

	ISO 16140	AOAC OMA	Health Canada	NordVal	FDA	Draft USDA/FSIS
QUALITATIVE methods	ISO 16140 Doc N 1199 (ISO CD 16140-2)PIV C2011-04-06 Pending revision of Part 2	AOAC OMA Draft revision document dated 3/24/11	Health Canada Draft Part 4 dated March, 2011	NordVal Protocol for the validation of alternative microbiological methods March 2009	FDA Guidelines FDA’s Qualitative Microbiology Methods Validation (ORA-LAB.7 version 1.2), pending revision (<i>proposed revision marked in red</i>).	Draft USDA/FSIS Guidelines Disclaimer: The use of the term “validation” is not intended to have any application to the implementation of 9 CFR 417.4(a)(1) on initial validation of HACCP plans. The Draft FSIS Guidelines deals exclusively with the evaluation of pathogen test kit methods.
General Principle for the VALIDATION of alternative method	Two phases – Methods comparison study and Interlaboratory study; compared to Reference method (Ref)	Three phases – Single Lab validation or Pre- collaborative, Independent Validation Study, and a Collaborative Study; compared to Ref	Methods comparison study for MFLP, Collaborative study for MFHPB (no earlier than 1 yr after MFLP is published); Relative to Ref Method CLARIFICATION NEEDED <i>Role of Comparative Study (3.3) is unclear when contrasted with Collaborative Study (3.2); also if it is applicable only to quantitative methods</i> For MMC same goal different tool. Comparative and Collaborative study are use to modify method status from MFLP to HPB. Comparative study most likely to use naturally contaminated sample while collaborative will use spike sample prepared by central lab. This is done through Government lab network. (I.I.)	Two phases – Methods comparison study and Interlaboratory study; compared to Reference method (Ref)	Two phases – Single lab validation study followed by inter-laboratory validation study; compared to Reference method (Ref) where possible.	One phase – a single lab validation study required for minor modifications to alternative method. For major modifications, follow AOAC-OMA guidance for inclusivity, exclusivity, repeatability, reproducibility and ruggedness testing. (A major modification to alternative method would include significant changes in the design or the component reagents for a screening test, for example, the introduction of a new antibody or oligonucleotide primer)

	ISO 16140	AOAC OMA	Health Canada	NordVal	FDA	Draft USDA/FSIS
Pre-Collaborative Phase(s)						
Overall	<p>Consists of:</p> <ol style="list-style-type: none"> 1)Relative Accuracy (RA)study 2)Relative Detection Limit (RLOD) study 3)Inclusivity/exclusivity study <p>All 3 performed by expert organizing lab</p> <p>For MicroVal and AFNOR these expert labs are independent from the test kit producer (PIV)</p>	<p>Consists of:</p> <ol style="list-style-type: none"> 1) Single Lab validation (SLV) by method developer or study director 2) Inclusivity and Exclusivity study by method developer or study director 2) Independent Validation Study (IV) by independent trained user 	<p>Consists of:</p> <ol style="list-style-type: none"> 1) Single lab food matrix comparison study 2) Inclusivity/exclusivity 3) LOD study (I.I.) <p>Provides for a Transfer Study (inter-laboratory Study) to demonstrate that the new method can be performed in another lab, but not subjected to full multi-site collaborative study (not required if Collaborative study was done)</p>	<p>Consists of:</p> <ol style="list-style-type: none"> 1)Relative Sensitivity (SE), Relative Specificity (SP), Relative Accuracy (RA), Agreement between the methods (kappa) 2) Detection Limit study 3)Inclusivity/exclusivity study <p>All 3 performed by expert organizing lab</p>	<p>Consists of:</p> <p>Single lab validation (SLV) by FDA personnel.</p> <ol style="list-style-type: none"> (a) Inclusivity/exclusivity studies. (b) Side-by-side comparison with reference method, if available. <p>Divided into 4 validation levels.</p> <p>Level 1, Urgent Usage SLV</p> <p>Level 2, Independent Lab Validation</p> <p>Level 3, Multiple Lab</p> <p>Level 4, Multiple Lab</p> <p>Each level is preceded by a SLV.</p>	<p>Validation of qualitative methods consisting of</p> <ol style="list-style-type: none"> 1) Relative recovery study (unpaired study) in portions inoculated to achieve fractional recovery 2) Determination of false negative rate (paired study) 3) If a major modification is introduced to the alternative method, then inclusivity, exclusivity, repeatability, reproducibility and ruggedness testing should also be performed, either through the direction of an independent organization, or by following guidance provided by the AOAC International Official Methods of Analysis Program. <p>Note: if a reference method is not available, false negative rate is determined in paired sample using “gold-standard” confirmatory procedure.</p> <p>Organization of SLV study:</p> <ul style="list-style-type: none"> • The work should be carried out in a laboratory that is independent of the manufacturer’s economic interest. For example, the study may

ISO 16140	AOAC OMA	Health Canada	NordVal	FDA	Draft USDA/FSIS
					<p>be carried out under contract to an academic laboratory, or a publicly-, or privately-owned laboratory that is not controlled by the test manufacturer.</p> <ul style="list-style-type: none"> • Alternatively, the validation may be performed through an independent organization such as AOAC, AFNOR, ISO, Microval, or NordVal. • The identity of the samples should be blinded to the analysts. • The study design should be reviewed by an outside party before initiating work. FSIS can review and comment on study design. • Study report and the associated raw data should be available for review by FSIS.

