1 2 3		-	]: AOAC INTERNATIONAL Methods Committee Guidelines for of Microbiological Methods for Cannabis and Cannabis Products
4 5	Table	of Cont	onts
6	1	Scope	
7	2	•	ability
, 8	2	•••	and Definitions
9	5	3.1	Analyte
10		3.2	Candidate Method
11		3.3	Candidate Method Result
12		3.4	Collaborative Study (CS)
13		3.5	Composite Test Portion
14		3.6	Confirmatory Identification Method
15		3.7	Confirmatory Phase
16		3.8	Confirmed Result
17		3.9	Decontamination
18		3.10	Enrichment Pool
19		3.11	Exclusivity
20		3.12	Fractional Recovery
21		3.13	Inclusivity
22		3.14	Limit of Detection50 (LOD50)
23		3.15	Limit of Quantitation (LOQ)
24		3.16	Matched Analyses
25		3.17	Material
26		3.18	Matrix
27		3.19	Method Developer Validation or Single-Laboratory Validation (SLV or
28			Precollaborative) Study
29		3.20	Precision
30		3.21	Presumptive Phase
31		3.22	Presumptive Result
32		3.23	Probability of Detection (POD)
33		3.24	Qualitative Method
34		3.25	Quantitative Method
35		3.26	Reference Method
36		3.27	Remediation
37		3.28	Repeatability
38		3.29	Repeatability Conditions
39		3.30	Reproducibility
40		3.31	Reproducibility Conditions
41 42		3.32	Robustness Study
42 42		3.33	Sterilization Test Portion
43		3.34	Test Portion

44		3.35	Unmatched Analyses		
45	4	Quali	Qualitative Methods—Technical Protocol for Validation		
46		4.1	Method Developer Validation or SLV (Precollaborative) Study		
47			4.1.1 Scope		
48			4.1.2 Inclusivity/Exclusivity Study		
49			4.1.3 Matrix Study		
50			4.1.4 Robustness Study [Performance Tested Methods (PTM) submissions only]		
51		4.2	Independent Validation Study		
52			4.2.1 Scope		
53			4.2.2 Reference Method		
54			4.2.3 Matrices		
55			4.2.4 Study Design		
56		4.3	Collaborative Study (CS)		
57			4.3.1 Scope		
58			4.3.2 Number of Laboratories		
59			4.3.3 Reference Method		
60			4.3.4 Matrix Selection		
61			4.3.5 Levels of Contamination		
62			4.3.6 Number of Test Portions		
63			4.3.7 Test Portion Size, Compositing and Pooling		
64			4.3.8 Source of Contamination		
65			4.3.9 Preparation of Artificially Contaminated Samples		
66			4.3.10 Preparation of Naturally Contaminated Samples		
67			4.3.11 Confirmation of Test Portions		
68			4.3.12 Data Analysis and Reporting		
69	5	Ouant	titative Methods—Technical Protocol for Validation		
70		5.1	Method Developer Validation or SLV (Precollaborative) Study		
71			5.1.1 Scope		
72			5.1.2 Inclusivity/ Exclusivity		
73			5.1.3 Matrix Study		
74			5.1.4 Robustness Study (PTM submissions only)		
75		5.2	Independent Validation Study		
76			5.2.1 Scope		
77			5.2.2 Reference Method		
78			5.2.3 Matrices		
79			5.2.4 Study Design		
80		5.3	Collaborative Study (CS)		
81			5.3.1 Scope		
82			5.3.2 Number of Laboratories		
83			5.3.3 Reference Method		
84			5.3.4 Food Categories		
85			5.3.5 Levels of Contamination		
86			5.3.6 Number of Test Portions		
87			5.3.7 Enumeration of Specific Microorganisms		

88		5.3.8 Source of Contamination					
89		5.3.9 Preparation of Artificially Contaminated Samples					
90		5.3.10 Use of Artificially and Naturally Contaminated Test Samples					
91		5.3.11 Confirmation of Test Portions					
92		5.3.12 Data Analysis and Reporting					
93	6	Confirmatory Methods					
94	7	Matrix Categorization					
95	8	Decontamination of Matrix					
96	9	Suitability of Testing Media					
97	10	In-Silico Analysis					
98	11	Safety					
99	12	References					
100	13	Annexes					
101		A MPN Analysis of Contaminated Matrix					
102		B Raw Format Data Table Template and Example for Qualitative Method Single					
103		Laboratory and Collaborative Studies					
104		C Calculation of POD and dPOD Values from Qualitative Method Single Laboratory Data					
105		D Summary Data Table for Qualitative Method Single Laboratory Studies					
106		E Example of Graph of POD Values from Qualitative					
107		Method Single Laboratory Data					
108		F Calculation of LPOD and dLPOD Values from Qualitative Method Collaborative Study					
109		Data					
110		G Data Summary Table Template and Example for					
111		Qualitative Method Collaborative Studies					
112		H Logarithmic Transformation of Data from Quantitative Method Single Laboratory and					
113		Collaborative Data					
114							
115	1	Scope					
116	The p	purpose of this document is to provide comprehensive AOAC INTERNATIONAL (AOAC)					
117	techi	nical guidelines for conducting microbiological validation studies for analysis methods of					
118	cann	abis and cannabis products submitted for AOAC® Official Methods of Analysis (OMA)					
119	statu	is, Performance Tested Methods (PTM), and/or Reviewed and Recognized ( $R^2$ )					
120	certi	fication.					
121							
122							
123	2	Applicability					
124	These	e guidelines are applicable to the validation of any candidate method, whether					
125	prop	rietary or nonproprietary, that is submitted to AOAC for OMA status, PTM, or $R^2$					
126	certi	fication. Circumstances, unforeseen by AOAC, may necessitate divergence from the					
127	guide	elines in certain cases. The PTM and R <sup>2</sup> Programs require a Method Developer Study and					
128	an In	dependent Laboratory Study. The OMA Program requires a Single-Laboratory Validation					
129	. ,	Study (also known as the Precollaborative Study), an Independent Validation Study, and					
130	a Col	laborative Study. A harmonized PTM-OMA or R <sup>2</sup> -OMA program can be followed in which					
131	PTM	or R <sup>2</sup> 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 132 133 toward OMA status. See Table 1 for more detail.

- 134
- 135
- 136 Table 1 137

			Relevant Guid	deline Sections
AOAC Program	Study Requirements	Qualita tive	Quantita tive	Confirmatory Identification
PTM/R <sup>2</sup>	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 <sup>2</sup> - 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

141

138 139

- 140
- 142 3.1
- 143 Microorganism or associated biochemicals (e.g., DNA, proteins, or lipopolysaccharides) 144 measured or detected by the method of analysis.
- 145 3.2 Candidate Method
- The method submitted for validation. 146

Analyte

- 3.3 147 Candidate Method Result
- 148 The final result of the qualitative or quantitative analysis for the candidate method.
- 149 For methods with a confirmation phase, only presumptive positive results that confirm
- 150 positive are considered as positive for the candidate method. All other results are 151 considered as negative for the candidate method.

450	
152	3.4 Collaborative Study (CS)
153	A validation study performed by multiple laboratories to estimate critical candidate
154	method performance parameters.
155	3.5 Composite Test Portion
156	Test portions taken from multiple samples of the same matrix combined together.
157	3.6 Confirmatory Identification Method
158	Method of analysis whose purpose is to determine the identity of an analyte.
159	(Biological Threat Agent Method; BTAM)
160	3.7 Confirmatory Phase
161	A procedure specified in some qualitative assays whereby a preliminary presumptive
162	result is confirmed by a subsequent and different method.
163	3.8 Confirmed Result
164	The qualitative response from the confirmatory phase of a candidate method.
165	3.9 Decontamination
166	The process of removing pathogenic microorganisms from products to allow for safe
167	handling and consumption
168	3.10 Enrichment Pool
169	A pool comprised of aliquots from multiple test portion enrichments.
170	3.11 Exclusivity
171	The nontarget strains, which are potentially cross-reactive, that are not detected by
172	the method.
173	3.12 Fractional Recovery
174	Validation criterion that is satisfied when an unknown sample yields both positive and
175	negative responses within a set of replicate analyses. The proportion of positive
176	responses should fall within 25 and 75% and should ideally approximate 50% of the
177	total number of replicates in the set. A set of replicate analyses are those replicates
178	analyzed by one method (either candidate or reference). Only one set of replicates
179	per matrix is required to satisfy this criterion.
180	An alternate plan acceptable to the Statistics Committee can be used.
181	3.13 Inclusivity
182	The strains or isolates of the target analyte(s) that the method can detect.
183	3.14 Limit of Detection50 (LOD50)
184	The analyte concentration at which the probability of detection (POD) is equal to 50%.
185	3.15 Limit of Quantitation (LOQ)
186	The lowest amount of analyte in a laboratory sample which can be quantitatively
187	determined with a defined confidence.
188	3.16 Matched Analyses
189	Two or more analyses or analytical results on the same unknown material, which can
190	be traced to the same test portion.
191	3.17 Material
192	The batch of matrix from which replicate test portions are removed for analysis. The
193	material (naturally contaminated, uncontaminated, or inoculated) contains analyte, if
194	present, at one homogeneous concentration.
195	3.18 Matrix

