AOAC INTERNATIONAL
Presidential Task Force on
Best Practices for Microbiological Methodology
US FDA Contract #223-01-2464, Modification #12

Executive Summary
Method/Matrix Extension Working Group (MEWG)

A. **METHOD VALIDATION - BIOLOGICAL VARIATION OF MICROORGANISMS AND TOXINS**
Contract Objective being addressed:
Objective 5
What are the scientific/statistical bases for developing validation protocols that adequately take into account the biological variation that exist within both the microorganisms and toxins produced by these microorganisms for which methods are developed [and the foods which will be analyzed]?

Summary of Recommendation
The MEWG has compiled a list of species and strains, taking into account immunological and genetic variation, that it recommends to be used in method validation studies. As new variants are discovered and made available, they can be added to the list.

Details of Recommendation
In order to assess the ability of a method to detect or quantify its target specifically, the method must be challenged with a variety of target and non-target organisms or molecules. The details of the challenge will depend on two main factors:

1. The method type – For example, immunoassay, molecular method or metabolic-based method (e.g., chromogenic agar) and
2. The target – For example, genus (e.g., *Salmonella* spp.), species (e.g., *Listeria monocytogenes*), a group of organisms (Enterohemorrhagic *Escherichia coli*), a single molecule or a group of molecules (e.g., staphylococcal enterotoxins).

Challenge studies will be designed to test variations of the target as appropriate to the method type, as well as test the most common food and environmental strains. For example, the presence of *E. coli* O157:H7 can be presumptively targeted by detecting the O157 antigen, the verotoxin genes or the verotoxins. Challenge studies could include non-motile strains, genetic variants, verotoxin expression variants (various levels of expression), strains that produce VT1, VT2 and VT1+2 and strains that produce VT1 variants and/or VT2 variants, depending on the method type.

In order to compare the performance of one method to another, a set of common strains must be used for inclusivity. Appendix 9 is a table of microorganism strains (including bacterial, viral and parasitic strains) and toxin types that can be used as reference strains for challenge studies during the initial validation of a method. This table takes into account known genetic and immunological variations of microorganisms and toxins. It is

recognized that shipping microorganisms can be problematic and, therefore, the table is comprised of reference strains found in the American Type Culture Collection (ATCC), the National Center for Type Cultures (NCTC) as well as other collections. An increasing number of strains are being labeled as bioterrorism agents, and in this case it may be beneficial to use a contract laboratory with the proper facilities and licenses to obtain, maintain and use these strains.

Inclusivity studies, therefore, would comprise a small number of appropriate reference strains chosen from Appendix 9 and a larger number of food isolates for validation of the target analyte(s) claim. The reference strains will allow comparison of one validation study to another, but it is recognized that food isolates are the most relevant strains for validating the claims of a method. The food isolates must, however, be well characterized relative to the method technology (genotype and/or phenotype characterization). By testing a sufficient number of variants within the target group, using reference strains and food isolates, one can be more confident in the comparative test results between methods and in being able to extend the method to additional strains.

The number of target organisms or toxins required for inclusivity testing is dependent on the target scope and the known variants available and, therefore, cannot be generalized. For inclusivity studies, AOAC currently recommends 100 strains for validation of methods for the detection of *Salmonella*, and 50 strains for methods for detection of pathogens (and other organisms) other than *Salmonella.* For some pathogens, for which number and/or availability of strains may be limited (for example, hepatitis A virus), or which have been highly characterized on the genetic level, it may be appropriate to use less than 50 strains for inclusivity testing. It is recognized, however, that inclusivity testing for a method targeting a genus should logically require more strains than a method targeting a species.

B. METHOD VALIDATION - VARIATION OF FOODS

Contract Objective being addressed:
Objective 5
What are the scientific/statistical bases for developing validation protocols that adequately take into account the [biological] variation that exist within [both the microorganisms and toxins produced by these microorganisms for which methods are developed and] the foods which will be analyzed?

Summary of Recommendation
Food commodity groups proposed by ISO and AOAC were extensively re-categorized based on physical structure, chemical parameters, bacterial load or other factors that would likely impact microbiological recovery and hence require different analytical approaches (See Appendices 1-8). The new food classification schemes are recommended to be used as the basis for method validation.

Details of Recommendation
The food categories found in ISO 16140 and the AOAC Guidelines are sub-categorized on the basis of broad food categories and microbial load and recovery. To make a broad

food claim, the AOAC Guidelines require 20 foods covering at least 6 of the 9 food categories in the precollaborative or single lab validation. ISO 16140 requires testing of three food types from each of five categories for an "all foods" claim. It has been observed, however, that methods validated with such broad claims do not necessarily perform well with new matrices that were not included in the validation study. Often times, matrix extension is not predictable within a food category. Therefore, the categories and sub-categories were redefined in an effort to make matrix extension more predictable.