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-Reference Method	<p>-Defined in ISO 16140-1</p> <p>-1st priority is ISO method, 2nd priority is CEN method, if neither exists, then 3rd priority is other recognized methods</p> <p>Note: definition still under discussion at ISO level to open up for non ISO/CEN methods (PIV)</p>	<p>-Can be various pre-existing recognized analytical methods e.g. AOAC OMA, ISO, FDA BAM, FSIS MLG and Health Canada</p> <p>-If no appropriate Ref can indicate “NA” in summary tables for POD</p>	<p>-Acceptable Ref published by HC (Part 1)</p> <p>-May include any methods from methods organizations, such as AOAC, BAM, APHA, ICMSF, IDF, ISO etc.</p> <p>-Where no Ref exists, MMC assess on case by case basis</p>	<p>ISO, CEN, NMKL, BAM, etc. It is up to the applicant; however, as the EU regulation in EC 2073/2005 Microbiological criteria states EN ISO methods, these are most frequently used.</p>	<p>-Must be BAM, unless there is no BAM reference method.</p> <p>-If there is no BAM reference method, but if there is a nationally/internationally recognized reference method, then FSIS MLG, AOAC, ISO, and Health Canada are all potential reference methods. APHA, ICMSF, and IDF methods also may be used as reference methods.</p>	<p>For FSIS regulated products, the current FSIS method, which is found in the Microbiology Laboratory Guidebook (MLG), is the most appropriate reference cultural method for validating methods used by FSIS-regulated establishments. FDA BAM, or methods referenced by ISO or <i>Codex Alimentarius</i> may be appropriate. Non-cultural methods applicable in some circumstances.</p>
-Selection of food	<p>RA</p> <p>-5 categories for all foods applications, 3 food types per category (see below)</p> <p>-Feed, environmental samples and primary production samples (PIV) are additional categories</p> <p>RLOD</p> <p>Same, except 1 food type per category (if possible) a different food type</p>	<p>SLV</p> <p>-All claimed matrices must be included in the study, in other words, no defined categories, and “all foods” claim not applicable</p> <p>-Environmental surfaces claim require 3-7 different surfaces (# required is under review (RF))</p> <p>IV</p> <p>At least 1 matrix that was tested in the SLV. For every 5 foods claimed, 1 food matrix must be included</p>	<p>-5 categories for all foods applications, 3 food types per category (Table 1).</p> <p>-Environmental samples is additional category</p>	<p>RA, SE, SP, Kappa</p> <p>-5 categories for all foods applications, 3 food types per category (see below)</p> <p>-Feed, environmental samples are additional categories</p> <p>LOD</p> <p>Same, except 1 food type per category (if possible) a different food type</p>	<p>The selection of foods is determined by FDA’s regulatory needs.</p>	<p>Matrices commonly sampled in FSIS regulated establishments: meat, poultry, and egg products, and environmental samples (sponges, swabs, brines)</p> <p>All claimed matrices must be included in the study. Contains proposal to create matrix categories based on intrinsic properties. “All Foods” claim not applicable</p>

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- Food category/type/item	<p>Each Food type can be made of various relevant food items. Annex B provides guidance (not mandatory) These are then grouped together to meet the sample number requirement of a food type, i.e. 20 samples.</p> <p><i>This is allow for the use of naturally contaminated samples (BL)</i></p>	<p>Only one single food item is accepted to meet the sample size requirement of a food type, i.e. 20 replicates.</p>	<p>Each food type can be made of various relevant food items. Table 1.</p> <p>CLARIFICATION NEEDED <i>Can these be grouped together to meet the sample number requirement of a food type, in this case 20 samples?</i> Yes, they can be group together to meet the sample number requirement. This notion has been introduce to allow for heterogeneity with in a food type. Products in a type may vary greatly in origin, composition, preparation processes, natural background; all those small variabilities could have an influence on the detectability of the target organism. (I.I.)</p>	<p>Each Food type can be made of various relevant food items. At NordVal’s homepage (www.nmkl.org) provides a list of food categories These are then grouped together to meet the sample number requirement of a food type, i.e. 20 samples.</p>	<p>Currently, foods are validated individually and there are no category claims. There are no “All Foods” claims.</p>	<p>Only one food item would represent each matrix category to meet the sample size requirement.</p>
-Natural or artificial contamination sample	<p>-Naturally contaminated preferred -OK to artificial contamination (Annex C and D)</p> <p>No specification on bulk or individual inoculation (PIV)</p>	<p>-Naturally contaminated preferred -OK to artificially inoculated , use one strain for each matrix , microorganisms must be stressed and then stabilized prior to inoculation</p>	<p>Foods can be either naturally contaminated or inoculated</p>	<p>-Naturally contaminated preferred if available also at medium and high contamination level -OK to artificial contamination -Vital that the contamination is low, medium and high</p>	<p>Naturally contaminated foods are preferred. <i>It is proposed that levels can be adjusted by dilution with uncontaminated product or increased with temperature abuse.</i></p>	<p>Artificial contamination only. Microorganisms must be stressed before testing is initiated.</p>
-Naturally occurring background microflora	<p>Undefined MicroVal: should be present, if not then added (PIV)</p>	<p>Undefined</p>	<p>Should be 10 times higher than the target organism</p>	<p>Undefined</p>	<p>Unspecified. Customarily, background microflora remains intact.</p>	<p>Typical levels (guidance provided). APC levels should be measured and reported</p>

