Welcome to section 2 of module 9, where we talk about method validation. Using validated methods and ensuring that they reliably produce the right result in our laboratory is an important aspect of the job of the analyst.

Validation

Quality Assurance

Reference	Validation Definition
Codex CAC/GL 74	Process to establish the **performance characteristics** and limitations of an analytical method: which analytes, in what kind of matrices, in the presence of which interference. Result = **precision and trueness values** of a certain analytical method under the examined conditions.
ISO 16140-1	Establishment of the **performance characteristics** of a method and provision of **objective evidence** that the performance requirements for a specified intended use are fulfilled.
USDA FSIS	Process to measure **performance characteristics** of a particular test, with the goal of determining whether the test is equivalent to the reference test for the intended conditions of use. "Equivalent" = the performance characteristics are **statistically indistinguishable**.
US FDA	Demonstration that **adequate confidence** is provided when the results obtained by the alternative method i.e., the commercially available kit, are **comparable to or exceed** those obtained using the reference method using the **statistical criteria** contained in the approved validation protocol.
Health Canada	Evaluation of the **performance parameters** of a new method in comparison to an accepted reference method using paired or unpaired samples. In the context of relative validation, the results of the reference method are assumed to reflect the true microbiological status of the samples and the performance parameters of the alternative method are **calculated in relation** to this.
ISO 17025:2017	Provision of objective evidence that a given item fulfills specified requirements, while **Validation** is **defined** as **verification**, where the specified requirements are adequate for an intended use

Much like many of the definitions in quality control, validation itself is defined differently by various organizations. The table shown here showcases some examples of definitions for the word "validation" as adopted by Codex, ISO and number of regulatory agencies.

Codex defines validation as the process to establish the performance characteristics and limitations of an

analytical method: This includes which analytes, in what kind of matrices, in the presence of which interference.

The result is the precision and trueness values of a certain analytical method under the examined conditions.

ISO 16140-1 defines validation as the establishment of the performance characteristics of a method and provision of objective evidence that the performance requirements for a specified intended use are fulfilled.

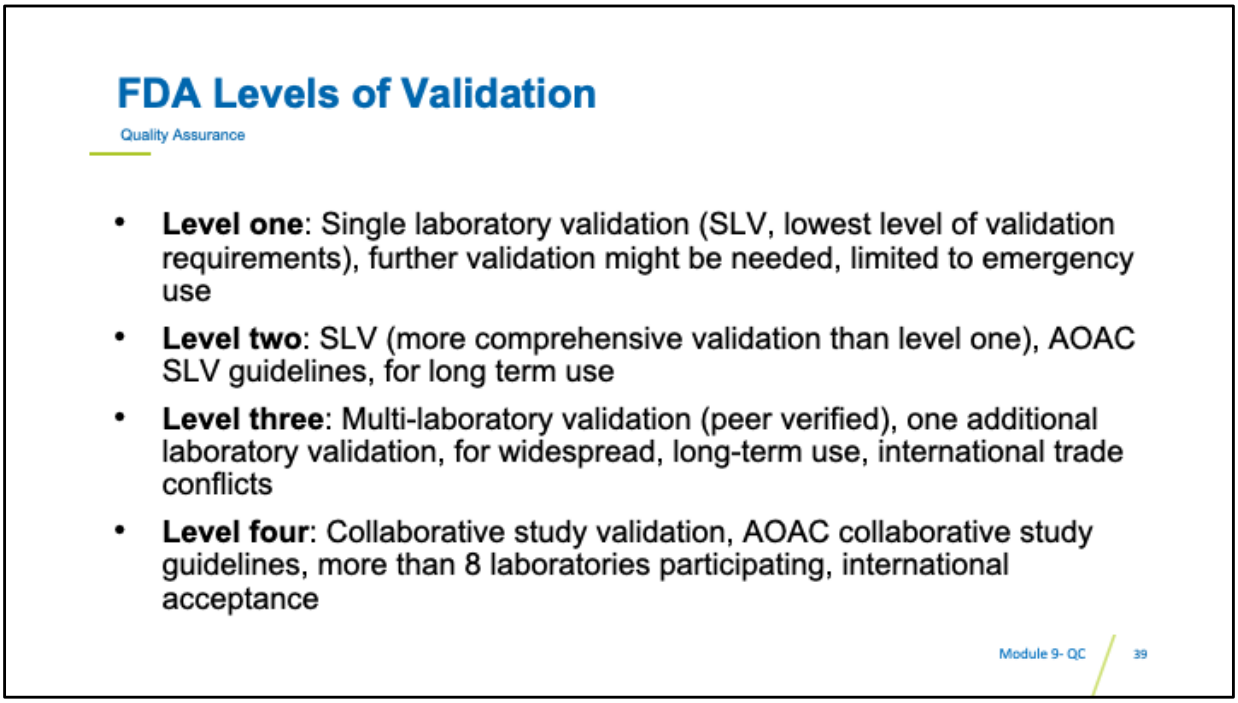
The USDA Food Safety and Inspection Service, defines it as the process to measure performance characteristics of a particular test, with the goal of determining whether the test is equivalent to the reference test for the intended conditions of use. "Equivalent" means the performance characteristics are statistically indistinguishable.

