**DRAFT AOAC SMPR 2021.XXX; Version 7; March 05, 2021**

**Method Name:** **Standard Method Performance Requirements for Viable Total Yeast and Mold Count Enumeration**

**Purpose:** AOAC *Standard Method Performance Requirements*$^{SM}$ (SMPRs) describe the minimum recommended performance characteristics to be used during the evaluation of a method. The evaluation may be an on-site verification, a single-laboratory validation, or a multi-site collaborative study. SMPRs are written and adopted by AOAC stakeholder panels composed of representatives from industry, regulatory organizations, contract laboratories, test kit manufacturers, and academic institutions. AOAC SMPRs are used by AOAC expert review panels in their evaluation of validation study data for method being considered for *Performance Tested Methods*$^{SM}$ or AOAC *Official Methods of Analysis*$^{SM}$ and can be used as acceptance criteria for verification at user laboratories.[1]

Approval Body: AOAC Cannabis Analytical Science Program
Approval Date:
Final version date:

1. **Intended Use**: Consensus-based Reference method.

2. **Applicability**: Candidate methods used to detect and quantify viable yeast and mold in cannabis and/or cannabis products. Candidate methods may be validated for specific matrices, categories or broader claims.

   See Table 4 for matrix/category claim acceptance criteria.

3. **Analytical Technique**: Any analytical technique that meets the method performance requirements is acceptable.

4. **Definitions**:

   ***Candidate Method*.**— The method submitted for validation [Appendix J: *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces*, *Official Methods of Analysis of AOAC INTERNATIONAL*, (2019) 21$^{st}$ Ed., AOAC INTERNATIONAL, Rockville, MD, USA]

   ***Candidate Method Presumptive Result*.**—Preliminary result for a test portion produced by following a candidate method's instructions for use.

   ***Candidate Method Confirmed Result*.**—Final result obtained for a test portion after cultural confirmation of a candidate method.

   ***Cannabis***.—genus of flowering plants within the Cannabinaceae family that commonly contain 9-tetrahydrocannabinol (THC), cannabidiol (CBD), and other cannabinoids and terpenes. Cannabis includes, but is not limited to, high-THC and high-CBD cultivars.

   ***Cannabis Concentrates***.—Extracts (primarily composed of cannabinoids and/or terpenes) manufactured through the extraction and concentration of compounds derived from the cannabis plant or flower. Final products can be many forms including oils, wax, or hash (Category II).

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***Cannabis Infused Edibles***.—Food and drinks containing extracts of cannabis and/or cannabis materials (Category III).

***Cannabis Infused Non-Edibles***.—Products containing extracts of cannabis and/or cannabis materials intended to be applied to the human body or any part thereof. Final products can be many forms including creams, ointments, cosmetics and therapeutic pads (Category IV).

***Cannabis Plant and Flower***.—General terms for the structural and flowering unadulterated parts of the cannabis plant (Category I).

***Cannabis Products***.—Products (Edible, and non-edible) extracted or infused with compounds derived from the cannabis plant including but not limited to CBD and THC.

***Confidence Interval.*** —The estimated range in which an obtained result should enclose the actual concentration. For the purpose of this SMPR, a 90% confidence interval is used.

***Probability of detection (POD)***.—The portion of positive analytical outcomes for a semi-quantitative method for a given matrix at a given analyte level or concentration. This difference in POD values between presumptive and confirmed results is termed $dPOD_{CP}$.

***Enumeration. –*** The determination of viable microorganisms in a given test portion.

***Exclusivity***.—Study involving pure nontarget strains, which are potentially cross-reactive, that shall be not detected or enumerated by the candidate method. *See* Table 8 for a list of recommended nontarget strains.[2]

***Inclusivity***.—Study involving pure target strains that shall be detected or enumerated by the candidate method. *See* Table 7 for a list of recommended target strains.[2]

***Laboratory probability of detection (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.[3]

***LCL***.—Lower confidence limit.

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

***Limit of Quantitation.*** - lowest analyte concentration that can be quantified with an acceptable level of precision and trueness under the conditions of the test.

***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.[2]

***Test portion***.—The test portion is the sample size used in most validation studies. For cannabis flower/plant and cannabis infused non-edible products a 10 g test portion is used. For cannabis concentrates, a 5 g test portion is used. For cannabis infused edibles, a 25 g test portion is used. A

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larger test portion can be used in validation studies when appropriate.[4,5] *See* Table 3 for minimum test portion requirements.

