codex alimentarius commission

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

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Agenda Item 3 **CX/MAS 10/31/3**

JOINT FAO/WHO FOOD STANDARDS PROGRAMME

CODEX COMMITTEE ON METHODS OF ANALYSIS AND SAMPLING
Thirty-first Session
Budapest, Hungary, 8 - 12 March 2010

PROPOSED DRAFT GUIDELINES ON CRITERIA FOR METHODS FOR DETECTION, IDENTIFICATION AND QUANTIFICATION OF SPECIFIC DNA SEQUENCES AND SPECIFIC PROTEINS, IN PARTICULAR IN FOODS DERIVED FROM MODERN BIOTECHNOLOGY

(At Step 3)

The Proposed Draft Guidelines, as included in the Report of the Working Group in Annex II, are hereby circulated at Step 3 for comments and consideration by the 31st Session of the Committee on Methods of Analysis and Sampling. Governments and international organizations wishing to provide comments should do so in writing, preferably by email, to the Secretariat, Codex Alimentarius Commission, Joint FAO/WHO Food Standards Programme, FAO, Viale delle Terme di Caracalla, 00153 Rome, Italy, Fax: +39 06 5705 4593, E-mail: codex@fao.org, with a copy to the Hungarian Codex Contact Point, Hungarian Food Safety Office, H-1097 Gyáli út 2-6. Budapest Hungary, Fax:+36 13879400, e-mail: HU_CodexCP@mebih.gov.hu, **before 10 February 2010**.

REPORT OF THE WORKING GROUP ON THE *"PROPOSED DRAFT GUIDELINES ON CRITERIA FOR METHODS FOR DETECTION, IDENTIFICATION AND QUANTIFICATION OF SPECIFIC DNA SEQUENCES AND SPECIFIC PROTEINS, IN PARTICULAR IN FOODS DERIVED FROM MODERN BIOTECHNOLOGY".*

August 17th – November 20th 2009 (electronic working group)

The Codex Committee on Methods of Analysis and Sampling (CCMAS) held its thirtieth session from 9 to 13 March, 2009 in Balatonalmádi, Hungary. During the 30th CCMAS session, it was agreed to establish an electronic working group to revise (at step 2 of the procedure) the "*Proposed Draft Guidelines on Criteria for Methods for Detection, Identification and Quantification of specific DNA Sequences and specific Proteins, in particular in Foods derived from Modern Biotechnology*", taking into account comments submitted and raised during the session (ALINORM 09/32/23).

The aforementioned electronic Working Group (eWG) began its activities on August 17th, and finished on November 20th, 2009. It was co-chaired by Argentina, United Kingdom and Germany. Argentina acted as the Host and provided an Internet/email-based platform for the functioning of the eWG[1]. The working language was English, and the eWG was open to all Members and Observer Organizations. The eWG was composed of 81 Participants, distributed in 31 Delegations from Member Countries, Member Organizations or International Organizations. The detailed list of Participants is included in this report as Annex I.

A total of 243 interventions were exchanged during the span of the eWG debates, which were structured as described next.

From August 17th to 21st there was a general debate on the overall document and the organization of the work. During their initial interventions, some Delegations referred to the applicability of the document to different food-related applications, and proposed to revise the new title and scope paragraph introduced during the 30th CCMAS meeting. In turn, other Delegations were opposed to the revision of these texts, since they represented a recent compromise and there was an interest in starting the review of the technical content of the draft without further delay.

For the sake of progressing, it was proposed that there would be a first revision or "reading" of the guideline text, where the title and scope paragraph would not be modified, and the subsidiary references to the scope along the text would be bracketed and left aside temporarily. This would allow focusing on technical details and adjusting the guidance to the new enlarged scope. After the eWG would have reviewed the whole text once in the light of the new scope, then there would be a renewed debate to consider the need of any adjustment on the title and scope language. Finally, after that specific debate, there would be a short second reading of the whole guideline to finish streamlining the text.

The first reading of the draft occurred from August 24th to October 19th. Consideration of the document was performed sequentially, in rounds of about one week focused on different sections. The first reading was based on an updated guideline draft distributed in advance, which included changes agreed during the 30th CCMAS. These changes were (a) the new title and scope paragraph, (b) structure changes originally proposed by Japan (CX/09/30/8-Add.1) and (c) suppression of language related to Codex procedural matters, because it was decided that the guideline was intended to be used by Governments

Pending issues submitted and raised during the earlier CCMAS session were addressed during the first reading. These included: the need of improvements in regards to protein-based methods, the update of

[1] For this purpose, the host developed an *ad hoc* implementation of the free, open-source software Moodle (http://moodle.org/), specifically adapted to the purposes of supporting a Codex electronic Working Group. Electronic Working Groups greatly facilitate the involvement of Developing Country Members as participants or hosts of working groups between Committees sessions. The Codex Procedural Manual indicates that preferred consideration should be given to the establishment of electronic working groups, in the search for worldwide consensus and greater acceptability of Codex Standards. Therefore, the template files and expertise generated during this experience are available for other interested members, and the website will remain on display until April, 2010 at www.agrobiotecnologia.gov.ar/ccmas.

methodologies and technical/scientific references, the estimation of measurement uncertainty, terminology harmonization and editorial amendments.

In addition, earlier terminology that restricted the applicability of the guidance to biotechnology products was updated to a more general terminology applicable to any specific DNA sequence and/or specific protein of interest in foods, according to the new scope. However, a few guidance details were only applicable or needed in relation to foods derived from biotechnology, therefore the specific terminology was maintained in those cases.

From October 19th to November 6th there was a debate on the scope-related language. It involved the title, scope paragraph, purpose paragraph and a standard formula for the subsidiary references to the scope along the text. Some Delegations alleged that the way that Foods derived from Modern Biotechnology were mentioned in those key sections induced the misconception that the guideline should only be applied to methods designed for those products. As a consequence, they proposed an alternative language. However, other Delegations were not satisfied with the alternative language, and stated their position in favor of maintaining the language and some key allusions to biotech foods as they emerged from the last CCMAS session.