The factors taken into consideration for sub-categorization include those that can affect microbial recovery or detection. Immunological and molecular methods can be affected by different factors, so both were considered. The factors used to sub-categorize foods included lipid or fat, protein, fiber, water activity or moisture level, presence of PCR inhibitors, microbial load, type of processing if any, presence of preservatives, surface structure, pH, salt and sugar. See Appendices 1-8 for the breakdown of each food category.

The concept of matrix extension is complicated and there are no hard and fast rules about how food products can or should be categorized for this purpose. Furthermore, this is a new way of thinking for most traditional food microbiologists. As we move away from qualitative assays towards quantitative, molecular-based methods, there will certainly be developments on this front. This document was constructed using the input of food microbiologists with expertise in a wide variety of matrices, as applied to many different detection methods, and is meant to be a guideline for future deliberations. Irrespective of how closely related a non-validated matrix may be to a validated matrix, the Matrix Extensions working group recommends that there needs to be some type of in-house verification conducted before using the alternative method on any previously un-validated matrix. This is particularly important when results are to be used for regulatory purposes.

To validate a category of foods, it is proposed that one matrix from each sub-category must be tested. This will no doubt increase the amount of work required to claim certain food categories, but will also increase the likelihood that the method is applicable to all types of foods in that category.

C. METHOD/MATRIX EXTENSION

Contract Objective being addressed:

Objective 1

Once a microbiological method has been validated for an array of specific foods and specific strains of a microorganism:

a) *To what extent can these results be extrapolated to other foods and other strains?*

b) *Are there abbreviated but scientifically/statistically appropriate procedures/protocols by which a validation can be expanded to include additional foods and/or strains?*

    c) *How can methods be applied to specific foods, where no validation has been performed?*

Summary of Recommendations
By using the food sub-categorization schemes shown in Appendices 1-8, matrix extension is simplified. The degree of validation required to extend a method to a new matrix is dependent on (1) how closely related the new matrix is to those that have been included in the initial validation, and (2) the level of validation initially performed (single lab, multi-lab, harmonized collaborative).

Details of Recommendations
With the exception of a few key methods (e.g., culture-based detection of *Salmonella* in "all foods," and and culture-based methods for the detection of *Listeria* spp. and *Escherichia coli* O157:H7 in broad categories of foods), when a method is validated by AOAC INTERNATIONAL or by the AOAC Research Institute, the claim is limited to those foods actually tested in the single lab validation (SLV), multi-lab validation (MLV) and/or in the harmonized collaborative validation (HCV). With sufficient representation within a food category, a claim can be made for that food category, although the actual foods tested must be clearly stated. There is a clear need to provide additional guidelines for matrix extension after appropriate laboratory validation has been completed.

When extending a validated method to a new matrix, then, it is logical to propose that the more closely related a new matrix is to a validated matrix, the higher the probability that the new matrix will perform similarly. The Matrix Extension Working Group has expended great effort to sub-categorize foods on the basis of their impact on microbial growth and recovery, as well as potential inhibitory effects, on rapid method technologies. These new sub-categorization schemes will be the basis for investigating proper protocols for matrix extension.

There are three situations to consider:
1. The new matrix is within the same sub-category or group (where there is no additional sub-category) as a validated matrix
2. The new matrix is in a new sub-category/group, but within the same class as a validated matrix
3. The new matrix is in a new class not previously validated

Table 1. Data Requirements for Matrix Extension

If new matrix is:	Then data required are:		
	For SLV Method	For MLV Method	For HCV Method
Situation 1: within the same sub-category/group as a validated matrix	None	None	None
Situation 2: in a new sub-category/group, but within the same class as a validated matrix	Verification	Verification	Single Lab Validation
Situation 3: in a new class not previously validated	Single Lab Validation	Multiple Lab Validation	Harmonized Collaborative Validation

The data set required to extend a validated method to a new matrix is summarized in Table 1. The extension of a validated method to a new matrix in Situation 1 should be the most predictable and, therefore, require no further experimentation. Due to the proposed scheme of sub-categorization of foods, all foods within the same sub-category are expected to perform equivalently. Therefore, if the proposed new matrix falls into the same sub-category (see the appended tables) as a previously validated matrix, the proposed matrix does not require a verification or validation study. The method can be applied to the new matrix without further study. While formal verification is not required in situation 1, it is good laboratory practice to perform some preliminary experiments to demonstrate that the method performs as expected with any new matrices being analyzed by the laboratory.

