum ≤ 100 CFU Temperature: 30–35 °C Incubation time: ≤ 3 days	-
Candida albicans	ATCC 10231 NCPF 3179 IP 48.72 NBRC 1594	Casein soya bean digest agar Inoculum: ≤ 100 CFU Temperature: 30–35 °C Incubation time: ≤ 5 days **- MPN is not applicable -**	Sabouraud-dextrose agar Inoculum: ≤ 100 CFU/ Temperature: 20–25 °C Incubation time: ≤ 5 days

Aspergillus brasiliensis	ATCC 16404 IMI 149007 IP 1431.83 NBRC 9455	Casein soya bean digest agar Inoculum: ≤ 100 CFU Temperature: 30–35 °C Incubation time: ≤ 5 days **- MPN is not applicable -**	Sabouraud-dextrose agar Inoculum: ≤ 100 CFU/ Temperature: 20–25 °C Incubation time: ≤ 5 days

9.3.1 Preparation of the Sample

In general, the physical and chemical features of the product under examination influence the correct sample preparation (1-8). Please note sample amounts can vary depending on the product category (please consider Table 3). With reference to the descriptions, preparations, sampling, and testing procedures concerning all mentioned media and broths in this document, please use USP <2021> (Microbial enumeration tests – nutritional and dietary supplements) and <2022> (Microbiological procedures for absence of specified microorganisms – nutritional and dietary supplements) as reference guidelines (9-10). The following procedures can be recommended (a microbial concentration of about $1 \cdot 10^8$ CFU/g is suggested):

- Water-soluble products: the recommended procedure is dissolution or dilution in the following diluents: buffered sodium chloride-peptone solution pH 7.0, phosphate buffer sterile pH 7.2 or casein soya bean digest broth (1 in 10 dilution, and subsequent dilutions are prepared with the same diluent). If needed, pH value may be adjusted until it reaches 6-8.

- Non-fatty and water-insoluble products: the recommended procedure is suspension in the following media: buffered sodium chloride-peptone solution pH 7.0, phosphate buffer sterile pH 7.2 or casein soya bean digest broth (1 in 10 dilution, and subsequent dilutions are prepared with the same diluent). If needed, pH value may be adjusted until it reaches 6-8. The suspension may be difficult enough. Consequently, polysorbate 80 (1 gram per liter) may be added

- Fatty products: the recommended procedure is: initial addition of a surface-active substance such as sterile polysorbate 80 to the sample (heating may be needed until 40 °C). Subsequently, dissolution in sterilized (by filtration) isopropyl myristate R (1:10 dilution) and mixing while temperature remains constant (a water-bath may be useful) until the incipient formation of an emulsion. Subsequent serial 10-fold dilution can be prepared with the same diluent, provided that a surface-active substance such as sterile polysorbate 80 is added

- Aerosolized products: these products should be transferred aseptically into a membrane filter apparatus or a sterile container, before a subsequent sampling operation is carried out.

9.3.2 Inoculation and Dilution

The microbial suspension for suitability testing has to be added to the prepared sample and to a control (sample is absent). The inoculum has to be ≤ 100 CFU/g, and it should be ≤ 1 % of the total volume of the diluted product. In addition, microbial recovery can be acceptably demonstrated on condition that the prepared sample is tested with the lowest possible dilution factor, unless antimicrobial effects or poor solubility are demonstrable (1-8)

Should antimicrobial effects need to be eliminated (see point **3.3**), an adequate sample treatment – dilution, filtration, neutralization, or inactivation – would be necessary before inoculum.

Should plate counts be used, each dilution would be correlated with the result of two plates (test in duplicate).

9.3.3 Neutralization/removal of antimicrobial activity

The possible inhibition of bacterial growth is demonstrable if there is a factor reduction > 2 considering (1-8):

a) The count of recovered microorganisms from the sample, and

b) The count of recovered microorganisms from the control.

With reference to solid culture media, the factor reduction should take into account the calculated value for the standardized inoculum.

With reference to MPN methods, the calculated number from inoculum has to be within 95 %-confidence limits (K =2) of obtained results with the control test.

Should the inhibition be observed, the following strategies may be recommended:

1) Augment the volume of used diluents (against these antimicrobials: alcohol, phenolics, aldehydes, sorbate)

2) Augment the volume of used culture media

3) Addition of a neutralizing agent to the diluents or culture media (sterilization is required – a blank test is required with neutralizer and without the tested sample)

4) Membrane filtration

5) A combination of above-mentioned strategies.