From October 26th to November 16th, a second reading of the updated draft was performed. The second reading considered the whole guideline simultaneously, which allowed for cross-section improvements, enhancements on fine technical details and editorial streamlining of the document[2].

Although best efforts were made, it was not possible to arrive to a final solution regarding scope-related language. However, there was consensus and a clear sense of progress regarding the remainder majority of the text. As a consequence the title and scope paragraph from 30th CCMAS, which were used as a reference during the technical revision, are presented in the revised draft as the main option, while the alternative wordings and options for subsidiary references to the scope are included in brackets.

Finally, the present report was elaborated from 16 to 20 November. The Working Group agreed to submit the Revised Draft Guideline to the CCMAS Chairperson and the Codex Secretariat, in order to facilitate to follow the next steps anticipated in the 30th CCMAS report: the circulation of the Revised Draft for comments at Step 3, in preparation for its consideration at the 31st CCMAS session.

To complement the present report, the records of the eWG debates will remain available in full at the eWG website (www.agrobiotecnologia.gov.ar/ccmas) until the next CCMAS meeting.

The revised document *"Proposed Draft Guidelines on Criteria for Methods for Detection, Identification and Quantification of specific DNA Sequences and specific Proteins, in particular in Foods derived from Modern Biotechnology"* is included in this report as **Annex II**.

[2] In different instances of the revision some Delegations suggested that, after the significant modifications introduced, a new reorganization of the document would further improve its quality. However, the details and merits of such reorganization were not debated in order to finish the eWG task on time.

ANNEX I

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PROPOSED DRAFT GUIDELINES ON CRITERIA FOR METHODS FOR DETECTION, IDENTIFICATION AND QUANTIFICATION OF SPECIFIC DNA SEQUENCES AND SPECIFIC PROTEINS, IN PARTICULAR IN FOODS DERIVED FROM MODERN BIOTECHNOLOGY.

[Alternative Title I:
PROPOSED DRAFT GUIDELINES ON PERFORMANCE CRITERIA AND VALIDATION OF METHODS FOR DETECTION, IDENTIFICATION AND QUANTIFICATION OF SPECIFIC DNA SEQUENCES AND SPECIFIC PROTEINS IN FOODS]

[Alternative Title II:
PROPOSED DRAFT GUIDELINES ON CRITERIA FOR METHODS FOR THE DETECTION AND IDENTIFICATION OF FOODS DERIVED FROM MODERN BIOTECHNOLOGY]

SECTION 1 – INTRODUCTION

1. Molecular and immunological analytical methods are currently the recognized tools for determination of DNA and protein analytes in foods [derived from modern biotechnology]. 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 definitions, validation of quantitative PCR methods, validation of qualitative PCR methods, validation of protein-based methods, and proficiency testing.

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 [derived from modern biotechnology] that produce results with comparable reproducibility when performed at different laboratories

5. The guidelines are aimed to give 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 will specify the relevant criteria and give explanations on how to consider these criteria, i.e.:
-by giving 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 for the validation of methods for the detection, identification, and quantification of specific DNA sequences and specific proteins in foods derived from modern biotechnology. These Guidelines may also provide information on the validation of methods for other specific DNA sequences and proteins of interest in other foods.

6 alternative [These guidelines provide information 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 and that will be used by laboratories responsible for food analysis. These methods can provide molecular and immunological approaches for, including among other uses, tests

for food authenticity, and biomarkers for foods containing material derived from recombinant-DNA organisms.].

SECTION 3 – DEFINITIONS

7. There are a number of terms related to the methods of analysis in the Codex Procedural Manual and other sources, which may also be applicable to the analysis of [DNA sequences and proteins of interest in foods], [foods derived from modern biotechnology]. Suggested definitions of these terms are given in Annex II.

SECTION 4 – METHOD VALIDATION

8. The Codex Alimentarius Commission places an emphasis on the acceptance of methods of analysis which have been "fully validated" through a collaborative trial conforming to an internationally accepted protocol. In a number of sectors, there are few methods of analysis which have been fully validated. As a result, Codex is also endorsing by reference single-laboratory validation protocols. In this area there may be pressure 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] [foods derived from modern biotechnology] are able to be, and intended to be performed at, multiple laboratories and should therefore be validated by multi-laboratory collaborative studies as soon as practicable.

Section 4.1 – Criteria Approach

9. Codex Alimentarius Commission has accepted the "criteria approach" for methods of analysis. It is necessary to ensure that this approach is incorporated into these guidelines.

Section 4.2 – General Method Criteria

10. The conventional criteria that have been adopted by Codex for the evaluation of methods of analysis are:

- trueness
- applicability (matrix, concentration range and preference given to 'general' methods)
- limit of detection
- limit of quantification
- precision; repeatability intra-laboratory (within laboratory), reproducibility inter-laboratory (within laboratory and between laboratories)
- selectivity
- sensitivity
- linearity
- robustness

Section 4.3 – Validation Process

11. Method validation is a process of establishing the performance characteristics and limitations of an analytical method and the identification of the influences, which may change these characteristics - and to what extent. 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.

12. The method validation process accepted by Codex includes the definition of the requirements for the method, testing that the method meets these requirements when carried out, for instance, by different laboratories in different countries, and documentation of the method performance and measurement uncertainty.

13. 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 may be recommended but 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

compliance with the requested performance or regulations. 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.

