

1 | TECHNICAL COMMUNICATIONS

2 | **AOAC INTERNATIONAL Methods Committee**
3 | **Guidelines for Validation of Microbiological Methods**
4 | **for Food and Environmental Surfaces**

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6 | This document provides guidelines for the AOAC validation of microbiological methods
7 | for food and environmental surfaces. It includes terms and their definitions associated
8 | with the *Official Methods of Analysis*SM and *Performance Tested Methods*SM programs and
9 | validation requirements for qualitative, quantitative, and identification methods.

10 | The guideline working group consisted of Sharon Brunelle, Robert LaBudde, Maria Nelson, and
11 | Paul Wehling. The guidelines were reviewed by the AOAC Methods Committee for
12 | Microbiological Methods.
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189 **1 Scope**

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

195

196

197 **2 Applicability**

198

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

208

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

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212 **3 Terms and Definitions**

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214 **3.1 Analyte**

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

217 **3.2 Bias**

218 The difference between the expectation of the test result or measurement result and the true value

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- 219 (ISO 3534-2).
- 220 | **3.3 Candidate Method**
- 221 The method submitted for validation.
- 222 | **3.4 Candidate Method Result**
- 223 The final result of the qualitative or quantitative analysis for the candidate method. For methods
- 224 with a confirmation phase, only presumptive positive results that confirm positive are considered
- 225 as positive for the candidate method. All other results are considered as negative for the candidate
- 226 method.
- 227 | **3.5 Collaborative Study (CS)**
- 228 A validation study performed by multiple laboratories to estimate critical candidate method
- 229 performance parameters.
- 230 | **3.6 Composite Test Portion**
- 231 Test portions taken from multiple samples of the same matrix combined together.
- 232 | **3.7 Confirmatory Phase**
- 233 A procedure specified in some qualitative assays whereby a preliminary presumptive result is
- 234 confirmed by a subsequent and different method.
- 235 | **3.8 Confirmed Result**
- 236 The qualitative response from the confirmatory phase of a candidate method.
- 237 | **3.9 Enrichment Pool**
- 238 A pool comprised of aliquots from multiple test portion enrichments.
- 239 | **3.10 Exclusivity**
- 240 The non-target strains, which are potentially cross-reactive, that are not detected by the method.
- 241 | **3.11 Fractional Recovery**
- 242 Validation criterion that is satisfied when an unknown sample yields both positive and negative
- 243 responses within a set of replicate analyses. The proportion of positive responses should fall
- 244 within 25% and 75% and should ideally approximate 50% of the total number of replicates in the
- 245 set. A set of replicate analyses are those replicates analyzed by one method (either candidate or
- 246 reference). Only one set of replicates per matrix is required to satisfy this criterion.
- 247 | **3.12 Identification Method**
- 248 Method of analysis whose purpose is to determine the identity of an analyte. (BTAM)
- 249 | **3.13 Inclusivity**
- 250 The strains or isolates of the target analyte(s) that the method can detect. (BTAM)
- 251 | **3.14 Limit of Detection₅₀ (LOD₅₀)**
- 252 The analyte concentration at which the probability of detection (POD) is equal to 50%.
- 253 | **3.15 Matched Analyses**
- 254 Two or more analyses or analytical results on the same unknown sample, which can be traced to
- 255 the same test portion.
- 256 | **3.16 Matrix**
- 257 The food, beverage, or environmental surface material to be included in the validation as per the
- 258 intended use of the method.
- 259 | **3.17 Method Developer Validation Study or SLV (Precollaborative) Study**
- 260 A validation study performed by a single laboratory in order to systematically estimate critical
- 261 candidate method performance parameters. The method developer study is usually performed by
- 262 the organizing laboratory or study director.

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- 263 **3.18 Presumptive Phase**
 264 The initial qualitative determination of the analyte in a test portion. In some qualitative
 265 microbiological assays, confirmation of results is required as specified in the method.
- 266 **3.19 Presumptive Result**
 267 The qualitative response from the presumptive phase of a candidate method that includes a
 268 confirmatory phase.
- 269 **3.20 Probability of Detection (POD)**
 270 The proportion of positive analytical outcomes for a qualitative method for a given matrix at a
 271 given analyte level or concentration. POD is concentration dependent. Several POD measures
 272 can be calculated, e.g., POD_R (reference method POD), POD_C (confirmed candidate method
 273 POD), POD_{CP} (candidate method presumptive result POD) and POD_{CC} (candidate method
 274 confirmation result POD). Other POD estimates include:
- 275 $dPOD$ – the difference between any two POD values
- 276 $LPOD$ – the POD value obtained from combining all valid collaborator data sets for a
 277 method for a given matrix at a given analyte level or concentration
- 278 $dLPOD$ – the difference between any two LPOD values
- 279 **3.21 Qualitative Method**
 280 Method of analysis whose response is either the presence or absence of the analyte detected either
 281 directly or indirectly in a specified test portion.
- 282 **3.22 Quantitative Method**
 283 Method of analysis whose response is the amount (count or mass) of the analyte measured either
 284 directly (e.g., enumeration in a mass or a volume), or indirectly (e.g., color absorbance,
 285 impedance, etc.) in a specified test portion.
- 286 **3.23 Reference Method**
 287 Pre-existing recognized analytical method against which the candidate method will be compared.
 288 (BTAM)
- 289 **3.24 Repeatability**
 290 Precision under repeatability conditions (ISO 5725-1).
- 291 **3.25 Repeatability Conditions**
 292 Conditions where independent test results are obtained with the same method on equivalent test
 293 items in the same laboratory by the same operator using the same equipment within short intervals
 294 of time.
- 295 **3.26 Reproducibility**
 296 Precision under reproducibility conditions (ISO 5725-1).
- 297 **3.27 Reproducibility Conditions**
 298 Conditions where independent test results are obtained with the same methods on equivalent test
 299 items in different laboratories with different operators using separate instruments.
- 300 **3.28 Robustness Study**
 301 A study which tests the capacity of a method to remain unaffected by small but deliberate
 302 variations in method parameters and which provides an indication of its reliability during normal
 303 usage (USP 31).
- 304 **3.29 Sample**
 305 The batch of matrix from which replicate test portions are removed for analysis. The sample

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306 (naturally contaminated, uncontaminated, or inoculated) contains analyte, if present, at one
307 homogeneous concentration.

308 **3.30 Test Portion**

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

310 **3.31 Unmatched Analyses**

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

313

314

315 **4 Qualitative Methods—Technical Protocol for Validation**

316

317 **4.1 Method Developer Validation Study or SLV (Precollaborative) Study**

318 **4.1.1 Scope**

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

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

332

333 **4.1.2 Inclusivity/Exclusivity Study**

334 **4.1.2.1 Species/Strain Selection**

335 The choice of inclusivity strains should reflect the genetic and/or serological and/or biochemical
336 diversity of the organisms involved, as well as other factors such as virulence, frequency of
337 occurrence and availability. Select at least 30 pure strains of the target organism(s) to be analyzed
338 as pure culture preparations. For *Salmonella* methods, the number of target organisms is increased
339 to at least 100 serovars that are selected to represent the majority of known somatic groups and
340 subtypes of *Salmonella*.

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

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

346 **4.1.2.2 Study Design**

347 Inclusivity strains are cultured by the candidate method enrichment procedure. The target
348 concentration for testing is 100 times the LOD₅₀ of the method. Test one replicate per strain.

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

351 Inclusivity and exclusivity evaluations shall be performed together as one study. Inclusivity and
352 exclusivity test samples must be blind coded, randomized and intermingled so the analysts cannot

Comment [SB1]: OMB recommended having the matrix study done in an independent lab for OMA methods. Committee H members suggested adding the PTM Independent study to all OMA submissions instead.

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353 know the identity, sequence or concentration of the test samples.

354 4.1.2.3 Data Reporting

355 Report inclusivity data as number of strains detected. For example, “Of the 30 specific inclusivity
356 strains tested, 27 were detected and 3 were not detected. Those strains not detected were the
357 following:...”

358 Report exclusivity data as number of strains not detected. For example, “Of the 30 specific
359 exclusivity strains tested, 28 were not detected and 2 were detected. Those detected were the
360 following:...”

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

364

365 4.1.3 Matrix Study

366 4.1.3.1 Reference Method

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

370 The reference method replicates may be contained within the Most Probable Number (MPN)
371 analysis scheme. See Appendix X-B for more details.

372 4.1.3.2 Food Categories

373 Refer to the Recommended Food Categories and Food Types for Microbiological Methods
374 contained in Appendix X-A. AOAC INTERNATIONAL recognizes claims for only the range of
375 food categories or specific food types included in the Method Developer Study, or the PCS and
376 CS. The number of different matrices required for testing depends on the applicability of the
377 method. All claimed matrices must be included in the Method Developer Study and the PCS.

378 4.1.3.3 Environmental Surfaces

379 The number of different surface types required for testing depends on the applicability of the
380 method. For methods claiming “Environmental Surfaces”, nine different surfaces must be tested:
381 stainless steel, plastic (polyethylene, polypropylene, or polycarbonate), ceramic (glazed earthen
382 material or glass), rubber, food grade painted surfaces, wood, sealed concrete (a commercially
383 available product that “seals concrete pores”), cast iron (coated to prevent rusting), and air filter
384 material. Alternatively, specific surfaces may be claimed. All claimed surface types must be
385 included in the Method Developer Study or the PCS.

386 For surfaces to be sampled with a swab, each test area should measure 1” x 1”. For surfaces to be
387 sampled with a sponge, each test area should measure 4” x 4”.

388 4.1.3.4 Levels of Contamination

389 Each matrix (food, beverage, or surface material) is divided into at least 3 samples. One sample
390 serves as the uncontaminated level, one or more samples are contaminated at levels that will
391 produce at least one reference method POD (POD_R) or candidate method POD (POD_C) in the
392 range of 0.25 – 0.75, and one sample is contaminated at POD_C of 1.00. Depending on the
393 laboratory’s confidence in satisfying this validation criterion, it may be advisable to prepare a
394 fourth sample targeting the fractional POD range. Contamination levels for which the two
395 methods yield statistically significant POD differences, but fall outside the 0.25 – 0.75 POD range,
396 are also considered acceptable. All outcomes for each contamination level tested, whether
397 fulfilling the POD requirement or not must be reported.

398 The target concentration for the fractional POD range is 0.2 – 2 cfu/ test portion for foods and
399 beverages, depending on the matrix. The target concentration for $POD = 1.00$ is approximately 5
400 cfu/ test portion for foods and beverages. Target concentrations for fractional PODs on

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401 environmental surfaces can be in the range $10^4 - 10^6$ cfu/surface area, depending on the surface,
402 organism, and environmental conditions of the testing area.

403 A 5-tube 3-level MPN estimation of contamination levels (1) must be conducted on the day that
404 the analysis of test samples is initiated. The MPN analysis scheme may also make use of the
405 reference method replicates. See Appendix X-B for details.

