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Application of ISO 16140-3:2021 in Your Laboratory

Introduction

Food microbiology contract laboratories are asked to test a wide array of foods and beverages. Laboratories need to ensure that the methods used to conduct testing are validated and verified for use with the specific sample types they routinely test in their laboratories.

The International Organization for Standardization (ISO) this year published a new standard focused on demonstrating user competency to perform and implement the methods used in laboratories: ISO 16140-3:2021 Microbiology of the food chain — Method validation — Part 3: Protocol for the verification of reference methods and validated alternative methods in a single laboratory.[1] Verification of the methods routinely used in laboratories is a requirement for laboratories accredited to ISO/IEC 17025:2017 General requirements for the competence of testing and calibration laboratories.[2]

This paper is a high-level overview of ISO 16140-3:2021 regarding verification and its intent is to orient you with key concepts within the standard.

Two 3M validated methods are used as examples to apply these concepts: the 3M™ Molecular Detection Assay-2 *Salmonella* and the 3M™ Petrifilm™ Enterobacteriaceae Count Plate. This primer is an overview and is not intended to replace the use of the ISO standard to correctly implement this standard within your laboratory.

After reviewing this paper, 3M strongly recommends reviewing the following additional sources of available training to deepen understanding and application of the standard:

- 3M Webinar: Application of the ISO 16140-3:2021 Standard in Your Lab[3]
- ISO TC34/SC9 Website[4], under "Supporting materials":
    - Recording of an ISO Webinar from March 2, 2020
    - Other supporting material available from ISO

General concepts within the standard

Fully validated methods

The ISO 16140-3:2021 standard on method verification can be applied to methods that have been "fully" validated, meaning a validation that included both a comparative study and an inter-laboratory study compared to a reference method, as described below:

- **Comparative study** – a method compared to a reference method, usually conducted within a single laboratory
- **Inter-laboratory study**– a method compared to a reference method, conducted between many laboratories

Validation vs verification

The terms ***validation*** and ***verification*** are often incorrectly used interchangeably, so it is important to understand their distinction and definitions. Method verification always follows validation. A method is first ***validated*** – demonstrating that the method performs equivalently to the reference method, based on key, defined method criteria; and then, before a laboratory puts the method into routine use, they need to ***verify*** that they can correctly use the method to achieve those key method criteria. In simple terms:

- **Validation** is proof that the ***method*** "works"
- **Verification** is proof that ***the user*** can perform that method correctly

Distinction Between Scopes

Scope specifies the different categories, types, and items for which a method can be applied. There is a distinction between the scope of a method, the scope of the validation of that method, and the scope of the laboratory's application of that method.

The scope of the method are those products for which the method claims it can be applied. It is often assumed that the scope of the method for a reference method means it is applicable to use with all products. However, not all reference methods have been validated for use with all products.

The scope of validation refers to those products for which the method claims it has been validated for use, via a validation study.

The scope of laboratory application, would be those products that are within the scope of validation of the method, and routinely tested within that laboratory.

Food and non-food Categories

A method may claim to be valid for use with all foods, as many reference methods do, but it is not possible to validate a method for ALL the types of foods found globally, in order to make such a claim. Because of this, both AOAC INTERNATIONAL and ISO have agreed to use the phrase "broad range of foods" versus the claim "all foods," for method validation studies. To support this terminology, each organization has together defined the general grouping of all foods into fifteen (food) categories (plus three 'other' non-food categories), as shown in Figure 1.