197       as per the intended use of the method.         198       3.19       Method Developer Validation Study or Single-Laboratory Validation (SLV or         199       Precollaborative) Study         200       A validation study performed by a single laboratory in order to systematically         201       estimate critical candidate method performance parameters. The method developer         202       study is usually performed by the organizing laboratory or Study Director.         203       3.20       Precision         204       The closeness of agreement between independent test results under stipulated         205       conditions. (ISO 5725-1)         206       3.21       Presumptive Phase         207       The initial qualitative determination of the analyte in a test portion. In some         208       qualitative microbiological assays, confirmation of results is required as specified in         209       3.22       Presumptive Result         211       The qualitative response from the presumptive phase of a candidate method that         212       includes a confirmatory phase.         213       3.23       Probability of Detection (PDD)         214       The proportion of positive analyte level or concentration. POD is concentration dependent.         216       Several POD measures can be calculated, e.g., PDDR (reference method POD),	196	The food, beverage, or environmental surface material to be included in the validation
199Precollaborative) Study200A validation study performed by a single laboratory in order to systematically201estimate critical candidate method performance parameters. The method developer202study is usually performed by the organizing laboratory or Study Director.2033.20Precision204The closeness of agreement between independent test results under stipulated205conditions. (ISO 5725-1)2063.21Presumptive Phase207The initial qualitative determination of the analyte in a test portion. In some208qualitative microbiological assays, confirmation of results is required as specified in209the method.2103.22Presumptive Result211The qualitative response from the presumptive phase of a candidate method that212includes a confirmatory phase.2133.23Probability of Detection (POD)214The proportion of positive analytical outcomes for a qualitative method for a given215matrix at a given analyte level or concentration. POD is concentration dependent.216Several POD measures can be calculated, e.g., PODR (reference method POD), PODC217(confirmed candidate method POD), PODCP (candidate method presumptive result218POD) and PODCC (candidate method confirmation result POD). Other POD estimates219include:220dPOD - the difference between any two POD values LPOD - the POD value obtained216from combining all valid222collaborator data sets for a method for a given matrix a	197	as per the intended use of the method.
200A validation study performed by a single laboratory in orderto systematically201estimate critical candidate method performance parameters. The method developer202study is usually performed by the organizing laboratory or Study Director.2033.20Precision204The closeness of agreement between independent test results under stipulated205conditions. (ISO 5725-1)2063.21Presumptive Phase207The initial qualitative determination of the analyte in a test portion. In some208qualitative microbiological assays, confirmation of results is required as specified in209the method.2103.22Presumptive Result211The qualitative response from the presumptive phase of a candidate method that212includes a confirmatory phase.2133.23Probability of Detection (POD)214The proportion of positive analytical outcomes for a qualitative method for a given215matrix at a given analyte level or concentration. POD is concentration dependent.216Several POD measures can be calculated, e.g., PODR (reference method POD), PODC217(confirmed candidate method POD), PODC (candidate method presumptive result218POD) and PODCC (candidate method for a given matrix at a given219include:220dPOD - the difference between any two POD values LPOD - the POD value obtained221from combining all valid222collaborator data sets for a method for a given matrix at a given223analyte level or concen	198	3.19 Method Developer Validation Study or Single-Laboratory Validation (SLV or
201estimate critical candidate method performance parameters. The method developer202study is usually performed by the organizing laboratory or Study Director.2033.20Precision204The closeness of agreement between independent test results under stipulated205conditions. (ISO 5725-1)2063.21Presumptive Phase207The initial qualitative determination of the analyte in a test portion. In some208qualitative microbiological assays, confirmation of results is required as specified in209the method.2103.22Presumptive Result211The qualitative response from the presumptive phase of a candidate method that212includes a confirmatory phase.2133.23Probability of Detection (POD)214The proportion of positive analytical outcomes for a qualitative method for a given215matrix at a given analyte level or concentration. POD is concentration dependent.216Several POD measures can be calculated, e.g., PODR (reference method POD), PODC217(confirmed candidate method POD), PODC (candidate method presumptive result218POD) and PODCC (candidate method for a given matrix at a given219include:220dPOD - the difference between any two POD values LPOD - the POD value obtained221from combining all valid222collaborator data sets for a method for a given matrix at a given223analyte level or concentration224Method of analysis whose response is either the presence or absence of the anal	199	Precollaborative) Study
201estimate critical candidate method performance parameters. The method developer202study is usually performed by the organizing laboratory or Study Director.2033.20Precision204The closeness of agreement between independent test results under stipulated205conditions. (ISO 5725-1)2063.21Presumptive Phase207The initial qualitative determination of the analyte in a test portion. In some208qualitative microbiological assays, confirmation of results is required as specified in209the method.2103.22Presumptive Result211The qualitative response from the presumptive phase of a candidate method that212includes a confirmatory phase.2133.23Probability of Detection (POD)214The proportion of positive analytical outcomes for a qualitative method for a given215matrix at a given analyte level or concentration. POD is concentration dependent.216Several POD measures can be calculated, e.g., PODR (reference method POD), PODC217(confirmed candidate method POD), PODC (candidate method presumptive result218POD) and PODCC (candidate method for a given matrix at a given219include:220dPOD - the difference between any two POD values LPOD - the POD value obtained221from combining all valid222collaborator data sets for a method for a given matrix at a given223analyte level or concentration224Method of analysis whose response is either the presence or absence of the anal	200	A validation study performed by a single laboratory in order to systematically
2033.20Precision204The closeness of agreement between independent test results under stipulated205conditions. (ISO 5725-1)2063.21Presumptive Phase207The initial qualitative determination of the analyte in a test portion. In some208qualitative microbiological assays, confirmation of results is required as specified in209the method.2103.22Presumptive Result211The qualitative response from the presumptive phase of a candidate method that212includes a confirmatory phase.2133.23Probability of Detection (POD)214The proportion of positive analytical outcomes for a qualitative method for a given215matrix at a given analyte level or concentration. POD is concentration dependent.216Several POD measures can be calculated, e.g., PODR (reference method POD), PODC217(confirmed candidate method POD), PODCP (candidate method presumptive result218POD) and PODCC (candidate method confirmation result POD). Other POD estimates219include:220dPOD - the difference between any two POD values LPOD - the POD value obtained211from combining all valid222collaborator data sets for a method for a given matrix at a given223analyte level or concentration224Qualitative Method225dLPOD - the difference between any two LPOD values2262273.24228Method of analysis whose response is either the presence or absence of the analyte2	201	
204The closeness of agreement between independent test results under stipulated205conditions. (ISO 5725-1)2063.21207The initial qualitative determination of the analyte in a test portion. In some208qualitative microbiological assays, confirmation of results is required as specified in209the method.2103.22211The qualitative response from the presumptive phase of a candidate method that212includes a confirmatory phase.2133.23214The proportion of positive analytical outcomes for a qualitative method for a given215matrix at a given analyte level or concentration. POD is concentration dependent.216Several POD measures can be calculated, e.g., PODR (reference method POD), PODC217(confirmed candidate method POD), PODC (candidate method presumptive result218POD and PODCC (candidate method confirmation result POD). Other POD estimates219include:220dPOD - the difference between any two POD values LPOD - the POD value obtained211from combining all valid222collaborator data sets for a method for a given matrix at a given223analyte level or concentration224Wethod of analysis whose response is either the presence or absence of the analyte225dLPOD - the difference between any two LPOD values226227228Method of analysis whose response is the amount (count or mass) of the analyte229detected either directly (e.g., enumeration in a mass or a volume), or indirectly </td <td>202</td> <td>study is usually performed by the organizing laboratory or Study Director.</td>	202	study is usually performed by the organizing laboratory or Study Director.
205conditions. (ISO 5725-1)2063.21Presumptive Phase207The initial qualitative determination of the analyte in a test portion. In some208qualitative microbiological assays, confirmation of results is required as specified in209the method.2103.22Presumptive Result211The qualitative response from the presumptive phase of a candidate method that212includes a confirmatory phase.2133.23Probability of Detection (POD)214The proportion of positive analytical outcomes for a qualitative method for a given215matrix at a given analyte level or concentration. POD is concentration dependent.216Several POD measures can be calculated, e.g., PODR (reference method POD), PODC217(confirmed candidate method POD), PODCP (candidate method presumptive result218POD) and PODCC (candidate method confirmation result POD). Other POD estimates219include:220dPOD - the difference between any two POD values LPOD - the POD value obtained218from combining all valid229collaborator data sets for a method for a given matrix at a given221analyte level or concentration222223223analyte level or concentration224224225dLPOD - the difference between any two LPOD values226227228Method of analysis whose response is either the presence or absence of the analyte229detected either directly or indirectly in a specified test portion.<	203	3.20 Precision
2063.21Presumptive Phase207The initial qualitative determination of the analyte in a test portion. In some208qualitative microbiological assays, confirmation of results is required as specified in209the method.2103.22Presumptive Result211The qualitative response from the presumptive phase of a candidate method that212includes a confirmatory phase.2133.23Probability of Detection (POD)214The proportion of positive analytical outcomes for a qualitative method for a given215matrix at a given analyte level or concentration. POD is concentration dependent.216Several POD measures can be calculated, e.g., PODR (reference method POD), PODC217(confirmed candidate method POD), PODCP (candidate method presumptive result218POD) and PODCC (candidate method confirmation result POD). Other POD estimates219include:220dPOD - the difference between any two POD values LPOD - the POD value obtained211from combining all valid222collaborator data sets for a method for a given matrix at a given223analyte level or concentration224detected either directly or indirectly in a specified test portion.2303.25Qualitative Method231S.25Qualitative Method232detected either directly (e.g., enumeration in a mass or a volume), or indirectly233measure either directly (e.g., enumeration in a mass or a volume), or indirectly234Reference Method235P	204	The closeness of agreement between independent test results under stipulated
2063.21Presumptive Phase207The initial qualitative determination of the analyte in a test portion. In some208qualitative microbiological assays, confirmation of results is required as specified in209the method.2103.22Presumptive Result211The qualitative response from the presumptive phase of a candidate method that212includes a confirmatory phase.2133.23Probability of Detection (POD)214The proportion of positive analytical outcomes for a qualitative method for a given215matrix at a given analyte level or concentration. POD is concentration dependent.216Several POD measures can be calculated, e.g., PODR (reference method POD), PODC217(confirmed candidate method POD), PODCP (candidate method presumptive result218POD) and PODCC (candidate method confirmation result POD). Other POD estimates219include:220dPOD - the difference between any two POD values LPOD - the POD value obtained211from combining all valid222collaborator data sets for a method for a given matrix at a given223analyte level or concentration224dLPOD - the difference between any two LPOD values225dLPOD - the difference between any two LPOD values2263.24Qualitative Method2273.25Qualitative Method238Method of analysis whose response is either the presence or absence of the analyte229detected either directly or indirectly in a specified test portion.230	205	
208qualitative microbiological assays, confirmation of results is required as specified in209the method.2103.22Presumptive Result211The qualitative response from the presumptive phase of a candidate method that212includes a confirmatory phase.2133.23Probability of Detection (POD)214The proportion of positive analytical outcomes for a qualitative method for a given215matrix at a given analyte level or concentration. POD is concentration dependent.216Several POD measures can be calculated, e.g., PODR (reference method POD), PODC217(confirmed candidate method POD), PODCP (candidate method presumptive result218POD) and PODCC (candidate method confirmation result POD). Other POD estimates219include:220dPOD - the difference between any two POD values LPOD - the POD value obtained221from combining all valid222collaborator data sets for a method for a given matrix at a given223analyte level or concentration224dLPOD - the difference between any two LPOD values225dLPOD - the difference between any two LPOD values2262273.24Qualitative Method231Method of analysis whose response is either the presence or absence of the analyte229detected either directly or indirectly in a specified test portion.2333.25Quantitative Method231Method of analysis whose response is the amount (count or mass) of the analyte232measured either directly (e.g., enu	206	
209the method.2103.22Presumptive Result211The qualitative response from the presumptive phase of a candidate method that212includes a confirmatory phase.2133.23Probability of Detection (POD)214The proportion of positive analytical outcomes for a qualitative method for a given215matrix at a given analyte level or concentration. POD is concentration dependent.216Several POD measures can be calculated, e.g., PODR (reference method POD), PODC217(confirmed candidate method POD), PODCP (candidate method presumptive result218POD) and PODCC (candidate method confirmation result POD). Other POD estimates219include:220dPOD - the difference between any two POD values LPOD - the POD value obtained221from combining all valid222collaborator data sets for a method for a given matrix at a given223analyte level or concentration224225225dLPOD - the difference between any two LPOD values2263.242273.24228Method of analysis whose response is either the presence or absence of the analyte229detected either directly or indirectly in a specified test portion.2313.25Qualitative Method231Method of analysis whose response is the amount (count or mass) of the analyte232(e.g., color absorbance, impedance, etc.) in a specified test portion.233(e.g., color absorbance, impedance, etc.) in a specified test portion.2333.26R	207	The initial qualitative determination of the analyte in a test portion. In some
<ul> <li>3.22 Presumptive Result</li> <li>The qualitative response from the presumptive phase of a candidate method that</li> <li>includes a confirmatory phase.</li> <li>3.23 Probability of Detection (POD)</li> <li>The proportion of positive analytical outcomes for a qualitative method for a given</li> <li>matrix at a given analyte level or concentration. POD is concentration dependent.</li> <li>Several POD measures can be calculated, e.g., PODR (reference method POD), PODC</li> <li>(confirmed candidate method POD), PODCP (candidate method presumptive result</li> <li>POD) and PODCC (candidate method confirmation result POD). Other POD estimates</li> <li>include:</li> <li>dPOD - the difference between any two POD values LPOD - the POD value obtained</li> <li>from combining all valid</li> <li>collaborator data sets for a method for a given matrix at a given</li> <li>analyte level or concentration</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD value</li></ul>	208	qualitative microbiological assays, confirmation of results is required as specified in
211The qualitative response from the presumptive phase of a candidate method that212includes a confirmatory phase.2133.23Probability of Detection (POD)214The proportion of positive analytical outcomes for a qualitative method for a given215matrix at a given analyte level or concentration. POD is concentration dependent.216Several POD measures can be calculated, e.g., PODR (reference method POD), PODC217(confirmed candidate method POD), PODCP (candidate method presumptive result218POD) and PODCC (candidate method confirmation result POD). Other POD estimates219include:220dPOD - the difference between any two POD values LPOD - the POD value obtained221from combining all valid222collaborator data sets for a method for a given matrix at a given223analyte level or concentration224225225dLPOD - the difference between any two LPOD values2262273.24Qualitative Method238Method of analysis whose response is either the presence or absence of the analyte229detected either directly or indirectly in a specified test portion.231Method of analysis whose response is the amount (count or mass) of the analyte239measured either directly (e.g., enumeration in a mass or a volume), or indirectly231Method of analysis whose response is the amount (count or mass) of the analyte232measured either directly (e.g., enumeration in a specified test portion.233(e.g., color absorbance, impedance, etc.)	209	the method.
212includes a confirmatory phase.2133.23Probability of Detection (POD)214The proportion of positive analytical outcomes for a qualitative method for a given215matrix at a given analyte level or concentration. POD is concentration dependent.216Several POD measures can be calculated, e.g., PODR (reference method POD), PODC217(confirmed candidate method POD), PODCP (candidate method presumptive result218POD) and PODCC (candidate method confirmation result POD). Other POD estimates219include:220dPOD - the difference between any two POD values LPOD - the POD value obtained221from combining all valid222collaborator data sets for a method for a given matrix at a given223analyte level or concentration224225225dLPOD - the difference between any two LPOD values226227228Method of analysis whose response is either the presence or absence of the analyte229detected either directly or indirectly in a specified test portion.2313.25Qualitative Method231Method of analysis whose response is the amount (count or mass) of the analyte232measured either directly (e.g., enumeration in a mass or a volume), or indirectly233(e.g., color absorbance, impedance, etc.) in a specified test portion.233(e.g., color absorbance, impedance, etc.) in a specified test portion.2343.26Reference Method235Preexisting recognized analytical method against which the candidate method will be<	210	3.22 Presumptive Result
2133.23Probability of Detection (POD)214The proportion of positive analytical outcomes for a qualitative method for a given215matrix at a given analyte level or concentration. POD is concentration dependent.216Several POD measures can be calculated, e.g., PODR (reference method POD), PODC217(confirmed candidate method POD), PODCP (candidate method presumptive result218POD) and PODCC (candidate method confirmation result POD). Other POD estimates219include:220dPOD - the difference between any two POD values LPOD - the POD value obtained221from combining all valid222collaborator data sets for a method for a given matrix at a given223analyte level or concentration224225225dLPOD - the difference between any two LPOD values2262273.24Qualitative Method228Method of analysis whose response is either the presence or absence of the analyte229detected either directly or indirectly in a specified test portion.2303.25Quantitative Method231Method of analysis whose response is the amount (count or mass) of the analyte232measured either directly (e.g., enumeration in a mass or a volume), or indirectly233(e.g., color absorbance, impedance, etc.) in a specified test portion.2333.26Reference Method234Preexisting recognized analytical method against which the candidate method will be2353.27Remediation236The process of removing or reduc	211	The qualitative response from the presumptive phase of a candidate method that
214The proportion of positive analytical outcomes for a qualitative method for a given215matrix at a given analyte level or concentration. POD is concentration dependent.216Several POD measures can be calculated, e.g., PODR (reference method POD), PODC217(confirmed candidate method POD), PODCP (candidate method presumptive result218POD) and PODCC (candidate method confirmation result POD). Other POD estimates219include:220dPOD - the difference between any two POD values LPOD - the POD value obtained221from combining all valid222collaborator data sets for a method for a given matrix at a given223analyte level or concentration224225225dLPOD - the difference between any two LPOD values2262273.24Qualitative Method231Method of analysis whose response is either the presence or absence of the analyte229detected either directly or indirectly in a specified test portion.231Method of analysis whose response is the amount (count or mass) of the analyte232measured either directly (e.g., enumeration in a mass or a volume), or indirectly233(e.g., color absorbance, impedance, etc.) in a specified test portion.2332.262343.26235Preexisting recognized analytical method against which the candidate method will be2362373.27Remediation2373.27238The process of removing or reducing the level of microbial contamination in a product	212	includes a confirmatory phase.
215matrix at a given analyte level or concentration. POD is concentration dependent.216Several POD measures can be calculated, e.g., PODR (reference method POD), PODC217(confirmed candidate method POD), PODCP (candidate method presumptive result218POD) and PODCC (candidate method confirmation result POD). Other POD estimates219include:220dPOD - the difference between any two POD values LPOD - the POD value obtained221from combining all valid222collaborator data sets for a method for a given matrix at a given223analyte level or concentration224225225dLPOD - the difference between any two LPOD values2262273.24228Method of analysis whose response is either the presence or absence of the analyte229detected either directly or indirectly in a specified test portion.2303.25Quantitative Method231Method of analysis whose response is the amount (count or mass) of the analyte232measured either directly (e.g., enumeration in a mass or a volume), or indirectly233(e.g., color absorbance, impedance, etc.) in a specified test portion.2343.26235Preexisting recognized analytical method against which the candidate method will be236compared.2373.273.27Remediation238The process of removing or reducing the level of microbial contamination in a product	213	3.23 Probability of Detection (POD)
<ul> <li>Several POD measures can be calculated, e.g., PODR (reference method POD), PODC</li> <li>(confirmed candidate method POD), PODCP (candidate method presumptive result</li> <li>POD) and PODCC (candidate method confirmation result POD). Other POD estimates</li> <li>include:</li> <li>dPOD - the difference between any two POD values LPOD - the POD value obtained</li> <li>from combining all valid</li> <li>collaborator data sets for a method for a given matrix at a given</li> <li>analyte level or concentration</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the differe</li></ul>	214	The proportion of positive analytical outcomes for a qualitative method for a given
<ul> <li>217 (confirmed candidate method POD), PODCP (candidate method presumptive result</li> <li>218 POD) and PODCC (candidate method confirmation result POD). Other POD estimates</li> <li>219 include:</li> <li>220 dPOD - the difference between any two POD values LPOD - the POD value obtained</li> <li>221 from combining all valid</li> <li>222 collaborator data sets for a method for a given matrix at a given</li> <li>223 analyte level or concentration</li> <li>224</li> <li>225 dLPOD - the difference between any two LPOD values</li> <li>226</li> <li>227 3.24 Qualitative Method</li> <li>228 Method of analysis whose response is either the presence or absence of the analyte</li> <li>229 detected either directly or indirectly in a specified test portion.</li> <li>3.25 Quantitative Method</li> <li>231 Method of analysis whose response is the amount (count or mass) of the analyte</li> <li>232 measured either directly (e.g., enumeration in a mass or a volume), or indirectly</li> <li>233 (e.g., color absorbance, impedance, etc.) in a specified test portion.</li> <li>234 3.26 Reference Method</li> <li>235 Preexisting recognized analytical method against which the candidate method will be</li> <li>236 compared.</li> <li>237 3.27 Remediation</li> <li>238 The process of removing or reducing the level of microbial contamination in a product</li> </ul>	215	matrix at a given analyte level or concentration. POD is concentration dependent.
218POD) and PODCC (candidate method confirmation result POD). Other POD estimates219include:220dPOD - the difference between any two POD values LPOD - the POD value obtained221from combining all valid222collaborator data sets for a method for a given matrix at a given223analyte level or concentration224225225dLPOD - the difference between any two LPOD values2262272273.24228Method of analysis whose response is either the presence or absence of the analyte229detected either directly or indirectly in a specified test portion.2303.25231Method of analysis whose response is the amount (count or mass) of the analyte232measured either directly (e.g., enumeration in a mass or a volume), or indirectly233(e.g., color absorbance, impedance, etc.) in a specified test portion.2343.26235Preexisting recognized analytical method against which the candidate method will be236compared.2373.273.27Remediation238The process of removing or reducing the level of microbial contamination in a product	216	Several POD measures can be calculated, e.g., PODR (reference method POD), PODC
<ul> <li>219 include:</li> <li>220 dPOD - the difference between any two POD values LPOD - the POD value obtained</li> <li>221 from combining all valid</li> <li>222 collaborator data sets for a method for a given matrix at a given</li> <li>223 analyte level or concentration</li> <li>224</li> <li>225 dLPOD - the difference between any two LPOD values</li> <li>226</li> <li>227 3.24 Qualitative Method</li> <li>228 Method of analysis whose response is either the presence or absence of the analyte</li> <li>229 detected either directly or indirectly in a specified test portion.</li> <li>230 3.25 Quantitative Method</li> <li>231 Method of analysis whose response is the amount (count or mass) of the analyte</li> <li>232 measured either directly (e.g., enumeration in a mass or a volume), or indirectly</li> <li>233 (e.g., color absorbance, impedance, etc.) in a specified test portion.</li> <li>234 3.26 Reference Method</li> <li>235 Preexisting recognized analytical method against which the candidate method will be</li> <li>236 compared.</li> <li>237 3.27 Remediation</li> <li>238 The process of removing or reducing the level of microbial contamination in a product</li> </ul>	217	(confirmed candidate method POD), PODCP (candidate method presumptive result
<ul> <li>dPOD - the difference between any two POD values LPOD - the POD value obtained</li> <li>from combining all valid</li> <li>collaborator data sets for a method for a given matrix at a given</li> <li>analyte level or concentration</li> <li>dLPOD - the difference between any two LPOD values</li> <li>3.24</li> <li>Qualitative Method</li> <li>Method of analysis whose response is either the presence or absence of the analyte</li> <li>detected either directly or indirectly in a specified test portion.</li> <li>3.25 Quantitative Method</li> <li>Method of analysis whose response is the amount (count or mass) of the analyte</li> <li>measured either directly (e.g., enumeration in a mass or a volume), or indirectly</li> <li>(e.g., color absorbance, impedance, etc.) in a specified test portion.</li> <li>3.26 Reference Method</li> <li>Preexisting recognized analytical method against which the candidate method will be</li> <li>compared.</li> <li>3.27 Remediation</li> <li>The process of removing or reducing the level of microbial contamination in a product</li> </ul>	218	POD) and PODCC (candidate method confirmation result POD). Other POD estimates
221from combining all valid222collaborator data sets for a method for a given matrix at a given223analyte level or concentration224225225dLPOD - the difference between any two LPOD values2262273.24Qualitative Method228Method of analysis whose response is either the presence or absence of the analyte229detected either directly or indirectly in a specified test portion.2303.25231Method of analysis whose response is the amount (count or mass) of the analyte232measured either directly (e.g., enumeration in a mass or a volume), or indirectly233(e.g., color absorbance, impedance, etc.) in a specified test portion.2343.26235Preexisting recognized analytical method against which the candidate method will be236compared.2373.273.28The process of removing or reducing the level of microbial contamination in a product	219	include:
<ul> <li>collaborator data sets for a method for a given matrix at a given</li> <li>analyte level or concentration</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>3.24 Qualitative Method</li> <li>Method of analysis whose response is either the presence or absence of the analyte</li> <li>detected either directly or indirectly in a specified test portion.</li> <li>3.25 Quantitative Method</li> <li>Method of analysis whose response is the amount (count or mass) of the analyte</li> <li>measured either directly (e.g., enumeration in a mass or a volume), or indirectly</li> <li>(e.g., color absorbance, impedance, etc.) in a specified test portion.</li> <li>3.26 Reference Method</li> <li>Preexisting recognized analytical method against which the candidate method will be</li> <li>compared.</li> <li>3.27 Remediation</li> <li>The process of removing or reducing the level of microbial contamination in a product</li> </ul>	220	dPOD - the difference between any two POD values LPOD - the POD value obtained
<ul> <li>analyte level or concentration</li> <li>analyte level or concentration</li> <li>dLPOD - the difference between any two LPOD values</li> <li>dLPOD - the difference between any two LPOD values</li> <li>3.24 Qualitative Method</li> <li>Method of analysis whose response is either the presence or absence of the analyte</li> <li>detected either directly or indirectly in a specified test portion.</li> <li>3.25 Quantitative Method</li> <li>Method of analysis whose response is the amount (count or mass) of the analyte</li> <li>measured either directly (e.g., enumeration in a mass or a volume), or indirectly</li> <li>(e.g., color absorbance, impedance, etc.) in a specified test portion.</li> <li>3.26 Reference Method</li> <li>Preexisting recognized analytical method against which the candidate method will be</li> <li>compared.</li> <li>3.27 Remediation</li> <li>The process of removing or reducing the level of microbial contamination in a product</li> </ul>	221	from combining all valid
224225dLPOD - the difference between any two LPOD values2262273.24Qualitative Method228Method of analysis whose response is either the presence or absence of the analyte229detected either directly or indirectly in a specified test portion.2303.25Quantitative Method231Method of analysis whose response is the amount (count or mass) of the analyte232measured either directly (e.g., enumeration in a mass or a volume), or indirectly233(e.g., color absorbance, impedance, etc.) in a specified test portion.2343.26Reference Method235Preexisting recognized analytical method against which the candidate method will be236compared.2373.27Remediation238The process of removing or reducing the level of microbial contamination in a product	222	collaborator data sets for a method for a given matrix at a given
225dLPOD - the difference between any two LPOD values2262273.24Qualitative Method228Method of analysis whose response is either the presence or absence of the analyte229detected either directly or indirectly in a specified test portion.2303.25Quantitative Method231Method of analysis whose response is the amount (count or mass) of the analyte232measured either directly (e.g., enumeration in a mass or a volume), or indirectly233(e.g., color absorbance, impedance, etc.) in a specified test portion.2343.26235Preexisting recognized analytical method against which the candidate method will be236compared.2373.27238The process of removing or reducing the level of microbial contamination in a product	223	analyte level or concentration
<ul> <li>3.24 Qualitative Method</li> <li>Method of analysis whose response is either the presence or absence of the analyte</li> <li>detected either directly or indirectly in a specified test portion.</li> <li>3.25 Quantitative Method</li> <li>Method of analysis whose response is the amount (count or mass) of the analyte</li> <li>measured either directly (e.g., enumeration in a mass or a volume), or indirectly</li> <li>(e.g., color absorbance, impedance, etc.) in a specified test portion.</li> <li>3.26 Reference Method</li> <li>Preexisting recognized analytical method against which the candidate method will be</li> <li>compared.</li> <li>3.27 Remediation</li> <li>The process of removing or reducing the level of microbial contamination in a product</li> </ul>	224	
2273.24Qualitative Method228Method of analysis whose response is either the presence or absence of the analyte229detected either directly or indirectly in a specified test portion.2303.25Quantitative Method231Method of analysis whose response is the amount (count or mass) of the analyte232measured either directly (e.g., enumeration in a mass or a volume), or indirectly233(e.g., color absorbance, impedance, etc.) in a specified test portion.2343.26Reference Method235Preexisting recognized analytical method against which the candidate method will be236compared.2373.27Remediation238The process of removing or reducing the level of microbial contamination in a product	225	dLPOD - the difference between any two LPOD values
228Method of analysis whose response is either the presence or absence of the analyte229detected either directly or indirectly in a specified test portion.2303.25Quantitative Method231Method of analysis whose response is the amount (count or mass) of the analyte232measured either directly (e.g., enumeration in a mass or a volume), or indirectly233(e.g., color absorbance, impedance, etc.) in a specified test portion.2343.26235Preexisting recognized analytical method against which the candidate method will be236compared.2373.27238The process of removing or reducing the level of microbial contamination in a product	226	
229detected either directly or indirectly in a specified test portion.2303.25231Method of analysis whose response is the amount (count or mass) of the analyte232measured either directly (e.g., enumeration in a mass or a volume), or indirectly233(e.g., color absorbance, impedance, etc.) in a specified test portion.2343.26Reference Method235Preexisting recognized analytical method against which the candidate method will be236compared.2373.27Remediation238The process of removing or reducing the level of microbial contamination in a product	227	3.24 Qualitative Method
<ul> <li>3.25 Quantitative Method</li> <li>Method of analysis whose response is the amount (count or mass) of the analyte</li> <li>measured either directly (e.g., enumeration in a mass or a volume), or indirectly</li> <li>(e.g., color absorbance, impedance, etc.) in a specified test portion.</li> <li>3.26 Reference Method</li> <li>Preexisting recognized analytical method against which the candidate method will be</li> <li>compared.</li> <li>3.27 Remediation</li> <li>The process of removing or reducing the level of microbial contamination in a product</li> </ul>	228	Method of analysis whose response is either the presence or absence of the analyte
<ul> <li>Method of analysis whose response is the amount (count or mass) of the analyte</li> <li>measured either directly (e.g., enumeration in a mass or a volume), or indirectly</li> <li>(e.g., color absorbance, impedance, etc.) in a specified test portion.</li> <li>3.26 Reference Method</li> <li>Preexisting recognized analytical method against which the candidate method will be</li> <li>compared.</li> <li>3.27 Remediation</li> <li>The process of removing or reducing the level of microbial contamination in a product</li> </ul>	229	detected either directly or indirectly in a specified test portion.
<ul> <li>measured either directly (e.g., enumeration in a mass or a volume), or indirectly</li> <li>(e.g., color absorbance, impedance, etc.) in a specified test portion.</li> <li>3.26 Reference Method</li> <li>Preexisting recognized analytical method against which the candidate method will be</li> <li>compared.</li> <li>3.27 Remediation</li> <li>The process of removing or reducing the level of microbial contamination in a product</li> </ul>	230	3.25 Quantitative Method
<ul> <li>(e.g., color absorbance, impedance, etc.) in a specified test portion.</li> <li>3.26 Reference Method</li> <li>Preexisting recognized analytical method against which the candidate method will be</li> <li>compared.</li> <li>3.27 Remediation</li> <li>The process of removing or reducing the level of microbial contamination in a product</li> </ul>	231	Method of analysis whose response is the amount (count or mass) of the analyte
<ul> <li>3.26 Reference Method</li> <li>235 Preexisting recognized analytical method against which the candidate method will be</li> <li>236 compared.</li> <li>237 3.27 Remediation</li> <li>238 The process of removing or reducing the level of microbial contamination in a product</li> </ul>	232	measured either directly (e.g., enumeration in a mass or a volume), or indirectly
<ul> <li>Preexisting recognized analytical method against which the candidate method will be</li> <li>compared.</li> <li>3.27 Remediation</li> <li>The process of removing or reducing the level of microbial contamination in a product</li> </ul>		
<ul> <li>236 compared.</li> <li>237 3.27 Remediation</li> <li>238 The process of removing or reducing the level of microbial contamination in a product</li> </ul>		
<ul> <li>237 3.27 Remediation</li> <li>238 The process of removing or reducing the level of microbial contamination in a product</li> </ul>		
238 The process of removing or reducing the level of microbial contamination in a product		•
239 to a level of compliance.		
	239	to a level of compliance.

