AOAC SMPR 2014.017

Standard Method Performance Requirements for Detection of *Salmonella* species in Romaine Lettuce and Baby Spinach

Intended Use: Routine Surveillance and Monitoring by a Trained Technician

1 Purpose

AOAC *Standard Method Performance Requirements*SM (SMPRs) describe the minimum recommended performance characteristics to be used during the evaluation of a method. The evaluation may be an on-site verification, a single-laboratory validation, or a multi-site collaborative study. SMPRs are written and adopted by AOAC stakeholder panels composed of representatives from industry, regulatory organizations, contract laboratories, test kit manufacturers, and academic institutions. AOAC SMPRs are used by AOAC expert review panels in their evaluation of validation study data for method being considered for *Performance Tested Methods*SM or AOAC *Official Methods of Analysis*SM, and can be used as acceptance criteria for verification at user laboratories. [Refer to Appendix F: *Guidelines for Standard Method Performance Requirements*, *Official Methods of Analysis of AOAC INTERNATIONAL* (2012) 19th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA.]

2 Applicability

Alternative methods used to detect *Salmonella* species and their serovars in preharvest field samples of romaine lettuce and baby spinach.

3 Analytical Technique

Any analytical technique that meets the method performance requirements is acceptable.

4 Definitions

Alternative method.—Method of analysis that demonstrates or estimates, for a given category of products, the same analyte as is measured using the corresponding reference method. The method can be a proprietary or noncommercial, and does not need to cover an entire analysis procedure, that is from the preparation of samples to the test report. [FDIS 16140-2 (2014): Microbiology of the food chain—Method validation—Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method]

An ISO term used to denote the method to be evaluated against a "reference" method. AOAC uses the term "candidate" method for the same purpose in its guidelines. The terms "alternative" and "candidate" methods are interchangeable for the purposes of this SMPR.

Acceptable minimum detection level (AMDL).—The predetermined minimum level of an analyte, as specified by an expert committee, which must be detected by the alternative method with an estimated 5% lower confidence limit on the probability of detection (POD) of 0.95 or higher. The AMDL is dependent on the intended use. [FDIS 16140-1 (2014): Microbiology of the food chain—Method validation—Part 1: Vocabulary]

Baby spinach.—Spinach (*Spinacia oleracea* L.) that has been harvested during a fairly early stage of plant growth, typically 6–8 true leaves, usually between 20–35 days after planting.

Differential probability of detection (dPOD).—The POD for any two methods can be compared by difference at a given analyte concentration. This difference in POD values is termed dPOD.

Exclusivity.—Study involving pure nontarget strains, which are potentially cross-reactive, that shall be not detected or enumerated by the tested method. (Ibid) *See* Annex II for a list of recommended nontarget strains.

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

Inclusivity.—Study involving pure target strains that shall be detected or enumerated by the alternative method. (Ibid) *See* Annex I for a list of recommended target strains.

Laboratory probability of detection (LPOD).—The POD value obtained from combining all valid collaborator data sets for a method for a given matrix at a given analyte level or concentration. [Appendix H: *Probability of Detection (POD) as a Statistical Model for the Validation of Qualitative Methods*, *Official Methods of Analysis of AOAC INTERNATIONAL*, (2012) 19th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA]

LCL.—Lower confidence limit.

Preharvest field sample.—Prior to and within 7 days of harvest.

Probability of detection (POD).—The proportion of positive analytical outcomes for a qualitative method for a given matrix at a given analyte level or concentration. [Appendix H: *Probability of Detection (POD) as a Statistical Model for the Validation of Qualitative Methods*, *Official Methods of Analysis of AOAC INTERNATIONAL*, (2012) 19th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA]

Romaine lettuce (*Lactuca sativa L. var longifolia*).—A columnar heading-type lettuce with firm, spoon-shaped leaves possessing a prominent mid-rib typical surrounding a compact set of elongated central leaves or 'heart.' Typical maturation takes 65 to 80 days in main season and up to 125–130 days during winter production. Plant spacing and preharvest interval varies depending on target for crop market…heads vs. hearts. Romaine lettuce may also be grown for marketing as baby or tender greens, harvested at a nonheading stage (35–45 days post-emergence) with 5–9 true leaves. Both green and red-pigmented varieties are produced.