- ***Full validation of the method.*** Full 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 5 – SPECIFIC CONSIDERATION FOR THE VALIDATION OF METHODS FOR THE DETECTION, IDENTIFICATION AND QUANTIFICATION OF [DNA SEQUENCES AND PROTEINS] [FOODS DERIVED FROM MODERN BIOTECHNOLOGY]

Section 5.1 – Method Development to Formal Validation

14. Before methods are accepted for use, they should be validated to ensure that they are fit-for-purpose.

15. Common methodologies for DNA-based analysis (Anklam *et al*., 2002; Poms *et al.*, 2004) are PCR-based methods used to detect a specific (targeted) DNA sequence (ISO21570:2005; Asensio 2007; Holst-Jensen & Berdal, 2004; Lipp *et al.* 2005; Miraglia *et al.*, 2004). Common approaches for protein utilize ELISA and lateral flow devices (ISO21572:2004; Grothaus *et al.*, 2006). 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 5.1.1 – Method Acceptance Criteria (Required condition for full validation)

16. In order to evaluate a method prior to full validation, information concerning both the method and the method testing is required. Information on this is given in Annex I.

17. The method will be evaluated based on the information provided. The 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 5.1.2 – Applicability of the Method

18. 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, a method that can be applied to single raw matrix cannot be necessarily applied to the complex matrices and/or the processed food, since the DNA and protein will be denatured easily.

19. [This is a particularly important criterion in the analysis of foods derived from modern biotechnology]. In principle the method should be applicable to the matrix of concern within the Codex system. If [the method is used for analyzing DNA sequences and proteins, then information should be provided] [this is a specific food derived from modern biotechnology then there is merit in requiring those seeking endorsement to provide information] on the method of analysis appropriate to the specific product and, ideally, the matrix in which it is likely to be used. In the case of "general purpose" methods to identify and quantify [DNA sequences and proteins] [foods derived from modern biotechnology] in a range of food matrices, at least one extraction method applicable to a general food matrix should be available.

20. The amount and nature of measurable target DNA and protein present in food and food ingredients may be significantly affected by processing steps. The changes that occur to a protein during processing may lead to denaturation, and while protein-based testing can be applied to processed food or feed, care should be taken to ensure that the test is validated and fit for the intended purpose. Typically, protein-based testing has been applied to minimally processed products (e.g. maize and wheat grain and flour), but specific applications have been developed for highly processed products like toasted soy meal and protein isolate. Processing may have a similar influence on the ability to detect target DNA.

Section 5.1.3 – Principle condition

21. DNA-based methods should detect, identify and quantify the relative levels of specific target and taxon-specific DNA sequences whilst protein-based methods should detect and quantify the level of specific protein in the product.

[21 alt. The provision of the detection method is aimed to serve mainly the requirements for the measurement of products derived from modern biotechnology. To serve these purposes, the method can detect and quantify the specific target and taxon-specific DNA sequence or the protein derived there from in the product; this may be achieved in most cases using either protein-based or DNA-based methods.]

22. In the execution of the assay reference materials should be utilized that have been prepared from a matrix containing the specific analyte, when available. When control samples or reference materials are not available, a plasmid containing the appropriate target and taxon-specific DNA sequences could be developed and used as an appropriate control if the targeted DNA sequence information is available.

23. 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 when using a gel-based method.
- appropriate control samples and standards.
- descriptions of calculations used to derive the result.

24. Protein based methods typically consist of a quantitative or qualitative method. The former is usually an ELISA system, and consists of the following:

- an antibody-coated micro plate,
- an enzyme-conjugated secondary antibody,
- standards,
- controls,
- an enzyme substrate for color development, and
- washing buffer and sample extraction buffer.

25. Quantification is done by comparing the amount of the specific protein found in the extract(s) with the amount of total extractable protein present in the extract or the total protein in the food matrix. This measurement may need to be corrected for extraction efficiency.

26. Whereas, the qualitative method may consist of an ELISA, or a lateral flow device which consists of the following:

- a sample pad,
- a conjugate pad,
- a nitrocellulose membrane, and
- a wicking pad assembled on a thin plastic backing.

27. The method provider should demonstrate that the method fulfils 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 that are used to detect multiple transformation events should allow for unequivocal detection and identification of a target DNA sequence which is common to a number of transformation events.
- DNA-based target-specific methods that are used for detection or relative quantification of a single transformation event should allow for unequivocal detection, identification and quantification of a target DNA sequence that is unique or specific to that transformation event.
- 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.

[27 alt. Should PCR be selected as the analytical method, the method should target a DNA sequence which is not present in the food being examined. Currently, the best choice concerning event-specificity PCR should be the chosen technique, because it is targeting a event-specific genomic region using a set of oligonucleotides (primers) that trigger the amplification of such a region. Among various types of event-specific genomic regions, the one relative to the junction between the recombinant insert and the host genomic DNA will probably be the location of choice. However, when a unique DNA sequence can be found within the recombinant insert, such a sequence (generally called construct specific) can also be targeted by appropriate oligonucleotide primers and amplified through a PCR. Identification of the amplified fragment, by e.g. probe hybridization or any appropriate equivalent method, is recommended]

Section 5.1.4 – Modular Approach to Method Validation

28. 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 ELISA 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 (Chapela *et al.,* 2007; Holst-Jensen & Berdal, 2004; Turci *et al.,* 2009) to achieve economic efficiencies as long as the validated method processes remain the same.

29. 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 5.2 – Collaborative Trial Requirements

Section 5.2.1 – General Information

30. The purpose of a collaborative trial is to fully 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.

31. 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.

32. For Codex purposes the ISO 5725:1996 or the AOAC/IUPAC Harmonized Protocol (Horwitz, 1995) has been adopted. If a collaborative trial has already been conducted according to an internationally accepted protocol, then this information can be used to assess the acceptability of the method for Codex purposes.

Section 5.2.2 – Minimum Performance Requirements

33. 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. Thus, the collaborative trial should confirm the results obtained during the previous method evaluation phases and should provide additional information about the method performance in a multi-laboratory setting. In particular, the compliance with the criteria for sensitivity and repeatability/reproducibility standard deviations and trueness should be assessed.

34. 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. First, the definition and thereafter the requirements are described.

35. The endorsed 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 will also be complemented with practical information about the operational steps of the validation process.

Section 5.2.3 – Collaborative Trial Test Materials

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

37. 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 analysed, and if a purified protein or DNA was used as the target for the analysis, then this should be reported.