With reference to neutralizers, the following choices can be shown here as examples:

a) Sodium bisulfite (against glutaraldehyde)

b) Glycine or thiosulfate (against aldehydes)

c) Calcium and magnesium ions (against ethylenediaminetetraacetic acid or EDTA).

Should the above-mentioned strategies have no effects, it could be concluded that the tested product has some antimicrobial effect. In these conditions, the test should be repeated with the highest dilution factor which could be compatible for observable microbial growth (1-8). See 9.3.5 for how to interpret these results.

9.3.4 Recovery of microorganism in the presence of product

Membrane Filtration. The use of membrane filters with pore sizing ≤ 0.45µm is preferred (cellulose nitrate filters for aqueous, oily and weakly alcoholic solutions; cellulose acetate filters for strongly alcoholic solutions). One membrane filter is used for each control microorganism. The quantity of sample (representing approximately 1 gram of product, unless the calculable number of microorganism is excessive) has to be transferred and filtered immediately; subsequently, the membrane filter is rinsed with adequate amount of the used diluent (example: three 100-ml portions; maximum: five 100-ml portions). In this way, antimicrobial residues on filter membranes would be probably removed (1-8).

Subsequently, control microorganisms are placed (< 100 CFU/g) into the last portion of rinse diluent; then, aseptically cut the filter membrane is subdivided aseptically into

two equal parts. With relation to TAMC, the membrane filter sections are transferred to the surface of casein soya bean digest agar. With reference to TYMC, the membrane filter sections are transferred to the surface of Sabouraud-dextrose agar (1-8).

Incubation conditions are listed in Table 2.

Plate-Count methods: the testing has to be performed in duplicate for each medium and dilution. Incubation conditions are listed in Table 2 (1-8).

Pour-plate methods: 1 ml of the prepared sample is placed in Petri dishes (f 9 cm) with 15-20 ml of the chosen culture medium (Table 2). Should diameters be higher than 9 cm, the quantity of culture media would be increased accordingly. Recommended temperature: ≤ 45 °C. The testing has to be performed in duplicate for each medium and dilution. Incubation conditions are listed in Table 2 (1-8).

*Surface-spread methods***:** 15-20 ml of the chosen culture medium (Table 2) is placed in Petri dishes (f 9 cm) at temperature ≤ 45 °C. Should diameters be higher than 9 cm, the quantity of culture media would be increased accordingly. After media solidification, plates have to be placed into an incubator or into a laminar airflow cabinet (drying). Finally, 0.1 ml of prepared sample is spread on media surfaces, and the testing has to be performed in duplicate for each medium and dilution. Incubation conditions are listed in Table 2 (1-8).

*The MPN method***.** This method is recommended TAMC in absence of other good methods because accuracy and precision are not satisfactory. Should the MPN method be uses, a series of ≥ 3 serial 10-fold dilutions of cannabis-related product would be considered. For each of these dilutions, three aliquots of 1 g or 1 mL are sampled with the aim of inoculating three 9-10-ml tubes (casein soya bean digest broth). The use of surface-active agents such as polysorbate 80 may be justified, and the use of neutralizers against antimicrobial effects is also allowed if needed. All inoculated tubes are incubated according to conditions displayed in Table 2. Finally, the MPN per ml or per gram of tested product has to be examined (1-8)

9.3.5 Results and interpretation

When verifying the suitability of the membrane filtration method or the plate-count method a mean count of any of the test organisms not differing by a factor greater than 2 from the value of the control defined above under Inoculation and dilution in the absence of the product must be obtained. When verifying the suitability of the MPN method the calculated value from the inoculum must be within 95% confidence limits of the results obtained with the control. If the above criteria cannot be met for one or more of the organisms tested with any of the described methods the method and test conditions that come closest to the criteria are used to test the product (1-8).

9.3.6 Examination of the product

Membrane filtration. After incubation (Table 2), the number of CFU/g or /ml of tested product has to be determined. Only plates with the highest number of colonies < 100 colonies are considered.

Plate-count (pour-plate and surface-spread) methods. After incubation, the mean value of results (CFU/g or /ml) of tested product has to be calculated taking into account only plates showing the highest number of colonies as follows: < 250 colonies for TAMC and < 50 colonies for TYMC.

The Most-probable-number method. After incubation, the number of tubes showing microbial spreading has to be considered for each dilution level. The MPN number has to be determined per gram or ml of the tested product. Useful tools can be found at the following web addresses:

- https://www.fda.gov/food/laboratory-methods-food/bam-appendix-2-most-probable-number-serial-dilutions (Bacteriological Analytical Manual, Appendix 2 – FDA)

- https://mpncalc.galaxytrakr.org (MPNcalc v1.2.0, by M. Ferguson and J. Ihrie).