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- Need for competitive microflora	Undefined	-Sufficient to demonstrate in one matrix -Inoculated competitor contamination level should be approx 10 times higher than the target	Undefined	Undefined	Must be at least 1 log greater than target analyte. This requirement can be fulfilled with naturally occurring microflora.	Naturally occurring microflora only.
-Estimation of contamination levels	For RLOD only, by MPN or other counting technique	MPN estimation of the levels	Undefined For LOD only by MPN (I.I.)	Artificial contamination, empirical determination	Unspecified. Customarily determined with Most Probable Number Analysis on the day analysis is initiated.	Before inoculation: direct plating to non-selective media. After inoculation: MPN
-No. of levels/samples	RA 20 samples per food type or 60 samples per category RLOD 3 levels -negative controls =5 samples -1 level (theoretical LOD, with fractional positive results (BL)) = 20 samples -Another level = at least 5 samples	SLV and IV 3 levels: -negative controls =5 samples -1 level with fractional positive results = 20 samples -Another (high) level = 5 or 20 samples (under review (RF))	3 levels: -negative controls =5 samples -1 level with fractional positive results = 20 samples -Another level up to 1 log higher= 20 samples	RA 20 samples per food type or 60 samples per category LOD 3 levels -negative controls =5 samples -1 level (theoretical LOD) = 20 samples -Another level = at least 5 samples	Level 1 , 6 replicates/level, single level Level 2 , 6 replicates/level, 1 inoculated level + 1 uninoculated level (5 replicates) Level 3 , 10 replicates/level, 1 inoculated level + 1 uninoculated level (5 replicates) Level 4 , 20 replicates/level, 1 inoculated level + 1 uninoculated level (5 replicates) <i>It is proposed that each of the 4 levels use 20 replicate test portions and that all levels have a negative control.</i>	For each matrix and analyte: 1) minimum 60 samples inoculated at fractional recovery level per alternative and reference method 2) 5-10 uninoculated samples per alternative and reference method
-Sample size	Undefined MicroVal: Is specified in the reference method, other (larger) samples size is allowed but specified in the certificate. (PIV)	Standard is 25 g or 25 mL, unless Ref method specified larger sample size	-25 g, but larger sample sizes are permitted -Sample size must be the same for alternate and Ref methods, consult MMC if testing composite samples	Undefined	25 g unless otherwise specified.	Application dependent. Portions should not be made larger without validation. Validation study conclusions from larger portions applicable to smaller portions.

	ISO 16140	AOAC OMA	Health Canada	NordVal	FDA	Draft USDA/FSIS
-Differentiation between Paired and unpaired samples	Yes	Yes, referred as matched or unmatched	Yes	Yes	Unspecified. Customarily, yes.	Yes. Unpaired samples used to measure relative recovery and paired samples from alternative method used to determine false negative rate.
-Confirmation of results It would be useful to indicate also how results are confirmed (BL)	- Paired; only the discrepant +/- results needs to be confirmed. - Unpaired: confirm all positive results	Whether matched or unmatched, all samples must be confirmed (need to confirm non-discrepant results at high level is under review (RF))	Paired or unpaired, all samples must be confirmed	- Paired; only the discrepant +/- results needs to be confirmed. - Unpaired: confirm all positive results	Yes, all positive results must be culturally confirmed.	Typically yes: all positive and negative samples must be confirmed using reference confirmatory procedure. However, guidance makes some exceptions, which are referred to as “levels” Level 1: All samples are confirmed. Levels 2 and 4: Alternative method results are confirmed. Level 3: No results are confirmed
-“True” positive : where alternate is confirmed positive and Ref is negative	For paired samples only This is regarded as true positive result since the presumptive positive determination by the alternate method was confirmed by enhanced confirmation techniques CLARIFICATION NEEDED <i>How are the true positive results incorporated into data analysis?</i> Comparison between methods are made both for confirmed and unconfirmed results to demonstrate the effect of confirmation and possible “true” results for the alternative method (see Tables 1	Alternate method can perform own confirmation in addition to reference method CLARIFICATION NEEDED <i>How are the true positive results incorporated into data analysis?</i>	If confirmation is positive under “sheer persistence”, an absolute positive results is scored for the alternate method, -For unpaired, that becomes the confirmed result, or +/+ -For paired, as Ref remains unchanged. that will be a FN for the Ref Report tables have columns for Presumptive, Confirmed and Final results	This is regarded as true positive result. NordVal operates with two tables when reporting the results, one table for obtained results of the screening and one table with the results after confirmation (this is not in the current ISO 16140). Two tables makes it more clear.	“True positives” only applies to paired samples, but the sample must be culturally confirmed.	“True” positive is screen positive by alternative method and confirmed positive by reference confirmatory procedure

