GUIDELINES ON PERFORMANCE CRITERIA AND VALIDATION OF METHODS FOR DETECTION, IDENTIFICATION AND QUANTIFICATION OF SPECIFIC DNA SEQUENCES AND SPECIFIC PROTEINS IN FOODS*

CAC/GL 74-2010

SECTION 1 – INTRODUCTION

1. Molecular and immunological analytical methods are currently the recognized tools for determination of DNA and protein analytes in foods. However, in order for the results obtained by such methods from different laboratories to gain wide acceptability and confidence as reliable, there is need for the analytical methods to satisfy certain quality criteria.

2. These guidelines provide appropriate criteria to validate the performance of methods developed to detect specific DNA sequences or specific proteins in foods.

3. Information relating to general considerations for the validation of methods for the analysis of specific DNA sequences and specific protein is given in the first part of these Guidelines. Specific annexes are provided that contain information on validation of quantitative Polymerase Chain Reaction (PCR) methods, validation of qualitative PCR methods and validation of protein-based methods**.**

SECTION 1.1 – PURPOSE AND OBJECTIVES

4. The goal of this document is to support the establishment of molecular and immunological methods for detection, identification and quantification of specific DNA sequences and specific proteins in foods, which produce results with comparable reproducibility when performed at different laboratories

5. The guidelines are aimed at providing guidance on how to establish methods to detect and identify specific DNA sequences and proteins in food by defining appropriate validation criteria, and whether or not a method complies with these criteria based on the performance characteristics of a method.

The guidelines specify the relevant criteria and give explanations on how to consider these criteria, i.e.:

-by providing the rationale for the most relevant criteria and

-by showing how to find out whether or not a method fulfils the given criteria requirements.

SECTION 1.2 SCOPE

6. These guidelines provide information on criteria for the validation of food analysis methods involving the detection, identification and quantification of specific DNA sequences and specific proteins of interest that may be present in foods, including those foods containing materials derived from modern biotechnology. These molecular and immunological methods are applicable to a wide range of uses such as tests for biomarkers in foods, including those derived from modern biotechnology and food authentication, and may be used by laboratories responsible for food analysis.

SECTION 2 – METHOD VALIDATION

7. The Codex Alimentarius Commission places an emphasis on the acceptance of methods of analysis which have been validated through a collaborative trial conforming to an internationally accepted protocol according to ISO 5725:1994 or the AOAC/IUPAC Harmonized Protocol. In this area there may be a need to adopt a formal single-laboratory validation as an interim measure in the absence of collaborative trial data. However, methods used for the analysis of DNA sequences and proteins, must be capable of being performed in many laboratories.

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* for applications such as food derived from modern biotechnology, food authentication, food speciation and other purposes

Section 2.1 – Criteria Approach

8. These guidelines apply the "criteria approach".

Section 2.2 – General Method Criteria

9. The general criteria for the selection of methods of analysis have been adopted in the Codex Procedural Manual. Such criteria are applied in this guideline. Additional criteria are described in the appropriate annexes.

Section 2.3 – Validation Process

10. Method validation is a process to establish the performance characteristics and limitations of an analytical method. The results of a validation process describe which analytes can be determined in what kind of matrices in the presence of which interference. The validation exercise results in precision and trueness values of a certain analytical method under the examined conditions.

11. Formal validation of a method is the conclusion of a long process, which includes the following main steps:

- ***Pre-validation of the method***. Pre-validation should be performed on a case-by case as needed. Pre-validation should ensure that a method performs in a manner, which allows a successful conclusion of the validation study, i.e. it should provide evidence about the suitability of the method for its intended purpose. Pre-validation should preferably be carried out by involving 2 - 4 laboratories. Statistical analyses (e.g. of "repeatability" and "reproducibility") should be made according to the validation procedure to be subsequently used.
- ***Validation of the method.*** Validation through a collaborative trial is expensive to undertake and usually follows only after the method has shown acceptable performance both in a single-laboratory and a pre-validation study.

SECTION 3 – SPECIFIC CONSIDERATION FOR THE VALIDATION OF METHODS FOR THE DETECTION, IDENTIFICATION AND QUANTIFICATION OF DNA SEQUENCES AND PROTEINS

Section 3.1 – Method Development to Formal Validation

12. Common methodologies for DNA-based analysis are PCR-based methods used to detect a specific (targeted) DNA sequence. Common approaches for protein utilize Enzyme-Linked Immuno-Sorbent Assay (ELISA) and lateral flow devices. For DNA-based analysis, the PCR approach is presently most widely applied, although other DNA-based methods that achieve the same objective may be employed if properly validated. Both DNA and protein-based approaches are considered here.

Section 3.1.1 – Method Acceptance Criteria (Required condition for validation)

13. In order to evaluate a method prior to validation, information concerning both the method and the method testing is required, as detailed in Annex I.

14. The method evaluation should verify that the principle preconditions for using the method for Codex purposes are fulfilled. This section describes the method acceptance criteria, which have to be fulfilled by the method in order to conduct a pre-validation and full collaborative trial.

Section 3.1.2 – Applicability of the Method

15. Applicability of the methods could be determined by confirming whether the methods may be used in the intended foods with the required performance and it should be clearly stated. Especially, in analysis of the DNA sequences and protein, some methods that can be applied to a single raw matrix may not be necessarily applicable to complex matrices and/or processed food, since the DNA and protein may be altered.

16. In principle the method should be applicable to the matrix of concern. In the case of "general purpose" methods to identify and quantify DNA sequences and proteins in a range of food matrices, at least one extraction method applicable to a general food matrix should be available.

Section 3.1.3 – Principle condition

17. DNA-based methods should detect, identify and may quantify the levels of specific DNA sequence(s). Protein-based methods should detect, identify and may quantify the level of a specific protein in the product.

18. Currently, the DNA-based detection method typically consists of PCR methodology and includes:

- a protocol describing an extraction method which is applicable to a relevant matrix;
- a protocol describing the conditions, including the apparatus used, under which PCR can be used to detect the target DNA sequence;
- a description of the oligonucleotide primer sequences which uniquely amplify the target DNA sequence;
- If applicable, a description of the fluorescent oligonucleotide probe sequence which uniquely identifies the target DNA sequence.
- a description of oligonucleotide primer sequences, which amplify a taxon-specific DNA sequence that should be present in the conventional food matrix irrespective of the presence of the specific analyte, in order to differentiate a negative result from failed extraction/amplification processes, and to quantify the amount of target DNA relative to the taxon-specific DNA.
- if applicable, a description of the fluorescent oligonucleotide probe sequence which uniquely identifies the taxon-specific DNA sequence.
- a description of the method used to detect the DNA
- appropriate control samples and standards.
- descriptions of calculations used to derive the result.