***UCL***. —Upper confidence limit.

## 5. System suitability tests and/or analytical quality control:

Positive and negative controls shall be embedded in assays as appropriate. Inhibition controls should be used for method verification for each new matrix. Manufacturer must provide written justification if controls are not appropriate to an assay.

## 6. Reference Material(s):

The use of live cultures and/or fungal spores (liquid stressed/non-stressed, lyophilized) is required for inclusivity and exclusivity testing and for inoculation of test matrices during the matrix studies. Extracted DNA is not suitable for use in validating methods against this SMPR but may be used to develop supplemental information.

## 7. Validation Guidance[2,6]:

At the time of the publication, no national reference method exists for viable yeast and mold count determination from cannabis products. Until a suitable reference method is established the following is recommended for method developers:

Natural contamination, artificial contamination, or a combination of both are acceptable for the validation study. Method developers must specify which mode of contamination is used for which sample subset upon method submission. If artificial contamination is used, a non-inoculated sample must be included.

When performing the validation, use of bulk contaminated test material or bulk inoculation of test material is required. In certain instances (ex. therapeutic patches) individual item inoculation may be required.

For the Single Laboratory Validation with artificial contamination, matrix naturally contaminated with non-target organisms (when available) shall be used.

It is recommended that in addition to the required confirmatory agar (DRBC), a secondary, non-selective agar is included in the cultural confirmation portion of the study and that suitability testing of media be performed.[4,7,8]

For quantitative methods, at least three contamination levels (low, medium, and high) shall be included. The concentration of target organisms in each contamination level may be determined by the method developer. Each contamination level should be prepared to have a concentration of target organisms that is approximately one logarithmic difference from adjacent contamination levels. It is expected that when applicable, contamination levels align with local regulatory requirements and thresholds.

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Data Analysis (reference Table 2):

For the data analysis portion of the method, the following guidance should be followed per method type:

*Quantitative Methods:*

a. Each lot of matrix must be analyzed separately for each candidate and confirmation method.
b. Perform a logarithmic transformation on the reported CFU/g: $Log_{10}$[CFU/g + 0.1]
    i. Where 0.1 provides an offset to allow inclusion of "0 CFU/g" data points.
c. Perform outlier tests (Cochran and Grubbs). Remove outliers from data analysis only if there is a justifiable cause.
d. Plot the candidate method result (y-axis) vs. the culture result (x-axis). Usually major discrepancies will appear.
e. Calculate repeatability as the standard deviation of replicates at each concentration of each matrix for each method.

$$s_r = \sqrt{\frac{\sum_{i=1}^{n}(X_i - \bar{X})^2}{n-1}}$$

f. Calculate the relative standard deviation:

$$RSD_r = [s_r/mean_{cand}] \times 100$$

g. Calculate the mean difference between the candidate and reference method $\log_{10}$ transformed results with 90% and 95% confidence intervals for matrix at each contamination level using the Least Cost Formulations, Ltd., Paired Method Analysis for Micro Testing Excel worksheet v1.2.
h. Prepare a summary table of mean, $s_r$, $RSD_r$ and difference of means with 90% and 95% CI data.

*Qualitative Methods:*

a. Analyze the data by Probability of Detection (POD) statistics according to the AOAC OMA Appendix J Guidelines.[2]
b. Analyze each contamination level separately.
c. Calculate the probability of detection (POD) as the number of positive outcomes divided by the total number of trials. Estimate the POD with a 95% confidence interval for:
    i. the candidate method presumptive results ($POD_{CP}$), and
    ii. the candidate method confirmed results ($POD_{CC}$).
d. Compare the presumptive and confirmed results by determining the $dPOD_{CP}$ at each contamination level and report results:

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

where $dPOD_{CP}$ is the difference in POD values between the candidate and reference methods. If the confidence interval of a dPOD does not contain zero, then the difference is statistically significant at the 5% level. For this validation, method comparisons are paired.

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199 **8. Method Performance Requirements:**
200 *See* Table 1 for contamination levels and replicates required for quantitative and qualitative
201 candidate methods.
202 *See* Table 2 for validation data analysis and acceptance criteria.
203 *See* Table 3 category test portion requirements.
204 *See* Table 4 for matrix claims acceptance criteria.
205 *See* Table 5 condition of inoculating culture and stabilization of matrix for inoculation.
206 *See* Table 6 for inclusivity and exclusivity guidance.
207 *See* Table 7 for the inclusivity panel.
208 *See* Table 8 for the recommended exclusivity panel.
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**Table 1. Contamination Levels and Replicates Required for Quantitative and Qualitative Candidate Methods**