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

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

410 | 4.1.3.5 Number of Test Portions

411 For matched test portions, the number of replicate test portions per contaminated level is 20 and
412 the number of replicate test portions of uncontaminated sample is 5. For unmatched test portions,
413 the number of test portions per contaminated level per method is 20 and the number of replicate
414 test portions of uncontaminated sample per method is 5.

415 | 4.1.3.6 Test Portion Size, Compositing and Pooling

416 The standard test portion size is 25 g or 25 mL, unless otherwise specified by the reference
417 method.

418 Test portion compositing is the combining of test portions prior to enrichment and can be
419 validated alongside the standard test portion size. The standard test portion size is validated for
420 both the candidate and reference methods and the standard test portion size can be mixed with X
421 uncontaminated test portions to create composite test portions for validation by the candidate
422 method. For example, if a matrix is to be validated for 375 g composited test portions (15 x 25 g),
423 then at the low contamination level test 20 replicates of 25 g for the candidate method, 20
424 replicates of 25 g for the reference method (either as matched or unmatched test portions) and 20
425 replicates of 25 g plus 350 g uncontaminated matrix for the candidate method. Repeat for the high
426 contamination level and test 5 replicates each for the uncontaminated level. MPNs are performed
427 only on the batch samples from which the 25 g test portions are taken. Acceptance criteria for
428 composited test portions are the same as for the standard test portion size.

429 Pooling is the post-enrichment combining of aliquots from more than one enriched test portion.
430 This is validated alongside the standard method by preparing replicate 25 g test portions for the
431 candidate method and replicate 25 g test portions for the reference method, either as matched or
432 unmatched test portions. At the conclusion of the enrichment procedure, test each enriched test
433 portion by the candidate and/or reference method as appropriate. In addition, pool an aliquot of
434 each test portion with X aliquots, as specified by the candidate method, of known negative
435 enriched test portions. Acceptance criteria for pooled enriched test portions are the same as for the
436 standard test portion analyses.

437 | 4.1.3.7 Source of Contamination

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

442 Numerous strains representing different serotypes or genotypes are required, if applicable.
443 Typically a different isolate, strain, biovar or species is used for each matrix. The product
444 inoculation should be conducted with a pure culture of one strain. Mixed cultures are not
445 recommended.

446 | 4.1.3.8 Preparation of Artificially Contaminated Samples

447 | 4.1.3.8.1 Food

448 Microorganisms in processed foods are typically stressed, thus the contaminating microorganisms
449 are also stressed for these types of foods. Microorganism stress may occur at the time of

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450 inoculation or during preparation of the food. Raw and cold-processed foods should be inoculated
451 with unstressed organisms, heat-processed foods with heat-stressed organisms (e.g., heat culture at
452 50°C for 10 min.), and dry foods with lyophilized culture. Mix well by kneading, stirring or
453 shaking as appropriate. Frozen foods should be thawed, inoculated, mixed and re-frozen.

454 The inoculum should be added to the sample, mixed well and allowed to equilibrate in the matrix
455 for 48-72 h at 4°C for refrigerated foods, for a minimum of two weeks at -20°C for frozen foods or
456 for a minimum of two weeks at room temperature for dried foods prior to analysis.

4.1.3.8.2 Environmental Surfaces

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

4.1.3.9 Preparation of Naturally Contaminated Samples

468 Naturally contaminated foods may be mixed with uncontaminated sample matrix or incubated to
469 achieve a level yielding fractionally positive results. Naturally contaminated surface materials
470 may be used as is, as long as the requirement for yielding fractionally positive results is achieved.

4.1.3.10 Need for Competitive Microflora

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

4.1.3.11 Environmental Surface Sampling

479 If all nine surfaces are being validated, five surfaces shall be sampled by sponge and the other four
480 surfaces sampled by swabs. The candidate method submitter will determine which surface will be
481 sampled by sponge or swab. An environmental sampling sponge is a porous moisture absorbing
482 matrix, approximately 2" (5 cm) x 3" (7.5 cm) often contained in a pre-sterilized sample bag. An
483 environmental swab is a sampling device comprised of synthetic (e.g. dacron) or cotton tips
484 affixed to a wood or polymeric stick, delivered in a pre-sterilized package.

485 Sponges and swabs are pre-moistened with a neutralizing broth, such as Dey-Engley (4), prior to
486 sampling. The entire sampling area is sponged or swabbed in both a horizontal and vertical
487 motion. Use the sponges to sample a 100 cm² (4"x4") area and swabs to sample a 5 cm² (1"x1")
488 area. Sponges/swabs containing samples are placed back into their individual respective bag or
489 tube and held at room temperature for 2 hours prior to initiation of testing.

4.1.3.12 Confirmation of Test Portions

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

4.1.3.13 Data Analysis and Reporting

494 Each level of each matrix must be analyzed and reported separately.

4.1.3.13.1 Raw Data Tables

496 For each matrix and level, report each result from each test portion separately. See Appendix X-C
497 for raw data table format.

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498 **4.1.3.13.2 Probability of Detection (POD)**

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

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

503 Estimate the POD with a 95% confidence interval for the candidate method, the reference method
504 and, if included, the presumptive and confirmed results. See Appendix X-D for details.

505 **4.1.3.13.3 Difference of Probabilities of Detection (dPOD)**

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

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

$$509 \quad dPOD_C = POD_C - POD_R$$

510 Estimate the $dPOD_{CP}$ as the difference between the candidate presumptive result POD (POD_{CP})
511 and the candidate confirmed result POD (POD_{CC}) values. Calculate the 95% confidence interval
512 on the $dPOD_{CP}$. See Appendix X-D for details.

$$513 \quad dPOD_{CP} = POD_{CP} - POD_{CC}$$

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

516 **4.1.3.13.4 Summary Data Tables**

517 For all matrices and levels, use the summary table from Appendix X-E.

518 **4.1.3.13.5 Graph of Data**

519 For each matrix, graph POD_R , POD_C and dPOD by level with 95% confidence intervals. See
520 example in Appendix X-F.

521 **4.1.3.13.6 Data Analysis and Reporting in the Absence of a Reference Method**

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

524

525 **4.1.4 Robustness Study (PTM submissions only)**526 **4.1.4.1 Strain Selection**

527 Robustness strains are prepared and analyzed as vegetative cells, spores or components thereof as
528 applicable to the candidate method. One material is tested at a level that yields fractional recovery,
529 and one non-target material is analyzed at the growth level achieved in a non-selective broth or at
530 a high inoculation level.

531 **4.1.4.2 Study Design**

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

535 The method developer, in conjunction with the AOAC Project Manager, is expected to make a
536 good faith effort to choose parameters that are most likely to affect the analytical performance and
537 determine the range of variations that can occur without adversely affecting analytical results. Use
538 a screening factorial experimental design.

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

540 **4.1.4.3 Data Analysis and Reporting**

541 The results are analyzed for variable detection due to changes in parameter settings. Report the
542 appropriate statistical measures of the measured variable(s) (e.g., Ct, absorbance, POD value, etc)

Deleted: Two target strains are

Deleted: using the candidate method
enrichment at approximately 10 times the
 LOD_{50} of the method

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and five replicates of the non-target are
tested for each factorial pattern

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543 | for each set of replicates for each treatment combination. This should include at least means,
544 | standard deviations, and confidence intervals where appropriate.

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545 | For continuous numerical outputs, use multiple linear regression to determine whether a change in
546 | response due to parameter variation is physically important.

547 | For binary sensors, calculate the POD value and confidence interval for each treatment
548 | combination. Analyze results using a generalized linear model.

Deleted: factorial pattern

549 |

550 | **4.1.5 Product Consistency and Stability Study (PTM submissions only)**

551 | **4.1.5.1 Strain Selection**

552 | Strains for product consistency and stability testing are prepared and analyzed as vegetative cells,
553 | spores or components thereof as applicable to the candidate method. One target strain, cultured by
554 | the candidate method enrichment procedure, is tested at a level that yields fractional recovery. One
555 | non-target organism is analyzed at the growth level achieved in a non-selective broth.

Deleted: Two

Deleted: s

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Deleted: using the candidate method enrichment

Deleted: 10 times the LOD₅₀ of the method

Deleted: and must show consistent results

556 | **4.1.5.2 Study Design**

557 | For the lot-to-lot consistency study, 6 lots of test kits (or test reagents) are tested,

558 | For test kit or reagent stability, either real-time or accelerated stability data will be accepted for
559 | initial PTM certification. If accelerated stability data are submitted, real-time stability data must
560 | be submitted within one year to support the shelf life of the kit.

Deleted: Five

561 | Ten replicates of a target organism and five replicates of a non-target organism are tested for each
562 | lot or time point.

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563 | Lot-to-lot and stability testing can be combined into one study by testing 6 lots spanning the shelf
564 | life of the product.

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565 | **4.1.5.3 Data Analysis and Reporting**

566 | The results are analyzed for variable detection between lots or time points. Report the appropriate
567 | statistical measures of the measured variable(s) (e.g., Ct, absorbance, POD value, etc) for each set
568 | of replicates for each product lot and between lots or for each stability time point. This should
569 | include at least means, standard deviations, confidence intervals and time slope, as appropriate.

570 | For binary sensors, calculate the POD value and confidence interval for each lot and each time
571 | point. For binary and continuous sensors, determine the standard deviation across lots and the
572 | time slope with confidence interval and % performance degradation across time points. For the
573 | combined approach, use a generalized mixed model fitting program to determine the lot to lot
574 | variability as a random effect and the time slope as a fixed effect.

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Deleted: s

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Deleted: using the candidate method enrichment

Deleted: 10 times the limit of detection

Deleted: and must show consistent results

Deleted: Five

Deleted: each

576 | **4.1.6 Between Instrument Variation Study (If applicable, PTM submissions only)**

577 | **4.1.6.1 Strain Selection**

578 | Strains for between instrument variation testing are prepared and analyzed as vegetative cells,
579 | spores or components thereof as applicable to the candidate method. One target strain, cultured by
580 | the candidate method enrichment procedure, is tested at a level that yields fractional recovery. One
581 | non-target organism is analyzed at the growth level achieved in a non-selective broth.

Deleted: variation

Deleted: product lot

582 | **4.1.6.2 Study Design**

583 | Six instruments are tested, Ten replicates of target and five replicates of the non-target are tested
584 | for each instrument.

Deleted: lots or for each stability time point

Deleted: and time slope

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585 | **4.1.6.3 Data Analysis and Reporting**

586 | The results are analyzed for variable detection between instruments. Report the appropriate
587 | statistical measures of the measured variable(s) (e.g., Ct, absorbance, POD value, etc) for each set
588 | of replicates for each instrument and between instruments. This should include at least means,
589 | standard deviations, and confidence intervals, as appropriate.

590 For binary sensors, calculate the POD value and confidence interval for each instrument. For
 591 binary and continuous sensors, determine the standard deviation across instruments.