19. Protein-based methods typically consist of a quantitative or qualitative method. These are usually immuno-sorbent analysis systems, and consist of the following:

- a protocol describing an extraction method which is applicable to a relevant matrix;
- a protocol describing the conditions, including the apparatus used, under which immuno-sorbent analysis can be used to detect the target protein;
- an antibody-coated support,
- an enzyme-conjugated secondary antibody,
- an enzyme substrate for colour development, and
- washing buffer and sample extraction buffer.
- a description of the method used to detect the protein
- appropriate control samples and standards.
- descriptions of calculations used to derive the result.

20. The method should fulfil the requirements below:

- Protein-based methods should allow for unequivocal detection, identification and/or quantification of a specific antigen or epitope.
- DNA-based screening methods are used to detect a target DNA present in multiple organisms. For instance, screening methods that are used to detect multiple transformation events should allow for detection of a target DNA sequence which is common to a number of transformation events.

- DNA-based specific methods that are used for unequivocal detection, identification and/or quantification of a specific organism which could be mixed with similar organisms should allow for the unequivocal detection, identification and/or quantification of a DNA sequence that is unique or specific to that organism. For instance, target-specific methods that are used for detection of a single transformation event should allow for unequivocal detection, identification and/or quantification of a DNA sequence that is unique or specific to that transformation event. For food authentication, the specific target sequence/s should uniquely define the taxon as required.
- DNA-based taxon-specific methods that are used for detection or relative quantification of target DNA should allow for unequivocal detection, identification and quantification of a DNA sequence that is unique or specific to that taxon
- For target and taxon-specific methods used in relative quantification, identification of the amplified fragment, by e.g. probe hybridization or any appropriate equivalent method, is recommended.

Section 3.1.4 – Unit of Measurement and reporting of results

21. Appropriate units of measurement (e.g. target copy numbers or molar equivalents), performance and data reporting criteria should be specified for each method prior to their use. For qualitative analysis, the results can be provided as present or not detected and for this reason there is no unit of measurement.

22. Measurements may be explicitly expressed as weight/weight or by relative percentage. However, none of the current methods (DNA or protein based) are able to measure them directly.

Section 3.1.5 – Measurement Uncertainty

23. As mentioned in the Codex Guideline on Measurement Uncertainty (CAC/GL 54-2004), laboratories are required to estimate the uncertainty of their quantitative measurements. Sample preparation and analytical methods are two significant sources for error that should be considered when evaluating an analytical measurement. Analysts using methods which have been validated according to these guidelines should have sufficient information to allow them to estimate the uncertainty of their result.

24. For details, refer to the Codex Guideline on Measurement Uncertainty (CAC/GL 54-2004), the section entitled "*The Use of Analytical Results: Sampling Plans, Relationship between the Analytical Results, the Measurement Uncertainty, Recovery Factors and Provisions in Codex Standard*" from the Codex Procedural Manual.

Section 3.1.6 – Modular Approach to Method Validation

25. The "method" refers to all the experimental procedures needed to estimate the measurand in a particular matrix. For a particular material this may include the processes for DNA or protein extraction and the final quantification in a PCR or Immuno-sorbent assay system, or a determination of the presence or absence of the analyte via a qualitative method. In such a case, the whole chain from extraction up to the analytical step constitutes a method. However, it may be possible to use the same sample preparation (e.g. grinding) method in combination with the same DNA or protein isolation process for several different subsequent analyses to achieve economic efficiencies as long as the validated method processes remain the same.

26. It would be inappropriate to substitute alternative processes, such as a different DNA or protein isolation process, into a validated method without conducting additional studies to show that the substitution does not affect the performance of the method.

Section 3.2 – Collaborative Trial Requirements

Section 3.2.1 – General Information

27. The purpose of a collaborative trial is to validate the data provided by previous testing in a pre-validation or a single laboratory exercise and to determine methodological precision in terms of repeatability and reproducibility.

28. The values of any performance parameters reported from validation studies should be interpreted and compared with care. The exact values and their interpretation may depend – besides the performance of the method - on the extent of the method.

29. If a collaborative trial has been conducted according to the ISO 5725:1994 or the AOAC/IUPAC Harmonized Protocol, then this information can be used to assess the acceptability of the method.

Section 3.2.2 – Minimum Performance Requirements

30. In a collaborative trial, the method performance should comply with the relevant parts of the method acceptance criteria and fulfil the method performance requirements specifically set below for the collaborative trial. In particular, the compliance with the criteria for sensitivity and repeatability/reproducibility standard deviations and trueness should be assessed.

31. In addition to the method acceptance criteria, at least the method performance requirements listed in Annex I should be evaluated from the experimental data of a collaborative trial.

32. The methods and their associated validation data will be revised on a regular basis as the scientific knowledge and experience gained in validation and collaborative trials evolve. These Guidelines are complemented with practical information about the operational steps of the validation process.

Section 3.2.3 – Collaborative Trial Test Materials

33. In principle, the method should be applicable to and tested on the matrix of concern (i.e. on which any specification has been made).

34. The effects of materials/matrices on the extraction step in a protocol are important to any analysis. When the results of a validation study are reported, it is important that the report includes details of which matrix was analyzed and whether a purified protein or DNA was used as the target for the analysis.

Section 3.2.4 – Specific Information on the Validation of Methods

35. Specific information on the validation of quantitative and qualitative PCR methods is given in Annexes II and III respectively.

36. Specific information on the validation of quantitative and qualitative protein-based methods is given in Annex IV.

SECTION 4 – QUALITY CONTROL REQUIREMENTS

Section 4.1 – Laboratory Quality

37. CAC/GL 27 provides guidance for laboratories involved in the import and export of foods. This guidance is based on compliance with ISO/IEC Standard 17025, proficiency testing and internal quality control as well as the use of methods of analysis validated according to Codex requirements.

Section 4.2 – Reference Material

38. A suitable reference material is generally required for the validation of a method. There are a number of matrices that can be used to develop reference materials or working standards for methods of detection of DNA sequences and proteins. Each has its own advantages and drawbacks for particular purposes. The physical form of the reference material determines its suitability for use with any given method. For ground materials, differences in particle size distribution between reference materials and routine samples may affect extraction efficiency of the target protein or DNA and method reproducibility due to sampling error.

39. Reference material for DNA based methods can be a matrix containing the analyte, DNA extracted from matrix containing the analyte, a plasmid containing the specific DNA, or if certified reference materials are not available, control sample materials, for example from proficiency testing schemes. Use of plasmid or amplicon DNA requires careful consideration of the choice to be incorporated into the plasmid or amplicon to ensure that the plasmid or amplicon DNA will be fit for the required purpose.

40. Reference materials for protein-based methods can be e.g. the protein itself purified from recombinant microbes (such as *E. coli*), a ground plant matrix (typically leaf or grain), or a processed food fraction.