9.3.7 Suitability testing methods for specified (general indicator) microorganisms

Suitability testing methods can also be realized with concern to general indicator microorganisms. In general, the following Table 3 can be considered (2-3, 9-10):

Table 3. General indicator microorganisms and recommended media for suitability testing methods (2-3, 9-10)

Non-sterile drugs and raw materials that are intended for inhalation use, and aqueous preparations for oral, oromucosal, cutaneous, or nasal administration	Non-sterile pharmaceutical products
Burkholderia cepacian (ATCC 25416)	Bile-Tolerant Gram-Negative Bacteria
Burkholderia cenocepacia (ATCC BAA-245)	*Pseudomonas aeruginosa* (ATCC 9027)
Burkholderia multivorans (ATCC BAA-247)	*Staphylococcus aureus* (ATCC 6538)
	Escherichia coli (ATCC 8739)
	Salmonella enterica (ATCC 14028)
	Candida albicans (ATCC 10231)
	Clostridium sporogenes (ATCC 11437)

With relation to these tests and the suitability of testing media, USP <61>, <62>, <2021>, and <2022> are recommended as specific references.

9.3.8 Suitability testing methods VS Cannabis-related Matrices

Table 4 shows four main cannabis-related product categories with minimum testing/sample amount and specific target microorganisms (11). On the left, the subdivision in different

products is offered; minimum testing/sample amount and corresponding target microorganisms are displayed on the right side. A useful reference is USP <2023> (12) when speaking of target microorganisms and related microbial levels in function of the classification of cannabis and cannabis-derived products (as natural sources of microbial contamination). Anyway, it has to be considered that certain products, especially food products, may have specific requirements. Consequently, mentioned target microorganisms serve as general target requirements, while some additional requirement may be found depending on the peculiar product.

Table 4. Cannabis and Cannabis Products. Minimum testing/sample amount and target microorganisms for suitability testing (11-12)

Product category and sub-category	n testing/sample amount	Target microorganism(s)
Cannabis Concentrates: Vape oil/cartridges - Live Resins Solventless (rosin, bubble hash) CO_2 Oil – Isolates Distillate Shatter Wax – Budder - Kief	5 grams	MC, TYMC, Staph, Pseudo, & Bile-tolerant gram neg bacteria
Cannabis Infused Edibles: Chocolate - Hard candies Soft candies Beverages Baked goods – Tinctures Ice cream Syrups - Capsules Orally dissolving strips Pills - Cooking oil	25 grams	TAMC, TYMC

Cannabis Infused Non-Edibles: Topicals - Cosmetic products Creams – Lotions - Chapstick Bath salts – Salves - Bath bombs Medicated patches - Lubes Suppositories - Inhalers	10 grams	TAMC, TYMC, Staph, Pseudomonas
Cannabis Plant and Flower: Joints/pre-rolls - Fresh/frozen Trim – Shake -Live plant material	10 grams	YMC, Bile-tolerant Gram-negative Bacteria, Salmonella spp, E. coli

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1713 **9.3.9 Suitability of Testing Media – Quality Control and Acceptance Parameters**

1714 With concern to suitability of testing media, it should be recognized that the U.S. general
1715 requirements are extremely detailed and the current state-of art, State by State. In addition,
1716 the efforts of the Industrial stakeholders in this ambit should be recognized. On the other side,
1717 the aim of this document is to give adequate and reliable information to a worldwide audience,
1718 also recognizing the pre-existing efforts of different Organizations in this ambit. Consequently,
1719 and with reference to the descriptions, quality-control and acceptance parameters required for
1720 suitability of testing media in this document, please use USP <61> (Microbiological Examination
1721 of Nonsterile Products: Microbial Enumeration Tests) as reference guideline which outlines
1722 passing requirements including lot-to-lot % (2).

1723 **9.3.10 Testing Media. Recommended Media for Selected Microorganisms**

Table 5 shows a selection of recommended media for selected microorganisms to be tested in the ambit of this document.