Section 5.2.4 – Specific Information on the Validation of Methods

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

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

Section 5.3 – Unit of Measurement

40. Appropriate units of measurement (e.g. target copy numbers per mg food/ molar equivalents, etc.), 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 absent (-) and for this reason there is no unit of measurement.

41. 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. In the case of a DNA-based method used for quantification of a specific DNA genome equivalents may typically be measured; notice that these may be influenced by a number of biological factors (Grothaus *et al.* 2007; Holst-Jensen & Berdal, 2004), depending on the part of the seed originally used for preparation of the flour or other components of the food (e.g. endosperm, germ), and whether the DNA or protein is retained in that portion. Protein methods measure the amount of a specific protein that is present and may be influenced by extraction efficiency.

Section 5.4 – Measurement Uncertainty

42. As mentioned in the Codex Guideline on Measurement Uncertainty (CAC/GL54-2004), laboratories are required to estimate 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 will have sufficient information to allow them to estimate the uncertainty of their result. Quantification based on the protein expressed can also significantly contribute to the uncertainty of the analysis.

43. For details, refer to the Codex Guideline on Measurement Uncertainty (CAC/GL54-2004), ~~and~~ 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 and (Trapman et al., 2009).

SECTION 6 – QUALITY CONTROL REQUIREMENTS

Section 6.1 – Laboratory Quality

44. The Codex Alimentarius Commission has adopted guidelines for the "quality" of laboratories involved in the import and export of foods. These quality characteristics are 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. These overarching guidelines provide information to and dictate requirements for laboratories working in the sector dealing with [DNA sequences and proteins] [foods derived from modern biotechnology].

45. It is required for laboratories to have standardized quality control practices in place, in order to avoid cross-contamination of material, which could result in false positive results. Several examples are available (Dieffenbach & Dveksler, 1993; Kwok & Higuchi, 1989; Mifflin, 2007; Newton, 1995). Other relevant guidance for DNA-based methods may be ISO 21569:2005 and ISO 21570:2005.

Section 6.2 – Guidance on the Laboratory Set-up and Operation

46. DNA-based methods for the analysis of [DNA sequences and proteins] [foods derived from modern biotechnology] require specific apparatus and handling techniques that differ from most chemical-analytical methods. The use of DNA based methods is consistently growing in other detection fields such as microbiology of food pathogens. It is necessary to provide information and instructions on the essential differences in laboratory set-up and handling techniques. Examples are available (ISO/DIS 24276:2006).

47. Immunological (protein-based) methods of analysis are well understood, and used in many laboratories for a number of analyses, and often come in kit form, simplifying their use; however, it is to be noticed that protein-based detection limits are below those of DNA-based methods**.**

48. In addition to the cross-contamination topic considered in the preceding section, compliance with minimal necessary biosafety directions (WHO, 2004) is recommended.

Section 6.3 – Reference Material

49. A suitable reference material is generally required for 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] [foods derived from modern biotechnology]. 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.

50. When analysing target- and taxon-specific plant DNA sequences, it is recommended to use a reference material prepared from 100% homozygous material or material with a certified level of zygosity, if possible. However, other material may be appropriate on a case-by-case basis.

51. Reference materials for protein detection methods can be either the protein itself purified from recombinant microbes such as E. coli, a ground plant matrix (typically leaf or grain), or a processed food fraction.

52. Where suitable reference materials are not available, the availability of quality control materials from proficiency testing schemes or from the use of plasmid or amplicon DNA may be considered. Use of plasmid or amplicon DNA requires careful consideration of the choice of target- and/or taxon-specific DNA to be

incorporated into the plasmid or amplicon to ensure that the plasmid or amplicon DNA will be fit for the required purpose.

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

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] [foods derived from modern biotechnology] are based on different principles. In PCR the targeted DNA is amplified in an exponential manner, in which a small difference in the beginning of the PCR process will lead to a big difference in the amplified amount of DNA after 35-45 cycles. Moreover, the quantification by real-time PCR is often based on two independent PCR systems: one for the target DNA and one for the taxon specific sequence. In contrast to PCR, immunological detection assays do not include multiple cycles in which the product of the previous amplification step is itself amplified.

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 analysed, 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:

- ***Amplicon length***

7. 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 sequence and the target sequence should be in a similar size range.

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

8. 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.

9. 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. Some real time instruments use laser technique for inducing fluorescence, others are equipped only with a lamp and filters for selecting a specific

wave length. The detection and quantification of the fluorescence could also vary according to the recording instruments and software used.

10. Qualitative methods may employ (for example) a gel-based system for interpreting results. In addition, qualitative methods generally tend to be less instrument-specific than quantitative methods.

11. Taking all the differences into account it is not appropriate to change the instrument without adaptation of the PCR method. Thus, because the methods are generally instrument and chemistries dependent they cannot be transferred to other equipment and chemistries without evaluation and/or modification.

12. This is in many ways equivalent to the Codex Type I method and should be considered in the same light.

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

13. Using more than one primer set in a single reaction is called multiplex PCR. The aim of using such approach is to reduce costs and time for the analysis of different targets in a single sample.

14. 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

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

Assay applicability

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

Hook Effect

17. 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.

Confirming method

18. For a quantitative immunoassay, antibodies may cross-react with other proteins present in the matrix; thus, it is necessary to demonstrate the credibility of assays. Another method such as HPLC, LC/MS, western blot or biological assay may be used as a confirming method. Empirical results from testing both methods with aliquots of the same analytical samples of known concentration may be provided.

INFORMATION ABOUT THE METHOD OPTIMISATION

Primer pairs

19. General methods have to provide the defined primer pairs and the sequence they target, as well as different sets of primers if available. 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.

Selectivity testing

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

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

22. 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

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

24. 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

25. Practical results from testing the method at different concentrations in order to test the sensitivity of the method should be provided. 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) should 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. This dilution effect will depend on the relative amount of taxon-specific DNA (e.g. soy-derived DNA) that is present in the total DNA following extraction from the food product. Some ingredients will contribute a large amount of DNA, such as wheat or maize flour or eggs, while other ingredients will not contribute any DNA, such as refined sugar, pure water or highly processed oils.