SECTION 5 – TECHNICAL AND METHODOLOGICAL INFORMATION

Technical and methodological aspects of DNA and protein-based methods are listed as references:

Allmann M, Candrian U, Hoefelein C and Luethy J (1993). Polymerase Chain Reaction (PCR): a possible alternative to immunochemical methods assuring safety and quality of food. *Lebensm. Unters. Forsch* 196:248-251.

Anklam E, Gadani F, Heinze P, Pijnenburg H and Van den Eede G (2002). Analytical methods for Detection and Determination of Genetically Modified Organisms (GMO's) in Agricultural Crops and Plant-derived Food Products. *European Food Research and Technology* 214:3-26.

Asensio L (2007). Review: PCR-based methods for fish and fishery products authentication. *Trends in Food Science & Technology* 18(11): 558-566.

Asensio L, Gonzalez I, Garcia T and Martin R (2008). Determination of food authenticity by enzyme-linked immunosorbent assay (ELISA). *Food Control* 19:1-8.

Carnegie, PR (1994). Quality control in the food industries with DNA technologies. *Australas. Biotechnol.* 4(3):146-9.

Chapela MJ, Sotelo CG, Pérez-Martín RI, Pardo MA, Pérez-Villareal B, Gilardi P and Riese J (2007). Comparison of DNA extraction methods from muscle of canned tuna for species identification. *Food Control.* 18(10):1211-1215

Codex Alimentarius Commission Procedural Manual. The Use of Analytical Results: Sampling Plans, Relationship between the Analytical Results, the Measurement Uncertainty, Recovery Factors and Provisions in Codex Standards.

CAC/GL 54-2004. Codex Guidelines on Measurement Uncertainty.

Colgan S, O'Brien LO, Maher M, Shilton N, McDonnell K and Ward S (2001). Development of a DNA-based assay for species identification in meat and bone meal. *Food Research International* 34(5):409-414.

Dahinden I, von Büren M and Lüthy J (2001). A Quantitative competitive PCR system to detect contamination of wheat, barley or rye in gluten-free food for coeliac patients. *European Food Research and Technology* 212(2):228-233.

Dieffenbach CW and Dveksler GS (1993). Setting up a PCR laboratory. *PCR Methods Appl.* 3(2):S2-7.

ISO 5725:1996 Accuracy (Trueness and Precision) of Measurement Methods and Results. Geneva: International Organization for Standardization.

ISO 21569:2005 Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - Qualitative nucleic acid based methods. Geneva: International Organization for Standardization.

ISO 21570:2005. Foodstuffs - Methods for the detection of genetically modified organisms and derived products - Quantitative nucleic acid based methods. Geneva: International Organization for Standardization.

ISO/DIS 24276:2006. Foodstuffs - Nucleic acid based methods of analysis for the detection of genetically modified organisms and derived products - General requirements and definitions. Geneva: International Organization for Standardization.

ISO/IEC Standard 17025:2005. General requirements for the competence of testing and calibration laboratories. Geneva: International Organization for Standardization.

Grothaus GD, Bandla M, Currier T, Giroux R, Jenkins R, Lipp M, Shan G, Stave J., and V. Pantella (2007). Immunoassay as an Analytical Tool in Agricultural Biotechnology. *Journal of AOAC International.* 85: 3, pp 780-786.

Holst-Jensen A. and Berdal KG (2004). The modular analytical procedure and validation approach and the units of measurement for genetically modified materials in foods and feeds. *Journal of AOAC International* 87(4):927-36.

Horwitz E. ISO/AOAC/IUPAC Harmonized Protocol for the Design, Conduct and Interpretation of Method-Performance Studies (1995). *Pure and Applied Chemistry* 67:331-343.

Kwok S and Higuchi R (1989). Avoiding false positives with PCR. *Nature* 339(6221):237-238.

Lipp M, Shillito R, Giroux R, Spiegelhalter F, Charlton S, Pinero D and Song P (2005) Polymerase Chain Reaction Technology as an analytical tool in Agricultural Biotechnology. *Journal of AOAC International* 88 (1):36-155.

Meyer R, Candrian U (1996). PCR-based DNA Analysis for the Identification and Characterization of Food Components. *Lebensmittel-Wissenschaft und-Technologie*. 29(1-2):1-9.

Mifflin TE (2007) Setting Up a PCR Laboratory. *Cold Spring Harbor Protocols* 14 (doi:10.1101/pdb.top14)

Miraglia M, Berdal KG, Brera C, Corbisier P, Holst-Jensen A, Kok EJ, Marvin HJP, Schimmel H, Rentsch J, van Rie JPPF and Zagon J (2004). Detection and traceability of genetically modified organisms in the food production chain. *Food and Chemical Toxicology* 42:1157-1180.

Newton CR, Herbitter A and Gubler U (1995). PCR: Essential Data. Hoboken (NJ): J. Wiley & Sons.

Olexova L, Dovičovičová L, Švec M, Siekel P, Kuchta T. (2006). Detection of gluten-containing cereals in flours and "gluten-free" bakery products by polymerase chain reaction. *Food Control* 17(3):234-237 .

Poms, RE; Klein CL, Anklam E (2004). Methods for allergen analysis in food: a review. *Food Addit. Contam*. 21(1):1-31.

Trapmann S, Burns M, Broll H, Macarthur R, Wood RKS, Žel Jana (2009). Guidance document on measurement uncertainty for GMO testing laboratories. *EUR - Scientific and technical research series*. Luxembourg: Office for official publications of the European communities.

Turci M, Sardaro MLS, Visioli G, Maestri E, Marmiroli, M and Marmiroli N (2010). Evaluation of DNA extraction procedures for traceability of various tomato products. *Food Control*. 21(2):143-149.

Williams R. (2005). Gene tests served up to tell fine foods from fakes. *Nature* 434:262.

Woolfe M and Primrose S (2004). Food forensics: using DNA technology to combat misdescription and fraud. *Trends in Biotechnology* 22(5):222-6.

ANNEX I: REQUIRED INFORMATION WHEN METHODS ARE TO BE CONSIDERED FOR VALIDATION

DESCRIPTION OF THE METHOD

1. A complete and detailed description of all the components of the method should be provided. The use of multiple plates for PCR and protein methods, as an example, should be explicitly addressed. The description should also include information on the scope of the method, and the unit of measurement should be clearly indicated, as well as the following:

Purpose and relevance of the method

2. The purpose of the method should be indicated in the method. The method should be fit for purpose for the intended use.

Scientific basis

3. An overview of the scientific principles on which the method is based (e.g., the molecular biology underlying the use of a real-time PCR method) should be provided.

Specification of the prediction model/mathematical model needed for the method

4. The DNA and protein-based techniques used to detect and quantify DNA sequences and proteins are based on different principles. In PCR the targeted DNA is amplified in an exponential manner. Moreover, the quantification by real-time PCR is often based on two independent PCR assays: one for the target DNA and one for the taxon specific DNA sequence. In contrast to PCR, immuno-sorbent assays involve binding one or more layers of antibodies to each initial target molecule, and amplification of the signal is proportional to the number of reporter molecules and, if applicable, the enzymatic reaction time.