| Type of Method | Contamination Type | Contamination Level | Replicates/ Method | Cultural Confirmation |
|---|---|---|---|---|
| Quantitative | Natural Contamination/ Artificial Contamination | Low | 5 | DRBC 25°C 5-7 days |
| | | Medium | 5 | |
| | | High | 5 | |
| Qualitative | Natural Contamination/ Artificial Contamination | Below Lowest Threshold[a] | 5 | |
| | | Low Action Limit[b] | 20 | |
| | | High Action Limit[b] | 20 | |
| | | Above Highest Threshold[c] | 5 | |

a. A lower contamination level targeted to be approximately one logarithmic difference from the lowest threshold determined in b.
b. Target threshold to determine a pass or fail result
c. A higher contamination level targeted to be approximately one logarithmic difference from the highest threshold determined in b.

**Table 2. Validation Data Analysis and Acceptance Criteria (Plants/Flowers, Concentrates, Infused Edibles, Infused Non-Edibles)**

| Type of Method | Contamination Level | Required Performance Characteristics | Acceptance Criteria/Confidence Interval |
|---|---|---|---|
| Quantitative | Low | Mean<br>Repeatability ($s_r$)<br>Relative Standard Deviation ($RSD_r$)<br>Difference of Means | Report Statistics at 90% & 95% Confidence Intervals |
| | Medium | | |
| | High | | |
| Qualitative | Below Lowest Threshold | Probability of Detection Statistics[a]<br><br>$dPOD_{CP} = POD_{CP} - POD_{CC}$ | POD of 0.00[c] |
| | Low Action Limit | | $dPOD_{cp}$ 95% CI: LCL < 0 < UCL[b] |
| | High Action Limit | | |
| | Above Highest Threshold | | POD of 1.00[c] |

a. Per AOAC OMA Appendix J Guidelines[2] (http://www.eoma.aoac.org/app_j.pdf)
b. The range between the lower and upper confidence interval should encompass 0, if not, the results must be investigated, and an explanation provided.
c. If acceptance criteria is not observed, results must be investigated, and an explanation provided.

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**Table 3. Category Test Portion Requirements**

| Category | Minimum Test Portion Size[a] |
|---|---|
| Plants & Flowers | 10 g |
| Concentrates | 5 g |
| Infused Edibles | 25 g |
| Infused Non-Edibles | 10 g |
| [a]Minimum test portion size required for validation. Alternatively, larger test portions may be validated. | |

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**Table 4. Acceptable Matrix Claims[9]**

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| Matrix Claim | Criteria | |
|---|---|---|
| | Number of Matrices | Minimum Number of Categories |
| Broad Range of Cannabis & Cannabis Products | 15 (minimum 3 matrices/category) | 4 categories |
| Variety of Cannabis & Cannabis Products | ≥ 10 (minimum 2 matrices/category) | 4 categories |
| Select Cannabis Products | ≥ 5 | 2 categories |
| Specific Category | ≥ 5 | 1 category |
| Specific Matrix (s) | ≥ 1 | 1 category |

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**Table 5. Condition of Inoculating Culture and Stabilization of Matrix**

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| Matrix | Inoculating Cells | Stabilization Conditions |
|---|---|---|
| Perishable product | Liquid non-stressed culture | 4°C, 48-72 h |
| Heat processed perishable product | Liquid heat stressed | 4°C, 48-72 h |
| Frozen Product | Liquid non-stressed culture (If frozen food is processed, cells must be heat stressed) | -20°C, 2 weeks |
| Shelf stable dry product | Dried culture | Ambient Temperature (20-25°C), 2 weeks |
| Shelf stable liquid product (heat processed) | Liquid non-stressed culture (If shelf stable product is processed, cells must be heat stressed) | Ambient Temperature (20-25°C), 2 weeks |

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249 **Table 6. Inclusivity/Exclusivity Performance Requirements**
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| Parameter | Parameter Requirements | Final Test Concentration (CFU/mL) | Minimum Acceptable Results |
|---|---|---|---|
| Inclusivity | Single-laboratory validation (SLV) study: target strains cultured by the candidate method enrichment procedure. A minimum of 50 strains is required. | 10-100 x limit of detection of the candidate method | 100% positive results[a] |
| Exclusivity | SLV study: At least 30 non-target organisms, (including those required in Table 8), cultured under optimal conditions for growth[b] | Overnight growth undiluted | 100% negative results[a] |

a 100% correct analyses are expected. All unexpected results are to be retested following internationally recognized guidelines.[2,6] Some unexpected results may be acceptable if the unexpected results are investigated, and acceptable explanations can be determined and communicated to method users

b In instances where an exclusivity culture produces a positive result by the candidate method, the culture may be reanalyzed after culture following the candidate method enrichment procedure. Both results (optimal growth conditions and candidate method enrichment) must be reported.