26. LOD should be determined for each method separately.

27. In the case of protein-based analyses, the limit of detection should be ascertained according to established procedures for immunoanalyses for each matrix.

Robustness testing

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

Cross-reactivity

29. The cross-reactivity, interferences and matrix effects should be evaluated.

Extraction efficiency

30. Empirical results from testing the protein 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

31. Indication of the matrix (e.g., processed food, raw materials, etc.), the type of samples (e.g., seeds, flour, pizza, cookies, etc.) and the range to which the method can be applied should be given. Relevant limitations of the method should also be addressed (e.g. inference 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

32. 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

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

Operator skills requirements

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

ANALYTICAL CONTROLS

35. The proper use of controls, when available, when applying the method should be indicated. 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.

36. 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 VALIDATION/PERFORMANCE

37. See the Codex “Check-list” (i.e. accuracy, applicability (matrix, concentration range and preference given to 'general' methods), detection limit, quantification limit, precision, recovery, selectivity, sensitivity, extraction efficiency and parallelism/linearity) and an assessment that the methods will be fit for purpose.

ANNEX II: DEFINITIONS APPLICABLE TO THE ANALYSIS OF [DNA SEQUENCES AND PROTEINS] [FOODS DERIVED FROM MODERN BIOTECHNOLOGY]

Accuracy

Accuracy is defined as the closeness of agreement between a test result or measured result and the true value. In practice the accepted reference value is substituted for the true value. The term accuracy, when applied to a set of test results or measurement results, involves a combination of random components and common systematic error or bias component.

Assay

Technical operation that consists in the determination of one or more features of a product, process or service according to a specific procedure.

Applicability

The analytes, matrices and concentrations for which a method of analysis may be used[3].

The analytes, matrices, and concentrations should be appropriate for the control purposes for which the method has been proposed. The description may also include warnings to known inferences by other analytes, or inapplicability to certain matrices and situations.

Authentication

Confirmation of the biological source of the test material.

Dynamic Range - Range Of Quantification

The interval of concentration within which the analytical procedure has been demonstrated by collaborative trial to have a suitable level of precision and trueness.

Identification

Set of operation having the object of deciding that the object is the specified one**.**

Limit of Detection (LOD)

Limit of detection is the lowest concentration or content of the analytes that can be detected reliably, but not necessarily quantified, as demonstrated by collaborative trial or single-laboratory validation. Alternatively it may be taken from the last value with reliable data used to determine the LOD. LOD is generally expressed as the amount of analyte at which the analytical method detects the presence of the analyte at least 95% of the time ($\leq$5% false negative results).

Limit of Quantification (LOQ)

The limit of quantification of an analytical procedure is the lowest amount or concentration of analyte in a sample, which can be quantitatively determined with an acceptable level of precision and trueness as demonstrated by satisfactory collaborative trial or single-laboratory[4] validation[5]. Alternatively, it may be taken from the last value with reliable data used for determining the LOQ.

Linearity

The ability of a test to obtain results (within a given range) that vary in a manner directly proportional to changes in the concentration (amount) of the analyte the sample, or by a well defined mathematical transformation.

Practicability

The ease of operations, in terms of sample throughput and costs, to achieve the required performance criteria and thereby meet the specified purpose[6].

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[3] Slightly modified from the definition provided in Codex CX/MAS 02/4: Proposed draft guidelines for evaluating acceptable methods of analysis. Version November 2002.

[4] e.g. Thompson et al. 2002. IUPAC Technical Report: Harmonised guidelines fro single-laboratory validation of methods of analysis. Pure Appl. Chem. 74(5): 835-855.

[5] Slightly modified from EN/ISO 24276:2006 (E).

[6] Adopted from EN/ISO 24276:2006 (E).

Generally, the method should be practical for its intended purposes.

Precision

The closeness of agreement between independent test/measurement results obtained under stipulated conditions.

Reference Material

Material or substance that has values of one or more properties are sufficiently homogeneous and well known to permit their use in calibration of an apparatus, assessing a measurement method or the attribution of value to other materials.

Repeatability standard deviation (RSD_r)

The standard deviation of test results obtained under repeatability conditions. Repeatability conditions are conditions where test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time.[7]

Reproducibility standard deviation (RSD_R)

The standard deviation of test results obtained under reproducibility conditions. Reproducibility conditions are conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment[8].

Recovery

Proportion of the amount of analyte, present in or added to the analytical portion of the test material, which is extracted and presented for measurement.

Ruggedness (Robustness)

Robustness refers to variations in the method as performed in different laboratories by different 'technicians'. The language used here is derived from "Ruggedness" which is the equivalent in the harmonized guidelines. Ruggedness should be demonstrated by the validation of the method in 8-12 laboratories as defined in the harmonized guidelines. It is preferable from a CODEX point of view, that these laboratories be distributed across several continent/trading blocks.

The robustness of an analytical method is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage[9].

Sensitivity

The sensitivity of a method is a measure of the magnitude of the response caused by a certain amount of analyte.

The method should be sensitive enough in order to be able to detect/quantify with respect to the thresholds as provided in the relevant legislation.

Since sensitivity is method- and purpose-dependent it should be specified in the protocol. A reasonable goal for sensitivity is that required to meet levels specified in contracts, with a reasonable certainty that the level does not exceed the required limit.

Sensitivity as a term is used in two different ways: LOD and instrument response. The use of "detection limit", or "limit of detection" is the preferred term to use as a measure of the ability of a method to detect a small amount of analyte. See also previous comments regarding sensitivity in this document.

Selectivity

Property of a method to respond exclusively to the characteristic or analyte of interest.

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[7] Definitions adopted from ISO 3534-1.