240		3.28 Repeatability		
241		Precision under repeatability conditions. (ISO 5725-1)		
242		3.29 Repeatability Conditions		
243		Conditions where independent test results are obtained with the same method on		
244		equivalent test items in the same laboratory by the same operator using the same		
245		equipment within short intervals of time.		
246		3.30 Reproducibility		
247		Precision under reproducibility conditions. (ISO 5725-1)		
248		3.31 Reproducibility Conditions		
249		Conditions where independent test results are obtained with the same methods on		
250		equivalent test items in different laboratories with different operators using separate		
251		instruments.		
252		3.32 Robustness Study		
253		A study which tests the capacity of a method to remain unaffected by small but		
254		deliberate variations in method parameters and which provides an indication of its		
255		reliability during normal usage. (USP 31)		
256		3.33 Sterilization		
257		The process of complete elimination or destruction of all form of microbial life (both		
258		vegetative cells and spores) which is carried out by various physical or chemical		
259		methods.		
260		3.34 Test Portion		
261		A specified quantity of the material that is taken for analysis by the method.		
262	3.35 Unmatched Analyses			
263	Two or more analyses or analytical results on the same unknown material, which			
264		cannot be traced to the same test portion.		
265				
266				
267	4	Qualitative Methods—Technical Protocol for Validation		
268				
269	4.1	Method Developer Validation Study or Single-Laboratory Validation (SLV or		
270	Preco	llaborative) Study		
271				
272	4.1.1	Scope		
273		·		
274	The M	ethod Developer Validation Study is intended to determine the performance		
275	charad	cteristics of the candidate method. The study is designed to evaluate performance		
276	param	eters including inclusivity, exclusivity, and probability of detection (POD). For PTM and		
277	R <sup>2</sup> sub	missions, robustness, product consistency, and stability are also included. The Method		
278	Develo	oper Study is normally conducted in a single laboratory, usually the method developer's		
279	labora	tory. Alternatively, the method developer can contract the work to an independent		
280	site.			
281	The SI	V or Precollaborative Study is a formal submission requirement for OMA microbiology		
282	methods and is normally conducted in the method developer laboratory. It precedes the			
283		orative 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.