5. If the derivation of the results relies upon a mathematical relationship this should be outlined and recorded (e.g., $\Delta\Delta$Ct method or a regression line or calibration curve obtained by other means). Instructions for the correct application of the model should be provided. These may include, depending on the method, a recommended number and range of levels to be analyzed, minimum number of replicates and/or dilutions to be included for routine analyses or the means and confidence intervals to evaluate the goodness-of-fit.

SPECIFIC INFORMATION REQUIRED FOR DNA-BASED METHODS

6. For DNA-based procedures, the following additional information should be supplied in particular:

Primer pairs

7. General methods have to provide the defined primer pairs and the sequence they target. Recommendations as to the efficiency/use of primer set have to be clearly stated, including if the primers are suitable for screening and/or quantification.

- ***Amplicon length***

8. Food processing will generally lead to a degradation of target DNA. The length of the amplified product may influence the PCR performance. Therefore the selection of shorter amplicon sizes (within reason) will increase the possibility to get a positive signal in the analysis of highly processed foodstuffs. In general the length of the amplified fragment for the taxon-specific DNA sequence and the target sequence should be in a similar size range.

- ***whether the method is instrument or chemistry specific***

9. At the moment a number of different types of real-time instruments and chemistries are available. These instruments and chemistries may have different performance such as stability of reagents, heating and cooling characteristics, which affects ramp rates and affects the time necessary for a whole PCR run.

10. Beside the differences in the heating and cooling system there are differences in the technique and software used to induce and subsequently to record the fluorescence. The detection and quantification of the

fluorescence could also vary according to the recording instruments and software used. Qualitative methods generally tend to be less instrument-specific than quantitative methods.

11. The methods are generally instrument and chemistries dependent and cannot be transferred to other equipment and chemistries without evaluation and/or modification.

- ***whether single- or multi-plex PCR amplifications are undertaken***

12. Using more than one primer set in a single reaction is called multi-plex PCR.

13. The information provided should demonstrate the robustness of the method for inter-laboratory transferability. This means that the method should have been tested by at least one other laboratory besides the laboratory which has developed the method. This is an important pre-condition for the success of the validation of the method.

SPECIFIC INFORMATION REQUIRED FOR PROTEIN-BASED METHODS

14. The following additional information should be supplied for protein-based procedures:

Assay applicability

15. Food processing will generally lead to degradation or denaturation of the target protein, which may result in a substantial change in immunoreactivity. Immunoassays should be evaluated for applicability to the target in processed products. Empirical results from testing the method for applicability for target in processed foods should be provided.

Hook Effect

16. In an antibody-based lateral flow device and plate format assay, a hook (saturation) effect could lead to a false negative result. A thorough demonstration that the working concentration range comfortably covers the practical need of target analytical samples is necessary. Therefore, empirical results from testing for a hook effect in target matrices should be provided.

Confirmatory method

17. For immunoassays**,** antibodies may cross-react with other proteins present in the matrix; thus, it is necessary to demonstrate the selectivity of assays. Another method may be used as a confirmatory method. Empirical results from testing both methods with aliquots of the same analytical samples of known concentration may be provided.

INFORMATION ABOUT THE METHOD PERFORMANCE.

Selectivity testing

18. The method has to be clear on the use of appropriate negative controls, such as animal and plant-derived material, different strains or target DNA sequence which should be used with this purpose, if those have been defined.

19. Empirical results from testing the method with DNA from non-target species/varieties and DNA from the reference species/variety material should be provided. This testing should include closely related materials and cases where the limits of the sensitivity are truly tested. In addition it might be appropriate, particularly for taxon-specific DNA sequence, to test other sources of similar foods to reduce the potential for obtaining a false positive.

20. Similarly, for protein methods, empirical results from testing the method with proteins from non-target and closely relevant species/varieties/traits, and purified target protein and/or reference positive control materials should be provided.

Stability testing

21. Empirical results from testing the methods (to detect both reference and target DNA sequences, or proteins) with different species, subspecies, varieties, cultivars, animal lines, or microbial strains as appropriate, may be provided in order to demonstrate, for instance, the stability of the copy number and sequence conservation of the taxon-specific gene DNA, or the stability of expression of the protein.

22. For protein methods, empirical results from testing the methods with target material and its derived/processed products, as appropriate, should be provided to demonstrate the stability of the immunoreactive form of the protein.

Sensitivity testing

23. Empirical results from testing the method at different concentrations in order to test the sensitivity of the method should be provided. Limits of detection (LOD) may be defined using samples comprising of single ingredients only. For food products made up of multiple ingredients, the actual sensitivity will be reduced, as total extracted DNA will be derived from more than one ingredient so that the starting amount of the actual measurand will be decreased.

24. LOD should be determined for each method and matrix, if necessary.

Robustness testing

25. Empirical results from testing the method against small but deliberate variations in method parameters should be provided.

Extraction efficiency

26. Empirical results from testing the method for its extraction efficiency in each matrix should be provided to demonstrate the extraction is sufficient and reproducible. For quantitative detection, the method of calibration for incomplete extraction may need to be provided.

PRACTICAL APPLICATION OF THE METHOD

Applicability

27. Indication of the matrix (e.g., processed food, raw materials, etc.), the type of samples and the range to which the method can be applied should be given. Relevant limitations of the method should also be addressed (e.g. interference by other analytes or inapplicability to certain situations). Limitations may also include, as far as possible, possible restrictions due to the costs, equipment or specific and non-specific risks implied for either the operator and/or the environment.

Operational characteristics and practicability of the method

28. The required equipment for the application of the method should be clearly stated, with regards to the analysis *per se* and the sample preparation. Information on costs, practical difficulties, and on any other factor that could be of importance for the operators should be also provided.

Experimental design

29. The experimental design, including the details about the number of runs, samples, replicates, dilutions etc. should be stated.

Operator skills requirements

30. A description of the practical skills necessary to properly apply the proposed method should be provided.

ANALYTICAL CONTROLS

31. The proper use of controls when applying the method should be indicated, when available. Controls should be clearly specified and their interpretation recorded. These may include positive and negative controls, their detailed contents, the extent into which they should be used and the interpretation of the obtained values.

32. The following should be stated:

- Types of analytical controls used:

  i. Positive and negative controls

  ii. Internal control used if applicable (competitive or non competitive).

  iii. Other types of controls like matrix control (to confirm sample was added to PCR) or extraction processing.

- Control samples.
- Reference materials used.

METHOD PERFORMANCE

33. Data on the criteria referred to in Section 2.2, "General Method Criteria" should be provided, as well as a general assessment that the method is fit for its intended purpose.