Deleted: lot and each time point
Deleted: lots and the time slope
Deleted: with confidence interval and % performance degradation across time points
Deleted: For the combined approach, use a generalized mixed model fitting program to determine the lot to lot variability as a random effect and the time slope as a fixed effect.
Deleted: (PTM submissions only)

593 **4.2 Independent Validation Study**

594 **4.2.1 Scope**

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

598 **4.2.2 Reference Method**

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

601 **4.2.3 Matrices**

602 The independent laboratory must test at least one matrix that was tested in the Method Developer
 603 Study. The total number of matrices to be evaluated by the independent laboratory is dependent
 604 on the claim of the candidate method. For every five foods claimed, one food matrix shall be
 605 included in the independent study and for every five environmental surfaces claimed, one surface
 606 shall be included in the independent study.

Deleted: and will be determined on a case by case basis

607 **4.2.4 Study Design**

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

615 **4.3 Collaborative Study (CS)**

616 **4.3.1 Scope**

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

620 **4.3.2 Number of Laboratories**

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

623 **4.3.3 Reference Method**

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

Deleted: all laboratories using the same study design as the candidate method
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Deleted: as the matrix
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627 **4.3.4 Matrix Selection**

628 At least one matrix per sample preparation procedure from those studied in the PTM or PCS shall
 629 be chosen for collaborative study. For methods with more than one sample preparation procedure,
 630 more than one matrix will be required in the collaborative study. Examples of what constitutes a
 631 different sample preparation procedure would include different test portion size, different
 632 enrichment media or conditions, different dilution volume and different homogenization
 633 equipment. The AOAC General Referee, Statistical Advisor and collaborative study protocol
 634 reviewers shall make the final selection of the matrix(es) with consideration of the PTM or PCS
 635 data and the relative importance of the matrices to food safety. The data from both the PCS and
 636 CS studies form the basis for defining the method applicability statement.

Deleted: In cases where the candidate method includes more than one sample preparation methodology, the General Referee, Statistical Advisor and protocol reviewers may select more than one matrix for the collaborative study.
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637 **4.3.5 Levels of Contamination**

638 Refer to Section 4.1.3.4. Use the reference method (or candidate method if there is no reference
 639 method) test portions with additional levels to estimate the MPN using the formula in Appendix
 640 X-B.

Deleted: across all labs

641 **4.3.6 Number of Test Portions**

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

645 **4.3.7 Test Portion Size, Compositing and Pooling**

646 The standard test portion size is 25 g or 25 mL, unless otherwise specified by the reference
 647 method. If composite test portions or pooling was included in the Method Developer Validation
 648 Study, it must also be included in the Collaborative Study.

649
 650 Test portion compositing is the combining of test portions prior to enrichment and can be
 651 validated alongside the standard test portion size. The standard test portion size is validated for
 652 both the candidate and reference methods and the standard test portion size can be mixed with X
 653 uncontaminated test portions to create composite test portions for validation by the candidate
 654 method. For example, if a matrix is to be validated for 375 g composited test portions (15 x 25 g),
 655 then at the low contamination level prepare 6 replicates of 25 g for the candidate method, 6
 656 replicates of 25 g for the reference method (either as matched or unmatched test portions) and 6
 657 replicates of 25 g plus 350 g uncontaminated matrix for the candidate method per collaborator.
 658 Repeat for the high contamination level and the uncontaminated level. MPNs are performed only
 659 on the batch samples from which the 25 g test portions are taken. Acceptance criteria for
 660 composited test portions are the same as for the standard test portion size.

661

662 Pooling is the post-enrichment combining of aliquots from more than one enriched test portion.
 663 This is validated alongside the standard method by preparing replicate 25 g test portions for the
 664 candidate method and replicate 25 g test portions for the reference method, either as matched or
 665 unmatched test portions. At the conclusion of the enrichment procedure, test each enriched test
 666 portion by the candidate and/or reference method as appropriate. In addition, pool an aliquot of
 667 each test portion with X aliquots, as specified by the candidate method, of known negative
 668 enriched test portions. Acceptance criteria for pooled enriched test portions are the same as for the
 669 standard test portion analyses.

670 **4.3.8 Source of Contamination**

671 Refer to 4.1.3.7.

672 **4.3.9 Preparation of Artificially Contaminated Samples**

673 Refer to 4.1.3.8.

674 **4.3.10 Preparation of Naturally Contaminated Samples**

675 Refer to 4.1.3.9.

676 **4.3.11 Confirmation of Test Portions**

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

679 **4.3.12 Data Analysis and Reporting**

680 Each concentration level of each matrix must be analyzed and reported separately. Data may be
 681 excluded due to an assignable cause if sufficient justification is provided. Excluded data must be
 682 reported, but should not be included in the statistical analysis.

683 **4.3.12.1 Raw Data Tables**

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684 For each matrix and concentration level, report each result from each test portion separately. See
685 Appendix X-C for raw data table format.

686 4.3.12.2 Estimate of Repeatability

687 Estimate the repeatability standard deviation (s_r) for qualitative methods according to Appendix X-
688 G.

689 4.3.12.3 Estimate of Reproducibility

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

694 For each matrix and level, calculate the standard deviation of the laboratory POD values (s_{POD})
695 and associated 95% confidence interval to estimate the reproducibility. See Appendix X-G for
696 details.

697 4.3.12.4 Cross-Laboratory Probability of Detection (LPOD)

698 Report the LPOD estimates by matrix and concentration with 95% confidence intervals for the
699 candidate method and, if included, the presumptive and confirmed results. See Appendix X-G for
700 details.

701 4.3.12.5 Difference of Cross-Laboratory Probability of Detection (dLPOD)

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

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

705 Estimate the $dLPOD_{CP}$ as the difference between the presumptive and confirmed LPOD values.
706 Calculate the 95% confidence interval on the $dLPOD_{CP}$. See Appendix X-H for details.

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

709 4.3.12.6 Summary Data Tables

710 For all matrices and levels, use the summary table from Appendix X-H.

711 4.3.12.7 Graph of Data

712 For each matrix, graph POD_R , $LPOD_C$ and $dLPOD_C$ by level with 95% confidence intervals. See
713 example in Appendix X-F.

714 4.3.12.8 Data Analysis and Reporting in the Absence of a Reference Method

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

717

718 5 Quantitative Methods—Technical Protocol for Validation

719

720 5.1 Method Developer Validation Study or SLV (Precollaborative) Study

721 5.1.1 Scope

722 The Method Developer Validation Study is intended to determine the performance of the
723 candidate method. The study is designed to evaluate performance parameters including inclusivity,
724 exclusivity, repeatability, bias, robustness, and reagent consistency and stability. The Method
725 Developer Study is normally conducted in a single laboratory, usually the method developer’s
726 laboratory. Alternatively, the method developer can contract the work to an independent site.

727 The SLV (Precollaborative) Study is a formal submission requirement for OMA microbiology
728 methods and is normally conducted in the method developer laboratory. It precedes the

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729 Collaborative Study. The purpose of an SLV (Precollaborative) Study is to define the applicability
 730 claims of a proposed OMA microbiology method by demonstrating the applicability of the method
 731 to various food categories. For OMA methods, the applicability statement immediately follows the
 732 method title. The applicability statement for microbiological methods is generally concerned with
 733 target analyte and food type coverage.

734 | 5.1.2 Inclusivity/ Exclusivity

735 This requirement is not applicable to total viable count or similar total enumeration methods that
 736 are not directed at specific microorganisms. The requirement applies to selective or differential
 737 quantitative methods.

738 5.1.2.1 Strain Selection

739 The choice of inclusivity strains should reflect the genetic and/or serological and/or biochemical
 740 diversity of the target organism(s). Select at least 30 pure strains of the target organism(s) to be
 741 analyzed as pure culture preparations. For *Salmonella* methods, the number of target organisms is
 742 increased to at least 100 serovars that are selected to represent the majority of known somatic
 743 groups and subtypes of *Salmonella*.

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

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

749 5.1.2.3 Study Design

750 | Inclusivity strains are cultured [in nonselective media](#). The target concentration for testing is 100
 751 times the LOD₅₀ of the method. Test one replicate per strain.

Deleted: by the candidate method enrichment procedure

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

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

757 5.1.2.4 Data Reporting

758 Report inclusivity data as number of strains detected. For example, “Of the 30 specific inclusivity
 759 strains tested, 27 were detected and 3 were not detected. Those strains not detected were the
 760 following:...”

761 Report exclusivity data as number of strains not detected. For example, “Of the 30 specific
 762 exclusivity strains tested, 28 were not detected and 2 were detected. Those detected were the
 763 following:...”

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

766 | 767 | 5.1.3 Matrix Study

768 5.1.3.1 Reference Method

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

772 | 5.1.3.2 Food Categories

773 Refer to the Recommended Food Categories and Food Types for Microbiological Methods
 774 contained in Annex A. AOAC INTERNATIONAL recognizes claims for only the range of food
 775 categories or specific food types included in Method Developer Study or the PCS and CS. The

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776 number of different matrices depends on the applicability of the method. All claimed matrices
777 must be included in the Method Developer Study and the PCS.

778 | 5.1.3.3 Levels of Contamination

779 For the artificially contaminated food types, 3 inoculated levels (high, medium, and low) and one
780 uninoculated level are required. The low level should be at the estimated limit of detection, and
781 the medium and high levels may be approximately 10 and 100 times higher, respectively.
782 Intermediate levels may be added, but they are not required.

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

786 | 5.1.3.4 Number of Test Portions

787 For each level, analyze 5 test portions by the candidate method and 5 test portions by the reference
788 method.

789 | 5.1.3.5 Source of Contamination

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

794 | 5.1.3.6 Preparation of Artificially Contaminated Samples

795 Microorganisms in processed foods are typically stressed, thus the contaminating microorganisms
796 are also stressed for these types of foods. Microorganism stress may occur at the time of
797 inoculation or during preparation of the food. Raw and cold-processed foods should be inoculated
798 with unstressed organisms, heat-processed foods with heat-stressed organisms (e.g., heat culture at
799 50°C for 10 min.), and dry foods with lyophilized culture. Mix well by kneading, stirring or
800 shaking as appropriate. Frozen foods should be thawed, inoculated, mixed and re-frozen.

801 The inoculum should be added to the sample, mixed well and allowed to equilibrate in the matrix
802 for 48-72 h at 4°C for refrigerated foods, for two weeks at -20°C for frozen foods or for two weeks
803 at room temperature for dried foods prior to analysis.

804 | 5.1.3.7 Use of Artificially and Naturally Contaminated Test Samples

805 Approximately 50% of the food types should be naturally contaminated unless the method is for a
806 specific microorganism that may not be naturally occurring in that number of food types. For the
807 food types that are naturally contaminated, 4 different lots are required per food type. There are no
808 uncontaminated levels required for the food types that are naturally contaminated.

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

810 | 5.1.3.8 Need for Competitive Flora

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

817 | 5.1.3.9 Confirmation of Test Portions

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

820 | 5.1.3.10 Data Analysis and Reporting

821 | 5.1.3.10.1 General Considerations

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

824 $\text{Log}_{10} [\text{CFU/unit} + (0.1)f]$

825 Where f is the reported CFU/unit corresponding to the smallest reportable result, and unit is the
826 reported unit of measure (e.g., g, mL, filter). For details, see Appendix X-I.