[8] Definitions adopted from ISO 3534-1

[9] Definition adopted from ICH Topic Q 2 A "Validation of analytical methods: definitions and terminology." The European Agency for the evaluation of medicinal products. CPMP/ICH/381/95. Version November 1994. http://www.emea.eu.int/pdfs/human/ich/038195en.pdf

Trueness

Trueness is defined as the closeness of agreement between the average of an infinite number of replicate measured quantity values and a reference quantity value.

Note: The measure of trueness is usually expressed in terms of bias or the ratio of the the average of an infinite number of replicate measured quantity values to a reference quantity value in this analytical sector.

Trueness has been referred to as "accuracy of the mean". This usage is not recommended.

In practice the accepted reference value is substituted for the true value.

Expectation is the expected value of a random variable, e.g. assigned value or long term average (ISO 5725-1).

Uncertainty

Parameter associated with the outcome of a measurement to characterize the dispersion of the values that could reasonably be attributed to him by measuring the magnitude.

ANNEX III: VALIDATION OF A QUANTITATIVE PCR METHOD

INTRODUCTION

1. DNA-based analysis is commonly performed using polymerase chain reaction (PCR). This technique amplifies a specific (short) segment of DNA to the extent that its quantity can be measured instrumentally (e.g., using fluorometric means in which case the minimal length of the segment that can be measured instrumentally has to be specified. 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. It is often the case that the results of a determination are 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 (e.g. a DNA sequence from another species) and that of the endogenous, or taxon-specific sequence (e.g. an endogenous maize gene 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 ruggedness 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 ruggedness (robustness). Other important factors are acceptance criteria and interpretation of results, and the issue of the units in which results are expressed.

7. Note: There is a general scientific discussion about the interpretation of the percentage values. It is recognised that so far there is no reliable weight/copy number relationship because of uncertainty in the correlation of weight of ingredient to number of molecules of DNA. Both the w/w and copy number/copy number calculations are acceptable provided it 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 reactions**.** These are given alphabetically, not necessarily in order of importance.

Trueness

9. 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.

10. Recommendation: The trueness should be within ± 25% of the accepted reference value over the whole dynamic range. This refers to the PCR-step provided that the modular approach has been applied.

Applicability

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

12. As an example it is required from an extraction method, independent of matrix to which it is to be applied, that it yields DNA or protein 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, detection of the protein) to be undertaken (e.g. Chapela et al. 2007; Turci et al. 2009).

13. 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 (Cankar et al. 2006). This is also the case for end-point PCR reactions carried out using fluorescent probes.

Dynamic Range - Range Of Quantification

14. 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.

15. 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 (Huebner *et al.*, 2001; Horwitz, 1995; Kay & Van den Eede, 2001).

16. If DNA is chosen to be used as calibrator, it is important that this calibrator needs to 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.

17. 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 variances in the values in this range.

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

18. If the validation of the quantitative PCR assay shows that the assay can measure [DNA] [foods derived from modern biotechnology] 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.

19. It is worth noting that a determination of an LOD or LOQ is not necessarily needed to establish the validity of a method for a given application. For example, it does not add much value if an LOD is determined to be 1ng/kg, while the scope of the method validation extends only for concentrations ranging in g/kg. In this and similar cases the reliability of the method will be proven by the other parameters and no efforts are included in the method validation to assess the LOD. However, the LOQ shall always be established and included in the validation study.

20. If the LOD is required, it is common practice to assume that it is the signal strength of a blank increased by three times the standard deviation of the blank. However, this method gives at best an estimate, may rely on a normal Gaussian distribution of the blank measurements around zero, and may give a lower value than the actual LOD. Its use is not valid in methods such as Quantitative PCR, in which the distribution of measurement values for blanks is typically truncated at zero and is thus not normally distributed. Thus the LOD needs to be experimentally determined unless the targeted concentrations are well above the LOD and the LOD therefore becomes irrelevant. 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 ($\leq$5% false negative results). In combination with the false positive rate, these are the only parameters required for a qualitative method other than selectivity and repeatability.

21. 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. Using the traditional approach, the LOQ can be expressed as the signal strength of a blank equal to the LOD increased by 6-10 times the standard deviation of the blank, unless it is known from other sources that the measured values range so high above the LOQ that its knowledge becomes irrelevant. However, this method to determine the LOQ leads only to an estimate of the true LOQ that may be an artificially high or low approximation.

22. 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 or proteins] [foods derived from modern biotechnology]

Practicability

23. The practicability of the method should be demonstrated.

Repeatability standard deviation (RSD_r)

24. Recommendation: The relative repeatability standard deviation should be ≤25% or as close as is practicable over the whole dynamic range of the method.

Reproducibility standard deviation (RSD_R)

25. Recommendation: The relative reproducibility standard deviation should be below 35% or as close as is practicable at the target concentration and over the majority of the dynamic range. $RSD_R \leq 50\%$ or as close as is practicable at the limit of quantification lower end.

Ruggedness (Robustness)

26. The evaluation of ruggedness (robustness) demonstrates the reliability of a method with respect to inadvertent variation in assay parameters. Variations that may be included are 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 triplicates. 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.

27. 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

28. 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%.

29. 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%).

30. 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.

Specificity

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

32. A taxon-specific assay should not recognize any sequence corresponding to phylogenetically related species, and should give similar Ct-values, not statistically different, when amplifying equal amounts of DNA from different varieties/cultivars of very different origins of the same taxon.

33. A species-specific assay should not recognize any sequence corresponding to closely related species, and should give similar Ct-values, not statistically different, when amplifying equal amounts of DNA from different varieties/cultivars of very different origins of the same species.

34. The adequacy of the testing needs to be analysed on a method-by-method basis.

35. Primer and probe design is the starting point for a method. Primers and probes should be checked against the known sequence of the target that the assay is designed to detect and pertinent sequences databases for possible homologies or against known target organism/pathogen sequences. After such a theoretical specificity assessment, selectivity should then be demonstrated experimentally.

36. For assays specific to the target DNA. Experimental evidence on specificity to 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.
- One sample from each source (an appropriate number of DNA samples) should be tested.
- Two replicates should be analyzed for each DNA sample, which shall give results within a Ct-value of 0.5.