ANNEX II: VALIDATION OF A QUANTITATIVE PCR METHOD

INTRODUCTION

1. DNA-based analysis is commonly performed using PCR. This technique amplifies a specific segment of DNA to the extent that its quantity can be measured instrumentally (e.g. using fluorometric means). Food processing operations (e.g. due to heat, enzymes and mechanical shearing), can result in degradation or reduction in the total amount of DNA. Methods should preferably be designed to amplify relatively short target- or taxon-specific DNA sequences.

2. Quantitative determinations are often expressed in terms of percent of a target-specific DNA sequence relative to a taxon-specific DNA sequence. In such a relative quantitative test, this measurement actually involves two PCR-based determinations – that of the target-specific DNA sequence and that of the endogenous, or taxon-specific sequence. Each of these determinations has its own uncertainties, and the two are likely to have different measurement characteristics. In most applications, the target DNA sequence will be present at low concentrations, and the taxon-specific DNA sequence will be present at concentrations 10 to 1000 times higher. It is thus important that both measurements are properly validated. In cases where the measurement is expressed directly as a percentage, these factors should be considered when validating the method. The results can be reported in other measure units such as copy numbers.

3. The consequence is that the analysis of DNA, especially in processed foods, aims at detecting a very small amount of target-specific DNA, often in the nanogram/gram range or lower. The result of a quantitative PCR analysis is often expressed in % as the relative amount of target DNA relative to the total amount of DNA of the comparator taxon/species DNA in a specific food matrix. The food matrix may also contain significant amounts of DNA from many other species/taxons.

4. Validation of methods consists of two phases. The first is an in-house validation of all of the parameters above except reproducibility. The second is a collaborative trial, the main outcome of which is a measure of the repeatability and reproducibility together with detailed information on the transferability of methods between laboratories. It is strongly recommended that a small-scale collaborative trial be performed to test the general robustness of a particular method before the expense of organizing a large-scale trial is incurred. In case any improvement of the method or the method description is needed, only limited expenses are incurred through the pre-trial, while a failure of a full interlaboratory method validation due to ambiguous method description is a very costly failure. Additionally, it may be pointed out that the implementation of an already validated method in a laboratory needs to include necessary experiments to confirm that the implemented method performs as well under local conditions as it did in the interlaboratory method validation. It is important to note that a method should be validated using the conditions under which it will be performed.

VALIDATION

5. A quantitative PCR assay should be validated for the intended use or application. The ISO 5725:1996 or AOAC/IUPAC Harmonized Protocol were developed for chemical analytical methods. These define the procedures necessary to validate a method. It is important to emphasize that all the principles and rules of the harmonized protocol are applicable to quantitative PCR methods.

6. A number of the parameters involved in validation of the performance of a quantitative PCR assay will be discussed in detail. These are scope, LOD and LOQ, trueness, precision, sensitivity and robustness. Other important factors are acceptance criteria and interpretation of results, and the issue of the units in which results are expressed.

7. There is a general scientific discussion about the interpretation of the percentage values. It is recognised that so far there is no reliable weight to copy number relationship because of uncertainty in the correlation of weight of ingredient to number of molecules of DNA. Both the weight to weight ratio and copy number to copy number ratio calculations are acceptable provided this is clearly stated when reporting results.

8. All parameters listed below, including selectivity and sensitivity, have to be assessed individually for each of the assays involved, including both reference and target specific PCR assays**.** These are given alphabetically, not necessarily in order of importance.

Applicability

9. The analytes, matrices and concentrations for which a method of analysis may be used should be stated.

10. It is required from an extraction method, independent of matrix to which it is to be applied, that it yields DNA of sufficient quantity, structural integrity and purity to allow a proper evaluation of the performance of the subsequent method steps (e.g. adequate amplification of DNA during the PCR step) to be undertaken.

11. In real-time PCR analysis, Ct-values can be used to estimate the efficiency of PCR. The efficiency can be tested, for example, by setting up a dilution series of the template DNA and determining the Ct-value (The threshold number of cycles at which the measured fluorescence signal crosses a user-defined threshold value) for each dilution. In the ideal situation, when amplification efficiency is 100%, a two-fold reduction in quantity of template DNA added to the PCR will result in an increase in the Ct value of one. Therefore, if DNA is diluted 10X, the theoretical difference in Ct values between the diluted and undiluted DNA should be approx 3.32. Theoretical numbers may not be achieved in real situations. Significant deviations from this relationship may indicate that the extracted DNA contains PCR inhibitors, that the DNA solution is not homogenous or the DNA quantity so low that stochastic variation in the amount of DNA in the reactions yield unreliable quantitative estimates. This is also the case for end-point PCR reactions carried out using fluorescent probes.

Dynamic Range - Range Of Quantification

12. The scope of the methods defines the concentration range over which the analyte will be reliably determined. The relative amount of taxon-specific DNA to total DNA in the DNA extract will vary depending on whether the DNA was extracted from a single ingredient or a complex food matrix. This desired concentration range defines the standard curves and a sufficient number of standards should be used, when applicable e.g. with calibration curves, to adequately define the relationship between concentration and response. The relationship between response and concentration should be demonstrated to be continuous, reproducible and should be linear after suitable transformation.

13. The range of a quantitative target-specific method can be designed to be from near zero to 100 percent relative to the taxon-specific DNA (w/w). However, it is common to validate a method for a range of concentrations that is relevant to the scope of the application. If a method is validated for a given range of values, the range may not be extended without further validation. For certain applications (e.g. food or grain analysis) the use of genomic DNA for the preparation of the standard curve (see discussion on the use of plasmid DNA below) may be considered. While it is easy to establish a nominal 100% standard it is difficult to reliably produce standard solutions below 0.1%. Additionally, the number of target sites (DNA sequence to be amplified) becomes so small that stochastic errors will begin to dominate and less reliable analysis is possible.

14. The DNA used as calibrator should be traced back (in its metrological meaning) to a reference of highest metrological order, e.g. a certified reference material. The range will be established by confirming that the PCR procedure provides an acceptable degree of linearity and trueness when applied to samples containing amounts of analyte within or at the extremes of the specified range of the procedure.

15. The unique characteristics of quantitative PCR impose particular restrictions on the low end of the dynamic range of a quantitative PCR. This is due to the difficulty in determining LOD and LOQ values due to the non-normal distribution of values in this range.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

16. If the validation of the quantitative PCR assay shows that the assay can measure DNA at (for example) 0.1% with acceptable trueness and precision, then it is often not necessary to determine the LOD and LOQ, as the method is only being applied above the range where these are relevant. However, if the method is

being used at concentrations close to the LOD and LOQ (typically 0.01-0.05%), then the assessment of the LOD and LOQ will become part of the validation procedure.