827 5.1.3.10.2 Initial Review of Data

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

832 5.1.3.10.3 Outliers

833 It is often difficult to make reliable estimations (average, standard deviation, etc.) with a small bias
834 in presence of outliers. Data should be examined to determine whether there exists an occasional
835 result that differs from the rest of the data by a greater amount than could be reasonably expected
836 or found by chance alone. Perform outlier tests (Cochran and Grubbs) in order to discard
837 significantly outlying values (2). There must be an explanation for every excluded result; no
838 results can be excluded on a statistical basis only. To view the data adequately, construct a stem-
839 leaf display, a letter-value display, and a boxplot (3).

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

842 5.1.3.10.4 Repeatability (s_r)

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

845 5.1.3.10.5 Bias

846 Calculate the mean of the transformed replicate values at each concentration of each matrix for
847 each method. Calculate the difference between the mean and the known concentration. Report
848 the bias with the 95% confidence interval.

849 5.1.3.10.6 Mean Difference between Candidate and Reference Where Applicable

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

853 854 5.1.4 Robustness Study (PTM submissions only)

855 5.1.4.1 Strain Selection

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

860 5.1.4.2 Study Design

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

865 The method developer, in conjunction with the AOAC Project Manager, is expected to make a
866 good faith effort to choose parameters that are most likely to affect the analytical performance and
867 determine the range of variations that can occur without adversely affecting analytical results.

868 Five replicates at each target concentration and 5 replicate of the non-target are tested for each

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869 factorial pattern.

870 **5.1.4.3 Data Analysis and Reporting**

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

874

875 **5.1.5 Product Consistency and Stability Study (PTM submissions only)**

876 **5.1.5.1 Strain Selection**

877 Strains for reagent consistency and stability testing strains are prepared and analyzed as vegetative
878 cells, spores or components thereof as applicable to the candidate method. One target strain is
879 tested using the candidate method enrichment at a high and low level within the quantitative range
880 of the candidate method. One non-target strain is enriched in a non-selective broth and tested at
881 the high level.

882 **5.1.5.2 Study Design**

883 For the lot-to-lot consistency study, 6 lots of test kits (or test reagents) are tested and must show
884 consistent results.

885 For test kit or reagent stability, either real-time or accelerated stability data will be accepted for
886 initial PTM certification. If accelerated stability data are submitted, real-time stability data must
887 be submitted within one year to support the shelf life of the kit. For real-time stability, data
888 demonstrating no statistical difference in bias and repeatability between time zero and the end of
889 the shelf life must be submitted.

890 Five replicates at each target concentration and 5 replicate of the non-target are tested for each lot
891 or time point.

892 Lot-to-lot and stability testing can be combined into one study by testing 6 lots spanning the
893 product shelf life.

894 **5.1.5.3 Data Analysis and Reporting**

895 The results are analyzed for variable bias between lots or time points. Report the mean and
896 standard deviation for each set of replicates at each concentration for each kit/reagent lot or
897 stability time point. Standard deviations (s_r) at each concentration are compared to determine if
898 the s_r varies by more than three-fold.

899

900 **5.1.6 Between Instrument Variability Study (If applicable, PTM submissions only)**

901 **5.1.6.1 Strain Selection**

902 Strains for between instrument variability testing are prepared and analyzed as vegetative cells,
903 spores or components thereof as applicable to the candidate method. One target strain is tested
904 using the candidate method enrichment at a high and low level within the quantitative range of the
905 candidate method. One non-target strain is enriched in a non-selective broth and tested at the high
906 level.

907 **5.1.6.2 Study Design**

908 Six instruments are tested and must show consistent results. Five replicates of each target
909 concentration and 5 replicates of a non-target are tested for each instrument.

910 **5.1.6.3 Data Analysis and Reporting**

911 The results are analyzed for variable bias between instruments. Report the mean and standard
912 deviation for each set of replicates at each concentration for each instrument. Standard deviations
913 (s_r) at each concentration are compared to determine if the s_r varies by more than three-fold.

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- 915 **5.2 Independent Validation Study (PTM submissions only)**
- 916 **5.2.1 Scope**
- 917 A validation study to corroborate the analytical results obtained by the method developer and to
918 provide additional single laboratory data. The independent validation study traditionally verifies
919 repeatability in the hands of an independent trained user. Required for PTM certification.
- 920 **5.2.2 Reference Method**
- 921 If there is a reference method, then the candidate method is compared to a reference method. The
922 reference method should be the same as that used in the method developer study.
- 923 **5.2.3 Matrices**
- 924 The independent laboratory must test at least one matrix that was tested in the Method Developer
925 Study. The total number of matrices to be evaluated by the independent laboratory is dependent
926 on the claim of the candidate method and will be determined on a case by case basis.
- 927 **5.2.4 Study Design**
- 928 The study design for validation of quantitative methods in the independent study follows the
929 Method Developer Validation Study design. Contamination levels, number of test portions,
930 source of contamination, preparation of samples, confirmation of test portions, and data analysis
931 and reporting are found in Section 5.1.3.
- 932
- 933 **5.3 Collaborative Study (CS)**
- 934 **5.3.1 Scope**
- 935 The Collaborative Study (CS) is a formal submission requirement for OMA methods and succeeds
936 the SLV (Precollaborative) Study. The purpose of the Collaborative Study is to estimate the
937 reproducibility and determine the performance of the candidate method among collaborators.
- 938 **5.3.2 Number of Laboratories**
- 939 A minimum of 8 laboratories reporting valid data for each food type is required. It is suggested
940 that at least 10–12 laboratories begin the analysis.
- 941 **5.3.3 Reference Method**
- 942 Candidate methods are compared to a reference method where applicable. The reference
943 method(s) used in the collaborative study must be the same as those used in the SLV
944 (Precollaborative) Study.
- 945 **5.3.4 Matrix Selection**
- 946 **5.3.5 One matrix from the PTM or PCS shall be chosen as the matrix for the collaborative study. The**
947 **AOAC General Referee, Statistical Advisor and collaborative study protocol reviewers shall make**
948 **the final selection of the matrix with consideration of the PTM or PCS data and the relative**
949 **importance of the matrices to food safety. In cases where the candidate method includes more**
950 **than one sample preparation methodology, the General Referee, Statistical Advisor and protocol**
951 **reviewers may select more than one matrix for the collaborative study. The data from both the**
952 **PCS and CS studies form the basis for defining the method applicability statement.** **Levels of**
953 **Contamination**
- 954 For artificially contaminated food types, 3 inoculated levels (high, medium, and low) and one
955 uninoculated level are required. The low level should be at the estimated limit of detection, and
956 the medium and high levels may be approximately 10 and 100 times higher, respectively.
957 Intermediate levels may be added, but they are not required.
- 958 If the method is intended to detect more than one target organism simultaneously from the same
959 test portion, the validation study should be designed so that target organisms are inoculated into a
960 common sample and the validation tests are performed in a simultaneous manner.
- 961 **5.3.6 Number of Test Portions**

Deleted: Food Categories

Deleted: The number of different food categories depends on the applicability of the method. If the method is specific to six or fewer matrices, then all matrices must be included in the CS. If the claimed applicability is for more than six matrices, then at least 6 matrices from the PCS must be included in the CS. The data from both the PCS and CS studies form the basis for defining the method applicability statement.¶

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- 962 | For each [contamination](#) level, 2 test portions are analyzed by the candidate method and 2 test
 963 | portions are analyzed by the reference method in each laboratory. Deleted: food type and
- 964 | **5.3.7 Enumeration of Specific Microorganisms**
- 965 | If the candidate method is for quantitation of a specific microorganism, it may be necessary to
 966 | include certain food types known to support the growth of such analytes. The inoculating
 967 | microorganisms must represent different genera, species and/or toxin-producing microorganisms
 968 | that are intended to be included in the method applicability statement. The choice of strains should
 969 | be broad enough to represent the inherent variation in the microorganisms of interest. Comment [SB2]: Delete?
- 970 | **5.3.8 Source of Contamination**
- 971 | Refer to section 5.1.3.5.
- 972 | **5.3.9 Preparation of Artificially Contaminated Samples**
- 973 | Refer to section 5.1.3.6.
- 974 | **5.3.10 Use of Artificially and Naturally Contaminated Test Samples**
- 975 | The use of both naturally and artificially contaminated test samples is strongly encouraged.
 976 | Because naturally contaminated foods are not always available particularly for methods applicable
 977 | to specific microorganisms, artificially contaminated test samples may be used.
- 978 | **5.3.11 Confirmation of Test Portions**
- 979 | Follow the reference method as written for isolation and confirmation of typical colonies from all
 980 | candidate method test portions.
- 981 | **5.3.12 Data Analysis and Reporting**
- 982 | For a detailed explanation of the quantitative method calculations to be performed, refer to the
 983 | OMA Program Manual Part 6, Appendix D (2).
- 984 | **5.3.12.1 General Considerations**
- 985 | Data often do not show a statistically normal distribution. In order to normalize the data, perform a
 986 | logarithmic transformation on the reported CFU/unit (including any zero results) as follows:
- 987 |
$$\text{Log}_{10} [\text{CFU}/\text{unit} + (0.1)f]$$
- 988 | Where f is the reported CFU/unit corresponding to the smallest reportable result, and unit is the
 989 | reported unit of measure (e.g., g, mL, 25 g). For details, see Appendix X-I.
- 990 | **5.3.12.2 Initial Review of Data**
- 991 | Plot the candidate method result versus the reference method result. The vertical y-axis
 992 | (dependent variable) is used for the candidate method and the horizontal x-axis (independent
 993 | variable) for the reference method. This independent variable x is considered to be accurate and
 994 | have known values. Usually major discrepancies will be apparent.
- 995 | Construct a Youden plot. For a given matrix-level combination, plot replicate pairs as first
 996 | replicate versus second replicate. Usually major discrepancies will be apparent: displaced means,
 997 | unduly spread replicates, outlying values, differences between methods, consistently high or low
 998 | laboratory rankings, etc.
- 999 | Only valid data should be included in the statistical analysis.
- 1000 | **5.3.12.3 Outliers**
- 1001 | It is often difficult to make reliable estimations (average, standard deviation, etc.) with a small bias
 1002 | and in presence of outliers. Data should be examined to determine whether any laboratory shows
 1003 | consistently high or low values or an occasional result that differs from the rest of the data by a
 1004 | greater amount than could be reasonably expected or found by chance alone. Perform outlier tests
 1005 | (Cochran and Grubbs) in order to discard the outlying values and to obtain a better estimate (2).
 1006 | There must be an explanation for every excluded data set; no data sets can be excluded on a

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1007 statistical basis only. To view the data adequately, construct a stem-leaf display, a letter-value
1008 display, and a boxplot (3).

1009 **5.3.12.4 Performance Indicators**

1010 Performance indicators for quantitative methods include repeatability and reproducibility standard
1011 deviations of the transformed data.