Extending a method to a matrix in a different sub-category/group within the same class(Situation 2) is less predictable than Situation 1 and, therefore, would require a basic level of experimentation. In Situation 2, a limited study to verify, rather than validate, the utility of the method for that matrix would be sufficient for SLV or MLV methods Verification would reveal gross effects on method performance such as the presence of inhibitors. An HCV would require a Single Lab Validation study for matrices in Situation 2.

Situation 3, in which a new class is being examined, would require full validation for SLV, MLV or HCV methods. Thus, an SLV method would require an SLV study, an MLV method would require an MLV study, and an HCV method would require an HCV study to extend the method to the new matrix.

The verification of method performance with a new matrix is intended to assure the user that the new matrix will produce neither high false positive rates (matrix is free from cross reactive substances) nor high false negative rates (matrix is free of inhibitory substances). To this end, a protocol is proposed in which the new matrix is spiked with a single strain of target organism chosen from the attached Strain list (Appendix 9) or a

single toxin type at a level 10 to 50 times higher than the LOD for the most similar validated matrix. Six replicates of the inoculated matrix and six replicates of the uninoculated matrix are tested and confirmed by both the alternative and the reference method. If no false positive or false negative results are obtained, then the new matrix is verified. If either false positive or false negative results are observed, then the study is expanded to a Single Laboratory Validation to define the operating characteristics of the method with the new matrix.

The Single Laboratory Validation (SLV) should follow the study design from the original validation study and should measure the 50% LOD for the new matrix being studied. The spike levels should be adjusted according to the expected LOD for the assay being evaluated and for the new matrix such that fractionally positive results are obtained for at least one of the levels.

For MLV and HCV method extension to a new food category, a Single Laboratory Validation is first carried out to determine the 50% LOD of the method with the new matrix as described above. These data provide the basis for the MLV or HCV study.

When extending a method to foods containing preservatives such as sodium benzoate, it is recommended that at least one verification study be performed in all cases.

All studies should be carried out in parallel with a reference method, when one is available, in order to compare the LOD_{50} values of the two methods. A test for statistical difference, such as Chi-Square, can be applied to compare the data sets where the same set of samples has been used for both methods (paired samples).

D. ACCEPTANCE CRITERIA FOR METHOD MODIFICATION
Contract Objective being addressed:
Objective 12
Can acceptance criteria be established for methods modification/substitution?

Summary of Recommendation
It is logical to say that when a method is modified, its performance should be at least as good as the original method. Recognizing that the modification of a method may have benefits other than enhanced performance parameters, such as time to result or ease of use, a modified method cannot be required to perform better than the original. Further, since there are many applications for methods (screening, regulatory action, process control, etc.) a modified method used for a different application may be acceptable even though its performance may be inferior to the original method. The MEWG, therefore, defers the subject of acceptance criteria to the Steering Committee.

Official Standards or Guidance Documents referenced:

1. Philip Feldsine, Carlos Abeyta and Wallace H. Andrews. 2002. AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Qualitative and Quantitative Food Microbiological Official Methods of Analysis. Journal of AOAC International 85 (5): 1188-1200.
2. ISO Standard 16140, *Protocol for the Validation of Alternative Methods*.
3. USDA Nutrient Data Laboratory http://www.nal.usda.gov/fnic/foodcomp/, April 2005

Appendix 1
Category 1 – Meat and Poultry

Class	Sub-category	Examples
A Water < 20%	None	Dehydrated Beef, Dehydrated Broth,
B Water between 20 – 80%	B.1 (Protein < 10%)	Most prepared foods, containing large amount of carbohydrates (15-30%), e.g. Frozen Entrées
	B.2 (Protein 10-30%, Lipid 10-30%, Cooked)	Hot Dogs, Bologna, Corned Beef Meat Patties
	B.3 (protein 10-20%, lipid 10-30%, raw)	Raw Chicken, Raw Beef, Raw Pork Ground Beef
	B.4 (protein 10-20%, lipid 10-30%, Marinated or spiced raw)	Raw Chicken, Raw Beef, Raw Pork
	B.5 (protein 10-35%, lipid < 10%, low fat, cooked)	Chicken Drumstick, Roast Beef- (Cured, Dried), Beef Brisket- Lean, Braised.
C Water 80-90%		Most Soups, Canned Baby Foods
D Water >90%		Most Broth.