Salmonella.—Straight rods, 0.7–1.5 × 2–5 µm. Gram negative. Usually motile by peritrichous flagella. Facultative anaerobic. Chemoorganotrophic, having both a respiratory and fermentative metabolism. D-glucose and other carbohydrates are catabolized with the production of acid and usually gas. Oxidase negative, catalase positive, indole and Voges-Proskauer negative, methyl red and Simmons citrate positive. Lysine and ornithine decarboxylase positive, there is a variable arginine dihydrolase reaction. H_2S is produced, urea is not hydrolyzed, growth on KCN and utilization of malonate are variable. Reduce nitrates. Carbohydrates usually fermented include L-arabinose, maltose, D-mannitol, D-mannose, L-rhamnose, D-sorbitol, trehalose, and D-xylose. Occur in humans, warm- and cold-blooded animals, food, and the environment. Pathogenic for humans and many animal species. Causative agent

of typhoid fever, enteric fevers, gastroenteritis, and septicemia. [*Bergey's Manual of Determinative Bacteriology,* 9th Ed., J.G. Holt (Ed.)]

Test portion.—The test portion is the sample size used in most validation studies and is typically 25 g. A different test portion can be used in validation studies when appropriate. When combining several test portion units into a composite sample, equivalency must be demonstrated in a validation study relative to the test portion of the reference method. For this validation scheme, refer to Appendix J: *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces* [*Official Methods of Analysis of AOAC INTERNATIONAL* (2012) 19th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA], or other acceptable international microbiology validation guidelines.

UCL. —Upper confidence limit.

5 System Suitability Tests and/or Analytical Quality Control

Positive and negative controls shall be embedded in assays as appropriate. Inhibition controls should be used for method verification for each new matrix.

Manufacturer must provide written justification if controls are not appropriate to an assay.

6 Reference Material(s)

None.

7 Validation Guidance

Use preharvest materials for method evaluation. Do not use processed materials.

Appendix J: *AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces* [*Official Methods of Analysis of AOAC INTERNATIONAL* (2012) 19th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, USA]; or ISO 16140:2003.

8 Maximum Time-to-Determination

Maximum time to complete an analysis starting from the test portion preparation to presumptive determination must be ≤24 h.

9 Method Performance Requirements

See Tables 1 and 2.

Approved by International Stakeholder Panel on Alternative Methods (ISPAM). Final Version Date: September 6, 2014. Effective Date: October 23, 2014.

Table 1. Matrix-dependent criteria

Parameter	Parameter requirements	Target test concentration[a]	Minimum acceptable results
Acceptable minimum detection level (AMDL)	Single-laboratory validation (SLV): Minimum of 20 replicates per food type, artificially inoculated as outlined in internationally accepted method validation guidelines	1–5 CFU/test portion	25 to 75% positive rate; and dPOD 95% CI, LCL < 0 < UCL[b]
High concentration	SLV: Minimum of five replicates per food type artificially inoculated as outlined in internationally accepted method validation guidelines at 10× the AMDL concentration	10–50 CFU/test portion	100% correct analyses are expected per food type[c]
Zero concentration	SLV: Minimum of five replicates per food type that have tested negative with the reference method in the validation study and have not been artificially inoculated	0 CFU/test portion	
LPOD	Multi-laboratory study	1–10 CFU/test portion	$0.15 \geq LPOD_C \geq 0.85$ dLPOD = 95% CI, LCL < 0 < UCL[b]
		10–50 CFU/test portion	LPOD[d] ≥ 0.95 dLPOD = 95% CI, LCL < 0 < UCL[b]
$LPOD_{(0)}$	Multi-laboratory study	0 CFU/test portion	LPOD[e] ≤ 0.05
RLOD	SLV	Combined levels	Paired study ≤1.5
	Multi-laboratory study		Unpaired study ≤2.5

[a] Confirm the target test concentration on the initiation of the method evaluation using the 3-level most probable number (MPN) procedure. *See* Annex III for further guidance.

[b] It is expected that the range between the lower and upper control limits should encompass 0. If not, the results must be investigated and an explanation provided.

[c] 100% correct analyses are expected. Some aberrations may be acceptable if the aberrations are investigated, and acceptable explanations can be determined and communicated to method users.

[d] At the 5% lower confidence limit. *See* Annex IV.

[e] At the 95% upper confidence limit. *See* Annex IV.