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**Table 7. Inclusivity Panel[a]**

| No. | Organism | Source | Origin | Results |
|---|---|---|---|---|
| 1 | *Alternaria alternata* | | | |
| 2 | *Arthrinium* species | | | |
| 3 | *Aspergillus aculeatus* | | | |
| 4 | *Aspergillus brasiliensis* | | | |
| 5 | *Aspergillus caesiellus* | | | |
| 6 | *Aspergillus flavus* | | | |
| 7 | *Aspergillus fumigatus* | | | |
| 8 | *Aspergillus niger* | | | |
| 9 | *Aspergillus oryzae* | | | |
| 10 | *Aspergillus terreus* | | | |
| 11 | *Aureobasidium* species | | | |
| 12 | *Botrytis cinerea* | | | |
| 13 | *Candida albicans* | | | |
| 14 | *Candida tropicalis* | | | |
| 15 | *Cladosporium* species | | | |
| 16 | *Cryptococcus laurentii* | | | |
| 17 | *Cryptococcus neoformans* | | | |
| 18 | *Erysiphe* species[b, c] | | | |
| 19 | *Fusarium proliferatum* | | | |
| 20 | *Fusarium oxysporum* | | | |
| 21 | *Fusarium solani* | | | |
| 22 | *Golovinomyces cichoracearum*[b, c] | | | |
| 23 | *Hyphodontia* species[c] | | | |
| 24 | *Microsphaera* species[b, c] | | | |
| 25 | *Mucor circinelloides* | | | |
| 26 | *Mucor hiemalis* | | | |
| 27 | *Paecilomyces* species | | | |
| 28 | Penicillium chrysogenum | | | |
| 29 | *Penicillium rubens* | | | |
| 30 | *Penicillium venetum* | | | |
| 31 | *Phytophthora infestans* | | | |
| 32 | *Podosphaera* species[b, c] | | | |
| 33 | *Purpureocillium* species | | | |
| 34 | *Rhizopus oryzae* | | | |
| 35 | *Rhizopus stolonifera* | | | |
| 36 | *Scopulariopsis acremonium* | | | |
| 37 | *Sphaerotheca* species[b, c] | | | |
| 38 | *Yarrowia lipolytica* | | | |
| 39 | *Talaromyces pinophilus* | | | |

[a]Method developers must test 50 total species to meet inclusivity requirements

[b]Organisms associated with powdery mildew

[c]Organism availability may be limited. Every effort to obtain these strains should be made. Non-traditional culture collections, such as the Cornell University Plant Pathology Herbarium, should be contacted for availability. In cases where strains are not available, different species may be supplemented. Expert reviewers will make the final determination if species analyzed is sufficient for certification.

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262 **Table 8. Exclusivity Panel**

| List of suggested organisms method developers can use to validate their methods. A minimum of 30 non-target organisms are required for AOAC adoption. Organisms utilized should be well characterized and information provided must include source, strain numbers and origin (if available) | |
|---|---|
| *Organism* | Reference ID (where applicable) |
| *Acinetobacter baumanii* | |
| *Aeromonas hydrophila* | |
| *Burkholderia cepacia* | |
| *Bacillus subtilis* | |
| *Citrobacter braakii* | |
| *Citrobacter farmeri* | |
| *Edwardsiella tarda* | |
| *Enterobacter aerogenes* | |
| *Enterobacter cloacae* | |
| *Erwinia amylovora* | |
| *Escherichia coli* | |
| *Escherichia coli* O157:H7 | |
| *Escherichia hermanii* | |
| *Escherichia vulneris* | |
| *Hafnia alvei* | |
| *Klebsiella oxytoca* | |
| *Klebsiella pneumonia* | |
| *Listeria monocytogenes* | |
| *Morganella morganii* | |
| *Pantoea agglomerans* | |
| *Proteus mirabilis* | |
| *Pseudomonas aeruginosa* | |
| *Pseudomonas fluorescens* | |
| *Pseudomonas putida* | |
| *Ralstonia insidiosa* | |
| *Rahnella aquatilis* | |
| *Salmonella* Agona | |
| *Stenotrophomonas maltophilia* | |
| *Staphylococcus aureus* | |
| *Serratia marcescens* | |
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**References**


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