17. In quantitative PCR, the distribution of measurement values for blanks is not Gaussian and typically follows a Poisson distribution. If the LOD is required, it should be experimentally determined. For quantitative methods the LOD is the amount of analyte at which the analytical method detects the presence of the analyte at least 95% of the time (≤5% false negative results)

18. For a quantitative method, it is important to know whether the LOQ for a particular matrix is close to the values to be measured. The LOQ needs to be experimentally determined, since the distribution measurement for quantitative PCR is not normally distributed.

19. In practice, two procedures have been employed to determine the LOQ. The first approach is to assay a number of conventional samples that have been supplemented (spiked) with known amounts of analyte. The LOQ is then the level at which the variability of the result meets certain preset criteria (such as +/- 2 SD from the lowest calibration data point, etc.). DNA extraction, however, may be difficult from some matrices, e.g. starches or ketchup, and lower extraction efficiencies may have to be accepted. When extraction efficiencies are low, this should be stated in the validation data and in the analytical report. A more complete approach is to test the method using a number of samples that contain known amounts of analyte. This is more complicated as it requires access to significant quantities of reference materials that contain a known range of concentrations of the DNA sequences of interest.

Practicability

20. The practicability of the method should be assessed by considering parameters such as: the quantity of samples that can be processed within a given time, estimated fixed costs to implement the method and the approximate cost per sample, practical difficulties on daily use or under particular conditions, as well as other factors that could be of importance for the operators.

Repeatability standard deviation (RSD_r)

21. The relative repeatability standard deviation for the PCR step should be ≤25% over the whole dynamic range of the method.

Reproducibility standard deviation (RSD_R)

22. The relative reproducibility standard deviation for the PCR step should be below 35% over the majority of the dynamic range, except at the limit of quantification, where the RSD_R could be higher.

Robustness

23. Robustness is a measure of the capacity of an analytical procedure to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Examples of such variations include: reaction volumes (e.g., 29 vs. 30µl), annealing temperature (e.g., +/- 1°C) and/or other relevant variations. The experiments need to be performed at least in triplicate. The response of an assay with respect to these small changes should not deviate more than ±35% in reproducibility experiments from the response obtained under the original conditions.

24. The adequacy of the robustness testing needs to be demonstrated on a method-by-method basis. For instance, for a real-time PCR method, the following factors and their origin / source should ideally be taken into account: different thermal cycler models, DNA polymerase, uracyl-n-glycosylase, magnesium chloride concentration, primer forward and reverse concentration, probe concentration, temperature profile, time profile, dNTP (including dUTP, if applicable) concentrations.

Sensitivity

25. For a quantitative PCR method, a linear relationship of the Ct as a function of the logarithm of the template concentration should be obtained across the range of the method. The correlation coefficient, y-intercept and slope of the regression line should be reported. The % of residual for each of the calibrators should preferably be ≤30%.

26. Besides reporting the curve parameters, it is suggested to define which range of slope values is acceptable in order to conduct the quantification as it is also important to calculate the reaction efficiency.

(Eg. -2.9 to -3.3 for DNA detection or the corresponding optimal values which indicate amplification efficiency close to 100%).

27. In cases where the ΔCt-method is employed by a laboratory instead of a calibration based quantitative method, it will be the responsibility of the analyst to ensure that the overall amount of DNA is well within the range for which the assay was validated.

Selectivity

28. The selectivity of the method should be demonstrated by providing experimental evidence. This demonstration should include analysis of samples containing a mixture of target DNA and non-target DNA where the limits of the detection (if appropriate to the dynamic range) are truly tested. As the method should be selective for the target DNA, it should only give a positive result with a food matrix containing the target DNA.

29. Primers and probes should have been checked against pertinent sequence databases for possible homologies with other sequences potentially present in the expected matrices, according to the intended use After such an assessment, selectivity should then be demonstrated experimentally.

30. For assays selective for the target DNA. Experimental evidence of selectivity for the target DNA should include:

- Assays of at least ten samples from different lots or batches of foods or ingredients lacking target DNA sequences, although the samples should contain taxon-specific DNA. All of these assays should have a negative result. For example, if the target DNA corresponds to a specific recombinant-DNA plant transformation event, samples could be derived from other (non-target) transformation events, as well as non-recombinant-DNA plants belonging to the same plant species.
- An appropriate number of DNA samples from each source should be tested.
- Two replicates should be analyzed for each DNA sample, which shall give results within a Ct-value of 0.5.

31. Test results should clearly indicate that no significant instrument reading or chemistry effects are observed.

32. For assays on taxon-specific DNA sequences. Experimental evidence of taxon selectivity should include:

- Assays of at least ten samples from different lots or batches of foods or ingredients derived from organisms belonging to the taxon of interest, but classified in different sub-taxon categories. All of these assays should have a positive result. For instance, if the taxon specificity supposedly corresponds to a plant species such as maize, the samples could correspond to maize varieties with different genetic origins.
- Assays of at least ten samples from different lots or batches of similar foods or ingredients derived from organisms not belonging to the taxon of interest, which may be present in the relevant food matrixes. All of these assays should have a negative result. For instance (and continuing with the earlier example) if the first ten assays were applied to different maize flours, in the second group of assays it could be appropriate to assay wheat/soy/rice flour.
- An appropriate number of DNA samples from each source should be tested.
- Two replicates should be analyzed for each DNA sample, which shall give results within a Ct-value of 0.5.

33. Test results shall clearly indicate that no significant instrument reading or chemistry effects are observed.

Trueness

34. As for any method, the trueness of a method should be determined by comparing results obtained from analysis of a reference material with the known or assigned value for that reference material. The impact of sample matrix effects, particularly when the sample matrix differs from that of the reference material, should be considered.

35. A trueness value of ± 25%, in regards to the PCR step, should be acceptable over the whole dynamic range.

REFERENCES FOR ANNEX II

AOAC (2002). International Methods Committee: Guidelines for Validation of Qualitative and Quantitative Food Microbiological Official Methods of Analysis.

Cankar K, Štebih D, Dreo T, Žel J, Gruden K (2006). Critical points of DNA quantification by real-time PCR-effects of DNA extraction method and sample matrix on quantification of genetically modified organisms. *BMC Biotechnol.* 6(37).

Chapela MJ, Sotelo CG, Pérez-Martín RI, Pardo MA, Pérez-Villareal B, Gilardi P and Riese J (2007). Comparison of DNA extraction methods from muscle of canned tuna for species identification. *Food Control*. 18(10):1211-1215.

Horwitz E. ISO/AOAC/IUPAC Harmonized Protocol for the Design, Conduct and Interpretation of Method-Performance Studies (1995). *Pure and Applied Chemistry* 67:331-343.

Huebner P, Waiblinger HU, Pietsch K and Brodmann P (2001). Validation of PCR methods for quantitation of genetically modified plants in food. *Journal of AOAC International* 84(6):1855-1864.

Kay S and Van den Eede G (2001). The limits of GMO detection. *Nature Biotechnology* 19(5):504.