288

289 4.1.2 Inclusivity/Exclusivity Study

290 291

292

306

308

319

321

4.1.2.1 Species / Strain Selection

293 The choice of inclusivity strains should reflect the genetic and/or serological and/or 294 biochemical diversity of the organisms involved, as well as other factors such as 295 virulence, frequency of occurrence and availability. Select at least 50 pure strains of 296 the target organism(s) to be analyzed as pure culture preparations. For Salmonella 297 methods, the number of target organisms is increased to at least 100 serovars that are 298 selected to represent the majority of known somatic groups and subtypes of 299 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.
- 304Species/strains specified for use must be traceable to the source. The source and305origin of each species/strain should be documented.
- 307 4.1.2.2Study Design

309 Inclusivity strains are cultured by the candidate method enrichment procedure. The 310 target concentration for testing is 100 times the LOD50 of the candidate method. 311 Test one replicate per strain. Exclusivity strains are cultured in nonselective media. 312 The target level is the growth limit of the organism. Test one replicate per strain. If 313 the cross reactive strain is detected repeat the analysis using the enrichment 314 conditions prescribed in the candidate method. Report all results. 315 Inclusivity and exclusivity evaluations shall be performed together as one study. 316 Inclusivity and exclusivity test samples must be blind coded, randomized and 317 intermingled so the analysts cannot know the identity, sequence or concentration of 318 the test samples.

320 4.1.2.3Data 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: ..."

328 329 330 331 332 333 334 335		<ul> <li>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.</li> <li>4.1.2.4 In-Silico Analysis</li> <li>For molecular methods, see section 10 for in-silico analysis guidelines that can serve as supplemental information for the inclusivity study.</li> </ul>
336 337	4.1.3	
338 339 340		4.1.3.1 Reference Method
341 342 343 344 345 346 347		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.
348 349 350 351 352 353		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.
354 355 356		4.1.3.2 Matrix Categories
357 358 359 360 361 362		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.
362 363 364		4.1.3.3 Levels of Contamination
365 366 367 368 369 370 371		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

372 prepare a fourth sample targeting the fractional POD range. All outcomes for each 373 contamination level tested, whether fulfilling the POD requirement or not must be 374 reported. The target concentration for the fractional POD range is typically 0.2-2 CFU/test 375 376 portion for cannabis and cannabis products, depending on the matrix. 377 A 5-tube 3-level Most Probable Number (MPN) estimation of contamination levels (1) 378 must be conducted on the day that the analysis of test samples is initiated. The MPN 379 analysis scheme may also make use of the reference method replicates. See Annex A 380 for details. 381 382 If the method is intended to detect more than one target organism simultaneously 383 from the same test portion, the validation study should be designed so that target 384 organisms are inoculated into a common sample and the validation tests are 385 performed in a simultaneous manner. 386 4.1.3.4 Number of Test Portions 387 388 389 The number of replicate test portions method per level is 5 for the high inoculation 390 level, 20 for the fractional positive level and 5 for the uncontaminated level. If 391 naturally contaminated material is used, 2 lots/batches of 20 replicates should be 392 analyzed, and one lot must produce fractionally positive results. 393 394 4.1.3.5 Test Portion Size, Compositing and Pooling 395 396 Sample sizes required are as written in each method and/or SMPR. 397 398 Test portion compositing is the combining of test portions prior to enrichment and can 399 be validated alongside the standard test portion size if desired. The standard test 400 portion size is utilized for the reference method and the standard test portion size is 401 mixed with X uncontaminated test portions to create composite test portions for 402 validation by the candidate method. For example, if a candidate method is to be 403 validated for 375 g composites ( $15 \times 25$  g analytical units), then, for each level, one 404 set of 20 composited test portions are made by combining twenty single 25 g 405 inoculated test portions with twenty 350 g uninoculated test portions to form the 406 twenty 375 g composited test portions. These 375 g candidate method composites are 407 then compared to the 25 g reference method test portions. MPNs are performed only 408 on the batch samples from which the reference method test portions are taken. 409 Acceptance criteria for composited test portions are the same as for the standard test 410 portion size. 411 Pooling is the post-enrichment combining of aliquots from more than one enriched test 412 portion. This is validated by preparing replicate test portions for the candidate 413 method and replicate test portions for the reference method, either as matched or 414 unmatched test portions. At the conclusion of the enrichment procedure, test each 415 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

423 Naturally contaminated matrix is preferred as a source of inoculum, if available. An 424 effort should be made to obtain naturally contaminated matrix as it is most 425 representative of the method usage environment. If naturally contaminated matrix 426 cannot be found, then pure culture preparations may be used for artificial inoculation. 427 Numerous strains representing different serotypes or genotypes are required, if 428 applicable. Typically a different isolate, strain, biovar or species is used for each 429 matrix. The product inoculation should be conducted with a pure culture of one strain 430 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

435 436 Microorganisms in cannabis products are typically stressed, thus the 437 contaminating microorganisms are also stressed for these types of products. 438 Microorganism stress may occur at the time of inoculation or during preparation 439 of the product. Raw and cold-processed cannabis products should be inoculated 440 with unstressed organisms, heat-processed cannabis products with heat-441 stressed organisms (e.g., heat culture at 50°C for 10 min), and dry cannabis 442 and cannabis products with lyophilized culture. Mix well by kneading, stirring 443 or shaking as appropriate. Frozen cannabis products should be thawed, 444 inoculated, mixed and refrozen. 445 The degree of injury caused by heat stressing should be demonstrated, for 446 nonspore-formers, by plating the inoculum in triplicate on selective and 447 nonselective agars. The degree of injury is calculated as follows: 448 449  $(1-(n_{select}/n_{nonselect})*100)$ 450 451 where  $n_{select}$  = mean number of colonies on selective agar and  $n_{nonselect}$ 

451 where helect - mean number of colonies on selective again and honselect
452 = mean number of colonies on nonselective agar. The heat stress
453 must achieve 50-80% injury of the inoculum. The inoculum should be added to
454 the sample, mixed well and allowed to equilibrate in the matrix for 48-72 h at
455 4C for refrigerated cannabis products, for a minimum of 2 weeks at -20C for
456 frozen cannabis products, or for a minimum of 2 weeks at room temperature
457 for dried cannabis and cannabis products prior to analysis.

458

420

421

422

431 432

433 434

460 4.1.3.8 Preparation of Naturally Contaminated Samples

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

466 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 sufficient to demonstrate this
recovery in one matrix. This requirement may be satisfied in the SLV
(Precollaborative) Study. The competitor contamination levels, which may be naturally
occurring or artificially introduced, should be 10 times higher than the target
microorganism.

4.1.3.10 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

486 Each level of each matrix must be analyzed and reported separately. The following 487 section describes the data analysis to be performed according to the POD model. It is 488 acceptable to analyze data according to the Chi Square statistical methodology for 489 paired studies, and the Relative Limit of Detection (RLOD) for unpaired studies, as 490 defined in the current revision of ISO 16140. Refer to ISO 16140 for detailed 491 descriptions of Chi Square and RLOD.

493

492

494 495

496

497 498

499

461

465

467

475

476

477

482 483 484

485

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)
- 500POD is the proportion of positive analytical outcomes for a qualitative method501for a given matrix at a given analyte level or concentration. POD is502concentration dependent.

503		The POD estimate is calculated as the number of positive outcomes divided by			
504		the total number of trials.			
505		Estimate the POD with a 95% confidence interval for the candidate method, the			
506		reference method and, if included, the presumptive and confirmed results. See			
507		Annex C for details.			
508					
509		4.1.3.11.3 Difference of Probabilities of Detection (dPOD)			
510					
511		Difference of probabilities of detection is the difference between any two POD			
512		values.			
513					
514					
515		Estimate the dPODC as the difference between the candidate method and			
516		reference method POD values. Calculate the 95% confidence interval on the			
517		dPODC.			
518		drobe.			
519		dPODC = PODC - PODR			
520		Estimate the dDODCD as the difference between the readileter means the			
521		Estimate the dPODCP as the difference between the candidate presumptive			
522		result POD (PODCP) and the candidate confirmed result POD (PODCC) values.			
523		Calculate the 95% confidence interval on the dPODCP. See Annex C for details.			
524					
525		dPODCP = PODCP - PODCC			
526					
527		If the confidence interval of a dPOD does not contain zero, then the difference			
528		is statistically significant at the 5% level.			
529					
530					
531		4.1.3.11.4 Summary Data Tables			
532					
533		For all matrices and levels, use the summary table from Annex D.			
534					
535		4.1.3.11.5 Graph of Data			
536					
537		For each matrix, graph POD <sub>R</sub> , POD <sub>c</sub> , and dPOD by level with 95% confidence			
538		intervals. See example in Annex E.			
539					
540					
541	<u> </u>	Robustness Study [Performance Tested Methods (PTM) or R <sup>2</sup> submissions only]			
542	7.1.7	Robustiless study [i erformance rested methods (i fm) of R submissions only]			
543		4.1.4.1 Strain Selection			
543 544		ד. ו. ד. ו שנו מווי שבובכנוטוו			
544 545		Pobustness strains are propared and analyzed as vegetative cells, speces or			
		Robustness strains are prepared and analyzed as vegetative cells, spores or			
546		components thereof as applicable to the candidate method. One material is tested at			

547		a level that yields fractional recovery and one nontarget material is analyzed at the		
548	growth level achieved in a nonselective broth or at a high inoculation level.			
549				
550		4.1.4.2 Study Design		
551				
552		Minor, reasonable variations in a method of a magnitude that might well be expected		
553		to occur when the method is used are deliberately introduced and tested. Variations in		
554		method parameters that can be influenced by the end user should be tested. Use a		
555		screening factorial experimental design.		
556		The method developer is expected to make a good faith effort to choose parameters		
557		that are most likely to affect the analytical performance and determine the range of		
558		variations that can occur without adversely affecting analytical results.		
559		Ten replicates of each material are tested for each treatment combination.		
560				
561		4.1.4.3 Data Analysis and Reporting		
562				
563		The results are analyzed for variable detection due to changes in parameter settings.		
564		Report the appropriate statistical measures of the measured variable(s) (e.g., Ct,		
565		absorbance, POD value, etc.) for each set of replicates for each treatment		
566				
	combination. This should include at least means, standard deviations, and confidence			
567 569		intervals where appropriate.		
568	4.2	Independent Validation Study		
569 570	4.2	Independent Validation Study		
570	4.2.4	( come		
571	4.2.1	Scope		
572	م ، رواند ا	detion study to complements the end time menute shteined by the method developer and		
573		dation study to corroborate the analytical results obtained by the method developer and		
574	-	vide additional single laboratory data. The independent validation study traditionally		
575		es POD in the hands of an independent trained user and is required for PTM or $R^2$		
576	certifi	cation and OMA approval.		
577				
578	4.2.2	Reference Method		
579				
580		e is a reference method, then the candidate method is compared to a reference		
581	method. The reference method should be the same as that used in the Method Developer			
582	Study.			
583				
584	4.2.3	Matrices		
585				
586	The in	dependent laboratory must test at least one matrix that was tested in the Method		
587	Develo	oper Study. The total number of matrices to be evaluated by the independent		
588	labora	tory is dependent on the claim of the candidate method. For every PTM or R <sup>2</sup> Validation		
589	Study,	one Independent Study is made by the appropriate method volunteer(s) in consultation		
590		he Study Director and relevant SMPRs.		

593

600

602

604

592 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.

- 601 4.3 Collaborative Study (CS)
- 603 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.

608 609

4.3.2 Number of Laboratories

610

611 At least 12 collaborators per matrix should be included due to potential failure to follow 612 protocol. A minimum of 10 valid laboratory data sets per matrix are required.

613

614 4.3.3 Reference Method

615

616 The reference method used in the Collaborative Study must be the same as that used in the 617 Method Developer Study or SLV (PCS). The reference method should be carried out by the 618 organizing laboratory and collaborators.

619

620 4.3.4 Matrix Selection

621

622 At least one matrix from those studied in the PTM or PCS shall be chosen by the appropriate 623 volunteer expert(s) in consultation with the Study Director for collaborative study. For 624 methods with more than one sample preparation/enrichment, one matrix per procedure may 625 be required in the collaborative study. The determination if the procedures differ 626 significantly to warrant expanding the collaborative study is made by the appropriate method 627 volunteer expert(s) in consultation with the Study Director. The Statistical Advisor and 628 reviewers can be consulted during this determination. Examples of what constitutes a 629 different sample preparation procedure would include different test portion size, different 630 enrichment media or conditions, different dilution volume and different homogenization 631 equipment. The AOAC appropriate method volunteer, Statistical Advisor and collaborative 632 study protocol reviewers shall make the final selection of the matrix(es) with consideration of 633 the PTM or PCS data and the relative importance of the matrices to food safety. The data

- 634 from both the PCS and CS studies form the basis for defining the method applicability 635 statement.
- 636

637 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.

643

645

644 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 blindcoded when sent to participating collaborators for analysis.

649

650 4.3.7 Test Portion Size, Compositing and Pooling

651

652 Sample sizes required are as written in each method.

653 Test portion compositing is the combining of test portions prior to enrichment and can be 654 validated alongside the standard test portion size if desired. The standard test portion size is 655 utilized for the reference method and the standard test portion size is mixed with X

656 uncontaminated test portions to create composite test portions for validation by the

657 candidate method. For example, if a candidate method is to be validated for 375 g

658 composites (15 × 25 g analytical units), then, for each level, one set of 20 composited test

659 portions are made by combining twenty single 25 g inoculated test portions with twenty 350 g

660 uninoculated test portions to form the twenty 375 g composited test portions. These 375 g

661 candidate method composites are then compared to the 25 g reference method test portions.

- 662 MPNs are performed only on the batch samples from which the reference method test 663 portions are taken. Acceptance criteria for composited test portions are the same as for the
- 664 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

672 for the standard test portion analyses.

673

674 4.3.8 Source of Contamination

675 Refer to 4.1.3.6.

676

677 4.3.9 Preparation of Artificially Contaminated Samples

678 679	Refer to 4.1.3.7.				
680 681	4.3.10 Preparation of Naturally Contaminated Samples Refer to 4.1.3.8.				
682					
683	4.3.11 Confirmation of Test Portions				
684	Follow the reference method or confirmation procedure as written for isolation and				
685	confirmation of typical colonies from all candidate method test portions regardless of				
686	presumptive result.				
687 682	4.2.42 Data Analysis and Danasting				
688	4.3.12 Data Analysis and Reporting				
689 600	Each concentration level of each matrix must be analyzed and reported separately. Data may				
690	be excluded due to an assignable cause if sufficient justification is provided. Excluded data				
691 692	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				
692 693	analyze data according to the Chi Square statistical methodology for paired studies, and the				
693 694	RLOD for unpaired studies, as defined in the current revision of ISO 16140. Refer to ISO 16140				
695	for detailed descriptions of Chi Square and RLOD.				
696					
697	4.3.12.1 Raw Data Tables				
698					
699	For each matrix and concentration level, report each result from each test portion				
700	separately. See Annex B for raw data table format.				
701					
702	4.3.12.2 Estimate of Repeatability				
703					
704	Estimate the repeatability standard deviation (sr) for qualitative methods according to				
705	Annex F.				
706					
707	4.3.12.3 Estimate of Reproducibility				
708					
709	Cross-laboratory estimates of probabilities of detection and their differences depend				
710	upon an assumption that the same performance is achieved in each laboratory. This				
711	assumption must be tested and the laboratory effect estimated. If the effect is large,				
712	method performance cannot be expected to be the same in two different laboratories.				
713	For each matrix and level, calculate the standard deviation of the laboratory POD				
714	values (sPOD) and associated 95% confidence interval to estimate the reproducibility.				
715	See Annex F for details.				
716					
717	4.3.12.4 Cross-Laboratory Probability of Detection (LPOD)				
718					
719	Report the LPOD estimates by matrix and concentration with 95% confidence intervals				
720	for the candidate method and, if included, the presumptive and confirmed results. See				
721	Annex F for details.				