At US FDA, it is the demonstration that adequate confidence is provided when

the results obtained by the alternative method i.e., the commercially available kit for example, are comparable to or exceed those obtained using the reference method using the statistical criteria contained in the approved validation protocol.

Health Canada defines validation as the evaluation of the performance parameters of a new method in comparison to an accepted reference method using paired or unpaired samples. In the context of relative validation, the results of the reference method are assumed to reflect the true for example microbiological status of the samples and the performance parameters of the alternative method are calculated in relation to this.

For ISO 17025, the latest edition in 2017, it is the provision of objective evidence that a given item fulfills specified requirements, while validation is defined as verification, where the specified requirements are adequate for an intended use.



Once again, I am using a specific example from the US FDA to explain a concept. I worked at the FDA for a couple of years, but I worked with them for about a decade, so I tend to have a lot of examples from there... We can discuss examples from other countries. during the in-person training.

The FDA defines four levels of validation. Level one is a single laboratory validation and it is limited to emergency use. This type of method is only acceptable when methods that have undergone inter laboratory validation are not available for an emerging contaminant or in an unexpected matrix.

For example, when melamine was first identified as a potential contaminant in cat food, there was no official method to measure it so an emergency method was developed, validated as a level 1 method, and used.

Level 2 validation is more comprehensive than level 1 and corresponds to the AOAC's single laboratory validation guidelines. This level of validation is enough for long term use within the agency's laboratory. A Level 3 method undergoes multi laboratory validation, which makes it peer verified, a more extensive process that is designed to ensure confidence for widespread long-term use and international trade.

Finally, a level 4 method has undergone collaborative validation studies typically under the auspices of the AOAC, where more than eight laboratories

participated. A level 4 method has international acceptance.

Level 1: Minimum Requirements

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Single matrix	Matrix	Samples 1 & 2	Samples 3 & 4	Samples 5 & 6	Samples 7 & 8
Same Day	Matrix Y (source 1)	Blank	Spiked (1/2 X)	Spiked (1 X)	Spiked (2 X)
	Matrix Y (source 2)	Blank	Spiked (1/2 X)	Spiked (1 X)	Spiked (2 X)

Example:
Analyte = Aflatoxins or aflatoxin B1
Target level = X (or regulatory level)

This table shows the number of matrices and sources that are needed as a minimum for a level 1 validation. This is the bare minimum to ensure that the method works in the matrix of interest. Obviously, there is only one matrix because this level is intended for emergency use only, so we shouldn't be dealing with too many matrices.

As usual, X represents the target level, which is the MRL if one exists. In an emergency, there often is no MRL since we are typically dealing with a completely unexpected contaminant. So X would then be decided based on the level that poses a health risk.

Level 2: Minimum Requirements

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Days	Single Matrix	Analyte Spiked Levels
Day 1 (source 1)	Corn 1, 2, 3, 4, 5	0, ½ X, 1X, 2X
Day 2 (source 2)	Corn 1, 2, 3, 4, 5	0, ½ X, 1X, 2X
Day 3 (source 3)	Corn 1, 2, 3, 4, 5	0, ½ X, 1X, 2X

Example: Matrix = Corn
Analyte = Aflatoxins or aflatoxin B1
X = Target level (or regulatory level)
Note: analyze CRM in replicate if it is available

The level 2 method, traditionally known as the single laboratory validation for routine use, requires the repetition of analysis from three sources of a matrix over three different days in at the for same concentrations as level one. If a certified reference material is available, it is recommended to include it in the replicate analysis.