Turci M, Sardaro MLS, Visioli G, Maestri E, Marmiroli, M and Marmiroli N (2010). Evaluation of DNA extraction procedures for traceability of various tomato products. *Food Control*. 21(2):143-149.

ANNEX III: VALIDATION OF A QUALITATIVE PCR METHOD

Introduction

1. A qualitative PCR should be validated as much as possible in the same way as it is intended to be used for routine analyses – that means the sensitivity of the method should be shown to be such that it can reliably detect a positive sample, and does not give rise to a significant number of false positives.

2. By their very nature, qualitative test results refer to the identification above/below a detection limit. Like the limit of detection for quantitative methods, the limit of detection for a qualitative method can be defined as the concentration at which a positive sample yields a positive result at least 95% of the time. This results in a rate of false negative results of 5% or less. This is also expressed as a ratio or percentage.

False Positive Rate

3. This is the probability that a known negative test sample has been classified as positive by the method. For convenience this rate can be expressed as percentage:

$$\% \text{ false positive results} = 100 \times \frac{\text{number of misclassified known negative samples}}{\text{total number of known negative samples}}$$

False Negative Rate

4. This is the probability that a known positive test sample has been classified as negative by the method. For convenience this rate can be expressed as percentage:

$$\% \text{ false negative results} = 100 \times \frac{\text{number of misclassified known positive samples}}{\text{total number of known positive samples}}$$

Note: since there are different definitions in use for the false positive and false negative rates, the validation report should clarify which one has been used.

5. In order to demonstrate the false negative rate for qualitative assay, a series of samples with a constant, known concentration of positive material in a pool of negative material have to be analysed and the results evaluated. It is important to note that the concept of confidence intervals and statistical uncertainty needs to be applied to the risk of false positive and/or false negative results as well. The desired level of confidence determines the size and number of pools that need to be tested.

Robustness

6. As with any validated method, reasonable efforts should be made to demonstrate the robustness of the assay. This involves careful optimisation and investigation of the impact of small modifications made to the method due to technical reasons, as described in the annex for quantitative PCR.

ANNEX IV: VALIDATION OF A PROTEIN-BASED METHOD

QUANTITATIVE TESTING

1. The following description of the procedure is only one of several possibilities to carry out an immunological detection assay for proteins of interest.

2. For example, in typical ELISA for proteins, the amount of the reporter substance from an enzymatic reaction is measured. The standard curve is generated by plotting the optical density (OD) on the y-axis against the concentration of the standards on the x-axis, obtaining a dose response curve using quadratic equation or other required curve fit model from the method. To obtain an accurate quantitative value, the OD for the sample solutions must pertain to the linear portion of the calibration curve. If the OD is too high, the sample solution must be diluted until the OD falls within the quantification range of the assay. The concentration of the protein analyte in the original sample is calculated by correcting for any dilution factor that was introduced in preparing the sample for application to the micro plate. The initial weight of the sample and the volume of extraction liquid, as well as any subsequent dilutions are used to calculate the dilution factor.

3. Various assay controls can be employed to demonstrate the performance of the assay. A blank sample such as an empty well or buffered solution can be run in parallel to determine any background response which shall be subtracted from sample and calibration responses if desired. A negative control sample (i.e. matrix extract solution known to contain no analyte) shall be used to demonstrate any non-specific response or matrix interference effects occurring in the assay. A positive control or matrix extract spiked with a known amount of the analyte can be run to demonstrate the accuracy of the test. Standards and samples can be run in an appropriate number of replicates to appreciate the precision of the test. Blanks, negative controls, positive controls, reference materials, and replicates can be run on each microplate to control for plate-plate variation.

REFERENCE MATERIALS

4. When applicable, the reference material consists of the same matrix as the target analytical sample to be tested. It typically includes negative control and positive reference materials. For example, if the matrix to be tested is soybean flour the standardized positive reference material would be soybean flour containing a known proportion of protein of interest. Alternatively, a pure sample or extract of the protein of interest may be used, providing the use of such protein reference materials has been validated against the matrix in question. In some cases the reference matrix, may be unavailable. Access to reference materials is important during the development, validation, and use of immunoassays for analysis of proteins in food matrix. The best available reference material should be used in order to comply with regulations and testing requirements.

5. Where food or food ingredients with and without the analyte are available, it is fairly straightforward to prepare a control sample with a known proportion of the target material. In other cases, generating control samples for certain matrices and analytes can be difficult. Stability and uniformity are important considerations. For example, if the matrix to be tested consists of a mixture of materials, the operator will need to combine materials in such a way as to achieve a homogeneous control sample with a known amount of the protein. The stability of these materials would need to be evaluated under storage and test conditions.

VALIDATION OF A QUANTITATIVE PROTEIN-BASED METHOD

6. The principles of method validation defined in the harmonized ISO/IUPAC/AOAC standard apply to protein methods.

7. Quantitative method validation parameters include accuracy/trueness, selectivity, extraction efficiency, sensitivity, range of quantification, precision, robustness, applicability and practicability.

8. Accuracy is demonstrated by measuring the recovery of analyte from spiked samples and is reported as the mean recovery at several levels across the quantitative range.

9. The recovery of proteins of interest should be determined by comparing results obtained from analysis of a reference material with the known or assigned value for that reference material. The impact of sample matrix

effects, particularly when the sample matrix differs from that of the reference material, should be considered. The recovery should be between 70 and 120%.

10. Extraction efficiency is a measure of how efficient a given extraction method is at separating the protein analyte from the matrix. It is expressed as percent analyte recovered from the sample. It can be difficult to truly demonstrate efficiency of the extraction procedure. There may not be an alternate detection method against which to compare the immunoassay results. One approach to addressing extraction efficiency is to demonstrate the recovery of the target protein analyte from each type of food fraction by exhaustive extraction, i.e. repeatedly extracting the sample until no more of the protein is detected.

11. The intra-assay precision describes how much variation occurs within an assay. It can be evaluated by determining the variation between replicates (% Coefficient of Variation) assayed at various concentrations on the standard curve and on the pooled variation (RSD_r) derived from absorbance values in standards from independent assays performed on different days. Inter-assay precision describes how much variation occurs between separate assays and can be measured by analysis of quality control samples on every microplate. The quality control samples required would consist of two pools of extracts, one extract from target analyte-containing samples and one from the control samples. If the protein is stable in extract, it can be stored frozen and a portion would be thawed and assayed on every microplate. Inter-assay precision can be evaluated over time and expressed as % Coefficient of Variation.

12. The relative repeatability standard deviation (RSD_r) should be ≤25% over the whole dynamic range of the method.

13. The relative reproducibility standard deviation (RSD_R) should be below 35% at the target concentration and over the majority of the dynamic range, excepting at the limit of quantification, where it could be greater.