700						
722		4 D 4 D F	Difference of Green Laboratory, Duchability of Datastics (JLDOD)			
723		4.3.12.5	Difference of Cross-Laboratory Probability of Detection (dLPOD)			
724 705		Difference a	republicity of detection is the difference between any two I DOD values			
725		Difference probability of detection is the difference between any two LPOD values.				
726			e dLPODC as the difference between the candidate and reference LPOD			
727			ulate the 95% confidence interval on the dLPODC.			
728			e dLPODCP as the difference between the presumptive and confirmed			
729			. Calculate the 95% confidence interval			
730			DCP. See Annex F for details. If the confidence interval of a dLPOD does			
731		not contain :	zero, then the difference is statistically significant.			
732		4 2 42 4	Construction Data Tables			
733		4.3.12.6	Summary Data Tables			
734						
735		For all matri	ices and levels, use the summary table from Annex G.			
736		4 2 4 2 7				
737		4.3.12.7	Graph of Data			
738						
739			trix, graph PODR, LPODC, and dLPODC by level with 95% confidence			
740		intervals. Se	e example in Annex E.			
741	-	<b>o</b>				
742	5	Quantitative	Methods—Technical Protocol for Validation			
743	F 4		alaman Malidatian Ctudu an CLM (Dracallahanativa) Ctudu			
744	5.1	Method Deve	eloper Validation Study or SLV (Precollaborative) Study			
745	<b>Г</b> 4 4	<b>C</b>				
746	5.1.1	Scope				
747		athed Develo	any Validation Chudu is intended to determine the performance of the			
748 740		-	per Validation Study is intended to determine the performance of the			
749 750			The study is designed to evaluate performance parameters including			
750 751			ity, repeatability, bias, and robustness. The Method Developer Study is			
751 752		•	I in a single laboratory, usually the method developer's laboratory.			
752		•	nethod developer can contract the work to an independent site.			
753 754		•	prative) Study is a formal submission requirement for OMA microbiology			
755	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					
756		•	of a proposed OMA microbiology method by demonstrating the			
756		•	e method to various food categories. For OMA methods, the applicability			
758		-				
759	statement immediately follows the method title. The applicability statement for microbiological methods is generally concerned with target analyte and food type coverage.					
760	mero	הוסנטצוכמו ווופו	הוסטה וה ברובי מוני נטונביוובט שונוו נמוצבי מומנענד מוט וטטט נעדב נטיפומצל.			
761	5.1.2	Inclusivity/	Fyclusivity			
762	5.1.2	inclusivity/ i				
763	This r	equirement is	not applicable to total viable count or similar total enumeration methods			
764		that are not directed at specific microorganisms. The requirement applies to selective or				
765	differential quantitative methods.					
, 00	unici	child quantity				

- 766
- 767 5.1.2.1 Strain Selection
- 768

769 The choice of inclusivity strains should reflect the genetic and/or serological and/or

770 biochemical diversity of the target organism(s). Select at least 50 pure strains of the target

771 organism(s) to be analyzed as pure culture preparations. For Salmonella methods, the number

772 of target organisms is increased to at least 100 serovars that are selected to represent the

- 773 majority of known somatic groups and subtypes of Salmonella.
- 774 The choice of exclusivity strains should reflect closely related, potentially cross-reactive 775 organisms. Other factors such as virulence, frequency of occurrence and availability should be
- 776 considered. Select at least 30 pure strains of potentially competitive organisms.
- 777 Species/strains specified for use must be traceable to the source. The source and origin of 778 each species/strain should be documented.
- 779 780 5.1.2.2

781

- 782 Inclusivity strains are cultured in nonselective media. The target concentration for testing is 783 100 times the LOD50 of the method. Test one replicate per strain.
- 784 Exclusivity strains are cultured in nonselective media. The target level is the growth limit of 785 the organism. Test one replicate per strain.
- 786 Inclusivity and exclusivity evaluations shall be performed together as one study. Inclusivity
- 787 and exclusivity test samples must be blind coded and intermingled so the analysts cannot 788 know the identity or concentration of the test samples.
- 789
- 790 5.1.2.3 Data Reporting

Study Design

791

- 792 Report inclusivity data as number of strains detected. For example, "Of the 50 specific 793 inclusivity strains tested, 47 were detected and 3 were not detected. Those strains not 794 detected were the following: ..."
- 795 Report exclusivity data as number of strains not detected. For example, "Of the 30 specific 796 exclusivity strains tested, 28 were not detected and 2 were detected. Those detected were 797 the following: ..."
- 798

799 The study report should include a table titled "Inclusivity/ Exclusivity Panel Results," which 800 lists all strains tested, their source, origin and essential characteristics plus testing outcome. 801

- 802
- 5.1.2.4 In-Silico Analysis 803
- 804 For molecular methods, see section 10 for in-silico analysis guidelines that can serve as 805 supplemental information for the inclusivity study.

806

807 5.1.3 Matrix Study

808

809 5.1.3.1 Reference Method

811 Candidate methods are compared to a cultural reference method, where applicable. The 812 following are examples of sources of acceptable reference methods: AOAC OMA, U.S. Food 813 and Drug Administration Bacteriological Analytical Manual (BAM), U.S. Department of 814 Agriculture-Food Safety and Inspection Service Microbiology Laboratory Guidebook (MLG) (for 815 meat and poultry products), International Organization for Standardization (ISO) and Health 816 Canada Compendium of Analytical Methods, USP, Dairy Standard Methods. 817 818 Recognizing that there may be a lack of reference methods available for the cannabis matrix, 819 guidance in AOAC Standard Method Performance Requirements should be followed in 820 conjunction with reputable reference method sources. When appropriate, Method developers 821 should coordinate with the study director and/or consultant for best practices to be followed. 822 823 5.1.3.2 Matrix Categories 824 825 AOAC INTERNATIONAL recognizes claims for the range of specific cannabis matrices 826 successfully validated in the Method Developer Study, or the PCS and CS. The number of 827 different matrices required for testing depends on the applicability of the method. All 828 claimed matrices must be included in the Method Developer Study and the PCS. See section 7 829 for guidance on matrix categorization. 830 831 5.1.3.3 Levels of Contamination 832 833 For the artificially contaminated food types, three inoculated levels (high, medium, and low) 834 and one uninoculated level are required. For naturally contaminated food, three 835 contamination levels (high, medium, and low) are required, and no uninoculated level. The 836 low level should be near the limit of detection, and the medium and high levels should cover 837 the analytical range of the candidate method. If the claimed range of the method is greater 838 than 4 logs, intermediate levels may be required at the discretion of the appropriate method 839 volunteer(s) in consultation with the Study Director. 840 If the method is intended to detect more than one target organism simultaneously from the 841 same test portion, the validation study should be designed so that target organisms are 842 inoculated into a common sample and the validation tests are performed in a simultaneous 843 manner. 844 845 5.1.3.4 Number of Test Portions 846 847 For each level, analyze five test portions by the candidate method and five test portions by 848 the reference method (or confirmatory method). 849 850 5.1.3.5 Source of Contamination 851 852 Naturally contaminated matrix is preferred as a source of inoculum, if available. Inoculating 853 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.

- 858 5.1.3.6 Preparation of Artificially Contaminated Samples
- 859

857

860 Microorganisms in cannabis products are typically stressed, thus the contaminating 861 microorganisms are also stressed for these types of products. Microorganism stress may occur 862 at the time of inoculation or during preparation of the product. Raw and cold-processed 863 cannabis products should be inoculated with unstressed organisms, heat-processed cannabis 864 products with heat-stressed organisms (e.g., heat culture at 50°C for 10 min), and dry 865 cannabis and cannabis products with lyophilized culture. Mix well by kneading, stirring or 866 shaking as appropriate. Frozen cannabis products should be thawed, inoculated, mixed and 867 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:

871

872  $(1-(n_{select}/n_{nonselect})*100$ 

873

874 where  $n_{select}$  = mean number of colonies on selective agar and  $n_{nonselect}$ 

875 = mean number of colonies on nonselective agar. The heat stress must achieve 50-80% injury
876 of the inoculum. The inoculum should be added to the sample, mixed well and allowed to
877 equilibrate in the matrix for 48-72 h at 4C for refrigerated cannabis products, for a minimum
878 of 2 weeks at -20C for frozen cannabis products, or for a minimum of 2 weeks at room

879 temperature for dried cannabis and cannabis products prior to analysis.

880 881

882 5.1.3.7 Use of Artificially and Naturally Contaminated Test Samples

883

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

886 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

- 889 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.
- 892
- 893 5.1.3.8 Need for Competitive Flora
- 894

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

898 899 900 901 902 903	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				
903 904	5.1.3.9 Confirmation of Test Portions				
905 906 907	Follow the reference method or confirmation procedure as written for isolation and confirmation of typical colonies from all candidate method test portions.				
908 909	5.1.3.10 Data Analysis and Reporting				
910 911	5.1.3.10.1 General Considerations				
912 913 914 915	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:				
916 917	Log10 [CFU/unit + (0.1)f]				
918 919 920	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.				
921 922	5.1.3.10.2 Initial Review of Data				
923 924 925 926 927	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.				
928 929 930	5.1.3.10.3 Outliers				
931 932 933 934 935 936 937 938 939 939 940 941	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.				

- 942 5.1.3.10.4 Repeatability (sr)
- 943
- 944 Calculate repeatability as the standard deviation of replicates at each concentration of each 945 matrix for each method.
- 946 947 5.1.3.10.5 Mean Difference Between Candidate and Reference Where Applicable 948 Report the mean difference between the candidate and reference method transformed 949 results and its 95% confidence interval. In addition, report the reverse transformed mean 950 difference and confidence interval in CFU/unit or spores/mL.
- 951 952
- 953 5.1.4 Robustness Study (PTM submissions only)
- 955 5.1.4.1 Strain Selection
- 956

- 957 Robustness strains are prepared and analyzed as vegetative cells, spores or components 958 thereof as applicable to the candidate method. One target strain is tested using the 959 candidate method enrichment at a high and low level within the quantitative range of the 960 candidate method. One nontarget strain is enriched in a nonselective broth and tested at the 961 high level.
- 962
- 963 5.1.4.2 Study Design
- 964
- 965 Minor, reasonable variations in a method of a magnitude that might well be expected to occur 966 when the method is used are deliberately introduced and tested. Variations in method 967 parameters that can be influenced by the end user should be tested. Use a screening factorial
- 968 experimental design.
- 969 The method developer is expected to make a good faith effort to choose parameters that are
- 970 most likely to affect the analytical performance and determine the range of variations that 971 can occur without adversely affecting analytical results.
- 972 Five replicates at each target concentration and five replicates of the nontarget are tested 973 for each factorial pattern.
- 974
- 975 5.1.4.3Data Analysis and Reporting
- 976
- 977 The results are analyzed for effects on bias and repeatability. Standard deviations (sr) at each 978 concentration are compared to determine if any robustness parameter value causes more 979 than a 3-fold increase in sr.
- 980
- 981 5.2 Independent Validation Study
- 982
- 983 5.2.1 Scope
- 984

985 A validation study to corroborate the analytical results obtained by the method developer and 986 to provide additional single laboratory data. The independent validation study traditionally 987 verifies repeatability in the hands of an independent trained user. 988 989 5.2.2 Reference Method 990 991 If there is a reference method (or confirmatory method), then the candidate method is 992 compared to a reference method. The reference method should be the same as that used in 993 the method developer study. 994 995 5.2.3 Matrices 996 997 The independent laboratory must test at least one matrix that was tested in the Method 998 Developer Study. The total number of matrices to be evaluated by the independent 999 laboratory is dependent on the claim of the candidate method. For every cannabis or 1000 cannabis product type claimed, one cannabis or cannabis product matrix shall be included in 1001 the independent study. The choice of matrices for the Independent Study is made by the 1002 appropriate method volunteer(s) in consultation with the Study Director. 1003 1004 5.2.4 Study Design 1005 1006 The study design for validation of quantitative methods in the independent study follows the 1007 Method Developer Validation Study design. Contamination levels, number of test portions, source of contamination, preparation of samples, confirmation of test portions, and data 1008 1009 analysis and reporting are found in Section 5.1.3. 1010 1011 5.3 Collaborative Study (CS) 1012 1013 5.3.1 Scope 1014 1015 The Collaborative Study (CS) is a formal submission requirement for OMA methods and 1016 succeeds the SLV (Precollaborative) Study. The purpose of the Collaborative Study is to 1017 estimate the reproducibility and determine the performance of the candidate method among 1018 collaborators. 1019 1020 5.3.2 Number of Laboratories 1021 1022 A minimum of eight collaborators reporting valid data for each cannabis or cannabis product 1023 type is required. It is suggested that at least 10-12 collaborators begin the analysis. 1024 1025 5.3.3 Reference Method 1026

1027 Candidate methods are compared to a reference method (or confirmatory method) where 1028 applicable. The reference method(s) used in the collaborative study must be the same as 1029 those used in the SLV (Precollaborative) Study.

1031 5.3.4 Matrix Selection

1032 1033

1034

1035

1036

1037

1038

1039

1040

1030

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

1041 or conditions, different dilution volume and different homogenization equipment. The 1042 appropriate AOAC method volunteer(s) shall make the final selection of the matrix(es) with 1043 consideration of the PTM or PCS data and the relative importance of the matrices to food 1044 safety. The data from both the PCS and CS studies form the basis for defining the method 1045 applicability statement.

- 1046
- 1047 5.3.5 Levels of Contamination

1048

1049 For the artificially contaminated cannabis or cannabis product types, three inoculated levels 1050 (high, medium, and low) and one uninoculated level are required. For naturally contaminated 1051 cannabis or cannabis product, three contamination levels (high, medium, and low) are 1052 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 1053 1054 claimed range of the method is greater than 4 logs, intermediate levels may be required at 1055 the discretion of the appropriate method volunteer(s) in consultation with the Study Director. 1056 If the method is intended to detect more than one target organism simultaneously from the 1057 same test portion, the validation study should be designed so that target organisms are 1058 inoculated into a common sample and the validation tests are performed in a simultaneous 1059 manner.

1060

1061 5.3.6 Number of Test Portions

1062

1063 For each contamination level, two test portions are analyzed by the candidate method and

1064 1065

1067

1066 5.3.7 Enumeration of Specific Microorganisms

1068 If the candidate method is for quantitation of a specific microorganism, it may be necessary 1069 to include certain cannabis or cannabis product types known to support the growth of such 1070 analytes. The inoculating microorganisms must represent different genera, species and/or

two test portions are analyzed by the reference method by each collaborator.