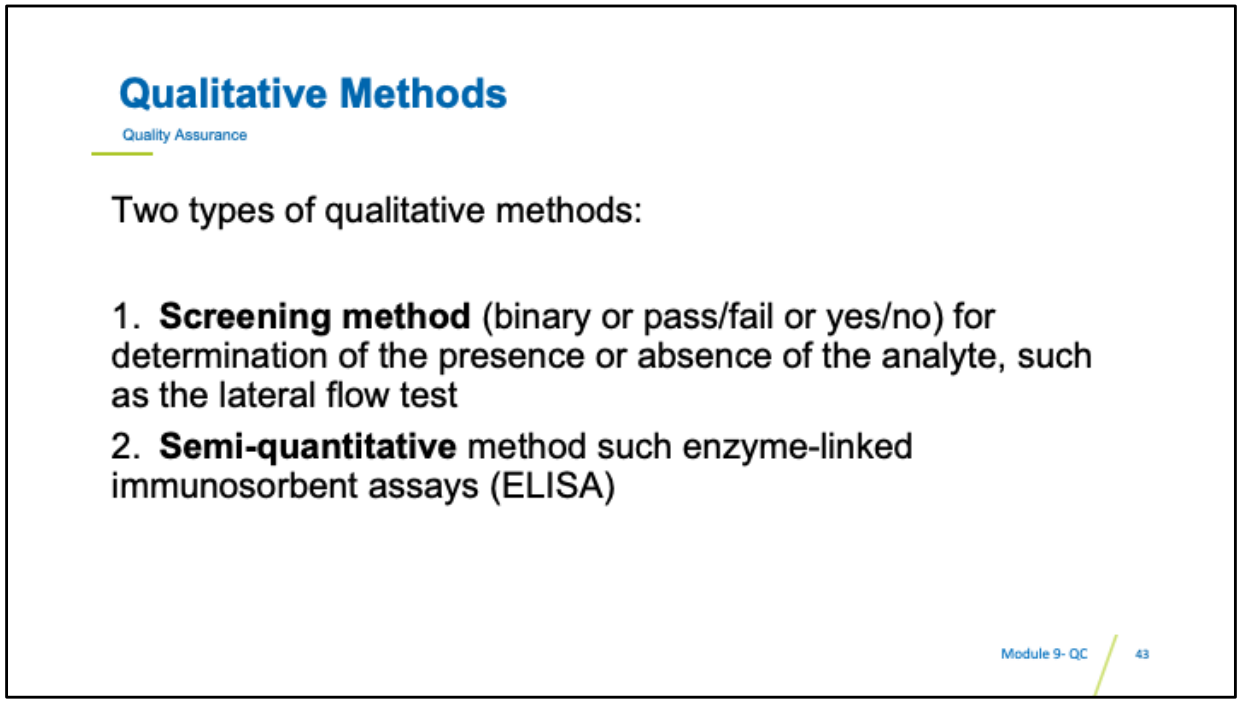
Level 3 and 4: Minimum Requirements

Quality Assurance

One Matrix, one analyte (total aflatoxins or aflatoxin B1)	Level 3 - Minimum Validation Requirements	Level 4 Minimum Validation Requirements
Number of participant laboratories	2 (one collaborating laboratory + originating laboratory)	8
Number of matrix source	3	3
Spike levels	4 (0, ½X, 1X, 2X	4 (0 ½X, 1X, 2X)
Replicates (for each matrix source)	3	2

X = Target level (or regulatory level)
Note: analyze CRM in replicate if it is available

Levels 3 and 4 differ in that they require measurements to be performed in more than one laboratory, a minimum of two laboratories for Level 3 and a minimum of eight laboratories for level 4. The number of sources of matrix goes up to three, but the spike levels remained the same, and each matrix source should be replicated three times for a Level 3 and twice for a level 4.



The acceptance criteria for the methods differ between qualitative and quantitative methods. Moreover, the two different types of qualitative methods have different acceptance criteria. A screening method, typically characterized by a pass or fail or yes/no answer, aims to determine the presence or absence of an analyte. The lateral flow tests discussed in module 2 are examples of screening methods. Some LC/MS (and /MS) and GC/MS methods are also implemented for screening purposes. Semi quantitative methods, such as ELISA, yield both a determination of presence and absence and a quantity, typically with a higher level of uncertainty.