14. Dilution agreement or linearity is used to evaluate that the assay is capable of giving equivalent results regardless of where in the quantitative range of the standard curve the sample OD interpolates. To conduct these experiments, samples that are positive for the target protein are ideally diluted such that at least three of the dilutions result in values that span the quantitative range of the curve. The Coefficient of Variation of the adjusted results from several dilutions of a single sample extract should ideally be ≤ 20%.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

15. It is worth noting that if the LOD or LOQ is established to be much lower than the range in which the method is intended to be used, a precise determination is not necessary. This would be the case, for example, when the LOD is in the range of 1 ng/kg, while the range of the method validation extends only for concentrations ranging in µg/kg.

16. It is common practice when estimating the LOD to assume that it is the signal strength of a blank increased by three times the standard deviation of the blank. This method gives at best an estimate, and relies on normal Gaussian distribution of the blank measurements around zero. This can generally be assumed for methods such as ELISA, but the LOD is best determined experimentally. Alternatively the LOD is commonly defined as a concentration equal to the lowest standard used in the assay, should a positive value be consistently obtained with that standard.

17. For a quantitative method, it is important to know whether the LOQ for a particular matrix is close to the values to be measured.

Cross-reactivity

18. The cross-reactivity is the degree to which analogs or other molecules can bind to the detection antibodies and therefore should be characterized and described in the method. The absence of cross-reactivity should be assessed using experimental results from testing the method with proteins or molecules from non-target and closely related taxa, purified target protein or reference positive control materials. The potential for interferences from reagents and labware can be evaluated by assaying extracts from analyte-free material.

Matrix effects

19. If the response of the method is affected by a substance in the final extract other than the specific protein analyte, the non-specific response is referred to as a matrix effect. One way to manage matrix effects is to demonstrate that the analytical method gives similar results with or without sample matrix present in the extract. In this approach, freedom from matrix effects would have to be demonstrated in all matrices for which the assay is to be used. Another approach (although less desirable) to managing matrix effects would be to prepare the standard solutions in extracts from analyte-free matrix. This would ensure that any matrix effects are consistent between the standards and the samples.

Robustness

20. Robustness is a measure of the capacity of an analytical procedure to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. Examples of such variations include: reaction volumes, incubation temperature (e.g. +/- 1°C for oven incubations and +/- 4°C for incubations at "room temperature") and/or other relevant variations. The experiments need to be performed at least in triplicate and the recovery needs to be calculated. The response of an assay with respect to these small changes should not deviate more than ±30% from the response obtained under the original conditions.

QUALITATIVE TESTING

21. Lateral flow devices are useful tools for on-site or field testing, although other immuno-sorbent assays such as traditional ELISA methods can also be used for qualitative testing. In order to ensure reliable results, assays should be validated and a description of the performance characteristics should include sensitivity, selectivity, applicability, limit of detection, robustness, matrix effects, and, if applicable, hook-effect.

VALIDATION OF A QUALITATIVE PROTEIN-BASED METHOD

22. The same principles apply to qualitative protein-based testing as to qualitative PCR testing. These approaches, including calculation of false positive and false negative rates, can therefore be applied to protein-based methods. In general, due to the reliable nature of protein-based lateral flow strip methods, they are not performed in duplicate on each sample. However, in ELISA testing (due to its quantitative nature), duplicate wells are typically used.

Applicability

23. The analytes, matrices and concentrations for which a method of analysis may be used should be stated.

24. Protein extraction can be a key factor in the performance of a protein method, and the buffers used can also affect the performance of the detection step. Thus careful optimization is required to ensure that protein detection methods are reliable. The criteria for determination of the LOD should be established for the method. For confirming the LOD of qualitative assays, fortification levels near to the LOD may be used, as long as one of the levels used meets the criteria of being above but close to the LOD. While such procedures can give an indication of the performance of the method, incurred samples with well known characteristics (if available) are the best matrix on which to establish the applicability of a method.

Practicability

25. The practicability of the method should be assessed by considering parameters such as: the quantity of samples that can be processed within a given time, estimated fixed costs to implement the method and the approximate cost per sample, practical difficulties on daily use or under particular conditions, as well as other factors that could be of importance for the operators.

REFERENCES FOR ANNEX IV

Grothaus GD, Bandla M, Currier T, Giroux R, Jenkins GR, Lipp M, Shan G, Stave JW and Pantella V. (2006). Immunoassay as an Analytical Tool in Agricultural Biotechnology. *AOAC International* 89:913-928

Guidelines for the Validation and Use of Immunoassays for Determination of Introduced Proteins in Biotechnology Enhanced Crops and Derived Food Ingredients. Lipton et al., *Food and Agricultural Immunology*, 2000, 12, 153-164.

Horwitz E. ISO/AOAC/IUPAC Harmonized Protocol for the Design, Conduct and Interpretation of Method-Performance Studies (1995). *Pure and Applied Chemistry* 67:331-343.

ISO 21572:2004. Foodstuffs-Methods for the detection of genetically modified organisms and derived products-protein based methods. Geneva: International Organization for Standardization.

Mihaliak CA and Berberich SA (1995). Guidelines to the Validation and Use of Immunochemical Methods for Generating Data in Support of Pesticide Registration, in: Nelson JO, Karu AE and Wong RB (eds.) Immunoanalysis of Agrochemicals: Emerging Technologies. *ACS Symposium Series 586*:288-300.

Stave JW (1999). Detection of the new or modified proteins in novel foods derived from GMO: future needs. *Food Control* 10:367-374.

Rogan GJ, Dudin YA, Lee TC, Magin KM, Astwood JD, Bhakta NS, Leach JN, Sanders PR and Fuchs RL (1999). Immunodiagnostic methods for detection of 5-enolpyruvylshikimate-3-phosphate synthase in Roundup Ready(R) soybeans. *Food Control* 10(6):407-414.

USDA (2004). U.S. Department of Agriculture/Grain Inspection, Packers and Stockyards Administration Directive 9181.2. [On line] http://archive.gipsa.usda.gov/reference-library/directives/9181-2.pdf.

ANNEX V ANALYTICAL CONTROL ACCEPTANCE CRITERIA AND INTERPRETATION OF RESULTS FOR QUANTITATIVE PCR METHODS

1. At a minimum, the following acceptance criteria are common to all quantitative PCR methods and applicable to each PCR run:

- The mean of the replicates of the positive DNA target control at a relevant concentration deviates less than 3 standard deviations from the assigned value. When applicable, a target DNA control is defined as reference DNA or DNA extracted from a certified reference material or known to be a positive sample representative of the sequence or organism under study. The control is intended to demonstrate what the result of analyses of test samples containing the target sequence should be.
- The amplification reagent control shall not result in an amplification signal above the background noise. The amplification reagent control is defined as control containing all the reagents, except extracted test sample template DNA. Instead of the template DNA, a corresponding volume of nucleic acid free reagent (such as water or buffer) is added to the reaction.

2. To accept the result of an unknown sample, the relative standard deviation of the sample replicates should be ≤35 %.