	ISO 16140	AOAC OMA	Health Canada	NordVal	FDA	Draft USDA/FSIS
	and 2 for paired data and 4 and 5 for unpaired data) (PIV)					
-Fractional positive	Can be achieved by either alternate or Ref. - All samples should not be all positive or all negative. -Ideal is 10 positive and 10 negative (50%) but any fractional results is acceptable	Can be achieved by either alternate or Ref. -proportion of positives 25% to 75%, ideal is approx 50% (10% to 90% is under review (RF))	Can be achieved by either alternate or Ref. -proportion of positives 25% to 75%,	Can be achieved by either alternate or Ref. - All samples should not be all positive or all negative. -Ideal is 10 positive and 10 negative (50%) but any fractional results is acceptable	Yes, one or both methods must give 40 – 90% positive results. <i>It is proposed that the percentage positive results be changed to 25 – 75%.</i>	defined as a range of 20-80% confirmed positive results using reference method
-Results analysis and criteria	RA -By type and by category -Relative accuracy AC, relative specificity SP, relative sensitivity SE -First by unconfirmed results, again by confirmed results -McNemar test as criteria, (for paired and unpaired) with caveats i.e. really not suitable for unpaired and “never be interpreted by only the McNemar test” RLOD -by category -LOD of alternate method divided by LOD of Ref For paired, no lower limit, but LOD alternate might not be > 2 times the LOD Ref For unpaired samples, no lower limit, the LOD alternate might	- by level and by matrix - by POD Probability of Detection 95% confidence interval for the alternate the Ref and presumptive and confirmed results -then by difference between POD alternate and POD Ref, confidence level must contain zero for method to be considered not different at 95% confidence - Chi Square is not required but “interesting”	Method Equivalence: -POD -one-tailed POD 95% confidence interval (I.I.) Performance parameters: - by level and by food, but only calculated for those that passed POD For Unpaired : -Performance parameters is the comparison of presumptive vs. confirmed results of the alternate method (not the Ref method results) -Specificity is based on presumptive results -Sensitivity is based on final (confirmed) results - Equivalence of alternate method and Ref can only be determined by the number of true positives in both sets, done by POD method For Paired: Use “absolute” results where Ref can have FN	RA -By type and by category -Relative accuracy AC, -Relative specificity SP, -Relative sensitivity SE - Kappa -First by unconfirmed results, again by confirmed results Criteria: SE ≥ 95% Kappa ≤ 0.80 LOD: fit for purpose	By level/individual experiment for each matrix. Per AOAC Microbiology guidelines, McNemar Chi Square statistics are used.	Performed for each matrix. <u>Unpaired study</u> : One sided chi-square test with alpha = 0.05. Criterion: indistinguishable or better performance than reference method. <u>Paired study</u> : Evaluate sensitivity with minimum 29 confirmed positive results. Zero false negative results from 29 confirmed positives would be consistent with a test having a sensitivity that met or exceeded 90% and zero negative results from 50 confirmed positives would be consistent with a test with a sensitivity that met or exceeded 94%. Criterion: none proposed

	ISO 16140	AOAC OMA	Health Canada	NordVal	FDA	Draft USDA/FSIS
	<p>not be >3 times the LOD Ref (In the ISO/CD 16140-2 version, I don't find any acceptability limit settled for unpaired samples, only specified for paired samples BL)</p> <p>The values of 2 (paired) and 3 (unpaired) are still tentative values!!!! (PIV)</p>		<p>Criteria: Sensitivity ≥98% Specificity ≥90.4% False negative rate < 2% False positive rate ≤ 9.6% Efficacy ≥94% LOD must be comparable or exceed the lower LOD of the Ref</p>			
Inclusivity and Exclusivity						
- Selection and no. of strains	<p>-Specified per target group (Annex F) -Inclusivity: 50 except 100 for <i>Salmonella</i> -Exclusivity: 30</p>	<p>-Inclusivity: 30 except 100 for <i>Salmonella</i> (50 under review (RF)) -Exclusivity: 30</p>	<p>-Inclusivity: 50 except 100 for <i>Salmonella</i> -Exclusivity: 30</p>	<p>-Specified per target group (Annex F) -Inclusivity: 50 except 100 for <i>Salmonella</i> -Exclusivity: 30</p>	<p><u>Inclusivity</u> Level 1, 10 (20 serotypes for <i>Salmonella</i>) Levels 2-4, 50 (100 serotypes for <i>Salmonella</i>) <u>Exclusivity</u> Level 1, 10 strains Levels 2-4, 30 strains</p>	<p>Refer to AOAC guidelines (Feldsine et al., 2002)</p>