Validation Procedure for Binary Test

Quality Assurance

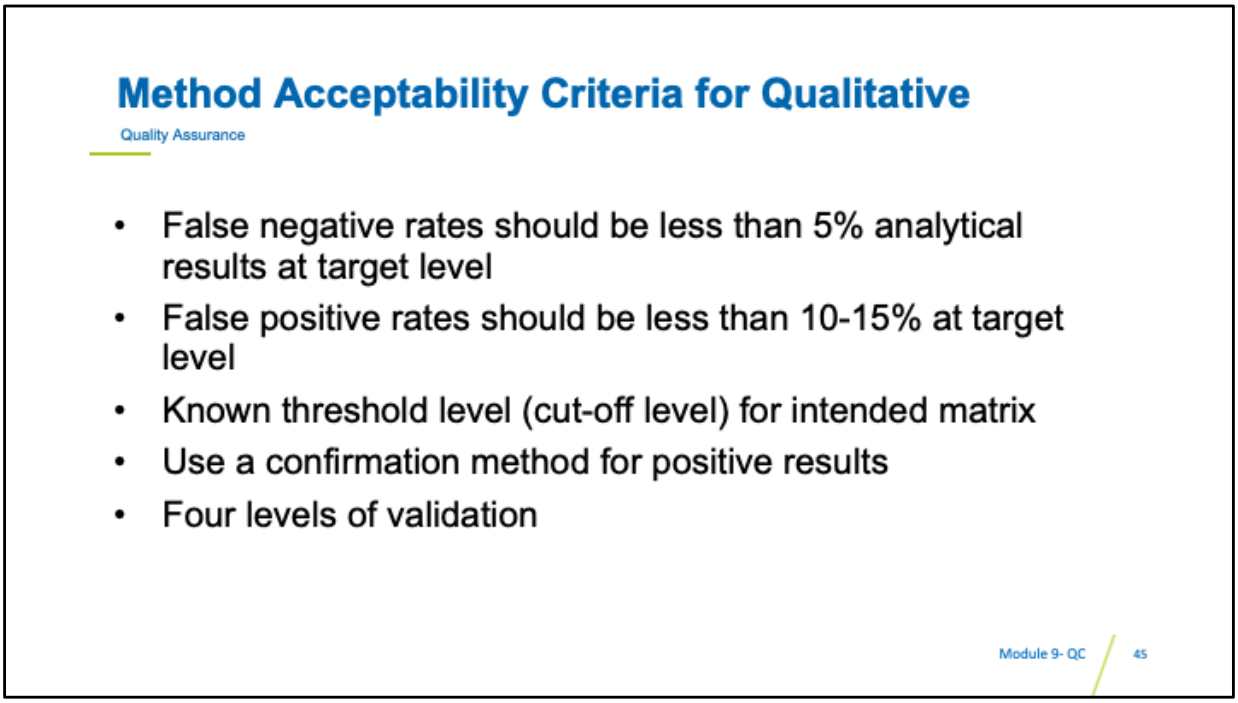
Matrix effect: analyze each matrix (0 spike level) with reference method and candidate method of each matrix, threshold level, calculate POD and 95% CI

Example: aflatoxins in corn (ppb)

Matrix	Spike level	Threshold level	Trial	Number +	+/trails	Calculated POD	95% CI	Ref. results
corn	0	5	30	0	0/30	0.00	0.00-0.11	<1
		10	30	0	0/30	0.00	0.00-0.11	
		20	30	0	0/30	0.00	0.00-0.11	
		40	30	0	0/30	0.00	0.00-0.11	