1012 **5.3.12.4.1 Repeatability (s_r)**

1013 Calculate repeatability as the standard deviation of replicates at each concentration of each matrix
1014 for each laboratory.

1015 **5.3.12.4.2 Reproducibility (s_R)**

1016 Calculate reproducibility as the standard deviation of replicates at each concentration for each
1017 matrix across all laboratories.

1018 **5.3.12.5 Mean Difference between Candidate and Reference Methods Where Applicable**

1019 Report the mean difference between the candidate and reference method transformed results and
1020 its 95% confidence interval. In addition, report the reverse transformed mean difference and
1021 confidence interval in CFU/unit.

1022 **5.3.12.6 Calculations**

1023 For details, refer to OMA Program Manual, Part 6 (2).

1024

1025 **6 Identification Methods**

1026

1027 **6.1 Method Developer Validation Study or SLV (Precollaborative) Study**

1028 **6.1.1 Scope**

1029 The Method Developer Study is intended to determine the performance of a microbiological
1030 identification method. The study is designed to evaluate performance parameters including
1031 inclusivity, exclusivity, robustness, and reagent quality consistency and stability. The Method
1032 Developer Study is normally conducted in a single laboratory, usually the method developer's
1033 laboratory. Alternatively, the method developer can contract the work to an independent site.

1034 The SLV (Precollaborative) Study is a formal submission requirement for OMA microbiology
1035 methods and is normally conducted in the method developer laboratory. It precedes the
1036 Collaborative Study. The purpose of an SLV (Precollaborative) Study is to define the applicability
1037 claims of a proposed OMA microbiology method. For OMA methods, the applicability statement
1038 immediately follows the method title.

1039 **6.1.2 Inclusivity/Exclusivity Study**

1040 **6.1.2.1 Species/Strain Selection**

1041 The choice of inclusivity strains should cover the genetic, serological, biochemical or physical
1042 diversity of the target agent group(s) as appropriate for the method. The number of organisms
1043 required for validation will be determined by the diversity of the target agent group(s) and the
1044 intended use claim. The number of strains tested should be no less than 30 for each pathogenic
1045 target species claimed where possible and no less than 3 strains for each non-pathogenic target
1046 species claimed. For *Salmonella* methods, the number of target organisms is increased to at least
1047 100 serovars that are selected to represent the majority of known somatic groups of *Salmonella*.

1048 The choice of exclusivity strains should include organisms not claimed by the identification
1049 method. The choice of exclusivity strains should reflect closely related, potentially competitive
1050 organisms. Other factors such as virulence, frequency of occurrence and availability should be
1051 considered. The number of species / strains tested should be no less than 30.

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1052 Species / strains selected for testing must be different than those used to develop the method if
 1053 possible. Species / strains specified for use must be traceable to the source. The source and origin
 1054 of each species / strain should be reported. Species / strains must have Certificate of Analysis
 1055 from the source documenting the identity and method(s) used to determine the identity or be well
 1056 characterized before use with documentation on file.

1057
 1058 The study designs presented are intended to be a suggested guideline. Specific study designs and
 1059 numbers of strains will be determined by the Methods Committee for Microbiology on a case by
 1060 case basis.
 1061

1062 6.1.2.2 Study Design

1063 Inclusivity strains are prepared and analyzed as vegetative cells on the media designated in the
 1064 candidate method. All media recommended for use with the candidate method must be validated.
 1065 Test one replicate per strain per medium using the candidate method.

1066 Exclusivity strains are prepared and analyzed as vegetative cells on the media designated in the
 1067 candidate method. All media recommended for use with the candidate method must be validated.
 1068 Test one replicate per strain per medium using the candidate method.

1069 Inclusivity and exclusivity evaluations shall be performed together as one study. Inclusivity and
 1070 exclusivity test samples must be blind coded and intermingled so the analysts cannot know the
 1071 identity of the test samples.

1072 6.1.2.3 Data Analysis and Reporting

1073 Analyze the data for correct identification, misidentification or unidentified organism. The data is
 1074 reported as number of species / strains correctly identified. For example, “Of the 30 specific
 1075 inclusivity strains tested, 28 were correctly identified and 2 were misidentified. Those strains
 1076 misidentified were the following:...” or “Of the 30 specific exclusivity strains tested, 27 were
 1077 correctly unidentified and 3 were misidentified. Those misidentified by the method were the
 1078 following:...”

1079 The study report should include a table titled “Inclusivity/Exclusivity Panel Results,” which lists
 1080 all species / strains tested their source, origin and essential characteristics plus testing outcome.
 1081

1082 6.1.3 Robustness Study (PTM submissions only)

1083 6.1.3.1 Strain Selection

1084 Robustness strains are prepared and analyzed as vegetative cells on agar(s) recommended by the
 1085 candidate method. Prepare ten inclusivity strains and five exclusivity strains for testing.

1086 6.1.3.2 Study Design

1087 Minor, reasonable variations in a method of a magnitude that might well be expected to occur
 1088 when the method is used are deliberately introduced and tested. Variations in method parameters
 1089 that can be influenced by the end user should be tested. Use a screening factorial experimental
 1090 design.

1091 The method developer, in conjunction with the AOAC Project Manager, is expected to make a
 1092 good faith effort to choose parameters that are most likely to affect the analytical performance and
 1093 determine the range of variations that can occur without adversely affecting analytical results.

1094 Test one replicate of each inclusivity and exclusivity organism for each factorial pattern.

1095 6.1.3.3 Data Analysis and Reporting

1096 The results are analyzed for the number of misidentifications when method parameters are altered.
 1097 Report the identification results for each factorial pattern.
 1098

1099 6.1.4 Product Consistency and Stability Study (PTM submissions only)

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- 1100 **6.1.4.1 Strain Selection**
- 1101 Strains for product quality testing are prepared and analyzed as vegetative cells on the agar(s)
- 1102 recommended for use with the candidate method. Prepare ten inclusivity and five exclusivity
- 1103 organisms for testing.
- 1104 **6.1.4.2 Study Design**
- 1105 For the lot-to-lot consistency study, 6 lots of test kits (or test reagents) are tested and must show
- 1106 consistent results.
- 1107 For product stability, either real-time or accelerated stability data will be accepted for initial PTM
- 1108 certification. If accelerated stability data are submitted, real-time stability data must be submitted
- 1109 within one year to support the shelf life of the kit. For real-time stability, data demonstrating no
- 1110 misidentifications must be submitted.
- 1111 Test one replicate of each inclusivity and exclusivity organism for each lot or time point.
- 1112 Lot-to-lot and stability testing can be combined into one study by testing 6 lots spanning the shelf
- 1113 life of the product.
- 1114 **6.1.4.3 Data Analysis and Reporting**
- 1115 The results are analyzed for the number of misidentifications between lots or time points. Report
- 1116 the identification results for each kit/reagent lot or stability time point.
- 1117
- 1118 **6.1.5 Between Instrument Variability Study (if applicable, PTM submissions only)**
- 1119 **6.1.5.1 Strain Selection**
- 1120 Strains for between instrument variability testing are prepared and analyzed as vegetative cells on
- 1121 the agar(s) recommended for use with the candidate method. Prepare ten inclusivity and five
- 1122 exclusivity organisms for testing.
- 1123 **6.1.5.2 Study Design**
- 1124 Six instruments are tested and must show consistent results. Test one replicate of each inclusivity
- 1125 and exclusivity organism for each instrument.
- 1126 **6.1.5.3 Data Analysis and Reporting**
- 1127 The results are analyzed for variable misidentifications between instruments.
- 1128
- 1129 **6.2 Independent Validation Study (PTM submissions only)**
- 1130 **6.2.1 Scope**
- 1131 A validation study to corroborate the analytical results obtained by the method developer and to
- 1132 provide additional single laboratory data. The independent validation study verifies the inclusivity
- 1133 and exclusivity in the hands of an independent trained user.
- 1134 **6.2.2 Study Design**
- 1135 Inclusivity and exclusivity strains are prepared and analyzed as vegetative cells on the media
- 1136 designated in the candidate method. All media recommended for use with the candidate method
- 1137 must be tested by the Independent laboratory. Test one replicate per strain per medium using the
- 1138 candidate method. For inclusivity, the independent laboratory must test at least 10 strains per
- 1139 pathogenic species claimed and at least 1 strain per non-pathogenic species claimed. For
- 1140 exclusivity, the independent lab must test at least 10 strains not claimed by the method. The
- 1141 strains selected should be different from those used to develop the method where possible.
- 1142 Inclusivity and exclusivity evaluations shall be performed together as one study. Inclusivity and
- 1143 exclusivity test samples must be blind coded and intermingled so the analysts cannot know the
- 1144 identity of the test samples.
- 1145 The study designs presented are intended to be a suggested guideline. Specific study designs and

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1146 numbers of strains will be determined by the Methods Committee for Microbiology on a case by
1147 case basis.

1148 **6.2.3 Data Analysis and Reporting**

1150 Analyze the inclusivity data for correct identification, misidentification and unidentified
1151 organisms. The data are reported as number of species / strains correctly identified. For example,
1152 “Of the 10 specific inclusivity strains tested, 9 were correctly identified and 1 was misidentified.
1153 The strain misidentified was the following:...”

1154 The study report should include a table titled “Inclusivity Panel Results,” which lists all species /
1155 strains tested, their source, origin and essential characteristics plus testing outcome.

1156 Analyze the exclusivity data for misidentifications and unidentified organisms. The data is
1157 reported as number of strains correctly unidentified. For example, “Of the 10 specific exclusivity
1158 strains tested, 7 were correctly unidentified and 3 were misidentified. Those misidentified by the
1159 method were the following:...”

1160 The study report should include a table titled “Exclusivity Panel Results,” which lists all strains
1161 tested, their source, origin and essential characteristics plus testing outcome.

1162

1163 **6.3 Collaborative Study**

1164 **6.3.1 Scope**

1165 The Collaborative Study is a requirement for OMA methods and succeeds the SLV
1166 (Precollaborative) Study. The purpose of the Collaborative Study is to estimate the reproducibility
1167 and determine the performance of the candidate method among collaborators.

1168 **6.3.2 Number of Collaborators**

1169 A minimum of 8 laboratories reporting valid data are required. The study director should plan on
1170 including additional laboratories due to potential invalid data sets.

1171 **6.3.3 Number of Tests**

1172 Each collaborator receives 12 replicates each of a subset of inclusivity and exclusivity organisms
1173 as recommended by the Methods Committee on Microbiology. Data collection at all test sites
1174 must begin on the same day to control for the age of the cultures.

1175 **6.3.4 Data Analysis and Reporting**

1176 Analyze the inclusivity data for correct identification, misidentification and unidentified
1177 organisms by laboratory. The data are reported as number of species / strains correctly identified
1178 by laboratory. For example, “Of the N specific inclusivity strains tested, N-2 were correctly
1179 identified and 2 were misidentified in Laboratory 1. Those strains misidentified were the
1180 following:...”