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-Inoculation and procedure	- Inclusivity : each pure culture strain performed once with alternate method only, using the alternate method enrichment protocol(s), if Neg, , first repeated using the ref method and still neg. (PIV) then consider adding a food matrix - Exclusivity: each pure culture grown in non-selective broth, if pos or doubtful then use complete enrichment	- Inclusivity : each pure culture strain performed once with alternate method only, using the alternate method enrichment protocol(s), if Neg, then consider adding a food matrix - Exclusivity: each pure culture grown in non-selective broth, if pos or doubtful then use complete enrichment	- Inclusivity and Exclusivity : each pure culture strain performed once with alternate method only, in the absence of a food matrix	- Inclusivity : each pure culture strain performed once with alternate method only, using the alternate method enrichment protocol(s), if Neg, then consider adding a food matrix - Exclusivity: each pure culture grown in non-selective broth, if pos or doubtful then use complete enrichment	Unspecified. Customarily, inclusivity testing is performed on the alternative method in pure culture at a level 1 log greater than the limit of detection of the alternative method. The method is followed as if it were actual sample analysis. For exclusivity, exclusivity cultures are treated identically to inclusivity cultures but testing is performed in non-selective media.	Refer to AOAC guidelines
Inter-laboratory Study						Applicable to alternative methods with a major modification, defined as any significant change in the design or the component reagents for a screening test, for example, the introduction of a new antibody or oligonucleotide primer. Follow guidance provided by the AOAC International Official Methods of Analysis Program
-Organized by	Expert lab only Expert Organizing lab only (beware: ISO/CD 16140-1 defines the organizing lab)	Method developer acceptable	Method developer acceptable	Expert lab only	FDA	

	ISO 16140	AOAC OMA	Health Canada	NordVal	FDA	Draft USDA/FSIS
	without the request of being independent from the test kit manufacturer, this is an additional requirement from the certification bodies, MicroVal and AFNOR Certification) (BL)					
-Differentiation between Paired and unpaired samples	Yes	Yes	Yes	Yes	Unspecified.	
- minimum no. of valid data sets/collaborators	-10; defined as individuals working independently using different sets of samples; from a min. of 5 different organizations, including organizing lab and different locations from same company	-10 valid lab data sets required - Specifies that 12 labs should start	Minimum of 8 labs reporting valid data, labs should be accredited per 17025 or demonstrate is functioning under equivalent quality system	-10; defined as individuals working independently using different sets of samples; from a min. of 5 different organizations, including organizing lab and different locations from same company	2 for a Level 2 study, 3 for a Level 3 study, and 10 for a level 4 study.	
-Sample size	NA Is defined by the protocol of the reference method (PIV)	Standard is 25 g or 25 mL, unless Ref method specified larger sample size	CLARIFICATION NEEDED <i>Consistent with Pre-collaborative?</i> Sample size is 25g unless otherwise specified by the method or need for larger size (to achieve enhance detectability, regulatory purpose or compositing) (I.I.)	NA	25 g unless otherwise specified.	
- number of foods	1; relevant food item, inoculated with target, using a challenging	1	At least 1	1; relevant food item, inoculated with target, using a	One or more.	

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	enrichment protocol			challenging enrichment protocol		
- number of levels	3; negative control, one level which produce fractional positive and another level	3; negative control, one level which produce fractional positive and another level	3; negative control, one level which produce fractional positive and another level about 10 times greater than the detection level	3; negative control, one level which produce fractional positive and another level	2 for a Level 2 study (1 inoculated and 1 uninoculated). 3 for Levels 3 & 4 (high, low, and uninoculated).	
- number of replicates	8; per level of contamination -minimum of 48 results per collaborator = 8 replicates x 3 levels x 2 methods -minimum of 480 results (48 from each collaborator) = (240 per method) for statistical analysis	12 per level of contamination - 72 results per collaborator = 12 replicates x 3 levels x 2 methods = 72 - minimum of 720 results (360 per method) for statistical analysis	8 per level - min of 24 results per collaborator (8 x3 levels) per method	8 laboratories; - 3 levels in duplicates 8 labs x 3 levels x 2 replicates x 2 methods	6	
-Confirmation	for Paired, only confirm the + Alt/- Ref, for Unpaired, confirm all enrichments	Matched or unmatched, confirm all samples	Confirm all samples	for Paired, only confirm the + Alt/- Ref, for Unpaired, confirm all enrichments	Yes.	
- Comparisons	Analyzed two ways: 1. Unconfirmed Alternate method results vs. confirmed Ref 2. Confirmed Alternate method results vs. confirmed Ref	By level and by matrix analyzed and reported separately	CLARIFICATION NEEDED <i>Consistent with Pre-collaborative?</i> By level and by matrix, all result confirmed Confirmed alternate method results vs reference (I.I.)		Alternative to reference method (if available).	

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-Parameters Calculated	Specificity (only for Neg controls) Sensitivity (only for inoculated levels) Relative Accuracy (% of agreements) RLOD of the different participants (BL)	Cross Lab Probability of Detection (LPOD) Difference between Alternate LPOD and Ref LPOD	CLARIFICATION NEEDED <i>Consistent with Pre-collaborative?</i> Yes, POD , dPOD determined for each matrix-level . All dPOD data is then used to assess the comparative performance of both methods All 5 method parameter(specificity, selectivity, FP, FN and method efficacy) calculated in one of two ways,, depending if sample is paired or un paired. (I.I.)	Rel Specificity Rel Sensitivity Rel Accuracy Kappa	Per AOAC guidelines, Sensitivity, Specificity, False Negative, and False Positive Rates.	
- Interpretation	McNemar test (chi square) RLOD is for information only : analysis of deviance test to assess the laboratory effect on RLOD then acceptability of RLOD global value (BL)	If confidence interval of dLPOD does not contain zero, then the diff is statistically significant	CLARIFICATION NEEDED <i>Consistent with Pre-collaborative?</i> Yes , dPOD one-tailed and method parameter requirement must be met. (I.I.)	Criteria: SE \geq 95% Kappa \leq 0.80 [LOD: fit for its purpose]	Per AOAC guidelines, McNemar Chi Square statistics.	