Table 5. Recommended testing media with relation to selected microorganisms (3, 5-6, 13)

Microorganism	Test strain (examples)	References: recommended testing media (TAMC)	References: recommended testing media (TYMC)
Staphylococcus aureus	**ATCC 6538** **NCIMB 9518** **CIP 4.83** **NBRC 13276**	**BAM Media M152: Trypticase (Tryptic) Soy Agar (https://www.fda.gov/food/laboratory-methods-food/bam-media-m152-trypticase-tryptic-soy-agar)**	
Pseudomonas aeruginosa	**ATCC 9027** **NCIMB 8626** **CIP 82.118**	**As above stated**	

	NBRC 13275		
Bacillus subtilis	**ATCC 6633** **NCIMB 8054** **CIP 52.62** **NBRC 3134**	**As above stated**	

Candida albicans	**ATCC 10231** **NCPF 3179** **IP 48.72** **NBRC 1594**	**As above stated**	**BAM Media M133: Sabouraud's Dextrose Broth and Agar** **(https://www.fda.gov/food/laboratory-methods-food/bam-media-m133-sabourauds-dextrose-broth-and-agar)**
Aspergillus brasiliensis	**ATCC 16404** **IMI 149007** **IP 1431.83** **NBRC 9455**	**As above stated**	**As above stated**
Specified (general indicator) microorganisms	**Test strain**	**Recommended testing media- references**	

Burkholderia cepacia	**ATCC 25416**	**Medium: *Burkholderia cepacia* agar (13)** **Australian Society of Microbiology (2021) Guidelines for Assuring Quality of Medical Microbiological Culture Media. Culture Media Special Interest Group for the Australian Society of Microbiology, Inc., 2nd Edition, July 2012. Available https://www.theasm.org.au/guidelines-reports. Accessed 28th July 2021**
Burkholderia cenocepacia	**ATCC BAA-245**	**As above stated**
Burkholderia multivorans	**ATCC BAA-247**	**As above stated**
Bile-Tolerant Gram-Negative Bacteria	**-**	**Enterobacteria Enrichment Broth Mossel (USP <62>)** **Violet red bile glucose agar (The International pharmacopeia)**
Pseudomonas aeruginosa	**ATCC 9027**	**BAM Media M37: Cetrimide Agar** **https://www.fda.gov/food/laboratory-methods-food/bam-media-m37-cetrimide-agar**

Staphylococcus aureus	**ATCC 6538**	**Mannitol Salt Agar (USP <62>)**
Escherichia coli	**ATCC 8739**	**MacConkey Agar (USP <62>)**
Salmonella enterica	**ATCC 14028**	**Rappaport Vassiliadis Salmonella Enrichment Broth** **Xylose Lysine Deoxycholate Agar (USP <62>)**
Candida albicans	**ATCC 10231**	**Sabouraud Dextrose Broth and/or Agar (USP <62>)**
Clostridium sporogenes	**ATCC 11437**	**Reinforced Medium for Clostridia Growth promoting** **Or** ***Cl. sporogenes* Columbia Agar (USP <62>)**

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10 In-Silico Analysis

10.1 Scope

In silico analysis for molecular methods is based on recommendations from AOAC OMA Appendix Q: *Recommendations for Developing Molecular Assays for Microbial Pathogen Detection Using Modern In Silico Approaches*, June 2020 (http://www.eoma.aoac.org/app_q.pdf). By utilizing available databases combined with modern bioinformatics and physical modeling tools, *in silico* analysis can be used to predict the selectivity of a molecular assay (e.g, PCR, RT-PCR, LAMP, NASBA) against tens of thousands of known sequences. Primers and probes are tested as applicable to each assay. *In silico* analysis is not intended to replace wet lab testing but can reduce wet lab testing allowing focus on potential false negative and false positive organisms.

10.2 Inclusivity/Exclusivity

Sequence quality refers to the likelihood that the given nucleotide is correctly specified at each position in a genome sequence. To reduce the potential for false negative reactions, use high-quality sequences in the inclusivity database. For the purposes of checking for false-positive amplifications, construct exclusivity and environmental background databases. For both the exclusivity and background databases, sequence quality is generally not an issue. Include partial sequences as well as complete genomes. Check primers for reactivity with all relevant organisms [such as the GenBank nonredundant (nr) or nucleotide (nt) databases] using a program such as *Primer-BLAST* (App Q reference 22), *Thermonucleotide BLAST* (App Q reference 23), or *ThermoBLAST* (App Q reference 14) to detect all off-target hits and amplicons. Test one genome per strain/isolate so as not to introduce bias.

Inclusivity – Include sequences for all known genetic variations of the target(s). Include all known full-length genomes (to reduce database size it can be helpful to remove identical sequences). For partial genomes, it is best to include only the partial sequences that contain the region of interest (i.e. the amplicon region). Report the number of genomes with 0, 1, 2, 3, or 4 mismatches for each primer and probe.