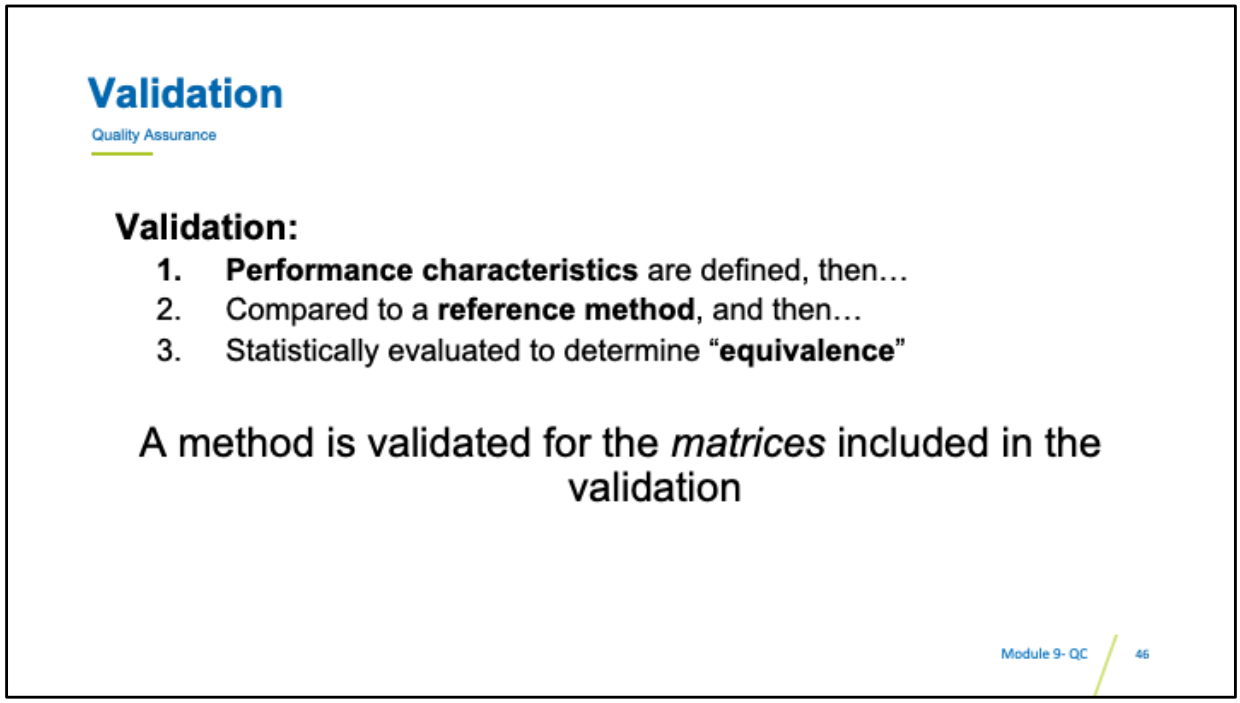
False positive rate ranges from 0 to 11% at 5 ppb level

The validation procedure for a binary test requires only the analysis of a blank matrix and one concentration, typically the target concentration or MRL. The false positive rate is calculated as the number of blank samples testing positive with a 95% confidence interval. The false negative rate is the proportion of test results for spiked samples at the target concentration that don't detect the analyte.



For qualitative methods, generally, a false negative rate of less than 5% at target level is acceptable. This number can differ depending on the actual target level because the standard deviation and uncertainty is higher for lower concentrations.

A 10 to 15% false positive rate at target test level is usually acceptable. A higher false positive rate is accepted because positive samples are verified using a confirmation method, while only a small proportion of negative samples are confirmed. From a health perspective, a false negative means that a food containing a contaminant recognized for posing a health risk is present at a concentration higher than the maximum residue limit allowed on the market. A false positive means that a sample that actually should not be a concern is being pulled from the market by mistake.



In summary, method validation is a measurement of performance characteristics compared to a reference method, and statistically evaluated to determine "equivalence".

A method is validated only for the *matrices* included in the validation work.