QUANTITATIVE methods	ISO 16140 Doc N 1199 C2011-04-06 Pending revision of Part 2	AOAC OMA Draft revision document dated 3/24/11	Health Canada Draft Part 3 dated April, 2011 (I.I.)	NordVal Protocol for the validation of alternative microbiological methods March 2009	FDA does not have quantitative methods validation guidelines. FDA would probably follow AOAC guidelines for quantitative methods except as specified below.	Not applicable to quantitative methods
General Principle for the VALIDATION of alternative method	Two phases – Methods comparison study and Interlaboratory study; Relative to Reference (Ref)	Two phases – Method comparison study, either Precollaborative or PTM and Collaborative	Two phases: Transferability study and comparison study. (I.I.)	Two phases – Methods comparison study and Interlaboratory study; Relative to Reference (Ref)		
I. Methods Comparison Study						
-Conducted by	Expert Lab	Method developer is acceptable	Method developer is acceptable. (I.I.)	Expert Lab	FDA	
I.1 Determination(s)	Beta-ETI tolerance intervals	Reproducibility and Mean Difference and 95% CI		Mean, Standard deviation, Confidence intervals		
-Reference Method	-Defined in ISO 16140-1 -1 st priority is ISO method, 2 nd priority is CEN method, -If neither exists, then 3 rd priority is other recognized methods	-Can be various pre-existing recognized analytical methods e.g. AOAC OMA, ISO, FDA BAM, FSIS MLG and Health Canada -If no appropriate Ref can indicate “NA” in summary tables for POD	-Acceptable Ref published by HC (Part 1) -May include any methods from methods organizations, such as AOAC, BAM, APHA, ICMSF, IDF, ISO etc. -Where no Ref exists, MMC assess on case by case basis	ISO, CEN, NMKL, BAM, etc. It is up to the applicant; however, as the EU regulation in EC 2073/2005 Microbiological criteria states EN ISO methods, these are most frequently used.	Must be BAM, unless there is no BAM reference method. -If there is no BAM reference method, but if there is a nationally/internationally recognized reference method, then FSIS MLG, AOAC, ISO, and Health Canada are all potential reference methods. APHA, ICMSF, and IDF methods also may be used as reference methods	
-Selection of food	-5 categories for all foods applications, 3 food types per category (see below)	-Claim only for range of food categories or specific food types in study	5 categories for all foods applications (1 type/category) 5 levels of the target	-5 categories for all foods applications, 3 food types per category (see below)		

	ISO 16140	AOAC OMA	Health Canada	NordVal	FDA	Draft USDA/FSIS
	<p>- Environmental samples, feed samples and primary production stage samples included as add. categories</p> <p>-Reduce categories if alternate method only validate specific</p>		<p>microorganism replicated the same number of time. (5 to 10) (I.I.)</p> <p>Environmental sample to be considered as a 6th category. (I.I.)</p>	<p>- Environmental samples, feed samples and primary production stage samples included as add. categories</p> <p>-Reduce categories if alternate method only validate specific</p>		
- Food category/type/item	<p>-Each Food type can be made of various relevant food items. Annex B provides guidance (not mandatory)</p> <p>-These are then grouped together to meet the sample number requirement of a food type, in this case 20 samples.</p>	<p>-Only one single food item is accepted to meet the sample size requirement of a food type, i.e. 20 replicates.</p> <p>(currently under review (RF))</p>	<p>-Each food type can be made of various relevant food items. Table 1.</p> <p>CLARIFICATION NEEDED</p> <p><i>Can these be grouped together to meet the sample number requirement of a food type, in this case 20 samples?</i></p> <p>-Each food type can be made of various relevant food items. Table 1.</p> <p>CLARIFICATION NEEDED</p> <p><i>Can these be grouped together to meet the sample number requirement of a food type, in this case 20 samples?—(I.I.)</i></p> <p>At least one relevant food matrix should be tested. (I.I.)</p>	<p>-Each Food type can be made of various relevant food items.</p> <p>- A list of matrices is given at www.nmkl.org under NordVal.</p>		