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

38. For assays on endogenous (taxon-specific) DNA sequences. Experimental evidence on specificity for endogenous or taxon-specific assays 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.
- One sample from each source (an appropriate number of DNA samples) should be tested.
- Two replicates should be analyzed for each DNA sample, which shall give results within a Ct-value of 0.5.

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

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ANNEX IV ANALYTICAL CONTROL ACCEPTANCE CRITERIA , PROFICIENCY TESTING AND INTERPRETATION OF RESULTS FOR QUANTITATIVE PCR METHODS

1. A validated method also includes values of criteria on which the validity of an observed measurement result can be assumed. It is important to follow these criteria and to observe the decision support system for data analysis and interpretation. In the case that it may be desired to deviate from said criteria and rules a new method validation study would be needed in order to demonstrate the validity of the new decision support system and procedures.

2. 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 is ≤LOD (Therefore, it is important to always determine LOD for validation of the method): 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 water is added to the reaction.
- The % of residual for each of the standards should be ≤30% or as close as is practicable.

3. To accept the result of an unknown sample, the relative standard deviation of the sample replicates should be ≤35 % or as close as is practicable.

4. The amplification process used in quantitative PCR determinations often commences with a small number of copies of the target DNA and the sampling process at this initial stage, together with the log-linear nature of the PCR calibration function, leads to a positive skew in results. As predicted, the study results consistently follow a positively skewed distribution (Thompson et al., 2006). Log-transformation prior to calculating *z*-scores is effective in establishing near-symmetric distributions that are sufficiently close to normal to justify interpretation on the basis of the normal distribution. The consequence for proficiency testing schemes is outlined in the *RSC Analytical Methods Committee Technical Brief 18*.

REFERENCES FOR ANNEX IV

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ANNEX V: 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. A concept of using false-positive and false-negative rates to describe the accuracy and precision of a qualitative assay has been developed for microbial assays (AOAC 2002). This concept can be applied to qualitative PCR assays. A critical issue in the validation of this type of method is the availability of test materials that are known to be either positive or negative. The provision of negative reference materials is particularly important and critical in the case of a qualitative method.

2. By their very nature, qualitative test results refer to the identification above/below a detection limit. The measures of precision and trueness are the frequencies of false negative and/or false positive results at the detection limit. False negative results indicate the absence of a given analyte when in fact the analyte is present in the sample, while false positive results indicate the presence of an analyte that is not present in the sample. Due to the inherent nature of the analytical technique, an increase in false negative results will be observed when the amount of analyte approaches the LOD of the method. 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. During validation of a qualitative PCR assay, it is also important to determine the number of false positive results (a positive result obtained using a sample that is known to be negative). 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. The false positive rate is the number of misclassified known negatives divided by the total number of negative test samples (misclassified positives plus the number of correctly classified known negatives) obtained with the method:
For convenience this rate can be expressed as percentage:

$$\% \text{ false positive results} = x\ 1\ \frac{\text{number of misclassified known negative samples}}{\text{total number of negative test results [incl. misclassified]}}$$

False Negative Rate

4. This is the probability that a known positive test sample has been classified as negative by the method. The false negative rate is the number of misclassified known positives divided by the total number of positive test samples (misclassified positives plus the number of correctly classified known positives) obtained with the method.
For convenience this rate can be expressed as percentage:

$$\% \text{ false negative results} = x\ \frac{\text{number of misclassified known negative samples}}{\text{total number of positive test results [incl. misclassified]}}$$

Note: different sectors use different definitions here.

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. For example, 100 positive test results obtained from 100 independent measurements on truly positive samples lead to the conclusion that the level of false negative results is below 4.5% at a confidence level of 99% for the tested concentration.

Ruggedness

6. As with any validated method, reasonable efforts should be made to demonstrate the ruggedness of the assay. This involves careful optimisation and investigation of the impact of small modifications made to the method due to technical reasons.

Applicability

7. In the case of qualitative PCR methods that use gels as the data delivery method, it is advisable to operate the methods at levels well above the LOD if possible, to ensure that the data interpretation is easy and as objective as possible.

REFERENCES FOR ANNEX V

AOAC (2002). Official Methods Program Manual, Appendix X p14f. *AOAC International* [On line] http://www.aoac.org/vmeth/omamanual/htm.

ANNEX VI: VALIDATION OF A PROTEIN-BASED METHOD
QUANTITATIVE TESTING

1. Quantitative immunoassays are used to determine levels of the target protein analyte in food products or specific raw materials for food production. In order to perform any immunological detection method such as a microplate ELISA for quantitative determination of a protein analyte in food samples or edible tissues, it is first necessary to obtain a representative sample of the target matrix material. The sample amount and procedure to prepare test portions will influence the detection limit or sensitivity of the assay. The analyte is then extracted from the sample by adding the appropriate liquid and blending, agitating, or applying sheering or sonic forces. Typical liquids used are water or buffered salt solutions. Sometimes surfactants or additives such as BSA (bovine serum albumin) are added according to the validated test and matrices. It is important that the extraction methods do not denature the proteins so as to make them unrecognisable by the antibodies, unless the assay is designed to detect denatured proteins.

2 The following description of the procedure is only one of several possibilities to carry out an immunological detection assay for proteins [of interest] [expressed in organisms derived from recombinant DNA].

3. In typical immunological detection assay for proteins, the optical density (OD) of the product of immunological reaction is measured. The standard curve is generated by plotting the 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.

4. To obtain an accurate and precise quantitative value, the OD for the sample solutions should pertain to the linear portion of the calibration curve. If the OD is too high, the sample solution should 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 microplate. 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.

5. 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

6. 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] [foods derived from modern biotechnology]. 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.