1071	toxin-producing microorganisms that are intended to be included in the method applicability				
1072	statement. The choice of strains should be broad enough to represent the inherent variation				
1073	in the microorganisms of interest.				
1074					
1075	5.3.8 Source of Contamination				
1076					
1077	Refer to s	ection 5.1.3.5.			
1078					
1079	5.3.9 Pre	eparation of Artificially Contaminated Samples			
1080					
1081	Refer to se	ection 5.1.3.6.			
1082					
1083	5.3.10 Use	e of Artificially and Naturally Contaminated Test Samples			
1084					
1085	The use of	f both naturally and artificially contaminated test samples is strongly encouraged.			
1086	Because n	aturally contaminated cannabis and cannabis products are not always available			
1087	particular	ly for methods applicable to specific microorganisms, artificially contaminated test			
1088	samples m	nay be used.			
1089					
1090	5.3.11 Co	nfirmation of Test Portions			
1091					
1092	Follow the reference method (or confirmatory method) as written for isolation and				
1093	confirmation of typical colonies from all candidate method test portions.				
1094					
1095	5.3.12 Data Analysis and Reporting				
1096					
1097	For a detailed explanation of the quantitative method calculations to be performed, refer to				
1098	Appendix D (3).				
1099					
1100	5.3.12.1	General Considerations			
1101					
1102	Data ofter	n do not show a statistically normal distribution. In order to normalize the data,			
1103	perform a logarithmic transformation on the reported CFU/unit (including any zero results) as				
1104	follows:				
1105					
1106	Log10 [CF	U/unit + (0.1)f]			
1107					
1108	where f is	the reported CFU/unit corresponding to the smallest reportable result, and unit is			
1109	the reported unit of measure (e.g., g, mL, 25 g). For details, see Annex H.				
1110	the reported unit of measure (e.g., g, mL, 25 g). For details, see Annex H.				
1111	5.3.12.2	Initial Review of Data			
1112	5.5.12.2				
1113	Plot the c	andidate method result versus the reference method (or confirmatory method)			
1114		e vertical y-axis (dependent variable) is used for the candidate method and the			
-					

1115 1116	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				
1117	apparent.				
1118	Construct a Youden plot. For a given matrix-level combination, plot replicate pairs as first				
1119		rsus second replicate. Usually major discrepancies will be apparent: displaced			
1120	•	uly spread replicates, outlying values, differences between methods, consistently			
1121	high or low laboratory rankings, etc.				
1122	-	ata should be included in the statistical analysis.			
1123	only valid d	ata shouta be metadea in the statistical analysis.			
1124	5.3.12.3	Outliers			
1125	5.5.12.5	oddiers			
1126	lt is often di	ifficult to make reliable estimations (average, standard deviation, etc.) with a			
1127		nd in presence of outliers. Data should be examined to determine whether any			
1128		hows consistently high or low values or an occasional result that differs from the			
1120	•	data by a greater amount than could be reasonably expected or found by chance			
1130		orm outlier tests (Cochran and Grubbs) in order to discard the outlying values and			
1131		better estimate (3). There must be an explanation for every excluded data set; no			
1132		n be excluded on a statistical basis only. To view the data adequately, construct a			
1133	stem-tear u	splay, a letter-value display, and a boxplot (4).			
1134	E 2 42 4	Derfermence Indicators			
1135	5.3.12.4	Performance Indicators			
1136	Derfermene	a indiantary for an artitative mathada include reportability and reproducibility			
1137		e indicators for quantitative methods include repeatability and reproducibility			
1138	standard de	viations of the transformed data.			
1139					
1140	5.3.12.4.1	Repeatability (sr)			
1141					
1142	Calculate repeatability as the standard deviation of replicates at each concentration of each matrix for each laboratory.				
1143	matrix for e	ach laboratory.			
1144	F 2 42 4 2				
1145	5.3.12.4.2	• • • •			
1146	Calculate reproducibility as the standard deviation of replicates at each concentration for				
1147	each matrix	across all laboratories.			
1148	F 2 42 F				
1149	5.3.12.5	Mean Difference between Candidate and Reference Methods Where Applicable			
1150					
1151	•	mean difference between the candidate and reference method transformed			
1152		its 95% confidence interval. In addition, report the reverse transformed mean			
1153	difference a	Ind confidence interval in CFU/unit.			
1154	/ - /				
1155	5.3.12.6	Calculations			
1156					
1157	For details,	refer to Appendix D (3).			
1158					

1159	6	Confirmatory Methods
1160		
1161 1162		6.1 Reference ISO 16140-6:2019
1163	7	Matrix Categorization
1164	/	Mati IX Categorization
1165	7.1	Scope
1166	7.1	эсоре
1167	The f	ollowing matrix categories and subcategories are intended to provide a guidance for
1168		od validation study design. The list is non-exhaustive, and per SMPR guidance and
1169		od specific requirements, matrices and subcategories that are included in the study
1170		n may need to be extended or modified.
1171		
1172	7.2	Matrix Categories and Subcategories
1173		
1174		7.2.1 Plant/Flower
1175		Buds
1176		Kief*
1177		Joints (pre-rolls)
1178		Fresh Frozen
1179		Shake/Trim
1180		7.2.2 Concentrates
1181		Isolate
1182		Shatter/Wax
1183		Vape oil/cartridges
1184		Distillate
1185		Hash/Rosin
1186		Kief*
1187		7.2.3 Infused Edibles
1188		Chocolate bars
1189		Baked Goods
1190		Gummies
1191		Tinctures
1192		Capsules/pills
1193		Beverages
1194		7.2.4 Infused Non-Edibles Lotion
1195		
1196		Balm/salve Bath bombs/bath salts
1197 1198		Transdermal Patches
1198		Oils (topical)
1200		ons (topical)
1200		

1201 \*Matrix classification varies depending on regional designation. Matrix should be 1202 classified under the category appropriate to the study and can only qualify as a 1203 required matrix for a single matrix category. 1204 1205 Inclusion of matrices outside of those listed above may be used for validation 1206 purposes, however, these matrices will be considered supplemental to the minimum 1207 number of matrices required by method-specific SMPRs. 1208 1209 1210 Decontamination of Matrix 8 1211 1212 8.1 Scope 1213 1214 The conformity assessment programs of AOAC INTERNATIONAL require candidate 1215 methods to be evaluated for all matrices claimed in the method's scope. The 1216 decontamination and sterilization techniques described in this section are most 1217 applicable to cannabis plant material. Testing can be performed using materials 1218 naturally contaminated or artificially inoculated with the target analyte(s). Matrix 1219 study designs are based on requirements outlined in official validation guidelines (e.g., 1220 Official Methods of Analysis SM Appendix J: AOAC INTERNATIONAL Methods Committee 1221 Guidelines for Validation of Microbiological Methods for Food and Environmental 1222 Surfaces) and/or Standard Method Performance Requirements (SMPRSM). These 1223 validation studies are designed to challenge the methods at the Limit of Detection 1224 (LOD50) for gualitative methods or Limit of Quantitation (LOQ) for guantitative 1225 methods. 1226 Qualitative method analysis requires the evaluation of multiple lots of a single matrix 1227 when naturally contaminated or multiple contamination levels (control, 1228 low/fractional, high) of a single lot when artificially contaminated. In both instances, 1229 test portions producing fractional positive results, those in which the low level (or 1 of 1230 the 2 naturally contaminated lots) produces 25-75% positive results, is required. For 1231 quantitative methods, testing should challenge the method at the LOQ and data should 1232 be obtained that spans the range of 2 logs (e.g., ~10 CFU/g, ~100 CFU/g, and ~1,000 1233 CFU/g). Obtaining these levels can be difficult to achieve and may require matrix 1234 manipulation, including reducing the presence of naturally occurring microbial 1235 bioburden to achieve fractional results (qualitative) or low levels (quantitative). 1236 Decontamination processes need to be thorough enough to reduce the bioburden to the levels required for validation/verification but should not impact the overall 1237 1238 integrity of the matrix. For cannabis flower, this means ensuring that key components 1239 of the plant, potency, moisture content, terpene profiles are not drastically altered. 1240 This document discusses various approaches to decontaminating cannabis matrices for 1241 the purposes of microbial method validation/verification and provides

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

1244 The decontamination techniques presented here are well-established in other 1245 industries, have some baseline data to support their use in the cannabis industry, and 1246 can be performed without significantly altering the plant matrix.

1248	8.2 Decontamination	
1249	8.2.1 Irradiat	
1250	8.2.1.	1 Gamma irradiation
1251	i.	Uses gamma rays (high energy photons) produced during the
1252		decay of the radioisotope Cobalt 60 radiation.
1253	ii.	Treatment conditions vary from 0-20 kGy/min (kiloGray), and
1254		treatment times also vary.
1255	iii.	Pros: Effective kill rates, can penetrate organic matter for
1256		surface and internal decontamination, high sterility assurance,
1257		fewer variables to control, no significant impact on CBD and
1258		THC; no residue left on product
1259	iv.	Cons: Terpene losses up to 38% (Hazekamp 2016), expensive
1260	8.2.1.	2 Electron-beam (E-beam)
1261	i.	Product is bombarded with high-energy electrons produced by an
1262		electrical current; beta emitter
1263	ii.	Treatment times are usually shorter than gamma or X-ray;
1264		treatment conditions around 15 kW (KWs) and an energy
1265	iii.	Capacity of 5.25 megaelectronvolt (MeV)
1266	iv.	Pros: Effective kill rates, generally less harsh on/damaging, fast,
1267		high sterility assurance level, no residue left on product
1268	۷.	Cons: Limited penetration depth, expensive
1269	8.2.1.	3 X-ray irradiation
1270	i.	Uses an electron beam produced by a current and focus that
1271		beam on a specific metal, which creates X-rays (photons)
1272		through a process called Bremsstrahlung.
1273	ii.	Treatment conditions vary: Radsource suggests 3-7 hour
1274		treatment time with a total 2000 Gy dose
1275	iii.	Pros: Internal and external decontamination; CBD and THC
1276		preserved; no residue left on product, high sterility assurance
1277	iv.	Cons: Terpene content may be altered, expensive
1278	8.2.1.	4 UV light:
1279	i.	UV-C light is typically used for air sterilization as a preventative
1280		measure; however some DIY decontamination methods are to use
1281		UV-C light bulbs set up in the dry/cure room or in a UV-C
1282		disinfection chamber for 360 degree exposure.

1283	ii.	Pros: Easy to use, inexpensive, no residue left on produce,
1284	iii.	Cons: Varied microbial sensitivity, surface level only
1285		
1286	8.2.2 Electro	magnetic radiation (non-ionizing)
1287	8.2.2.	1 Microwave
1288	i.	Used infrequently but some processors combine microwave,
1289		vacuum and agitation to accelerate the dry/cure process
1290	ii.	Treatments vary in time and range from 3-30 GHz
1291	iii.	Pros: Good microbial reduction of external and internal
1292		microbial contaminants, no residue left on product
1293	iv.	Cons: Process creates heat, which can alter cannabinoid,
1294		terpene, and moisture content
1295	8.2.2.2	2 Radio frequency (RF)
1296	i.	Uses RF to create dipoles (molecules with separated positive and
1297		negative charges) and align in an electric field, causing rotation
1298		and heat
1299	ii.	Treatments conditions range from 100 MHz to <10 GHz (up to 10)
1300		and times vary
1301	iii.	Pros: Good microbial reduction of external and internal
1302		microbial contaminants, no residue left on product, longer
1303		wavelengths and penetration depth than microwaves
1304	iv.	Cons: Process creates heat, which can alter cannabinoid,
1305		terpene, and moisture content, process is dependent bound
1306		water
1307		
1308	8.2.3 Reactive	e oxygen species and ionized gases (surface decontamination
1309	methods)	
1310	8.2.3.	1 Ozone
1311	i.	Created by reacting oxygen through an electric current creating
1312		O3 (ozone)
1313	ii.	Treatment times vary and concentrations range from 10-1000
1314		ppm ozone. Generally 10-30 minute exposure.
1315	iii.	Pros: External microbial decontamination, does not alter
1316		cannabinoid or terpene profile, no residue left on product, fast
1317	iv.	Cons: Surface level decontamination, moisture loss I the flower
1318		matrix
1319	8.2.3.2	2 Hydrogen peroxide
1320	i.	Some cultivators submerge flower after harvest and allow to dry
1321	ii.	Vaporized H2O2 like TheBOX flash vaporizes aqueous hydrogen
1322		peroxide and distributes inside an enclosed chamber; treatment
1323		conditions and times vary
1324	iii.	Pros: surface contamination, no residual chemicals left on the
1325		plant matter, no significant impact on CBD, THC, or terpenes

1326	iv.	Cons: May increase water activity/moisture content to the plant		
1327		matter, may discolor product, mostly surface level		
1328		decontamination		
1329	8.2.3.3 Cold plasma			
1330	i.	Generated by high voltage current passed through air, creating a		
1331		mix of electrons, ions, photons, and free radicals; does not		
1332		exceed tens of degrees Celsius, making it "cold"		
1333	ii.	Treatment times and conditions vary		
1334	iii.	Pros: External microbial decontamination, no residue left on		
1335		product		
1336	iv.	Cons: Surface level decontamination		
1337				
1338	8.2.4 Heat tr	eatment		
1339	8.3.4.	1 Pasteurization		
1340	8.3.4.	2 Steam treatment		
1341	8.3.4.	3 Autoclaving		
1342	8.3.4.	4 Heating/baking: can decarboxylate flower and change		
1343	appea	rance and terpene profile		
1344				
1345	8.2.5 Cold te	mperature treatment		
1346	8.3.5.	8.3.5.1 Freeze drying		
1347	8.3.5.2 Fresh frozen			
1348				
1349	8.2.6 Carbon	dioxide, ethanol, butane, propane, etc. (extraction/remediation)		
1350	i.	Pros: These organic solvents will remove microbial contaminants		
1351		internally and externally		
1352	ii.	Cons: Extraction removes the majority of cannabinoids and		
1353		terpenes from the plant material, rendering mostly the insoluble		
1354		matter afterwards, which does not represent the original		
1355		material		
1356				
1357	8.3 Methods to Confi	irm Integrity of Product Maintained following Decontamination		
1358				
1359	To evaluate the effe	ctiveness of decontamination procedures, decontaminated		
1360	•	valuated using methods described below. It is recommended that		
1361	decontaminated product be analyzed with a minimum of the Microbial Burden,			
1362	Cannabinoid Profile and the Moisture Content/Water Activity in order to establish the			
1363	efficacy of the product decontamination and verification of the integrity of the			
1364	product post treatment. The Microbial Burden in the treated product should have a			
1365	reduction to a level	that meets the requirements of the method validation study		
1366	and/or is below the	regulatory limit of the governing body.		
1367	8.3.1 Microbi	al Burden		
1368				

1369 1370 1371 1372	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.
1373 1374 1375	8.3.1.1 Classical Determination of Microbiological Parameters in Cannabis Products
1376 1377 1378 1379 1380 1381 1382 1383 1384 1385 1386	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
1387 1388 1389 1390	8.3.1.2 Genetic Based Determination of Detection of species and/or groups known to be pathogenic
1391 1392 1393 1394 1395 1396 1397 1398 1399 1400 1401 1402 1403 1404 1405 1406	<ul> <li>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).</li> <li>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.</li> <li>iii. Indirect metabolite readings (e.g. Sol)</li> <li>iv. Sequencing</li> </ul>
1400 1407 1408 1409 1410 1411 1412	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

1413

1414 1415

1416 1417

1418

1419

1420

1421

1422

1423

1424 1425

1426

1427

1428

1429

1430

1431

1432

1433

1434

1435

1436

1437

1438

1439 1440

1441

1442

1443

1444

1445

1446

1447

1448

1449

1450

1451 1452 1453

1454

1455

1456

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

1457			iv.	Sensory evaluation
1458			٧.	Trichome integrity
1459				
1460				
1461	9	Suitability of Te	esting	g Media
1462		9.1 Scope		

1463 The present document is intended to give adequate information concerning the 1464 suitability of testing methods for cannabis-related products. The suitability test should 1465 demonstrate that tested products have no inhibitory effects on the growth of selected control microorganisms under aerobic conditions. In other terms, obtained results in 1466 recovery test media have to be completely representative: the designed suitability 1467 testing method should be able to neutralize all possible antimicrobial effects related to 1468 1469 the tested product. In numerical terms, the growth of control microorganisms has to be 1470 predictable and verifiable.