Figure 1. Food and non-food categories for AOAC and ISO method validation

Categories					
Raw milk and dairy products	Heat-processed milk and dairy products	Raw meat and ready-to-cook meat products (except poultry)	Ready-to-eat, ready-to-reheat meat products	Raw poultry and ready-to-cook poultry products	Ready-to-eat, ready-to-reheat meat poultry products
Eggs and egg products (derivatives)	Raw and ready-to-cook fish and seafoods (unprocessed)	Ready-to-eat, ready-to-reheat fishery products	Fresh produce and fruits	Processed fruits and vegetables	Dried cereals, fruits, nuts, seeds and vegetables
Infant formula and infant cereals	Chocolate, bakery products and confectionary	Multi-component foods or meal components	Pet food and animal feed	Environmental samples (food or feed production)	Primary production samples (PPS)

- **Broad range of foods:** To make a claim that a method is validated for the scope that includes "a broad range of foods," a defined number of (food) items must be tested from at least five of these 15 (food) categories.
- **Limited range of foods:** To make a claim that a method is validated for less than five (food) categories (defined as a "limited range of foods"), only the selected (food) categories from the 15 (food) categories are included in the method validation.
- **Other (Non-foods):** To claim validation for one or all three of the non-food categories, additional items from each of these categories would also need to be validated.

Verification is conducted in two stages:

1. **Implementation verification** is conducted first to demonstrate the user laboratory can conduct the method correctly. This is conducted using one (food) item.
    - **For qualitative methods**, this one (food) item must be an item that was tested during the validation study of the method and the same sample size must be used for this item as was used during the validation study
    - **For quantitative methods**, this one (food) item can be any (food) item from within the scope of the validation of the method
2. (**Food) item verification** demonstrates that the user laboratory can conduct the method with the types of (food) items that are routinely tested in the user's laboratory. The number of items needed to test will depend on the number of categories for which the laboratory routinely uses this method.

    Because not many (food) items are tested during verification, the standard prefers that the user chooses "challenging" foods that are ***claimed*** in the validation of the method but were not tested during the validation study. Challenging, meaning items that may have a low pH, a low water activity, a high number of particulate, etc. – anything that could perhaps be inhibitory (a challenge) to the performance of the method.

Conducting Method Verification - *Examples*

Verification of a QUALITATIVE Method: 3M™ Molecular Detection Assay-2 *Salmonella*

Step 1: Define the scope of laboratory application

As an example, a laboratory that would like to conduct verification of the 3M Molecular Detection Assay-2 *Salmonella* for use in their laboratory would begin by first looking at what (food) items are routinely tested their laboratory for ***Salmonella*** and determining how many (food) categories these items fall into, based on the categories in Figure 1 above. This laboratory (shown in Figure 2 below) tests (food) items that fall within one main (food) category: "raw poultry and ready to cook poultry products" as well as testing sponges collected from the manufacturing environment, which fall into one of the 'other' (non-food) categories: "Environmental samples".

The laboratory will need to test items from both of these categories to complete verification of this method for use in their laboratory.

Figure 2. The "User" Laboratory (food) items – Qualitative method. Items tested are highlighted in light orange.

The User Laboratory			
(Food) Category	**Location 1 Raw**	**Location 2 Frozen**	**Location 3 Ready-to-cook**
Raw poultry & ready-to-cook poultry products	**Ground chicken** Breast	**Boneless skinless** Breast, Thighs, Tenders	**Chicken wings** Seasoned 1, Seasoned 2, Seasoned 3
	Ground turkey 85% lean, 93% lean	**Thin sliced chicken breast** Seasoned 1, Seasoned 2, Seasoned 3	**Chicken tenders** Breaded, Seasoned 1, Seasoned 2, Seasoned 3
	Boneless skinless Chicken breast, Tenderloins, Thighs, Legs	**Seasoned breast pieces** Fajita, Spice garlic herb, Barbeque, Lemon pepper	**Nuggets** Breaded, Seasoned 1, Seasoned 2, Seasoned 3
	Bone/skin Chicken breast, Thighs, Drums, Wings, Leg		**Chicken strips** Breaded, Seasoned 1, Seasoned 2, Seasoned 3
Environmental samples (food and feed production)			**Sponges from equipment (EM)** w/Letheen broth

Step 2: Review method validation data, and choose (food) items for the verification study

The laboratory will next need to confirm that the food and non-food items they routinely test have been fully validated via either AOAC® *Official Method of Analysis*℠[5] and/or a Certification body (NF Validation by AFNOR Certification or MicroVal) that follows the protocol defined in ISO 16140-2:2016 Microbiology of the food chain - Method validation - Part 2: Protocol for the validation of alternative (proprietary) methods against a reference method.[6] The laboratory must then determine which items were included in the validation study(s).