Quality Assurance

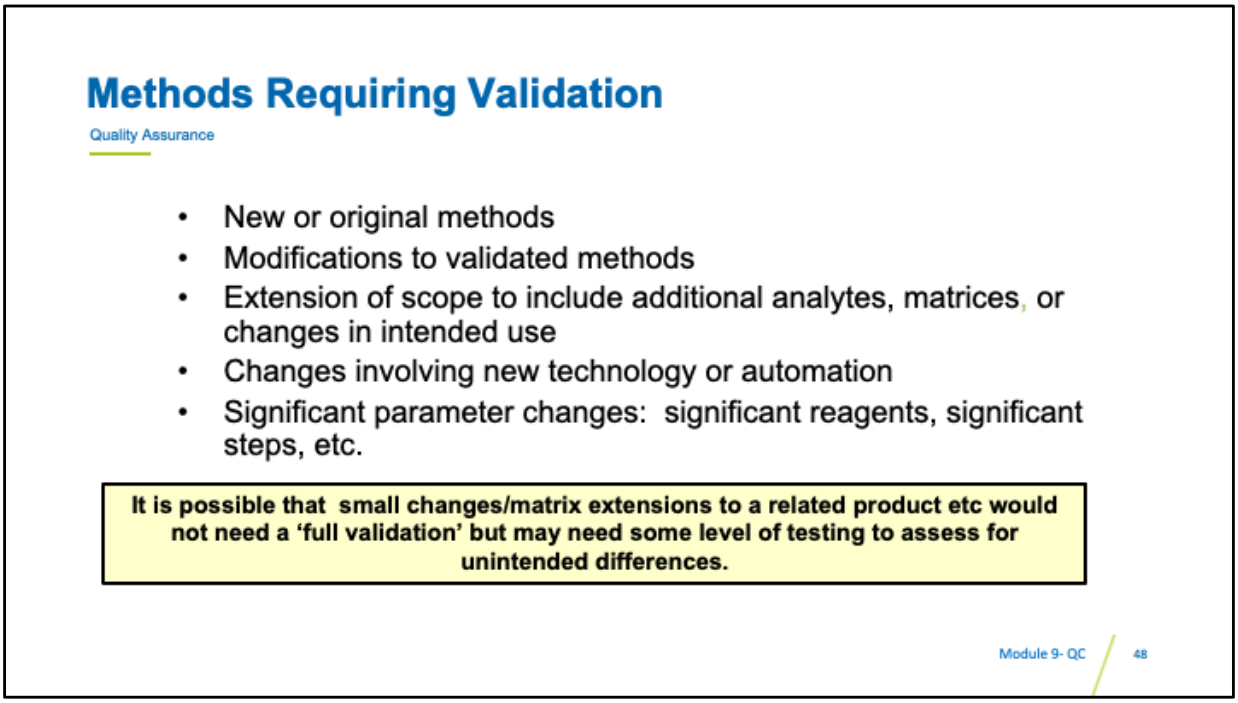
FDA Guidelines

Guidelines for the Validation of Chemical Methods for the FDA FVM Program, 3rd Ed.

Table 1. Key Validation Parameter Requirements for Chemical Methods

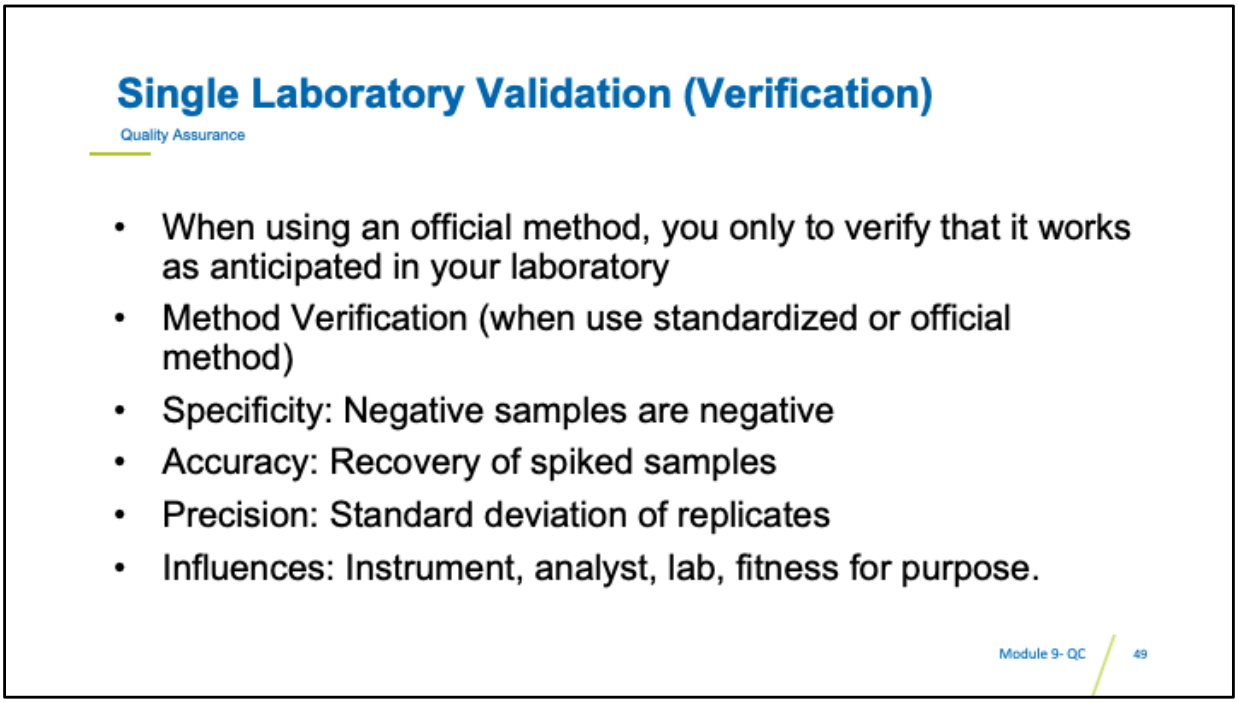
	Level One: Emergency/ Limited Use	Level Two: Single Laboratory Validation	Level Three: Multi-Laboratory Validation	Level Four: Full Collaborative Study
Number participating labs	1	1	≥ 2	8 (quantitative) 10 (qualitative)
Number of matrices*	≥1	≥3 recommended where available	≥3 recommended where available	≥3 recommended where available
Number of analyte(s) spike levels for at least one matrix source**	≥2 spike levels + 1 matrix blank	≥3 spike levels + 1 matrix blank	≥3 spike levels + 1 matrix blank	≥3 spike levels + 1 matrix blank
Replicates required per matrix source at each level tested per laboratory	≥2 (quantitative) ≥2 (qualitative)	≥2 (quantitative) ≥3 (qualitative)	≥2 (quantitative) ≥3 (qualitative)	≥2 (quantitative) ≥3 (qualitative)
Replicates required at each level tested per laboratory if only one matrix source used	≥4 (quantitative) ≥6 (qualitative)	≥6 (quantitative) ≥9 (qualitative)	≥3 (quantitative) ≥6 (qualitative)	≥2 (quantitative) ≥6 (qualitative)