- 1471 Should any antimicrobial feature be present in the cannabis-related product under 1472 examination, one of the below-mentioned options would be carried out on the product 1473 before suitability testing:
- 1474 a) Dilution 1475
  - b) Filtration
  - c) Neutralization
    - d) Inactivation.

1478 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, 1479 fungistatic substances, etc.). Consequently, should the suitability test give 1480 unsatisfactory results, all analytical evaluations based on the used method would be 1481 1482 questioned, and a new suitability test would be required with the aim of neutralizing 1483 the inhibitory agent(s).

- 1484 In addition, should any modification in the testing method and/or in the cannabis-1485 related product be introduced with potential influence on analytical performance, the suitability test would be obligatorily repeated (confirmation needed). 1486
- 1487 It has to be noted that growth promotion testing procedures must be conducted in order to then conduct suitability testing, taking into account that: 1488
- 1489

1490

1493 1494

1476

- 1) Growth promotion testing should demonstrate that culture media can effectively 1491 1492 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.
- 1495 This document has been written taking into account:
- a) The United States Pharmacopeia (USP <51>, <60>, <61>, <62>, and <1111>) with 1496 1497 reference to general concepts concerning suitability of testing methods;

- 1498b) The United States Pharmacopeia (USP <2021>, <2022>, and <2023>) with1499reference to microbiological features of cannabis and cannabis-derived products1500as non-sterile nutritional and dietary supplements;
- 1501 c) The Bacteriological Analytical Manual and Pharmaceutical Microbiology Manual 1502 of the Food and Drug Administration;
- 1503 d) The International Pharmacopoeia;
- 1504 e) The European Pharmacopoeia;
- 1505 f) The "Guidelines for Assuring Quality of Medical Microbiological Culture Media" 1506 (the Australian Society of Microbiology, Inc., 2nd Edition, July 2012)
- 1507 g) The recent AOAC SMPR 2021.009; Version 9; June 17, 2021 (Method Name: 1508 Standard Method Performance Requirements for Viable Yeast and Mold).
- 1509 h) Current Good Manufacturing Practice (cGMP).

1510All operations have to be performed with the aim of avoiding microbial contamination1511of the product under examination, and also assuring that control microorganisms are1512not negatively influenced.

1513

## 1514 9.2 Preparation of Test Strains

1515 The following Table 1 shows standard microorganisms for suitability testing, and 1516 correlated preparation procedures (1-8). The listed microorganisms are provided as 1517 examples, but may not be required for all methods or applications. Method developers 1518 should coordinate with the study director and/or consultant for best practices to be 1519 followed.

- 1520Table 1: standard microorganisms for suitability testing, and correlated preparation1521procedures (1-8)
- 1522

Microorganism	Test strain (examples)	Preparation
Staphylococcus aureus	ATCC 6538	Casein soya bean digest agar
	NCIMB 9518	or
	CIP 4.83	Casein soya bean digest broth
	NBRC 13276	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

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

1523 1524 1525 1526 1527	<ul> <li>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:</li> <li>1) Buffered sodium chloride-peptone solution at pH 7.0, or</li> <li>2) Phosphate buffer at pH 7.2.</li> </ul>
1528 1529 1530	An exception has to be mentioned with concern to <i>A. brasiliensis</i> : 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.
1531	
1532	9.3 Suitability of the Method in the Presence of the Tested Product
1533 1534 1535 1536	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).
1537 1538 1539 1540 1541	<ul> <li>The enumeration method can be one of the below-mentioned systems, taking into account the nature of the tested products (1-8):</li> <li>1) Membrane Filtration</li> <li>2) Plate-Count methods</li> <li>3) The Most- Probable-Number (MPN) method.</li> </ul>
1542 1543 1544 1545	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.
1546 1547	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	Casein soya bean digest Agar	
	CIP 4.83 NBRC 13276	MPN casein soya bean digest broth	-
		Inoculum: ≤ 100 CFU Temperature: 30–35 °C	
		Incubation time: ≤ 3 days	
Pseudomonas aeruginosa	ATCC 9027 NCIMB 8626	Casein soya bean digest agar/MPN	
	CIP 82.118	casein soya bean digest broth	-
	NBRC 13275	Inoculum: ≤ 100 CFU Temperature: 30–35 °C	
		Incubation time: ≤ 3 days	

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

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

1548

### 9.3.1 Preparation of the Sample

In general, the physical and chemical features of the product under examination 1549 influence the correct sample preparation (1-8). Please note sample amounts can vary 1550 depending on the product category (please consider Table 3). With reference to the 1551 1552 descriptions, preparations, sampling, and testing procedures concerning all mentioned 1553 media and broths in this document, please use USP <2021> (Microbial enumeration 1554 tests - nutritional and dietary supplements) and <2022> (Microbiological procedures for absence of specified microorganisms - nutritional and dietary supplements) as 1555 reference guidelines (9-10). The following procedures can be recommended (a 1556 1557 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.
- 1579
- 1580 9.3.2 Inoculation and Dilution

1581The microbial suspension for suitability testing has to be added to the prepared sample1582and to a control (sample is absent). The inoculum has to be  $\leq 100$  CFU/g, and it should1583be  $\leq 1$  % of the total volume of the diluted product. In addition, microbial recovery can be1584acceptably demonstrated on condition that the prepared sample is tested with the lowest1585possible dilution factor, unless antimicrobial effects or poor solubility are demonstrable (1-15868)

- 1587Should antimicrobial effects need to be eliminated (see point 3.3), an adequate sample1588treatment dilution, filtration, neutralization, or inactivation would be necessary1589before inoculum.
- 1590Should plate counts be used, each dilution would be correlated with the result of two1591plates (test in duplicate).
- 1592

#### 1593 9.3.3 Neutralization/removal of antimicrobial activity

- 1594The possible inhibition of bacterial growth is demonstrable if there is a factor reduction1595> 2 considering (1-8):
- a) The count of recovered microorganisms from the sample, and
- b) The count of recovered microorganisms from the control.
- 1598With reference to solid culture media, the factor reduction should take into account the1599calculated value for the standardized inoculum.
- 1600With reference to MPN methods, the calculated number from inoculum has to be within160195 %-confidence limits (K =2) of obtained results with the control test.

- 1602 Should the inhibition be observed, the following strategies may be recommended: 1603 1) Augment the volume of used diluents (against these antimicrobials: alcohol, 1604 phenolics, aldehydes, sorbate) 1605 2) Augment the volume of used culture media 1606 3) Addition of a neutralizing agent to the diluents or culture media (sterilization is 1607 required - a blank test is required with neutralizer and without the tested sample) 1608 4) Membrane filtration 1609 5) A combination of above-mentioned strategies. 1610 With reference to neutralizers, the following choices can be shown here as examples: 1611 a) Sodium bisulfite (against glutaraldehyde) 1612 b) Glycine or thiosulfate (against aldehydes) 1613 C) Calcium and magnesium ions (against ethylenediaminetetraacetic acid or EDTA). 1614 1615 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 1616 1617 repeated with the highest dilution factor which could be compatible for observable 1618 microbial growth (1-8). See 9.3.5 for how to interpret these results. 1619 1620 1621 9.3.4 Recovery of microorganism in the presence of product 1622 **Membrane Filtration.** The use of membrane filters with pore sizing  $\leq 0.45 \mu m$  is 1623 preferred (cellulose nitrate filters for aqueous, oily and weakly alcoholic solutions; 1624 cellulose acetate filters for strongly alcoholic solutions). One membrane filter is used 1625 for each control microorganism. The quantity of sample (representing approximately 1 1626 gram of product, unless the calculable number of microorganism is excessive) has to be 1627 transferred and filtered immediately; subsequently, the membrane filter is rinsed with 1628 adequate amount of the used diluent (example: three 100-ml portions; maximum: five 1629 100-ml portions). In this way, antimicrobial residues on filter membranes would be probably removed (1-8). 1630 1631 Subsequently, control microorganisms are placed (< 100 CFU/g) into the last portion of
- 1631Subsequently, control microorganisms are placed (< 100 CFU/g) into the last portion of</th>1632rinse diluent; then, aseptically cut the filter membrane is subdivided aseptically into

1633 two equal parts. With relation to TAMC, the membrane filter sections are transferred to 1634 the surface of casein soya bean digest agar. With reference to TYMC, the membrane 1635 filter sections are transferred to the surface of Sabouraud-dextrose agar (1-8). 1636 Incubation conditions are listed in Table 2. 1637 1638 Plate-Count methods: the testing has to be performed in duplicate for each medium 1639 and dilution. Incubation conditions are listed in Table 2 (1-8). 1640 Pour-plate methods: 1 ml of the prepared sample is placed in Petri dishes (f 9 cm) with 1641 1642 15-20 ml of the chosen culture medium (Table 2). Should diameters be higher than 9 1643 cm, the quantity of culture media would be increased accordingly. Recommended 1644 temperature:  $\leq$  45 °C. The testing has to be performed in duplicate for each medium 1645 and dilution. Incubation conditions are listed in Table 2 (1-8).

1646

1647Surface-spread methods: 15-20 ml of the chosen culture medium (Table 2) is placed in1648Petri dishes (f 9 cm) at temperature  $\leq$  45 °C. Should diameters be higher than 9 cm, the1649quantity of culture media would be increased accordingly. After media solidification,1650plates have to be placed into an incubator or into a laminar airflow cabinet (drying).1651Finally, 0.1 ml of prepared sample is spread on media surfaces, and the testing has to1652be performed in duplicate for each medium and dilution. Incubation conditions are listed1653in Table 2 (1-8).

1654

1655 The MPN method. This method is recommended TAMC in absence of other good methods 1656 because accuracy and precision are not satisfactory. Should the MPN method be uses, a 1657 series of  $\geq$  3 serial 10-fold dilutions of cannabis-related product would be considered. 1658 For each of these dilutions, three aliquots of 1 g or 1 mL are sampled with the aim of 1659 inoculating three 9-10-ml tubes (casein soya bean digest broth). The use of surfaceactive agents such as polysorbate 80 may be justified, and the use of neutralizers against 1660 1661 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 1662 tested product has to be examined (1-8) 1663

1664

1665 9.3.5 Results and interpretation

1666 When verifying the suitability of the membrane filtration method or the plate-count 1667 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 1668 absence of the product must be obtained. When verifying the suitability of the MPN 1669 1670 method the calculated value from the inoculum must be within 95% confidence limits of 1671 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 1672 conditions that come closest to the criteria are used to test the product (1-8). 1673

1674

## 1675 9.3.6 Examination of the product

- 1676Membrane filtration. After incubation (Table 2), the number of CFU/g or /ml of tested1677product has to be determined. Only plates with the highest number of colonies < 100</td>1678colonies are considered.
- 1679Plate-count (pour-plate and surface-spread) methods. After incubation, the mean value1680of results (CFU/g or /ml) of tested product has to be calculated taking into account only1681plates showing the highest number of colonies as follows: < 250 colonies for TAMC and</td>1682< 50 colonies for TYMC.</td>
- 1683The Most-probable-number method. After incubation, the number of tubes showing1684microbial spreading has to be considered for each dilution level. The MPN number has1685to be determined per gram or ml of the tested product. Useful tools can be found at the1686following web addresses:
- 1687• <a href="https://www.fda.gov/food/laboratory-methods-food/bam-appendix-2-most-probable-number-serial-dilutions">https://www.fda.gov/food/laboratory-methods-food/bam-appendix-2-most-</a>1688probable-number-serial-dilutions(Bacteriological Analytical Manual, Appendix16892 FDA)
- 1690• <a href="https://mpncalc.galaxytrakr.org">https://mpncalc.galaxytrakr.org</a> (MPNcalc v1.2.0, by M. Ferguson and J.1691Ihrie).

## 1692 9.3.7 Suitability testing methods for specified (general indicator) microorganisms

1693 Suitability testing methods can also be realized with concern to general indicator 1694 microorganisms. In general, the following Table 3 can be considered (2-3, 9-10):

1695 Table 3. General indicator microorganisms and recommended media for suitability testing1696 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)

- 1697 With relation to these tests and the suitability of testing media, USP <61>, <62>, <2021>, and <2022> are recommended as specific references.
- 1699

#### 1700 9.3.8 Suitability testing methods VS Cannabis-related Matrices

Table 4 shows four main cannabis-related product categories with minimum testing/sampleamount 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.

1710 **Table 4.** Cannabis and Cannabis Products. Minimum testing/sample amount and target 1711 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 <sub>2</sub> Oil – Isolates Distillate Shatter Wax – Budder - Kief	5 grams	IC, 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	ТАМС, ТҮМС

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

#### 1712

## 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

1724 Table 5 shows a selection of recommended media for selected microorganisms to be tested in 1725 the ambit of this document.