AOAC validation data

AOAC validation data for 3M Molecular Detection Assay-2 *Salmonella* is accessed in two parts. The comparative study data can be found in the AOAC® *Performance Tested Method*℠ Certificate 091501 for the method, available on the AOAC website. This certificate contains the list of (food) items tested in the comparative study, in addition to a summary of the data. The AOAC® *Official Method of Analysis*℠ 2016.01 is granted after an interlaboratory study of the method, and can be found on the 3M Food Safety website.

ISO 16140-2:2016 validation data

3M Molecular Detection Assay-2 *Salmonella* validation per the ISO 16140-2:2016 protocol was conducted via NF VALIDATION by AFNOR Certification. The AFNOR certificate 3M 01/16-11/16 and summarized validation study report can be found on the AFNOR Certification website.

(Food) item for implementation verification – the laboratory will need to select one (food) item tested during the validation study that also belongs within the scope of laboratory application of the user laboratory. For verification of this method, the laboratory chose raw ground chicken breast.

(Food) items for (food) item verification – only one (food) category is tested within the laboratory application, so the laboratory is only required to choose one (food) item for the (food) item verification study, as well as testing environmental sponges with Letheen broth to verify the "other" non-food category under their laboratory application. However, because there are different types of (food) items within the one (food) category of "Raw poultry and ready-to-cook poultry products" which may provide challenges to recovery of low number of ***Salmonella***, the laboratory may choose to include the testing of additional (food) items in their (food) item verification study, such as those (food) items highlighted in Figure 2 above.

Step 3: Implementation verification using $eLOD_{50}$ (Example: Protocol 1)

For qualitative methods, the ***estimated*** LOD_{50} is determined for ***both*** implementation and (food) item verification. Estimated LOD_{50} ($eLOD_{50}$) is a definition unique to ISO 16140-3:2021. The term LOD, level of detection, is used for qualitative methods in microbiology based on *replicate* analyses at three different inoculation levels of the target analyte in a matrix. The study performed for verification is called $eLOD_{50}$ because although the study evaluates replicates at different inoculation levels, the study will not test enough samples to meet the requirements for determination of an actual LOD_{50}, as is used for method validation.

The standard provides three protocols from which to choose to determine the $eLOD_{50.}$ For each protocol, test portions are prepared and inoculated. Protocols one and two require the use of a culture grown in the laboratory for inoculation of test portions at three levels: high, intermediate and low levels. Protocol three allows for the use of a standardized reference material – inoculating with a known concentration of the target microorganism – and requires inoculation of replicates at only a low level of inoculation.

As an example, if the laboratory chooses to follow protocol one, it would first review the validation study to see what the LOD_{50} was for raw ground chicken breast and determine then how to begin inoculation of their (food) items. A culture of *Salmonella* is grown overnight, serially diluted and plated to determine the concentration of the inoculum before preparing to dilute to the required concentrations of inoculation for each level (per the standard).

The standard states to aim for inoculation of 9x the LOD_{50} from the validation study for the high inoculation level, 3x LOD_{50} for the intermediate inoculation level and 1x the LOD_{50} for the low inoculation level. If an LOD_{50} was not provided in the validation study, the standard then says to assume an LOD_{50} of 1 for your low inoculation level, to help determine the three inoculation levels.

After inoculation, the test portions should be analyzed per the instructions for the method to be verified, in this case, the 3M Molecular Detection Assay-2 *Salmonella*. The laboratory should then record the number of positive results at each inoculation level and use the most probable number (MPN) tables provided within the standard to determine the multiplier that should be used for the low inoculation level to determine the $eLOD_{50}$ and assess whether it meets the Acceptability Limits as defined in the standard.