Table 2. Inclusivity/exclusivity

Parameter	Parameter requirements	Final test concentration (CFU/mL)	Minimum acceptable results
Inclusivity	Single-laboratory validation (SLV) study: At least 100 *Salmonella* serovars cultured by the candidate method enrichment procedure	10–100 × AMDL	100% positive results[a]
Exclusivity	SLV: At least 30 non-*Salmonella* species cultured in nonselective broth	Overnight growth undiluted	100% negative results[a]

[a] 100% correct analyses are expected. All aberrations are to be retested following internationally recognized guidelines (ISO 16140, AOAC OMA Appendix J, The Compendium of Analytical Methods of Health Canada). Some aberrations may be acceptable if the aberrations are investigated, and acceptable explanations can be determined and communicated to method users.

ANNEX I
Inclusivity Panel

List of suggested serovars method developers can use to validate their methods. A minimum of 100 serovars are required for AOAC adoption. Highlighted species/strains are required. Additionally, at least one of each subspecies (salamae, arizonae, diarizonae, houtenae, indica) and one of *Salmonella bongori* must be included in the validation study.

	Salmonella (serovar included)	Antigenic properties serotype		Year outbreak	CDC[a] top 20[b]	FDA[c] ranking top 40
		O	H			
1	*Salmonella bongori*, Serotype Brookfield	66	z_{41}:-			
2	*Salmonella bongori*	66				
3	*Salmonella enterica* subsp. Salamae	47				
4	*Salmonella enterica* subsp. Salamae	50				
5	*Salmonella enterica* subsp. salamae	53				
6	*Salmonella enterica* subsp. salamae	55				
7	*Salmonella enterica* subsp. salamae serovar Artis	56				
8	*Salmonella enterica* subsp. salamae	57				
9	*Salmonella enterica* subsp. salamae serovar Basel	58				
10	*Salmonella enterica* subsp. salamae	59				
11	*Salmonella enterica* subsp. salamae	60				
12	*Salmonella enterica* subsp. Arizonae[d]	40				41
13	*Salmonella enterica* subsp. Arizonae	51				
14	*Salmonella enterica* subsp. Arizonae	62				
15	*Salmonella enterica* subsp. Arizonae	63				
16	*Salmonella enterica* subsp. Arizonae	65				
17	*Salmonella enterica* subsp. diarizonae	35				
18	*Salmonella enterica* subsp. diarizonae	47				
19	*Salmonella enterica* subsp. Diarizonae[d]	48				29
20	*Salmonella enterica* subsp. diarizonae serovar Eilbek	61				
21	*Salmonella enterica* subsp. houtenae serovar Halmstad	3,{10}{15}{15,34}	g,s,t:-			
22	*Salmonella enterica* subsp. houtenae serovar Harmelen	51				
23	*Salmonella enterica* subsp. houtenae serovar Ochsenzoll	16				
24	*Salmonella enterica* subsp. Indica	1,6,14,25				
25	*Salmonella enterica* subsp. Indica	45				
26	*Salmonella enterica* subsp. enterica serovar Paratyphi A	1,2,12				
27	*Salmonella enterica* subsp. enterica serovar Agona[d]	1,4,[5],12	f,g,s:[1,2]	11	15	5
28	*Salmonella enterica* subsp. enterica serovar Heidelberg	1 ,4,[5],12	r:1,2	14, 13, 11	7	
29	*Salmonella enterica* subsp. enterica serovar Paratyphi B[d]	1,4,[5],12	b:1,2		16	34
30	*Salmonella enterica* subsp. enterica serovar Derby	1, 4,[5], 12	f,g:[1,2]			
31	*Salmonella enterica* subsp. enterica Typhimurium[d]	1,4 ,[5],12	i:1,2	13, 12, 11, 10	2	6
32	*Salmonella enterica* subsp. enterica serovar Saintpaul[d]	1,4,[5],12	e,h:1,2	13	12	15
33	*Salmonella enterica* subsp. enterica serovar Sandiego[d]	1,4,[5],12	e,h:e,n,z_{15}	13		24
34	*Salmonella enterica* subsp. enterica 1,4,[5],12:i:-	1,4,[5],12	i:-	10	5	
35	*Salmonella enterica* subsp. enterica Chester	1,4,[5],12	e,h:e,n,x	10		
36	*Salmonella enterica* subsp. enterica Stanley[e]	1,4,[5],12,[27]	d:1,2	14		31
37	*Salmonella enterica* subsp. enterica serovar Indiana	1,4,12	z;1,7			
38	*Salmonella enterica* subsp. enterica serovar Preston	1,4,12	z:l,w			
39	*Salmonella enterica* subsp. enterica serovar Bredeney	1,4,12,27	l,v:1,7	12		
40	*Salmonella enterica* subsp. enterica Vellore	1,4,12,27	z_{10}:z_{35}			
41	*Salmonella enterica* subsp. enterica serovar Schwarzengrund	1 ,4 ,12, 27	d:1,7			
42	*Salmonella enterica* subsp. enterica serovar Abortusequi	4,12	-:e,n,x			
43	*Salmonella enterica* subsp. enterica serovar Abortusovis	4,12	c:1,6			