This is a summary of the small tables presented before, as it appears in the document entitled Guidelines for the validation of chemical methods for the FDA Food and Veterinary Medicine Program.

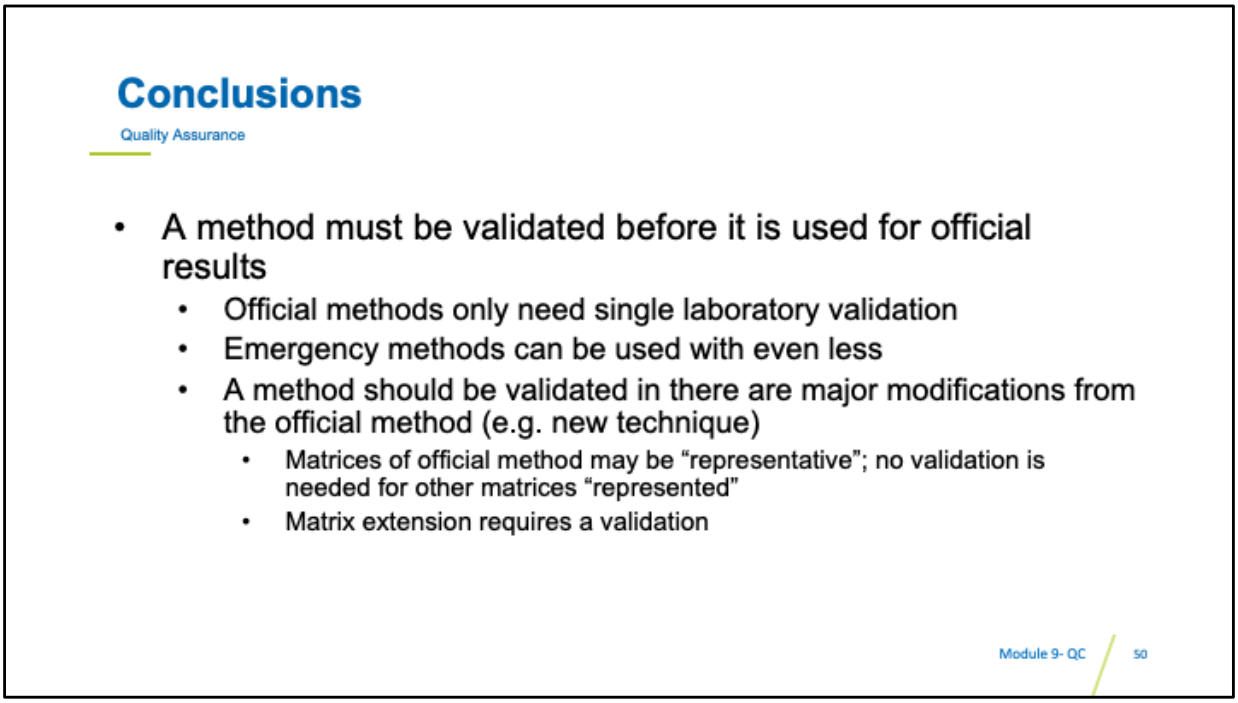


Original methods and methods deviating significantly enough from existing methods to be considered new, significant modifications to validated methods, extension of method scope to include additional analytes, highly differing matrices, or changing the intended use of the method, changes involving new technology or automation, and significant changes to important parameters such as critical reagents or steps all result in a requirement for validation.