Because there was no LOD_{50} listed in the 3M Molecular Detection Assay-2 *Salmonella* validation report, the laboratory assumed an LOD_{50} of 1, per the standard. To now determine if the test portions met the Acceptability Limit for verification of qualitative methods, the laboratory must look within the table in the standard (Table 16: Acceptability limits for the verification of validated methods) which states that the $eLOD_{50}$ should be ≤ 4x LOD_{50}.

ISO has provided an Excel®-based program (workbook) available on the ISO TC34/SC9 website to make calculations and determinations as so whether one's results meet Acceptability Limits.

Step 4: (Food) item verification using $eLOD_{50}$ (Example: Protocol 1)

Because estimated LOD_{50} is used for *both* implementation and (food) item verification, this same protocol is repeated for each of the (food) items needed to complete (food) item verification. For this laboratory that would be one (food) item and one 'other' non-food item to complete verification of each category this laboratory claims to test within its Scope of Application.

Verification of a Quantitative Method: 3M™ Petrifilm™ Enterobacteriaceae Count Plate

Step 1: Define the scope of laboratory application

For this example, a manufacturing plant that makes ice cream products, infant formula, frozen pizzas, refrigerated ready-to-cook pasta and collects environmental samples as part of their environmental monitoring program will be used. Sorting these items into the 15 food + three "other" categories using Figure 1 above helps determine that this laboratory tests (food) items from three different (food) categories: "Heat processed milk & dairy product," "Infant formula and infant cereals," "Multi component foods or meal components," and one non-food category, "Environmental samples."

The laboratory at this manufacturing plant will need to test items from all four categories to complete verification of this method for use.

Step 2: Review method validation data, and choose (food) items for the verification study

The laboratory will first need to confirm that the food and non-food items they routinely test have been fully validated via either AOAC® *Official Method of Analysis*℠ and/or a certification body (NF Validation by AFNOR Certification or MicroVal) that follows the protocol defined in ISO 16140-2:2016.

AOAC validation data

The AOAC® *Official Method of Analysis*℠ *2003.01* for 3M Petrifilm Enterobacteriaceae Count Plate can be found on the AOAC website. (This method has no Performance Tested Method (PTM) data because the method was fully validated prior to creation of the AOAC PTM program).

ISO 16140-2:2016 validation data

3M Petrifilm Enterobacteriaceae Count Plate validation per the ISO 16140-2:2016 protocol was validated via NF VALIDATION by AFNOR Certification. The AFNOR Certificate 3M 01/06-09/97 and summarized validation study report can be found on the AFNOR Certification website.

Food item for implementation verification – the laboratory will select one (food) item within the scope of the method validation which also belongs to the scope of the laboratory application. For verification of this method, the laboratory will choose vanilla ice cream because it is easily homogenized for dividing into test portions.

Food items for (food) item verification – the laboratory will select one challenging (food) item from each of the three identified (food) categories, in addition to the one non-food item category in order to complete verification of this method. For this example, the laboratory will choose vanilla ice cream with chocolate pieces and almonds, dehydrated milk powder, and ready-to-cook spinach and cheese tortellini, to verify each of the three (food) categories claimed to be validated for the method, and swabs with Letheen broth for verification of the one non-food category.

(Food) items chosen for both implementation and (food) verification are highlighted in Figure 3 below.

Figure 3. The "User" Laboratory (food) items – Quantitative method. Items tested are highlighted in the light orange boxes.

The User Laboratory				
(Food) Categories				
Heat Processed Dairy	**Infant Formula**	**Multi-Component/Composite Foods**		**Environmental Samples**
Vanilla ice cream	Infant cereal with wheat, oat, sugar, rice	Frozen cheese pizza	Ready-to-cook pasta	Sponges with Letheen broth
Vanilla ice cream with chocolate swirls	Dehydrated milk powder	Frozen supreme pizza	Ready-to-cook spinach and cheese tortellini	Swabs with Letheen broth
Vanilla ice cream with chocolate pieces and almonds	Whey-based dairy infant formula	Frozen sausage and anchovy pizza	Ready-to-cook cheese tortellini	

Step 3: Implementation verification - Inter-laboratory Reproducibility Standard Deviation (S_{IR})

Implementation verification for quantitative methods is achieved by determining the intralaboratory reproducibility standard deviation, expressed as S_{IR} (Intra - meaning within one lab, versus Inter – meaning between several labs, as is conducted during method validation).