Category 2 – Fruits and Vegetables   Appendix 2

Class	Group	Sub-category		
A: Fresh	*A.1:* *Low pH (<3.0-4.9)* most fruits, including citrus, berries, apples	A.1.1: Smooth product consistency	A.1.2: Rough/irregular product consistency	
	A.2: Reduced pH (5.0-7.0) melons, many vegetables	A.2.1: Smooth product consistency e.g. grapes, apples, squash	A.2.2: Rough/irregular product consistency e.g. berries, lettuce	
B: Frozen, and heat processed products	*B.1:* *Low pH (<3.0-4.9)* most fruits, including citrus, berries, apples	B.1.1: Smooth product consistency	B.1.2: Rough/irregular product consistency	
	B.2: Reduced pH (5.0-7.0) melons, many vegetables	B.2.1: Smooth product consistency e.g. grapes, apples, squash	B.2.2: Rough/irregular product consistency e.g. berries, lettuce	
C: Juice and Juice Concentrates	*C.1:* *Low pH (<3.0-4.9)* most fruit juices, including citrus, berries, apples, tomato	C.1.1: High °Brix (>60) high sugar fruit juice concentrates	C.1.2: Moderate °Brix (40-59) low sugar fruit juices	C.1.3: Low °Brix (<40) most fruit juices
	C.2: Reduced pH (5.0-7.0) most vegetable juices	C.2.1: High °Brix (>60) high sugar vegetable juice concentrates	C.2.2: Moderate °Brix (40-59) low sugar vegetable juices,	C.2.3: Low °Brix (<40) most vegetable juices
D: Dry and Low Moisture Products	D.1: Very low a_w (<0.60) (raisins, apricots)			
	D.2: Reduced a_w (>0..60) (dried vegetables, dried apples)			
E: Fermented fruit and vegetable products	(e.g., sauerkraut) No further sub-categorization			
F: Nutmeats	No further sub-categorization			

*Note: While compounds that can interfere with detection assays may be associated with many if not most food matrices, the inhibitory effect of fruit and vegetable matrices may be particularly troubling. Users are encouraged to consult the literature and perform preliminary experiments to demonstrate that the method performs as expected with new matrices of concern before routinely using the method on those matrices.

Category 3 – Dairy Products   Appendix 3

Class	Group by Water Content	Sub-category by Fat Content	Representative Examples
A. Fermented and Non-Fermented Products*	A.1: (<20%)	A.1.1 (<10%)	Milkshake powder, Buttermilk-dried, Dry non-fat milk, Dry Whey, casein**
		A.1.2 (10-30%)	Dry, whole milk, Grated Parm. Cheese
		A.1.3 (30-70%):	Powdered cream
		A.1.4 (>70%):	Butter, margarine
	A.2: (20-50%)	A.2.1 (<10%)	Canned Condensed milk
		A.2.2 (10-30%)	American cheese, pasteurized, Brie, Gouda, Monterey, Colby, Hard and Soft goat Cheese
		A.2.3 (30-70%):	Margarine
	A.3: (50-80%)	A.3.1 (<10%)	Ice Cream, Low-fat Yogurt, Ricotta, Milkshake, Evap. Milk
		A.3.2 (10-30%)	Sour Cream, Whipped cream, Mozzarella
		A.3.3 (30-70%)	Heavy Cream
	A.4: (>80%)	A.4.1 (<10%)	Fat free Half and Half, Whey-fluid, Plain Yogurt, Cottage cheese (reg and low fat, Milk substitute, buttermilk, Milk
		A.4.2 (>10%)	Half and Half reg.

*To interpret the table, the user must first categorize the dairy product in question as fermented or non-fermented. Thereafter, the sub-categorization based on water and fat content can be used. Note that the representative examples are not meant to be exhaustive and there are many other products which might fit into any one subcategory.

**The detection of certain pathogens in some products may differ based on methods of manufacture (e.g. *Salmonella* detection in non-fat dry milk or casein products). Consult the literature before applying matrix extension in these particular applications.