Exclusivity – Populate with genomes of near neighbors (organisms that are phylogenetically distinct but closely related to the target). Include full and partial sequences. Report % homology for each primer and probe.

Background organisms – Populate with organisms that may be present in the matrix or related to the intended use (e.g. the human genome, human RNA RefSeq, human microbiome, soil microbes, etc.). Report genomes with highest homology to each primer and probe.

Select sequences from the following:

Generalized databases

- National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov)
- GenBank (https://www.ncbi.nlm.nih.gov/genbank/)
- European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI, https://www.ebi.ac.uk)
- DNA Data Bank of Japan (DDBJ, https://www.ddbj.nig.ac.jp/index-e.html)

Curated pathogen genome databases

- Virus Pathogen Database and Analysis Resource (ViPR, https://www.viprbrc.org/brc/home.spg?decorator=vipr)
- NCBI Influenza Virus Database (https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-select.cgi?go=database)
- Los Alamos Hemorrhagic Fever Viruses Database (https://hfv.lanl.gov/content/index)
- Virulence Factor Database (VFDB, http://www.mgc.ac.cn/VFs/main.htm)
- Global Initiative on Sharing All Influenza Data (GISAID, https://www.gisaid.org)

Primer design software tools that utilize such databases as an integral part of their design, such as *BioVelocity* (App Q reference 9) and *PanelPlex* (DNA Software, Inc.), are recommended to simplify the task of database management.

10.3 Physical Chemistry Modeling

Perform thermodynamic folding simulations to determine if the primers and probe can bind to their targets without substantial unfolding of the target. Primers that require substantial unfolding of the target are often “fragile” and can give false negatives if a mutation occurs at a primer binding site or if the salt concentrations vary slightly (e.g., due to a bad master mix lot or user intentionally diluting reagents). Use a program [e.g., *MFOLD* (App Q reference 16), *RNAStructure* (App Q reference 17), or *Visual OMP* (App Q reference 14), etc.] to predict the secondary structure of the RNA or DNA target regions. Programs such as *AutoDimer* (App Q reference 19) and *ThermoBLAST* (App Q reference 14) can be used to check sets of primers to ensure that they do not form primer-dimer species involving the 3'-ends of the primers.

Unimolecular Folding – Determine all potential secondary structures of the regions of the target where the primers and probe binds, including approximately 150 extra bases on either end of the target region (typically we use the amplicon region with an extra 150 nts. on the 5’ and 3; sides). Both the sense and antisense strands should be folded to observe if any folding is present at the sites where the forward primer, reverse primer, and probe bind. Determine whether primer/probe binding requires high energy ($\triangle G^{\circ}_{T,}$ where T is the annealing temperature) for unfolding of these

structures. If the target site where the primer binds is unfolded, then it is safe to use the 2-state $\triangle G^{\circ}_{T}$ and Tm, to characterize the hybridization. However, if the primer-binding site on the target is highly folded, then it is necessary to compute the energy to unfold that region and to use a "multi-state coupled equilibrium model" (for further details see App Q reference 14. All of this is handled in programs such as *Visual OMP* (App Q reference 14) or *RNAStructure* (App Q reference 17). Identify potential inhibitory secondary structures which can cause primers to be fragile to minor variations in reagent quality or the presence of even a single mismatch within the binding region. Report $\triangle G^{\circ}_{T}$(unfolding) or the fraction bound for each primer and probe.

Bimolecular Thermodynamics (Hybridization) – Dependent on nucleotide composition, primer/probe length, strand concentration, salt conditions, and temperature. Report $\triangle G^{\circ}_{T}$ and Tm of primer/probe binding.

Note: Reporting specific sequences of proprietary primers/probes is not required.

Look for potential false negative variants, indicated by high number mismatches, highly folded regions, and/or weak primer/probe binding. Look for potential false positives when homology to any of the sequences in the exclusivity and background databases is >80% for primers and probes. Look for potential false amplicons (i.e. where there are two primer sites pointing in opposite directions and with a spacing between the primers < 1000 nts.). Such false amplicons may consume PCR reagents, which could lead to false negatives. In addition, report amplicon length. Follow up with wet lab testing to confirm all potential false positives and potential false negatives. Conduct ongoing monitoring of performance by *in silico* analysis as new isolates are sequenced, and new variants emerge.

11 Safety

Follow appropriate procedures for handling of microbial pathogens. 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.

12 References

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ANNEXES (Reference Appendix J for review; Insert for publication)

ANNEX A

ANNEX B

ANNEX C

ANNEX D

ANNEX E

ANNEX F

ANNEX G

ANNEX H