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/laborat ory-methods-food/bam-media- m152-trypticase-tryptic-soy-agar)	
Pseudomonas aeruginosa	ATCC 9027 NCIMB 8626 CIP 82.118	As above stated	

Table 5. Recommended testing media with relation to selected microorganisms (3, 5-6, 13) 1726 

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

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/laborat ory-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) microorganism s	Test strain	Recommended testin	ıg media- references

Burkholderia cepacia	ATCC 25416	Medium: <i>Burkholderia cepacia</i> 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., 2 <sup>nd</sup> Edition, July 2012. Available <u>https://www.theasm.org.au/guidelines- reports</u> . Accessed 28 <sup>th</sup> 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		Mannitol Salt Agar (USP <62>)
aureus	ATCC 6538	
Escherichia coli		MacConkey Agar (USP <62>)
	ATCC 8739	
Salmonella		Rappaport Vassiliadis Salmonella Enrichment Broth
enterica	ATCC 14028	Xylose Lysine Deoxycholate Agar (USP <62>)
Candida		Sabouraud Dextrose Broth and/or Agar (USP <62>)
albicans	ATCC 10231	
Clostridium		Reinforced Medium for Clostridia Growth promoting
sporogenes	ATCC 11437	Or
		<i>Cl. sporogenes</i> Columbia Agar (USP <62>)

# 1731 10 In-Silico Analysis

## 1733 **10.1 Scope**

1730

1732

In silico analysis for molecular methods is based on recommendations from AOAC OMA 1734 1735 Appendix Q: Recommendations for Developing Molecular Assays for Microbial 1736 Pathogen Detection Using Modern In Silico Approaches, June 2020 1737 (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 1738 1739 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 1740 1741 applicable to each assay. In silico analysis is not intended to replace wet lab testing 1742 but can reduce wet lab testing allowing focus on potential false negative and false 1743 positive organisms.

1744 **10.2 Inclusivity/Exclusivity** 

1745 Sequence quality refers to the likelihood that the given nucleotide is correctly 1746 specified at each position in a genome sequence. To reduce the potential for false 1747 negative reactions, use high-quality sequences in the inclusivity database. For the 1748 purposes of checking for false-positive amplifications, construct exclusivity and 1749 environmental background databases. For both the exclusivity and background 1750 databases, sequence quality is generally not an issue. Include partial sequences as 1751 well as complete genomes. Check primers for reactivity with all relevant organisms 1752 [such as the GenBank nonredundant (nr) or nucleotide (nt) databases] using a program 1753 such as Primer-BLAST (App Q reference 22), Thermonucleotide BLAST (App Q 1754 reference 23), or ThermoBLAST (App Q reference 14) to detect all off-target hits and 1755 amplicons. Test one genome per strain/isolate so as not to introduce bias.

- 1756 Inclusivity Include sequences for all known genetic variations of the target(s).
  1757 Include all known full-length genomes (to reduce database size it can be helpful to
  1758 remove identical sequences). For partial genomes, it is best to include only the partial
  1759 sequences that contain the region of interest (i.e. the amplicon region). Report the
  1760 number of genomes with 0, 1, 2, 3, or 4 mismatches for each primer and probe.
- 1761 *Exclusivity* Populate with genomes of near neighbors (organisms that are
  1762 phylogenetically distinct but closely related to the target). Include full and partial
  1763 sequences. Report % homology for each primer and probe.
- 1764Background organisms Populate with organisms that may be present in the matrix or1765related to the intended use (e.g. the human genome, human RNA RefSeq, human1766microbiome, soil microbes, etc.). Report genomes with highest homology to each1767primer and probe.

1768	Select sequences from the following:
1769	Generalized databases
1770	<ul> <li>National Center for Biotechnology Information (NCBI,</li> </ul>
1771	https://www.ncbi.nlm.nih.gov)
1772	<ul> <li>GenBank (<u>https://www.ncbi.nlm.nih.gov/genbank/</u>)</li> </ul>
1773	European Molecular Biology Laboratory-European Bioinformatics Institute
1774	(EMBL-EBI, <u>https://www.ebi.ac.uk</u> )
1775	• DNA Data Bank of Japan (DDBJ, <u>https://www.ddbj.nig.ac.jp/index-e.html</u> )
1776	Curated pathogen genome databases
1777	<ul> <li>Virus Pathogen Database and Analysis Resource (ViPR,</li> </ul>
1778	https://www.viprbrc.org/brc/home.spg?decorator=vipr)
1779	NCBI Influenza Virus Database
1780	(https://www.ncbi.nlm.nih.gov/genomes/FLU/Database/nph-
1781	<u>select.cgi?go=database</u> )
1782	Los Alamos Hemorrhagic Fever Viruses Database
1783	(https://hfv.lanl.gov/content/index)
1784	<ul> <li>Virulence Factor Database (VFDB, <u>http://www.mgc.ac.cn/VFs/main.htm</u>)</li> </ul>
1785	<ul> <li>Global Initiative on Sharing All Influenza Data (GISAID,</li> </ul>
1786	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.

1790 **10.3 Physical Chemistry Modeling** 

1791 Perform thermodynamic folding simulations to determine if the primers and probe can 1792 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 1793 1794 mutation occurs at a primer binding site or if the salt concentrations vary slightly 1795 (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 1796 1797 Visual OMP (App Q reference 14), etc.] to predict the secondary structure of the RNA 1798 or DNA target regions. Programs such as AutoDimer (App Q reference 19) and 1799 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. 1800

1801Unimolecular Folding - Determine all potential secondary structures of the regions of1802the target where the primers and probe binds, including approximately 150 extra1803bases on either end of the target region (typically we use the amplicon region with an1804extra 150 nts. on the 5' and 3; sides). Both the sense and antisense strands should be1805folded to observe if any folding is present at the sites where the forward primer,1806reverse primer, and probe bind. Determine whether primer/probe binding requires1807high energy ( $\Delta G^{\circ}_{T}$ , where T is the annealing temperature) for unfolding of these

- 1808 structures. If the target site where the primer binds is unfolded, then it is safe to use 1809 the 2-state  $\triangle G^{\circ}_{T}$  and Tm, to characterize the hybridization. However, if the primerbinding site on the target is highly folded, then it is necessary to compute the energy 1810 to unfold that region and to use a "multi-state coupled equilibrium model" (for further 1811 1812 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 1813 1814 inhibitory secondary structures which can cause primers to be fragile to minor 1815 variations in reagent quality or the presence of even a single mismatch within the 1816 binding region. Report 1817  $\Delta G^{\circ}_{T}$  (unfolding) or the fraction bound for each primer and probe.
- 1818Bimolecular Thermodynamics (Hybridization) Dependent on nucleotide composition,1819primer/probe length, strand concentration, salt conditions, and temperature. Report1820 $\triangle G^{\circ}_{T}$  and Tm of primer/probe binding.
- 1821 *Note*: Reporting specific sequences of proprietary primers/probes is not required.
- Look for potential false negative variants, indicated by high number mismatches, 1822 1823 highly folded regions, and/or weak primer/probe binding. Look for potential false 1824 positives when homology to any of the sequences in the exclusivity and background 1825 databases is >80% for primers and probes. Look for potential false amplicons (i.e. 1826 where there are two primer sites pointing in opposite directions and with a spacing 1827 between the primers < 1000 nts.). Such false amplicons may consume PCR reagents, 1828 which could lead to false negatives. In addition, report amplicon length. Follow up 1829 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 1830 1831 isolates are sequenced, and new variants emerge.
- 1832
- 1833 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.

- 1839
- 1840
- 1841 12 References
- 1842
- 1843 (1) U.S. Food and Drug Administration (FDA) (2010) Bacteriological Analytical Manual,
- 1844 http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/
- 1845 BacteriologicalAnalyticalManualBAM/ucm109656.htm
- 1846 (2) U.S. Food and Drug Administration (FDA) (2005) Bacteriological Analytical Manual,
- 1847 http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/
- 1848 BacteriologicalAnalyticalManualBAM/UCM064244

1849	
1850	(3) Official Methods of Analysis (2012) 19th Ed., Appendix D,
1851	AOAC INTERNATIONAL, Gaithersburg, MD
1852	
1853	(4) Tukey, J.W. (1977) Exploratory Data Analysis, Addison-
1854	Wesley, pp 8 ff, 33, and 39 ff
1855	
1856	(5) AOAC INTERNATIONAL Official Methods of Analysis Online,
1857	http://www.eoma.aoac.org/
1858	(6) U.S. Food and Drug Administration (FDA) (2011) Bacteriological Analytical Manual
1859	Online, http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/
1860	BacteriologicalAnalyticalManualBAM/default.htm
1861	(7) U.S. Department of Agriculture-Food Safety and Inspection Service (FSIS) Microbiology
1862	Laboratory Guidebook Online, http://www.fsis.usda.gov/Science/
1863	Microbiological_Lab_Guidebook/index.asp
1864	(8) International Organization for Standardization (ISO) http:// www.iso.org/iso/prods-
1865	services/ISOstore/store.html
1866	(9) Health Canada Compendium of Analytical Methods- Microbiological Methods,
1867	http://www.hc-sc.gc.ca/fn-an/ res-rech/analy-meth/microbio/index-eng.php
1868	(10) Feldsine, P., Abeyta, C, & Andrews, W.H. (2002) J. AOAC Int. 85, 1187-1200
1869	(11) International Organization for Standardization (ISO) Standard 16140, Microbiology of
1870	Food and Animal Feeding Stuffs – Protocol for the Validation of Alternative Methods,
1871	http://www.iso.org/iso/search.htm?qt=16140&searchSubmit
1872	=Search&sort=rel&type=simple&published=true
1873	
1874	(12) LaBudde, R.A. (2008) Statistical Analysis of Interlaboratory Studies, XX. Measuring the
1875	Performance of a Qualitative Test Method, TR290, Least Cost Formulations, Ltd, Virginia
1876	Beach, VA
1877	(13) LaBudde, R.A. (2009) Coverage Accuracy for Binomial Proportion 95% Confidence
1878	Intervals for 12 to 100 Replicates, TR297, Least Cost Formulations, Ltd, Virginia Beach, VA
1879	(14) LaBudde, R.A. (2009) Statistical Analysis of Interlaboratory Studies, XXII. Statistical
1880	Analysis of a Qualitative Multicollaborator Study as a Quantitative Study Under the Large
1881	Sample Approximation, TR296, Least Cost Formulations, Ltd, Virginia Beach, VA
1882	
1883	(15) Mohaptra, S. (2017). Sterilization and Disinfection. Essentials of Neuroanesthesia. 929-
1884	944 <u>10.1016/B978-0-12-805299-0.00059-2</u>
1885	
1886	(16) USDA AMS (2021). Remediation and Disposal Guidelines for Hemp Growing Facilities. U.S.
1887	Domestic Hemp Production Program
1888	https://www.ams.usda.gov/sites/default/files/media/HempRemediationandDisposalGuideli
1889	<u>nes.pdf</u>
1890	
1891	(17) US FDA (2021). Chemical Hazards. <u>https://www.fda.gov/animal-veterinary/biological-</u>
1892	chemical-and-physical-contaminants-animal-food/chemical-hazards

1893 1894	
1895 1896 1897 1898	(18) US FDA (2014). Bacterial Endotoxins/Pyrogens. <u>https://www.fda.gov/inspections-</u> <u>compliance-enforcement-and-criminal-investigations/inspection-technical-guides/bacterial-</u> <u>endotoxinspyrogens</u>
1899 1900 1901 1902	(19) Gamma Industry Processing Alliance. 2017. A comparison of Gamma, E-Beam, X-ray and Ethylene Oxide Technologies for the Industrial Sterilization of Medical Devices and Healthcare Products.
1903 1904	(20) University of Rochester Medical Center. 2020. Basics on Processing & Sterilization. https://www.urmc.rochester.edu/sterile/basics.aspx
1905 1906 1907	(21) Ghanem I, Orfi M, Shamma M. 2008. Effect of gamma radiation on the inactivation of aflatoxin B1 in food and feed crops. Braz J Microbiol <b>39:</b> 787-791.
1908 1909 1910	(22) Hazekamp A. 2016. Evaluating the Effects of Gamma-Irradiation for Decontamination of Medicinal Cannabis. Front Pharmacol <b>7</b> .
1911 1912 1913 1914	(24) Jerushalmi, Shachar & Maymon, Marcel & Dombrovsky, Aviv & Freeman, Stanley. (2020). Effects of cold plasma, gamma and e-beam irradiations on reduction of fungal colony forming unit levels in medical cannabis inflorescences. Journal of Cannabis Research. 2. 10.1186/s42238-020-00020-6.
1915 1916	(25) USP (2021) USP <51> - Preservative Challenge Test. United States Pharmacopeia, Rockville, MD, USA
1917 1918	(26) USP (2021) USP <61> - Microbiological Examination of Nonsterile Products: Microbial Enumeration Tests. United States Pharmacopeia, Rockville, MD, USA
1919 1920	(27) USP (2021) USP <62> - Microbiological Examination of Nonsterile Products: Tests for Specified Microorganisms. United States Pharmacopeia, Rockville, MD, USA
1921 1922	(28) USP (2021) USP <111> - Design and Analysis of Biological Assays. United States Pharmacopeia, Rockville, MD, USA
1923 1924	(29) WHO (2019) International Pharmacopeia, 9 <sup>th</sup> Edition. World Health Organization, Geneva, Switzerland
1925 1926	(30) U.S. FDA (2021) Bacteriological Analytical Manual BAM. U.S. Food and Drug Administration, Washington, D.C., USA
1927 1928	(31) U.S. FDA (2020) Pharmaceutical Microbiology Manual, Document Number: ORA.007. U.S. Food and Drug Administration, Washington, D.C., USA

1929 (32) European Pharmacopeia (2013) European Pharmacopeia 01/2005:20612, Microbiological
1930 examination of non sterile products (total viable aerobic count), European Pharmacopeia,
1931 Strasbourg, France

1932 (33) USP (2021) USP <2021> - Microbial Enumeration Tests - Nutrition and Dietary
 1933 Supplements. United States Pharmacopeia, Rockville, MD, USA

1934 (34) USP (2021) USP <2022> - Microbiological Procedures for Absence of Specified
 1935 Microorganisms - Nutrition and Dietary Supplements. United States Pharmacopeia, Rockville,
 1936 MD, USA

1937 (35) AOAC SMPR 2021.009; Version 9; June 17, 2021 (Method Name: Standard Method1938 Performance Requirements for Viable Yeast and Mold)

(36) USP (2021) USP <2023> - Microbiological Attribute of Nonsterile Nutritional and Dietary
 Supplements. United States Pharmacopeia, Rockville, MD, USA

(37) 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., 2<sup>nd</sup> Edition, July 2012. Available <u>https://www.theasm.org.au/guidelines-</u>
 reports. Accessed 28<sup>th</sup> July 2021

- 1945 (38) U.S. Department of Health and Human Services, Biosafety in Microbiological and
- 1946 Biomedical Laboratories (BMBL), 6th Edition, HHS Publication No. (CDC) 300859, Revised June 1947 2020; found at: Biosafety in Microbiological and Biomedical Laboratories—6th Edition
- 1948
- 1949 ANNEXES (Reference Appendix J for review; Insert for publication)
- 1950 1951 ANNEX A
- 1952
- 1953 ANNEX B
- 1954

1956

- 1955 ANNEX C
- 1957 ANNEX D
- 1958
- 1959 ANNEX E
- 1960 1961 ANNEX F
- 1962
- 1963 ANNEX G
- 1964
- 1965 ANNEX H