Category 4 – Egg Products   Appendix 4

Class	Group	Examples
A	< 5% salt or sugar added	shell eggs, whole eggs, egg yolks, egg whites, dried whole egg, dried egg yolk, dried egg whites, egg substitutes
B	≥ 5% salt or sugar added	whole eggs, yolks, or egg products

Category 5 – Miscellaneous Foods   Appendix 5

Class	Group	Examples
A. Cereals and Grains	A.1 Flour and dry mixes	
	A.2 Unbaked, viable-yeast leavened products	
	A.3 Dough, batter, and baked products	
B. Chocolate*	B.1 Fat < 20%	Cocoa powders, all Confectionery products, Ingredients, Coatings, Chocolate bars
	B.2 Fat > 20%	Cocoa powders, all Confectionery products, Ingredients, Coatings, Chocolate bars
C. Pasta	C.1 Raw, Fresh	
	C.2 Raw, Dried	
	C.3 Cooked	
D. Dressings, Condiments and Marinades	D.1 Do not require refrigeration for microbiological safety	Contain preservatives, Aw <0.85 or pH < 4.0
	D.2 Require refrigeration for microbiological safety	Specified by manufacturer (does not apply to products that need refrigeration after opening)
E. Soy Products	None	

*Note: While compounds that can interfere with detection assays may be associated with many if not most food matrices, the inhibitory effect of chocolate and chocolate products may be particularly troubling. Users are encouraged to consult the literature and perform preliminary experiments to demonstrate that the method performs as expected with new matrices of concern before routinely using the method on those matrices.

Category 6 – Seafood   Appendix 6

Class	Group	Sub-category						
A. Finfish	A.1 Fresh Water	A.1.1 Raw Fresh	A.1.2 Raw Frozen	A.1.3 Cooked	A.1.4 Dried	A.1.5 Cold Smoked, Marinated or Cured	A.1.6 Carbon Monoxide (CO) Treated	A.1.7 Fermented
	A.2 Salt Water	A.2.1 Raw Fresh	A.2.2 Raw Frozen	A.2.3 Cooked	A.2.4 Dried	A.2.5 Smoked, Marinated or Cured	A.2.6 CO Treated	A.2.7 Fermented
B. Molluscan Shellfish*		B.1 Raw Fresh	B.2 Raw Frozen	B.3 Cooked	B.4 Marinated or Hot Smoked	B.5 High Pressure Treated		
C. Crustaceans		C.1 Raw Fresh	C.2 Raw Frozen	C.3 Cooked				
D. Squid/Octopus		D.1 Raw Fresh	D.2 Raw Frozen	D.3 Cooked				

*Note: While compounds that can interfere with detection assays may be associated with many if not most food matrices, the inhibitory effect of molluscan shellfish is particularly well characterized. Users are encouraged to consult the literature and perform preliminary experiments to demonstrate that the method performs as expected with all molluscan shellfish matrices on which it is to be applied. In particular, the following are known to impact ability to recover target organisms: (1) differences (seasonal, storage, or processing related) in biochemical composition of the animal tissue; (2) differences in background flora arising from harvest water conditions (mostly seasonal) and temperature history of the product.

Category 7 – Animal Feed   **Appendix 7**

Class (Dry Matter)	Group (Crude Fiber)	Sub-category (Crude Protein)	Representative Examples
A. DM>75%	A.1 CF<10%	A.1.1 CP<20%	Cereal grains Dried bakery waste Dried whey
		A.1.2 CP>20%	Bean varieties Blood meal Soybean meal Distillers grains Feather meal Meat meal Meat & bone meal Poultry by-product
	A.2 CF>10%	A.2.1 CP<20%	Alfalfa hay Clover hay Barley hay Cottonseed hulls Dried beet pulp Dried apple pomace Wheat bran Oat hulls
		A.2.2 CP>20%	Canola meal Sunflower meal Cottonseed meal Coconut meal Avocado seed meal

B. DM<75%	B.1 CF<10%	B.1.1 CP<20%	Bread by-products High moisture corn Cane molasses Beet molasses Citrus molasses
		B.1.2 CP>20%	Wet distillers grain (corn)
	B.2 CF>10%	B.2.1 CP<20%	Fresh alfalfa Fresh clover Wet Apple pomace Wet beet pulp Fresh grasses Sugar beet tops Ensiled forages
		B.2.2 CP>20%	Wet distillers grain (sorghum, barley)

Category 8 – Spices*   Appendix 8

Class	Group
1	Black Pepper, White Pepper, Caraway Anise, Celery, Cumin, Dill, Fennel, Nutmeg, Coriander, Ginger, Paprika
2	Onion, Garlic
3	Oregano, Cinnamon, Allspice
4	Thyme, Marjoram, Basil, Sage, Rosemary
5	Red Pepper, Chili Pepper
6	Cloves

*Spices are a particularly troubling category as many contain uncharacterized naturally occurring compounds that can interfere with detection assays. Although this table can serve as a guideline, the user is strongly encouraged to consult the literature and perform preliminary experiments on each spice to demonstrate that the method performs as expected with new matrices before routinely using the method on those matrices.

Appendix 9 – See Excel Spreadsheet