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-Natural or artificial contamination sample	<p>-Naturally contaminated preferred (based on guessed analyte concentration) -Majority of total samples analyzed must be naturally contaminated -OK to artificially inoculated</p> <p>CLARIFICATION NEEDED <i>For pathogenic organisms, majority of samples will not be naturally contaminated; does the majority of samples still need to be naturally contaminated?</i></p> <p>MicroVal: No strict requirement. It is up to the MicroVal Technical Committee to decide on the percentage of naturally contaminated samples. This is done per category based on the information supplied by the expert lab. (PIV)</p>	<p>Natural samples preferred. -ONLY use artificial if target organism (i.e. <i>Staphylococcus</i>) is not routinely found in food type -50% of food types are naturally contaminated unless not naturally occurring</p>	<p>naturally contaminated samples, especially those that have been implicated in food illness investigations, otherwise it is acceptable to use spiked samples. (I.I)</p>	<p>-Naturally contaminated preferred -OK to artificially inoculated</p> <p>Vital to have samples with different levels of contamination, thus artificially contaminated most commonly used.</p>		
-No. of samples	<p>Per level: 2 samples for each Candidate & Ref (duplicate by preparing sub-samples) -4 replicates per sample -3 seed levels (low,</p>	<p>Per level: 5 samples for Candidate & Ref Artificially contaminated – 3 inoculation levels & one uninoculated; low level is above LOD</p>	<p>For each of the five food categories, at least five levels of the target analyte are measured by both the reference and the alternative method. Each sample must be replicated the same number of times</p>	<p>5 matrixes 5 level each matrix 5 replicates each level 2 methods</p>		

	ISO 16140	AOAC OMA	Health Canada	NordVal	FDA	Draft USDA/FSIS
	<p>med, high) -total analysis per food category is 72 (3 typeX3levelsX2samplesX4 replicates)</p> <p>-2 samples at each level may belong to same food type, but not necessarily sample food item</p> <p>-also of interest to determine limit of quantification (LOQ) -lower LOQ only used when the measurement principle of the alternate method is not based on visual observation of target organism -additional level is target to verify lower limit of quantification</p>	<p>Co-inoculate if method detects more than one organism Natural contaminated – 4 different lots per food type, no uninoculated level</p>	<p>(2 preferably 5 to 10) giving a total of 10 to 50 results per food category. sample concentrations of microorganisms should be selected to cover uniformly the whole range of interest, including regulatory levels for selected commodities For Small Ranges: Level 1 - zero organisms spiked (negative control) or not detected if naturally contaminated. Level 2 - $0 < LOD$ Level 3 - LOD Level 4 - LOQ Level 5 - Regulatory level Level 6 - Regulatory level + 1 Log_{10}</p> <p>For Wider Ranges: Level 1 - zero organisms spiked (negative control) Level 2 - $LOD < LOQ$ Level 3 - LOQ Level 4 - Regulatory level - 1 Log_{10} Level 5 - Regulatory level Level 6 - Regulatory level + 1 Log_{10}</p> <p>(I.I.)</p>			

	ISO 16140	AOAC OMA	Health Canada	NordVal	FDA	Draft USDA/FSIS
-Results and criteria	<p>-plot Log candidate vs. Ref -examine for outliers (visual); temp discard outliers and repeat</p> <p>Calculate -log transformed counts (reference values) -average for each sample -compute absolute bias for each sample -compute the upper and lower limits of the Beta-expectation tolerance intervals (Beta-ETI) -calculate upper and lower acceptability criterion -make a graphic representation of the computed results</p> <p>See Annex R for calculations</p>	<p>-plot Log candidate vs. Ref -examine for outliers (Cochran & Grubbs) -calculate repeatability -calculate mean difference and 95% CI -report reversed transformed mean difference and CI</p>	<p>MFLP status</p> <ul style="list-style-type: none"> • Linearity • Selectivity (inclusivity $\geq 98\%$, exclusivity $\leq 10\%$) • LOD • LOQ • Critical limit • Regulated specification includes in range study • Transferability achieved. (I.I.) 	<p>Calculate the - mean (log), - standard deviation (sd) - confidence level (+/- 2 x sd). Plot the results of the alternative and reference method (level vs obtained result). Plot the confidence level of the reference method. If the mean of the alternative method falls within the confidence level of the reference method, and the precision of both methods are satisfactory (sd <0.4 log), then the results can be considered equivalent and satisfactory.</p>		
<p>Inclusivity and Exclusivity NOTE: Requirement is applicable to quantitative methods for specific organisms only (i.e. <i>Listeria</i>) Not applicable to Total plate counts or Total yeast and mold methods)</p>						