1181 The study report should include a table titled “Inclusivity Panel Results,” which lists all species /
1182 strains tested, their source, origin and essential characteristics plus testing outcome by laboratory.

1183 Analyze the exclusivity data for misidentifications and unidentified organisms. The data are
1184 reported as number of strains correctly unidentified. For example, “Of the M specific exclusivity
1185 strains tested, M-3 were correctly unidentified and 3 were misidentified in Laboratory 1. Those
1186 misidentified by the method were the following:...”

1187 The study report should include a table titled “Exclusivity Panel Results,” which lists all strains
1188 tested, their source, origin and essential characteristics plus testing outcome by each laboratory.

1189

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Appendix X-A. Classification of Food Categories for Validation Studies

Food categories and/or food types that one would like to include in a validation study but that are not found on this list should be reviewed by the appropriate General Referee and the Methods Committee on Microbiology and Extraneous Materials.

Table X-A.1 Food categories relevant to foodborne pathogenic bacteria

Food type	<i>Yersinia</i> spp.	<i>Clostridium perfringens</i>	<i>Listeria monocytogenes</i>	<i>E.coli</i> O157 and VTEC	<i>Staphylococcus aureus</i>	<i>S.aureus</i> enterotoxins	<i>Campylobacter</i> spp.	<i>Salmonella</i> spp.	<i>Bacillus cereus</i>
A. Meat products									
Raw	x		x	x			x	x	x
Heat processed			x	x	x	x		x	
Frozen			x	x				x	
Fermented			x	x				x	
Cured		x	x		x	x		x	
Other		Dishes/gravy	Pâté					x	
B. Poultry									
Raw	x						x	x	
Heat processed								x	
Frozen								x	
Others		Dishes/gravy							
C. Fish and seafood products									
Raw	x		x	x			x	x	
Heat processed								x	
Frozen			x	x				x	
Shellfish	x			x			x	x	
Smoked		x	x		x	x		x	
Other								x	
D. Fruits and vegetable based products									
Unpasteurized juices				x				x	
Raw	x		x	x			x	x	
Heat processed		x							
Frozen			x					x	
Dry									x
Juice/concentrates				x				x	
Low moisture								x	
Nut meats			x	x				x	
Others						Processed mushrooms			
E. Dairy products									
Raw	x		x	x	x	x	x	x	x
Heat processed			x						x
Frozen			x	x	x	x		x	x
Fermented			x	x	x	x		x	
Dry					x	x		x	x
Other									
Ice cream			x					x	

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Cheese			x	x				x	
F. Chocolate/bakery products									
Low moisture								x	
Dry powder								x	
Milk chocolate								x	
Others					Pastry	Pastry			Custards
G. Animal feeds									
Low moisture								x	
Pet food								x	
H. Pasta									
Uncooked noodles								x	
I. Miscellaneous									
Dressings			x	x				x	
Spices		x						x	
Mayonnaise			x	x			x	x	
Flour			x				x	x	
Egg and derivatives				x				x	
Cereals/rice									x

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1241 Table X-A.2 Food categories relevant to nonpathogenic microorganisms

Food Type	Yeasts and molds	Lactic acid bacteria	Total viable counts	Coliforms	<i>Escherichia coli</i>
A. Meat products					
Raw	x	x	x	x	x
Heat processed		x	x	x	
Frozen	x		x	x	x
Fermented	x	x	x		
Cured		x	x		
B. Poultry products					
Raw	x	x	x	x	x
Heat processed		x	x	x	
Frozen	x		x	x	x
Others					
C. Fish and seafood products					
Raw	x	x	x	x	x
Heat processed		x	x	x	
Frozen	x		x	x	x
Smoked	x	x	x	x	
D. Fruits and vegetable based products					
Raw	x	x	x	x	x
Heat processed			x	x	
Frozen	x		x	x	
Dry	x		x	x	
Fermented	x		x		
Cured/salted	x		x		
Juice/concentrates	x	x	x		
Low moisture	x				
E. Dairy products					
Raw	x	x	x	x	x
Heat processed			x	x	
Frozen	x		x	x	x
Fermented	x				x
Dry			x	x	
F. Chocolate/bakery products					
Low moisture/IMF	x		x	x	
Dry			x	x	
Milk chocolate	x		x	x	
G. Animal feeds					
Low moisture	x		x	x	
Dry pet food	x			x	x
H. Pasta					
Uncooked noodles	x		x	x	

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I. Miscellaneous					
Dressings	x	x	x	x	x
Spices			x		x
Mayonnaise	x	x	x		x
Egg and derivatives			x	x	
Cereals/rice			x	x	

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Notes: Examples of representative food products contained in food categories listed in Annex A, Tables A.1 and A.2

Food Category	Example Matrices
A. Meat products	Ground beef, ground pork, meat by products, glandular products, frog legs, rabbit carcasses, lamb, sausage, frankfurters, lunch meat, beef jerky, meat substitutes
B. Poultry	Ground chicken, ground turkey, cooked chicken, raw chicken parts
C. Fish and seafood products	Raw shrimp, fish sticks, surimi, raw fish filet, raw oysters, raw mussels, raw clams, cooked crawfish, smoked fish, pasteurized crab meat
D. Fruits and vegetable based products	Fresh/frozen fruits or dried fruits, orange juice, apple juice, apple cider, tomato juice, melon cubes, berries
	Pecans, walnuts, peanut butter, coconut, almonds
	Lettuce, spinach, kale, collard greens, cabbage, bean sprouts, seed sprouts, spent water from bean sprouts and seed sprouts, peas, mushrooms, green beans
E. Dairy products	Yogurt, cottage cheese, hard and soft cheeses, raw or pasteurized liquid milk (skim, 2% fat, whole, buttermilk), infant formula, coffee creamer, ice cream, nonfat dry milk/dry whole milk, dried buttermilk, dried cheese spray
F. Chocolate/bakery products	Frosting and topping mixes, candy and candy coating, milk chocolate
G. Animal feed	Dry pet food, meat and bone meal, chicken and feather meal
H. Uncooked pasta	Uncooked noodles, macaroni, spaghetti
I. Miscellaneous	Shell eggs, liquid whole eggs, oral or tube feedings containing egg, dried whole egg or dried egg yolk, dried egg whites
	Oregano, pepper, paprika, black pepper, white pepper, celery seed or flakes, chili powder, cumin, parsley flakes, rosemary, sesame seed, thyme, vegetable flakes, onion flakes, onion powder, garlic flakes, allspice
	Wheat flour, casein, cake mixes, whey, nonfat dry milk/dry whole milk, corn meal, dried whole egg or dried egg yolk, dried egg whites, soy flour, dried yeast, cereals, dried buttermilk, dry cheese spray

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1248 **Appendix X-B. MPN Analysis of Contaminated Matrix**
 1249

1250 The most probable number (“MPN”), also known as the maximum likelihood estimate, is obtained as the
 1251 root of the following equation:

1252

$$1253 \sum_{k=1}^K \left[\frac{d_k m_k}{\exp(d_k \text{MPN}) - 1} - d_k (n_k - m_k) \right] = 0$$

1254

1255 where the summation over $k = 1, 2, \dots, K$ ranges over the serial dilution sets, and

1256

1257 d_k = the amount of sample used in the k -th dilution set

1258 m_k = the number of replicates in the k -th dilution set

1259 n_k = the number of positive results in the k -th dilution set

1260 MPN = the most probable number estimate

1261

1262 A 95% confidence interval for the MPN estimate can be obtained as the 2.5% and 97.5% quantiles of
 1263 sampling distribution of MPN generated by bootstrap resampling with 10,000 realizations. For bootstrap
 1264 resampling to be acceptable, at least one dilution set with fractional response must have 5 replicates or
 1265 more.

1266

1267 Approximate confidence intervals may also be found from one of the following formulas:

1268

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

1269 $\text{UCL, LCL} = \text{MPN} \pm \frac{1.9600}{\sqrt{I}}$, directly on MPN

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

1270

1271

1272 When an equal number of replicates in each set and a constant dilution ratio between sets are used, tables,
 1273 such as those in the FDA Bacteriological Analytical Manual Appendix 2, may be used to supply estimates
 1274 of MPN with 95% confidence intervals.

1275

1276 It is strongly recommended that no less than 5 replicates be used in each dilution set, and that the replicates
 1277 tested in the reference laboratory be included as one of the dilutions for each concentration level. Dilution
 1278 sets with fewer replicates supply unreliable estimates. For fractional detection concentration levels, a
 1279 dilution ratio of 1/2 or 1/3 is recommended instead of the customary 1/10.

1280

1281

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1283 EXAMPLE:

1284

1285 A candidate test method is evaluated at an expected 50% fractional detection concentration level. Twenty
 1286 replicates are done in the reference laboratory. During test portion preparation, 5 replicates are made each
 1287 of 3 and 1/3 times the desired concentration level. All 30 replicates are tested by the reference method in
 1288 the reference laboratory, with presence or absence results.
 1289

	Initial	Estimate	Bootstrap LCL	Bootstrap UCL
MPN:	0.055	0.053	0.034	0.086
		Direct:	0.027	0.079
		In based:	0.032	0.087

Series	Dilution Factor	Number Tubes	Number Positive	Dilution Estimate
1	3.00000	5	5	0.333
2	1.00000	20	15	0.024
3	0.33333	5	1	0.012

1290

1291

1292 “The MPN estimate is 0.053 MPN/g (1.3 MPN/25g) with a 95% confidence interval from bootstrap
 1293 resampling of 0.034 MPN/g (0.85 MPN/25g) to 0.086 MPN/g (2.2 MPN/25g).”

1294

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1296 **Appendix X-C. Raw Format Data Table Template and Example for Qualitative Method Single**
 1297 **Laboratory and Collaborative Studies**

1298

1299 The purpose of the Raw Format Data Table is to document in a software-friendly format all of the factors,
 1300 variables and measurements in the experiment. By matrix and concentration level, report each result from
 1301 each method for each test portion separately.

1302 Each row (record) in the Raw Format Data Table should contain the following columns (fields):

1303

- 1304 1. Matrix type: An identifier indicating the matrix involved, such as “EGGS”. The same exact
 1305 identifier must be used for the same matrix.
- 1306 2. Concentration level: The MPN/test portion for the level. (The MPN/test portion, and not MPN/g
 1307 or MPN/mL, is the relevant measure for statistical analysis of the data.)
- 1308 3. Laboratory: An identifier indicating the laboratory involved, such as “01”.
- 1309 4. Method: An identifier indicating the test method used, such as “REF “ for the reference method,
 1310 “C-P” for the candidate presumptive method or “C-C” for the candidate confirmation method.
- 1311 5. Replicate: A unique identifier for the test portion involved. If this identifier is common to two
 1312 rows in the Table, this implies the results are matched by test portion. Example identifiers might
 1313 be “01” or “001” or “A1”.
- 1314 6. Result: “0” for absence or “1” for presence (detection).