For this study, the laboratory will choose one (food) item within the scope of the method validation which also belongs to the scope of the laboratory application. The laboratory will first need to collect a minimum of ten different kinds of samples of vanilla ice cream in order to provide a variety of vanilla ice creams such as you would expect would come into the laboratory routinely. The samples can be ice creams from different batches or lots, manufacturers, production days, etc. More than ten samples can be collected to ensure enough samples/variety.

A culture of *Enterobacteriaceae* is grown overnight and then serial dilutions are made and plated to determine inoculation levels. Each of the samples of vanilla ice cream is thoroughly homogenized and then divided into two test portions: A and B. Each test portion set is then inoculated with a range of contamination as would typically be found in the samples routinely analyzed in the laboratory (between 30 cfu/g – 30,000 cfu/g). Because this study is being conducted in one laboratory, the laboratory needs to make the analysis of the test portions A and B as different in as many ways as possible: using different technicians, incubators, batches of culture media (different preparations from the same batch of media powder), etc.

Each of the sample test portions is then analysed using the 3M Petrifilm Enterobacteriaceae Count Plate method. Results are recorded and used to calculate the intra-laboratory reproducibility standard deviation S_{IR} using the formula provided in the standard or using the Excel®-based program (workbook) available on the ISO TC34/SC9 website.

Within the validation study report for the 3M Petrifilm Enterobacteriaceae Count Plate method, the laboratory will locate the lowest Inter-laboratory reproducibility standard deviation (S_R) mean value of the (food) items used in the validation study and compare this to the calculated S_{IR} obtained. In Figure 4 below, the lowest S_R mean value in the validation study for this method was found to be 0.125 (0.126 + 0.122 + 0.126 = 0.125).

The S_{IR} must be ≤ 2 x the lowest S_R to meet the implementation verification Acceptability Limits.

Figure 4. Alternative method 3M Petrifilm Enterobacteriaceae Count Plate AFNOR report accuracy profile

Alternative Method			
Levels	**Low**	**Medium**	**High**
Target value	2,274	3,238	4,191
Number of participants (K)	14	14	14
Average for alternative method	2,224	3,244	4,223
Repeatability standard deviation (sr)	0,122	0,110	0,104
Between-labs standard deviation (sL)	0,031	0,052	0,071
Reproducibility standard deviation (sR)	0,126	0,122	0,126

Step 4: (Food) item verification using eBias

(Food) item verification for quantitative methods is achieved by determining the estimated Bias (eBias), which is another definition unique to ISO 16140-3:2021. An accurate determination of the bias (as done in validation studies) is not possible because the number of samples that will be tested in the verification study is small. Therefore, the term ***eBias*** is used for the verification study.

To conduct (food) item verification, the laboratory will use the three challenging (food) items and one non-food item (swabs with Letheen broth) chosen from review of the categories in the scope of the laboratory application.

Each (food) item will be artificially contaminated at three inoculation levels that cover the range of use of the method as it is routinely used by the laboratory (for example: 30-300, 300-3,000 and 3,000-30,000 cfu/g). Each of these three levels will be performed in *duplicate*.

A culture of *Enterobacteriaceae* is grown overnight, and then serial dilutions are made and plated, to determine the correct inoculation levels. When diluting the inoculation suspension to prepare for inoculation in the (food) item duplicates, consider additional dilutions that will be needed to achieve counts within the countable range of the method for each of the three levels. As illustrated in Figure 5 below, the inoculum requires further dilution to achieve the correct dilution/counting levels for the high, intermediate and low levels; the inoculum, when mixed with the volume of the (food) item, requires less dilution.