	ISO 16140	AOAC OMA	Health Canada	NordVal	FDA	Draft USDA/FSIS
- Selection and no. of strains.	Inclusivity: 50 strains; Exclusivity: 30 strains	Inclusivity: 30 or 50 strains (currently under review (RF)) Exclusivity: 30 strains	Inclusivity: 30 strains Exclusivity: 20 strains (I.I.)	Inclusivity: 30 strains; Exclusivity: 20 strains		
-Inoculation and procedure	Inclusivity: -test in pure culture in alternative method media inoculated at 100 times the LOD50 -check with reference method if negative -repeat with food matrix if negative Exclusivity: test in pure culture in alternative method media inoculated from culture grown in nonselective media	Inclusivity: -test in pure culture in nonselective media at 100 times the LOD50 Exclusivity: -test in pure culture in nonselective media at growth limit	Inclusivity: -test in pure culture at 100 times the LOD Exclusivity: -test in pure culture at 100XLOD (I.I.)	Inclusivity: -test in pure culture in alternative method media inoculated at 100 times the LOD50 -check with reference method if negative -repeat with food matrix if negative Exclusivity: test in pure culture in alternative method media inoculated from culture grown in nonselective media		
2. Interlaboratory Study						
-Organized by	Expert lab	Organizing Lab	HC as an Organizing lab. (I.I.)	Expert lab		
- minimum no. of valid data sets/collaborators	8, from at least 8 participants	8, Suggest to start with 10-12 participants to begin analysis	Minimum of 8 participating accredited lab. (I.I.)	8 labs (suggest starting with 10-12)		
- number of foods	1; relevant food item, inoculated with target, using a challenging enrichment protocol	1 or more depending on number of different prep./enrichment methods (currently under review (RF))	At least one. (I.I.)	1		
-Natural or artificial contamination sample	-Naturally contaminated preferred -OK to artificial contaminate (Annex C and D)	-Natural samples preferred. -ONLY use artificial if target organism (i.e. <i>Staphylococcus</i>) is not routinely found in food type	- naturally contaminated preferred (if possible combination food-strain from out-brake). If naturally contaminated not available, spiking is acceptable. (I.I.)	-Naturally contaminated preferred -OK to artificial contaminate		

	ISO 16140	AOAC OMA	Health Canada	NordVal	FDA	Draft USDA/FSIS
- number of levels	3; cover lower, middle and upper levels of the entire range of alternative method -also include negative control	-Artificially contaminated – 3 inoculation levels & one uninoculated; low level is slightly above LOD -Co-inoculate is method detect more than one organism	Artificially contaminated-3 level which include the regulatory level. (I.I.)	4		
- number of replicates	Per level: 2 samples each for Candidate & Ref	Per level: 2 samples each for Candidate & Ref	Per level : 2 sub-samples from each level tested in duplicate, by reference and candidate method. (I.I.)	Per level: 2 samples each for Candidate & Ref		
- Comparisons	-use robust estimators, do not exclude data from laboratories unless microbiological reasons	Refer to Appendix D for detailed -plot Log candidate vs. Ref -construct a Youden plot -examine for outliers (Cochran & Grubbs)	<ul style="list-style-type: none"> • Linearity • Trueness: for each 3 level, bias estimates must demonstrate no significant difference (95% CI) • Reproducibility and repeatability limit (Horwitz criterion) (I.I.) 	-use robust estimators, do not exclude data from laboratories unless microbiological reasons		

	ISO 16140	AOAC OMA	Health Canada	NordVal	FDA	Draft USDA/FSIS
-Parameters Calculated	<p>Accuracy profile</p> <p>Settle upper and lower acceptability criterion (BL)</p> <p>Calculate</p> <p>-log transformed counts (reference values)</p> <p>-calculate “reference values”, the median of the log transformed counts</p> <p>-calculate reproducibility standard deviation</p> <p>-calculate Z, the global average of measurements made with the alternative method.</p> <p>--compute absolute bias for each sample</p> <p>-compute the Beta-expectation tolerance intervals (Beta-ETI)</p> <p>-calculate the “coverage factor” of given level k</p> <p>-compute differences between the limits of tolerance interval and target value X_k</p> <p>calculate upper and lower acceptability criterion-(BL)</p> <p>-calculate upper and lower acceptability criterion</p> <p>-make a graphic representation of the computed results</p>	<p>-calculate repeatability and reproducibility SD</p> <p>-calculate mean difference and 95% CI</p> <p>-report reversed transformed mean difference and CI</p>	<p>-Relative accuracy (Bias)</p> <p>-Repeatability standard deviation and repeatability limits (Sr, (r), RSD</p> <p>- reproducibility standard deviation and reproducibility limit</p> <p>-comparison of the between laboratory variance and within laboratory variance. (I.I.)</p>	<p>Calculate</p> <p>- Median</p> <p>- Repeatability</p> <p>- Reproducibility (from Reusseuw)</p> <p>Plot the data (graphically). See if the results are overlapping (the medians and the reproducibility)</p>		

	ISO 16140	AOAC OMA	Health Canada	NordVal	FDA	Draft USDA/FSIS
Interpretation	Beta-ETI values fall within the acceptable limits for all levels of contamination.		<p>Linearity: Intercept = 0 (estimation includes the value zero (95% CI) Slope = 1 (estimation includes the value of one (95% CI) 2. Selectivity: Inclusivity > 98% (n = 30 strains) Exclusivity < 10% (n = 20 strains) 3. Regulated specifications included in the range studied (see reference 8.5). 4. Transferability of the method achieved within specified limits.</p> <p>For collaborative studies: 1. Linearity : Verification of the model obtained from the transferability study. Intercept = 0 (estimation includes the value zero (95% CI) Slope = 1 (estimation includes the value of one (95% CI) 2. Trueness : For each of the three levels, bias estimates must demonstrate no significant differences (95% CI). 3. Reproducibility and repeatability limits are evaluated against Horwitz criterion (see reference 8.2) 4. Regulated specifications are included in the range studied. (I.I.)</p>	If the results of the alternative method fall within the uncertainty (reproducibility) of the reference method, the two methods provide equivalent results.		