1315

1316 In computer format, the Raw Format Data Table should be given either as: 1) a “fixed-format” file with
 1317 fixed column widths and blanks or tabs as separators and a file extension of “.txt” or “.xls”; or 2) a
 1318 “comma-separated value” file with commas as separators between columns and identifiers within quotes,
 1319 and a file extension of “.csv”.

1320

1321 It is desirable to include a “header” record as the first record in the file with identifiers for each column.

1322

1323 An example file named “ecoli.csv” might be:

1324

1325 “matrix”, “level”, “lab”, “method”, “replicate”, “result”

1326 “spinach”, “2.20”, “01”, “cpres”, “001”, 0

1327 “spinach”, “2.20”, “01”, “cconf”, “002”, 1

1328 “spinach”, “2.20”, “01”, “ref”, “003”, 1

1329 “spinach”, “2.20”, “01”, “cpres”, “004”, 1

1330 “spinach”, “2.20”, “01”, “cconf”, “005”, 1

1331 “spinach”, “2.20”, “01”, “ref”, “006”, 1

1332 etc.

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1333 **Appendix X-D. Calculation of POD and dPOD Values from Qualitative Method Single Laboratory**
 1334 **Data**

1335

1336 In general, four different probabilities detected (PODs) are to be calculated: POD_R (for the reference
 1337 method), POD_C (for the confirmed candidate method), POD_{CP} (for the candidate presumptive method) and
 1338 POD_{CC} (for the candidate confirmation method).
 1339

1340 For each of these four cases, calculate the POD as the ratio of the number positive (x) to total number
 1341 tested (N):
 1342

1343
$$POD = \frac{x}{N}, \quad \text{Where POD is } POD_C, POD_R, \text{ etc.}$$

1344

1345 The POD estimates and 95% confidence interval (LCL, UCL) estimates are given by:
 1346

1347 1. For the case where $x = 0$,
 1348

POD = 0

1349 LCL = 0

UCL = $3.8415 / (N + 3.8415)$

1350

1351 2. For the case where $x = N$,
 1352

POD = 1

1353 LCL = $N / (N + 3.8415)$

UCL = 1

1354

1355 3. For the case where $0 < x < N$,
 1356

POD = x / N

1357
$$LCL = \frac{x + 1.9207 - 1.9600 \sqrt{x - \frac{x^2}{N} + 0.9604}}{N + 3.8415}$$

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

1358 where $1.9600 = z$, the Gaussian quantile for probability 0.975, $1.9207 = z^2 / 2$, $0.9604 = z^2 / 4$ and $3.8415 =$
 1359 z^2 .
 1360

1361 Finally, if $x \leq 1$, set LCL = 0. If $x \geq N-1$, set UCL = 1.
 1362

1363 The confidence interval corresponds to the uncorrected Wilson-score method, modified for $x = 1$ and $x =$
 1364 $N-1$ to improve coverage accuracy on the boundary.

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1365

1366 The differences in proportions detected are estimated by:
1367

$$1368 \quad d\text{POD}_C = \text{POD}_C - \text{POD}_R$$

$$d\text{POD}_{CP} = \text{POD}_{CP} - \text{POD}_{CC}$$

1369

1370 The associated 95% confidence interval (LCL, UCL) for the expected value of $d\text{POD} = \text{POD}_1 - \text{POD}_2$
1371 is estimated by:
1372

$$\text{LCL} = d\text{POD} - \sqrt{(\text{POD}_1 - \text{LCL}_1)^2 + (\text{POD}_2 - \text{UCL}_2)^2}$$

1373

$$\text{UCL} = d\text{POD} + \sqrt{(\text{POD}_1 - \text{UCL}_1)^2 + (\text{POD}_2 - \text{LCL}_2)^2}$$

1374

1375 where (LCL₁, UCL₁) is a 95% confidence interval for POD₁ and (LCL₂, UCL₂) is a 95% confidence
1376 interval for POD₂, as determined above.
1377
1378
1379
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1383 | Appendix X-E. Summary Data Table for Qualitative Method Single Laboratory Studies

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1385

Table 1: Comparative results for the detection of *Listeria innocua* in raw shrimp

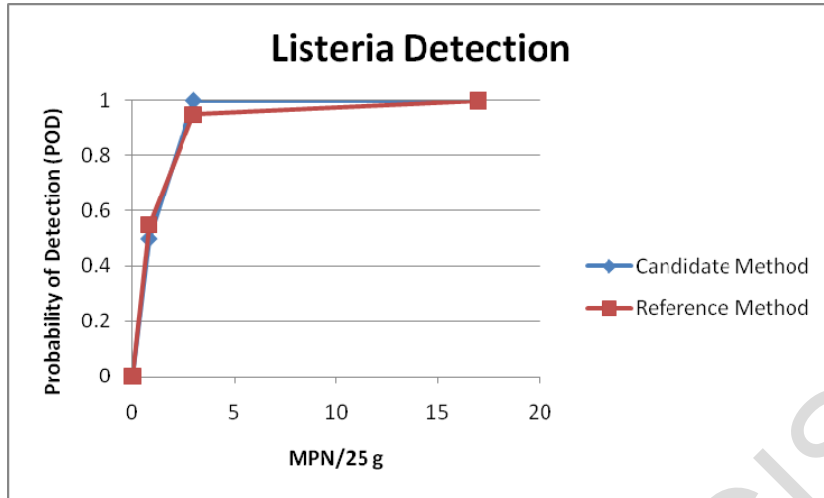
Statistic	Concentration MPN/25g	Candidate presumptive (CP)			Candidate confirmed (CC)			Candidate method (C)			Reference method (R)			C vs R		CP vs CC	
		N	x	POD(CP)	N	x	POD(CC)	N	x	POD(C)	N	x	POD(R)	dPOD(C,R)	dPOD(CP,CC)		
Estimate	0.00	20	0	0.00	20	0	0.00	20	0	0.00	20	0	0.00	0.00	0.00		
LCL	0.00		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

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Appendix X-F. Example of Graph of POD Values from Qualitative Method Single Laboratory Data



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1396

NOTES:

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

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Appendix X-G. Calculation of LPOD and dLPOD Values from Qualitative Method Collaborative Study Data

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1401

Calculations are done in 4 steps for both the candidate method and the reference method.

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1403
1404
1405
1406

Step 1. The overall fractional response (mean POD = LPOD) for the method is calculated from the pooled POD_j responses of the individual laboratories ($j = 1, 2, \dots, L$). Then the standard deviation of an individual x value, called the repeatability standard deviation s_r , is estimated. Next a 95% confidence interval on s_r is obtained.

1407
1408
1409
1410

Step 2. The standard deviation s_L of the laboratory variance component due to differences in detection among laboratories is estimated. s_L is called the “laboratory effect” standard deviation. A 95% confidence interval for s_L is obtained.

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1412
1413
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1415
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1417

Step 3. Both s_L and s_r are used to estimate a 95% confidence interval for the expected value of LPOD. Also estimated is the reproducibility standard deviation s_R , which is the standard deviation of measurement of a single x at a single laboratory, including both the laboratory-laboratory and repeatability error sources. A 95% confidence interval is estimated for the expected value of s_R . Also estimated is the “intraclass correlation coefficient” (or “ICC”) I_r for the repeatability effect with an associated 95% confidence interval. The I_r indicates the proportion of the total variance that is due to repeatability variance.

1418
1419
1420

Step 4. A T statistic based on the χ^2 distribution or Fisher randomization test of homogeneity of POD across laboratories is done to see if the observed laboratory effect is detectably greater than zero.

1421

LPOD

1422
1423
1424
1425

Report the LPOD estimates with 95% confidence intervals for the candidate method and, if included, the presumptive and confirmed results. LPOD estimates are determined as for the single-laboratory case, but are based on the composite data across laboratories.

1426

$$LPOD = \frac{\sum x_j}{\sum N_j} = \frac{x}{N} \quad j = 1, 2, 3, \dots, L$$

1427
1428
1429

where $N = \sum N_j$ is the total number of data, $x = \sum x_j$ is the total number of detections, and L is the number of laboratories.

1430
1431
1432
1433

The LPOD is the proportion detected as positive in the entire set of data across laboratories for the concentration level for the particular method. LPOD is distinguished from POD in that it includes between-laboratory variation as well as within-lab variation.

1434

Repeatability Standard Deviation (s_r)

1435
1436

Estimate the repeatability standard deviation:

$$s_r^2 = \frac{\sum \left[x_j - \frac{x_j^2}{N_j} \right]}{N - L}$$

1437

1438

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

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1439 where N is the total number of data and L is the number of laboratories.

1440

1441 **Laboratory Effect Standard Deviation (s_L)**

1442 Estimate the laboratory effect standard deviation:

1443

$$s_L^2 = \max \left\{ 0, s_{\text{POD}}^2 - \frac{s_r^2}{n} \right\}$$

where

1444
$$n = \left(N - \frac{\sum N_j^2}{N} \right) \left(\frac{1}{L-1} \right)$$

is the weighted average number of replicates per collaborator and

$$s_{\text{POD}}^2 = \frac{\sum (\text{POD}_j - \text{LPOD})^2}{L-1}$$

1445 is the observed variance in POD values across laboratories.

1446

1447

1448 **Reproducibility Standard Deviation (s_R)**

1449 Calculate the reproducibility standard deviation:

1450

1451
$$s_R^2 = s_r^2 + s_L^2$$

1452 If the laboratory effect s_L is near zero, then $s_r \approx s_R \approx \sqrt{\text{LPOD}(1 - \text{LPOD})}$

1453

1454

1455 **Intraclass Correlation Coefficient for Repeatability (I_r)**

1456

1457 The “intraclass correlation coefficient” (or “ICC”) I_r , measures the fraction of the variance in POD values
1458 due to repeatability s_r^2 , and is estimated by:

1459

1460
$$I_r = \frac{s_r^2}{s_r^2 + s_L^2} = \frac{s_r^2}{s_R^2}$$

1461

1462 **Confidence Intervals for LPOD, s_r , s_L and s_R :**

1463

1464 Methods for calculating confidence intervals for LPOD, s_r , s_L and s_R will be developed by the AOAC
1465 Committee on Statistics and incorporated into the Qualitative Collaborative Study software.

1466

1467

1468 **dLPOD Estimates and Confidence Intervals**

1469 The dLPOD statistics are differences between the LPOD estimates:

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1470 $dLPOD_c = LPOD_c - LPOD_R$
 1471 $dLPOD_{CP} = LPOD_{CP} - LPOD_{CC}$

1471 Confidence intervals for the dLPOD values may be obtained from the standard deviations of the paired
 1472 differences by laboratory:

1473

1474 $s_{dPOD} = \sqrt{\frac{\sum(dPOD_i - dLPOD)^2}{L-1}}$

1475 Methods for calculating confidence intervals for dLPOD will be developed by the AOAC Committee on
 1476 Statistics and incorporated into the Qualitative Collaborative Study software.