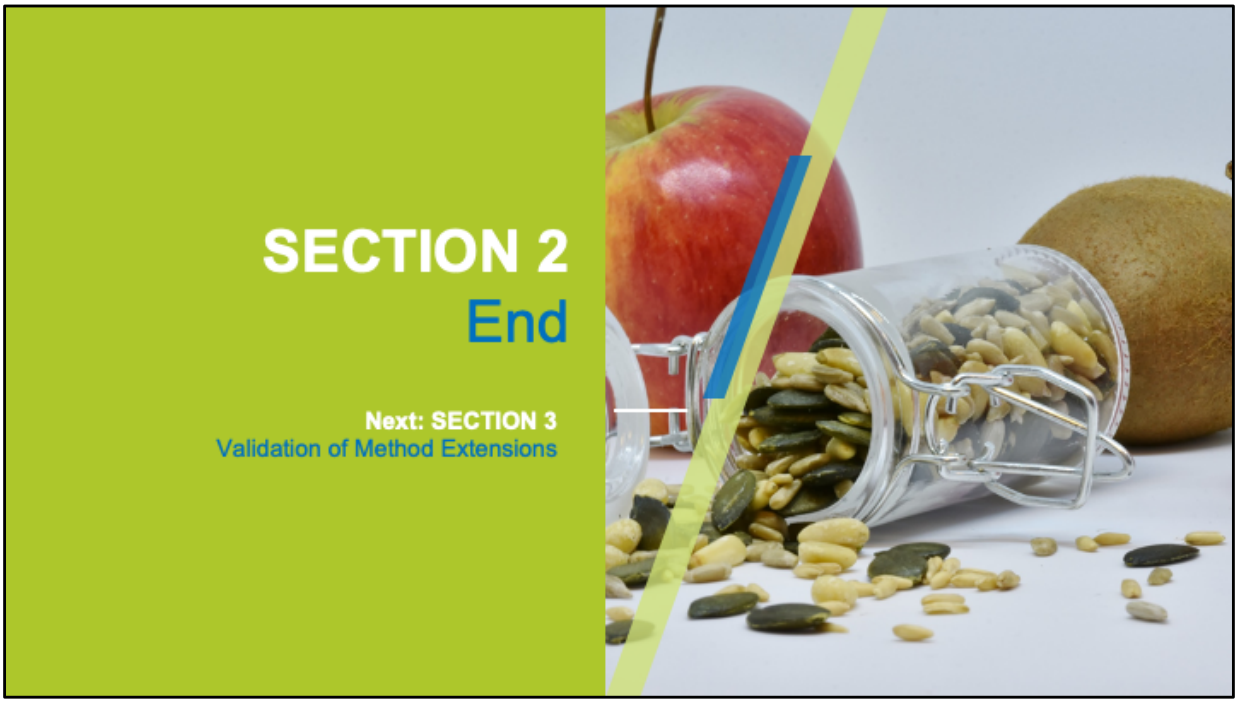
Matrix extensions may require lower level of verification limited to testing for unintended differences if the new matrices are not significantly different from the matrices included in the original method. Groupings of matrices have been developed and published to assist in the determination of whether a new matrix needs any validation at all. Methods are increasingly validated for representative matrices and all other commodities belonging to the same group would not need to be validated because they are considered included in the original method. We will discuss this further in the next section.



A single laboratory validation, also known as a verification, is sufficient when using an official method. Verification only aims to verify that it works as anticipated in one's laboratory. In a nutshell, the specificity of the method reassures us that negative samples are negative. The accuracy tells us how well it recovers the analytes from a spiked sample. The precision expresses the standard deviation of replicates and all of these factors are influenced not only by the procedures of the method but also by the instrument, analyst, laboratory conditions and fit for purpose.



A method must be validated before it is used for official purposes. The level of validation that is acceptable is related to the circumstances of the use of the method. Official methods only need single laboratory validation, while emergency methods can be used with even less. The level of validation should be increased with larger or more impactful modifications to a method. Matrices of official method may be "representative"; no validation is needed for other matrices belonging to the same group in this case. Matrix extension requires a validation.



You have reached the end of section 2. Section 3 discusses validation of method extensions.