ANNEX I
Inclusivity Panel (*continued*)

	Salmonella (serovar included)	Antigenic properties serotype		Year outbreak	CDC[a] top 20[b]	FDA[c] ranking top 40
		O	H			
44	*Salmonella bongori*, Serotype Brookfield	66	z_{41}:-			
45	*Salmonella enterica* subsp. enterica Hartford	6, 7	y:e,n,x	10		
46	*Salmonella enterica* subsp. enterica Braenderup[d]	6 , 7,14	Serovar is now recognized as Westhampton var 15+	12	10	40
47	*Salmonella enterica* subsp. enterica serovar Bareilly[d]	6,7,14	y:e,n,x	12	17	14
48	*Salmonella enterica* subsp. enterica serovar Infantis[d]	6,7,14	r:1,5	12	9	28
49	*Salmonella enterica* subsp. enterica serovar Lille	6,7,14	z_{38}:-	13,12		
50	*Salmonella enterica* subsp. enterica serovar Mbandaka[d]	6, 7, 14	z_{10}:e,n,z_{15}	13		2
51	*Salmonella enterica* subsp. enterica serovar Oranienburg[d]	6, 7, 14	m,t:[z_{57}]		11	10
52	*Salmonella enterica* subsp. enterica serovar Thompson[d]	6, 7, 14	K:1,5		14	19
53	*Salmonella enterica* subsp. enterica serovar Montevideo[d]	6 , 7,14,[54]	g,m,[p],s:[1,2,7]	13,12, 10	6	4
54	*Salmonella enterica* subsp. enterica serovar Hadar	6,8	z_{10}:e,n,x	12, 11		
55	*Salmonella enterica* subsp. enterica serovar Muenchen[d]	6, 8	d:1,2		8	12
56	*Salmonella enterica* subsp. enterica serovar Newport[d]	6, 8, 20	e,h:1,2	13,12, 10	3	1
57	*Salmonella enterica* subsp. enterica serovar Haardt	8	k:1,5			
58	*Salmonella enterica* subsp. enterica serovar Kentucky[d]	8, 20	i,z_6			18
59	*Salmonella enterica* subsp. enterica Panama[d]	1,9,12	l,v:1,5	11		39
60	*Salmonella enterica* subsp. enterica serovar Berta	1,9,12	[f],g,[t]:-		19	
61	*Salmonella enterica* subsp. enterica serovar Enteritidis[d]	1,9,12	g,m:-	12, 11, 10	1	17
62	*Salmonella enterica* subsp. enterica serovar Gallinarum	1,9,12	-:-			
63	*Salmonella enterica* subsp. enterica serovar Javiana[d]	1,9,12	i,z_{28}:1,5		4	13
64	*Salmonella enterica* subsp. enterica serovar Neasden	9,12	g,s,t:e,n,x			
65	*Salmonella enterica* subsp. enterica serovar Typhi	9,12[Vi]	d:-	10	18	
66	*Salmonella enterica* subsp. enterica Baildon	9,46	a:e,n,x	10		
67	Salmonella enterica subsp. enterica serovar Anatum[d]	3,{10}{15}{15,34}	e,h:1,6		20	8
68	*Salmonella enterica* subsp. enterica serovar Anatum var. 15+	3,{10}{15}	g,m,s:-			
69	*Salmonella enterica* subsp. enterica serovar Give[d]	3,{10}{15}{15,34}	l,v:1,7			11
70	*Salmonella enterica* subsp. enterica Nchanga	3 ,{10}{15}	l,v:1,2	12		
71	*Salmonella enterica* subsp. enterica serovar Krefeld	1,3,19	y;l,w			
72	*Salmonella enterica* subsp. enterica serovar Senftenberg[d]	1 ,3,19	g,[s],t:-			3
73	*Salmonella enterica* subsp. enterica serovar Abaetetuba[d]	11	k:1,5			38
74	*Salmonella enterica* subsp. enterica serovar Poona[d]	1,13,22	z:1,6	13		21
75	*Salmonella enterica* subsp. enterica Cubana[d]	1 ,13,23	z_{29}:-			27
76	*Salmonella enterica* subsp. enterica Mississippi	1 ,13,23	b:1,5		11	
77	*Salmonella enterica* subsp. enterica serovar Bristol	13,22	z:1,7			
78	*Salmonella enterica* subsp. enterica serovar Putten	13,23	d:l,w			
79	*Salmonella enterica* subsp. enterica serovar Kaitaan	1,6,14,25	m,t:-			
80	*Salmonella enterica* subsp. enterica serovar Schalkwijk	6 ,14,[24]	i:e,n,z_{15}			
81	*Salmonella enterica* subsp. enterica serovar Sundsvall	[1], 6,14,[25]	z:e.n.x			
82	*Salmonella enterica* subsp. enterica serovar Nottingham	16	d:e,n,z_{15}			
83	*Salmonella enterica* subsp. enterica serovar Matadi	17	k:e,n,x			
84	*Salmonella enterica* subsp. enterica serovar Cerro	6,14,18	z_4,z_{23}:[1,5]			
85	*Salmonella enterica* subsp. enterica serovar Minnesota	21	b:e,n,x			