1477

1478 **Test of Interlaboratory Variability**

1479 An interlaboratory effect causing POD variation will almost always be present at some level. The minimum
 1480 size of this effect can be judged by the estimate s_L above and how much its LCL exceeds zero. However,
 1481 “Heywood cases” where $s_L = 0$ make LCL = 0, which is then uninformative. To test the specific question
 1482 of whether or not an interlaboratory effect in POD is detectable in the study, a more direct test of this is by,
 1483 e.g., computing the usual test of homogeneity statistic
 1484

$$T = \sum \left[\frac{(x_i - N_i \cdot x/N)^2}{N_i \cdot x/N} + \frac{\left(N_i - x_i - \frac{N_i(N-x)}{N} \right)^2}{N_i(N-x)} \right]$$

1485

$$= \sum \frac{(x_i - N_i \cdot LPOD)^2}{N_i \cdot LPOD(1-LPOD)}$$

1486 which is approximately distributed as χ^2 for L-1 degrees of freedom. Alternatively, a Fisher “exact”
 1487 randomization test can be used on the 2 x L contingency table counts. The size of s_L should be assessed on
 1488 its own merit, and the test of T should be used solely to judge whether or not the study is large enough to
 1489 isolate the effect clearly (e.g., to choose number of replicates or laboratories in a study design). A
 1490 significance level of $\alpha = 0.10$ is recommended for the statistic T above, or $\alpha = 0.05$ if a Fisher “exact” test
 1491 is used.
 1492

1493 **Example:**

1494 Suppose the reference method in an interlaboratory study gave the following results when 12 replicate test
 1495 portions were tested in each of 10 laboratories:

1496

Method	Method	Method		
R	R	R		R
Lab	Positive	Neg	Total	POD
1	7	5	12	0.5833
2	9	3	12	0.7500
3	6	6	12	0.5000
4	10	2	12	0.8333
5	5	7	12	0.4167
6	7	5	12	0.5833

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7	5	7	12	0.4167
8	7	5	12	0.5833
9	11	1	12	0.9167
10	9	3	12	0.7500
All	76	44	120	

1497

1498

1499 Here, $x = 76$, $N = 120$ and $LPOD = 0.6333 (= 76/120)$.

1500

1501 The repeatability standard deviation

1502

$$s_r^2 = \frac{\sum \left[x_j - \frac{x_j^2}{N_j} \right]}{N - L} = \frac{\left[(7 - 49/120) + (9 - 81/120) + K + (9 - 81/120) \right]}{120 - 10}$$

1503

$$= 0.2242$$

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

1504

1505 And $\sqrt{LPOD(1 - LPOD)} = 0.4819$, suggesting s_L will be small compared to s_r .

1506

1507 The among laboratory standard deviation is

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

1508

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

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

$$= 0.01093$$

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1509

1510 and $s_L = \sqrt{0.01093} = 0.1045$, which is noticeably less than s_r , as expected.

1511

1512 The reproducibility standard deviation is

1513

$$s_R^2 = s_r^2 + s_L^2$$

1514

$$= 0.01093 + 0.2242$$

$$= 0.2351$$

1515

1516 so $s_R = \sqrt{0.2351} = 0.4849 \approx s_r$

1517

1518

1519

The results are summarized here:

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

1520

1521

1522

1523

1524

1525

1526

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

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1527 | Appendix X-H. Data Summary Table Template and Example for Qualitative Method Collaborative Studies
 1528
 1529

Table 1: Comparative results for the detection of *Listeria innocua* in raw shrimp by the Candidate and Reference methods in an interlaboratory study.

Statistic	Concentration MPN/25g	Laboratory	Candidate presumptive (CP)			Candidate confirmed (CC)			Candidate result (C)			Reference method (R)			C vs R		CP vs CC	
			N	x	POD(CP)	N	x	POD(CC)	N	x	POD(C)	N	x	POD(R)	dLPOD(C,R)	dLPOD(CP,CC)		
		01	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00		
		02	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00		
		03	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00		
		04	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00		
		05	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00		
		06	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00		
		07	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00		
		08	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00		
		09	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00		
		10	12	0	0.00	12	0	0.00	12	0	0.00	12	0	0.00	0.00	0.00		
Estimate	0.00	All	120	0	0.00	120	0	0.00	120	0	0.00	120	0	0.00	0.00	0.00		
LCL	0.00				0.00			0.00			0.00			0.00	-1.00	-1.00		
UCL	0.02				0.17			0.03			0.03			0.03	0.03	0.03		
s_r					0.00			0.00			0.00			0.00				
LCL					0.00			0.00			0.00			0.00				
UCL					0.17			0.17			0.17			0.17				
s_L					0.00			0.00			0.00			0.00				
LCL					0.00			0.00			0.00			0.00				
UCL					0.03			0.03			0.03			0.03				
s_R					0.00			0.00			0.00			0.00				
LCL					0.00			0.00			0.00			0.00				
UCL					0.24			0.24			0.24			0.24				
I_r					1.00			1.00			1.00			1.00				
LCL					0.00			0.00			0.00			0.00				
UCL					1.00			1.00			1.00			1.00				
P_T					1.0000			1.0000			1.0000			1.0000				

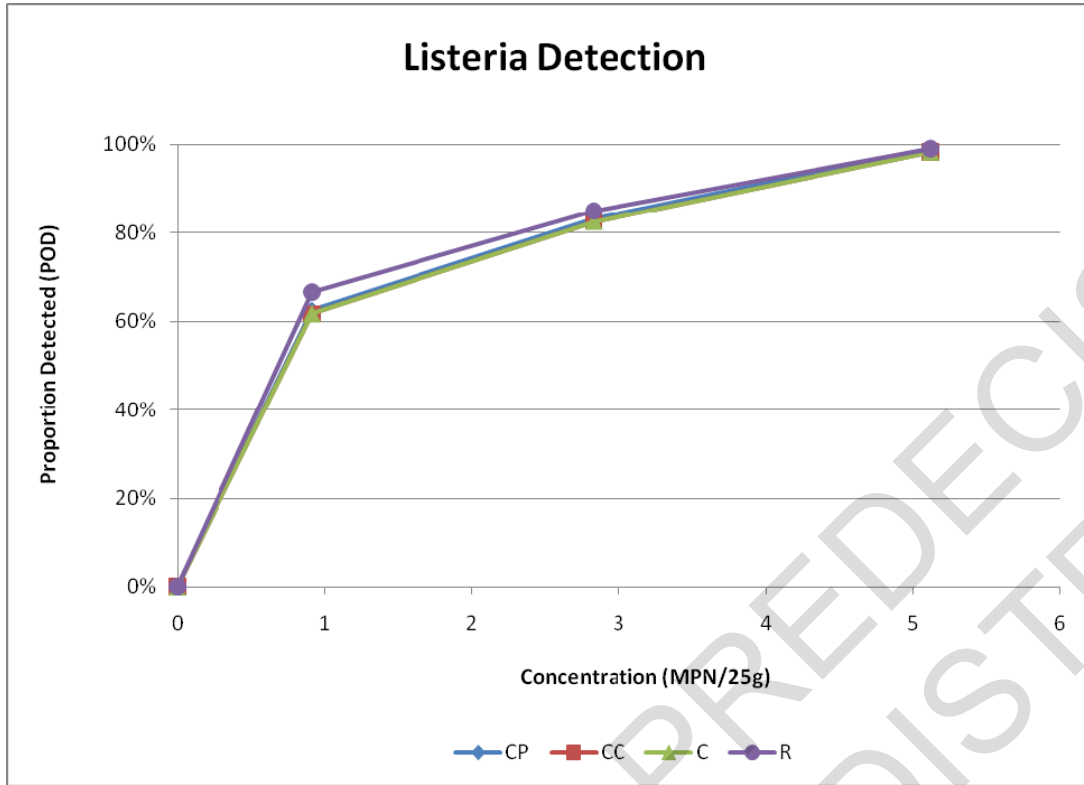
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		01	12 8	0.67	12 8	0.67	12 8	0.67	12 7	0.58	0.08	0.00
		02	12 9	0.75	12 8	0.67	12 8	0.67	12 7	0.58	0.08	0.08
		03	12 8	0.67	12 8	0.67	12 8	0.67	12 6	0.50	0.17	0.00
		04	12 6	0.50	12 6	0.50	12 6	0.50	12 10	0.83	-0.33	0.00
		05	12 7	0.58	12 7	0.58	12 7	0.58	12 7	0.58	0.00	0.00
		06	12 6	0.50	12 6	0.50	12 6	0.50	12 8	0.67	-0.17	0.00
		07	12 8	0.67	12 8	0.67	12 8	0.67	12 6	0.50	0.17	0.00
		08	12 7	0.58	12 7	0.58	12 7	0.58	12 11	0.92	-0.33	0.00
		09	12 8	0.67	12 8	0.67	12 8	0.67	12 9	0.75	-0.08	0.00
		10	12 8	0.67	12 8	0.67	12 8	0.67	12 9	0.75	-0.08	0.00
Estimate	0.92	All	120 75	0.63	120 74	0.62	120 74	0.62	120 80	0.67	-0.05	0.01
LCL	0.73			0.53		0.53		0.53		0.58	-0.36	-0.37
UCL				0.72		0.71		0.71		0.76	-0.04	0.12
s_r				0.50		0.50		0.50		0.47		
LCL				0.44		0.44		0.44		0.42		
UCL				0.52		0.52		0.52		0.52		
s_L				0.00		0.00		0.00		0.04		
LCL				0.00		0.00		0.00		0.00		
UCL				0.13		0.11		0.11		0.22		
s_R				0.50		0.50		0.50		0.47		
LCL				0.45		0.45		0.45		0.42		
UCL				0.52		0.52		0.52		0.52		
I_r				1.00		1.00		1.00		0.99		
LCL				0.99		1.00		1.00		0.82		
UCL				1.00		1.00		1.00		1.00		
P_T				0.9634		0.9867		0.9867		0.3711		
etc.												

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1534 | Appendix X-I. Logarithmic Transformation of Data from Quantitative Method Single Laboratory and
1535 Collaborative Data

1536

1537 Quantitative microbiological count data from experiments spanning multiple dilutions often do not show a
1538 Poisson nor a Gaussian statistical distribution. When the underlying physical mechanism allows for
1539 “clustering”, typically a logarithmic transformation will normalize the data.

1540

1541 Perform a logarithmic transformation on the reported CFU/unit (including any zero results) as follows:

1542

$$1543 \quad Y = \log_{10} [\text{CFU/unit} + (0.1)f]$$

1544

1545 where f is the reported CFU/unit corresponding to the smallest reportable result, and “unit” is the reported
1546 unit of measure (e.g., g, mL, 25 g).

1547

1548 EXAMPLES:

1549

1550 1. For the control concentration, the CFU/g is reported as “< 0.003”. So CFU/unit = 0.0, and Y =
1551 $\log_{10} [0.0 + (0.1)(0.003)] = -3.52$.

1552 2. For the low concentration, the CFU/g is 0.042. So $Y = \log_{10} [0.042 + (0.1)(0.003)] = -1.37$.

1553 3. For the high concentration, the CFU/g is 0.231. So $Y = \log_{10} [0.231 + (0.1)(0.003)] = -0.64$.

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