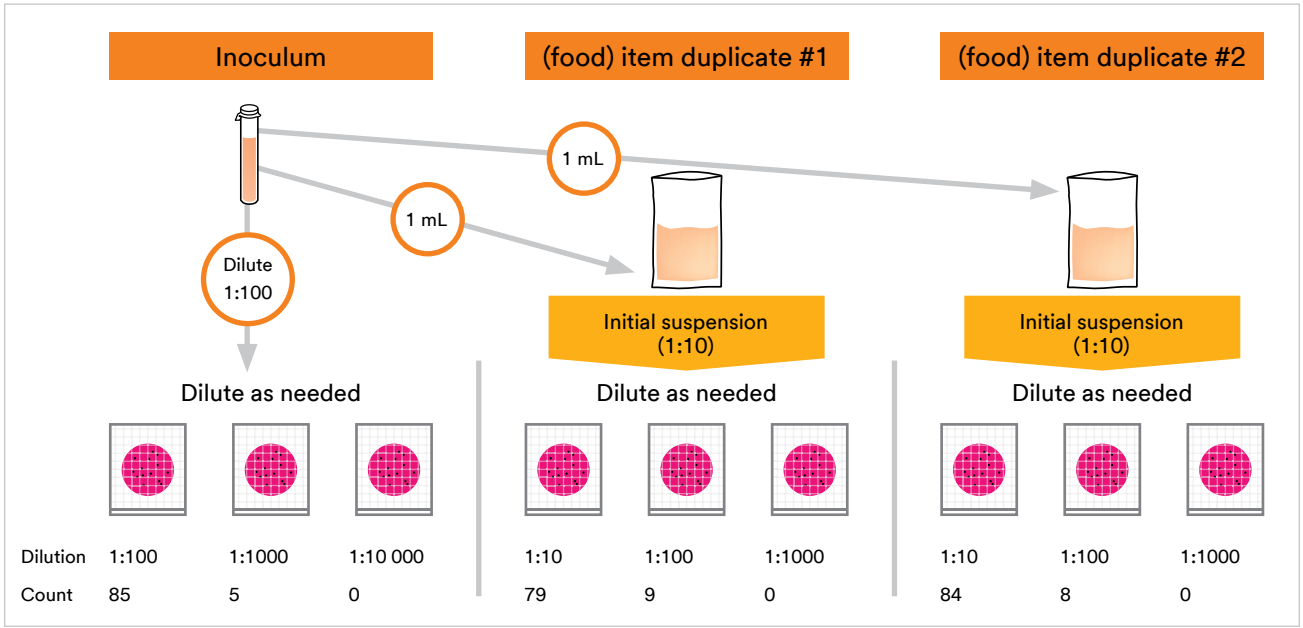
To calculate the eBias, enumeration is conducted and recorded for:

- The **inoculum** at all three levels
- The **(food) item *with*** inoculum at all three levels
- The **(food) item *without*** inoculum in duplicate (as a negative control) to determine the background microbiota level (if any) in the (food) item.

For each of the three levels, the counts of the (food) item duplicates are averaged and a $\log_{10}$ transformation is done to determine $\log_{10}$ cfu/g for each level. These results are then expressed in $\log_{10}$ cfu/***test portion*** for each level and compared to the log transformation on the count of that *same* inoculum level determined for each level *without* the (food) item. The eBias is the absolute difference in results between the inoculated (food) item and the inoculum.

To meet the Acceptability Limits for eBias per the standard, the absolute difference for each level must be ≤ 0.5 $\log_{10}$/ml. Again, the Excel®-based program (workbook) is available on the ISO TC34/SC9 website for you to insert your data and help complete all calculations to determine if you meet the Acceptability Limits.

Figure 5. Quantitative (Food) item verification protocol with 3 levels of inoculum



Further help:

For help applying this new standard in your laboratory, please contact 3M and access the support materials available to you on the ISO TC34/SC9 website.

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References

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5. AOAC INTERNATIONAL. 2012. Appendix J: AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces
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7. ISO TC34/SC9 Website (accessed June 24, 2021): Excel®-based program (workbook) on the ISO TC34/SC9 website under 'Supporting materials'

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