ANNEX I
Inclusivity Panel (*continued*)

	Salmonella (serovar included)	Antigenic properties serotype		Year outbreak	CDC[a] top 20[b]	FDA[c] ranking top 40
		O	H			
86	*Salmonella enterica* subsp. enterica Pomona[d]	28	y:1,7	13		37
87	*Salmonella enterica* subsp. enterica Urbana[d]	30	b:e,n,x			33
88	*Salmonella enterica* subsp. enterica serovar Adelaide	35	f,g:-			
89	*Salmonella enterica* subsp. enterica serovar Inverness	38	k:1,6			
90	*Salmonella enterica* subsp. enterica serovar Champaign	39	k:1,5			
91	*Salmonella enterica* subsp. enterica serovar Johannesburg	1,40	b:e,n,x	11		
92	*Salmonella enterica* subsp. enterica serovar Waycross	41	z_4,z_{23}:[e,n,z_{15}]			
93	*Salmonella enterica* subsp. enterica serovar Kahla	1, 42	z_{35}:1,6			
94	*Salmonella enterica* subsp. enterica serovar Houten	43	z_4,z_{23}:-			
95	*Salmonella enterica* subsp. enterica serovar Niarembe	44	a:l,w			
96	*Salmonella enterica* subsp. enterica serovar Deversoir	45	c:e,n,x			
97	*Salmonella enterica* subsp. enterica serovar Dahlem	48	k:e,n,z_{15}			
98	*Salmonella enterica* subsp. enterica serovar Wassenaar	50				
99	*Salmonella enterica* subsp. enterica Utrecht	52	d:1,5			
100	*Salmonella enterica* subsp. enterica serovar Uccle	54	g,s,t:-			
101	*Salmonella enterica* subsp. enterica Tranora	55	k:z_{39}			
102	*Salmonella enterica* subsp. enterica serovar Crossness	67	r:1,2			
103	*Salmonella enterica* subsp. enterica serovar Weltevreden[d]	3 , {10}{15}	r:z_6			7
104	*Salmonella enterica* subsp. enterica serovar Tennessee[d]	6,7,14	z_{29}:[1,2,7]			9
105	*Salmonella enterica* subsp. enterica serovar Rubislaw[d]	11	r:e,n,x			16
106	*Salmonella enterica* subsp. enterica serovar Virchow[d]	6,7,14	r:1,2			20
107	*Salmonella enterica* subsp. enterica serovar Hvittingfoss[d]	16	b:e,n,x			22
108	*Salmonella enterica* subsp. enterica serovar Gaminara[d]	16	d:1,7			23
109	*Salmonella enterica* subsp. enterica serovar Aberdeen[d]	11	i:1,2			25
110	*Salmonella enterica* subsp. enterica serovar Mgulani	38	i:1,2			26
111	*Salmonella enterica* subsp. enterica serovar Havana[d]	1,13,23	f,g,[s]:-			30
112	*Salmonella enterica* subsp. enterica serovar Wandsworth[d]	39	b:1,2			32
113	*Salmonella enterica* subsp. enterica serovar Caracas[d]	[1],6,14,[25]	g,m,s:-			35
114	*Salmonella enterica* subsp. enterica serovar Rissen[d]	6,7,14	f,g:-			36
115	*Salmonella enterica* subsp. enterica serovar Michigan[d]	17	l,v:1,2			42
116	*Salmonella enterica* subsp. enterica serovar Meleagridis[d]	3,{10}{15}{15,34}	e,h;l,w			43

[a] CDC = Centers for Disease Control and Prevention.

[b] Number indicates the numerical position on CDC list.

[c] FDA = U.S. Food and Drug Administration.

[d] Serotypes isolated from vegetable products, including spices. Courtesy of FDA-CFSAN.

[e] Strains identified from outbreaks, shorten date as (2011 = “11”; 2012 = “12”).

ANNEX II
Exclusivity Panel

Organism

Aeromonas hydrophila
Additional *Aeromonas* species
Burkholderia species
Campylobacter jejuni
Citrobacter braakii
Citrobacter farmerii
Citrobacter freundii
Citrobacter youngae
Additional *Citrobacter* species
Edwardsiella tarda
Enterobacter aerogenes
Enterobacter amnigenus
Enterobacter cancerogenus
Enterobacter cloacae
Enterobacter gergoviae
Enterobacter sakazakii
Erwinia species
Escherichia coli
Escherichia coli O157:H7
Escherichia fergusonii
Escherichia hermanii
Escherichia vulneris
Hafnia species
Klebsiella oxytoca
Klebsiella pneumonia
Morganella morganii
Pantoea species
Proteus hauseri
Proteus mirabilis
Proteus vulgaris
Pseudomonas aeruginosa
Pseudomonas fluorescens
Pseudomonas species
Ralstonia species
Rhanella species
Serratia marcesens
Shigella dysenteriae
Shigella flexneri
Shigella sonnei
Vibrio vulnificus
Yersinia species

ANNEX III
Example Most Probable Number (MPN) Procedures

For 25 Grams

(*1*) Perform a 3-level MPN on the low and high inoculation levels of test material. Prepare five test portions of 50 g and five test portions of 10 g. To the 50 g test portions, add 450 mL of BAM method enrichment broth. To the 10 g test portions, add 90 mL of the BAM method enrichment broth. Follow the BAM reference method for the matrix through to confirmations.

(*2*) Use the twenty 25 g replicate low-level test portions or the five 25 g replicate high-level test portions analyzed by the reference method in the matrix study as the third level for the MPN.

(*3*) Use the number of positives from the 50 g portions, 25 g portions, and 10 g portions to calculate the MPN for each inoculated level of each matrix. Access the MPN calculator (http://www.lcfltd.com/customer/LCFMPNCalculator.exe) to determine the MPN values and 95% confidence intervals.

For 325 Grams

(*1*) For foods with a 325 g reference method test portion, prepare five test portions at 650 g (50 g primary sample + 600 g uninoculated matrix) and five test portions at 130 g (10 g primary sample + 120 g uninoculated matrix). Add the appropriate reference method enrichment broth to give a 1:3 ratio of test portion to broth. Follow the reference method through to confirmations.

(*2*) Use the 20 replicate test portions (low level) or five replicate test portions (high level) analyzed by the reference method in the matrix study as the third level for the MPN.

(*3*) Use the number of positives from the five large portions, 20 (low level) or five (high level) nominal portions, and five small portions to calculate the MPN for each inoculated level of each matrix. Access the MPN calculator (http://www.lcfltd.com/customer/LCFMPNCalculator.exe) to determine the MPN values and 95% confidence intervals. *Note*: Data must be entered in test portion size order from large to small.

Annex IV: LPOD Tables

Table 1. LPOD ≥ 0.95 (5% lower confidence limit)

Equivalent to ≥ x positive results for N analyses	
X	N
119	120
129	130
138	140
148	150

Table 2. LPOD ≤ 0.05 (95% upper confidence limit)

Equivalent to ≤ x positive results for N analyses	
X	N
1	120
2	140
3	165