7. Where the positive and negative commodities are available, it is fairly straightforward to prepare a reference sample with a known proportion of the target material. In other cases, generating reference 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 negative control and positive reference materials in such a way as to achieve a homogeneous reference sample with a known amount of the protein. The stability of these materials would need to be evaluated under storage and test conditions. In any case, it is useful to have negative and positive reference materials available to use as negative and positive controls.

VALIDATION OF A QUANTITATIVE PROTEIN-BASED METHOD

8. The principles of method validation defined in the harmonized ISO/IUPAC/AOAC standard (Horwitz 1995) apply to both PCR and protein methods. ISO has developed specific international guidelines for validation of immunoassays for the detection and quantification of GM foodstuffs (ISO21572:2004). These guidelines are equally applicable to other foodstuffs.

9. Quantitative method validation parameters include accuracy/trueness, specificity, extraction efficiency, sensitivity, range of quantification, precision, ruggedness, applicability, practicability and parallelism (Grothaus *et al.*, 2006).

10. Accuracy: Accuracy is demonstrated by measuring the recovery of analyte from fortified samples and is reported as the mean recovery at several levels across the quantitative range. Ideally, quantitative methods will have demonstrated recoveries between 70 and 120% and a coefficient of variation (CV) of less than 20% for measured recoveries at each fortification level (Mihaliak & Berberich, 1995).

11. The recovery of proteins [expressed in organisms derived from recombinant DNA] 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.

Recommendation: The Recovery should be between 70 and 120%.

12. Repeatability standard deviation (RSDr)

Recommendation: The relative repeatability standard deviation should be ≤25% or as close as is practicable over the whole dynamic range of the method.

13. Reproducibility standard deviation (RSDR)

Recommendation: The relative reproducibility standard deviation should be below 35% or as close as is practicable at the target concentration and over the majority of the dynamic range. $RSD_R \leq 50\%$ *or as close as is practicable at the limit of quantification lower end.*

Extraction efficiency

14. 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 (Stave, 1999).

15. Precision: Intra-assay precision describes how much variation occurs within an assay. It can be evaluated by determining the variation (% CV) between replicates assayed at various concentrations on the standard curve and on the pooled variation (% CV) 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 % CV (Rogan *et al*, 1999).

16. Dilution agreement or parallelism 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 CV of the adjusted results from several dilutions of a single sample extract should ideally be ≤ 20%.

Sensitivity

17. The sensitivity of the assay could be defined as the amount of analyte that can be measured by an absorbance reading of two standard deviations above background absorbance (Rogan *et al*, 1992). The detection limit could be expressed as the lowest dilution of the protein of interest that can be detected when combined with the rest of protein extracted from the sample (Rogan *et al.*, 1999).

Dynamic Range - Range Of Quantification

18. The scope of the methods defines the concentration range over which the analyte will be accurately determined. This desired concentration range defines the standard curves and a sufficient number of standards should be used to adequately define the relationship between concentration and test's response. The relationship between response and concentration should be demonstrated to be continuous, reproducible and should be linear after suitable transformation.

19. Quantitative protein methods generally give an estimate of the concentration of the protein of interest in the matrix . For GMO the interpretation of the percentage values (e.g. dynamic range from 10% to 500% the target value) can be difficult when using quantitative methods, due to variations in the expression of the amount of protein in different tissues of plants or among cultivars, and within the same tissue at different locations. Care should be taken to employ a method which can detect the specific protein in the analyzed matrix. For example, it is believed that proteins undergo modification or degradation due to processing to a greater degree than DNA, and thus loss of signal due to food processing effects should be considered.

20. 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 g/kg.

Limit of Detection (LOD)

21. LOD is defined in annex II. Proteins are usually present in foods [derived from modern biotechnology] at higher concentrations than the target DNA for PCR methods. Thus stochastic effects have less influence on the determination of the LOD than when using PCR.

22. 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.

Limit of Quantification (LOQ)

23. For a quantitative method, it is important to know whether the LOQ for a particular matrix is close to the values to be measured. Using the traditional approach, the LOQ can be expressed as the signal strength of a blank equal to the LOD increased by 6-10 times the standard deviation of the blank, unless it is known from other sources that the measured values range so high above the LOQ that its knowledge becomes irrelevant. However, this method to determine the LOQ leads only to an estimate of the true LOQ that may be an artificially high or low approximation.

24. 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 and percent recovery of the analyte meet certain preset criteria. For small molecules, these criteria have typically been a RSDr of ≤20% and 70-120% recovery (Mihaliak & Berberich, 1995). Protein recovery, however, may be difficult from some matrices, e.g. starches or oils, and lower recovery efficiencies may have to be accepted. When recovery 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 target. This is more complicated as it requires access to significant quantities of reference materials that contain a known range of concentrations of the [protein of interest] [foods derived from modern biotechnology]. Procedures for assessing LOD and LOQ during the validation of quantitative PCR methods are also discussed in annexes III and IV.

Cross-reactivity

25. The cross-reactivity or assay specificity 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. Cross-reactivity should be demonstrated by showing experimental results from testing the method with proteins or molecules from non-target and closely related species/variety, and purified target protein and/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

26. 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 free analyte matrix, This would ensure that any matrix effects would be consistent between the standards and the samples.

Ruggedness (Robustness)

27. The evaluation of ruggedness (robustness) demonstrates the reliability of a method with respect to inadvertent variation in assay parameters. Variations that may be included are reaction volumes incubation temperature (e.g., plus and minus 5-10^{o}C) and/or other relevant variations. The experiments need to be performed at least in triplicates 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. Experiments which may be performed to establish ruggedness include repeated analysis of a sample or samples on several days and measurement of trueness and precision in fortified samples using control material from several sources.

QUALITATIVE (THRESHOLD) TESTING

28. Lateral flow devices are useful tools for on-site or field threshold testing. Traditional ELISA methods can also be used for qualitative testing. In order to ensure reliable results, the manufacturers of such assays should conduct a method validation and provide a description of the performance characteristics of the product in the package insert, including sensitivity, specificity, applicability, and hook-effect. If this has been completed there is generally no need for validation studies to be performed by users of Lateral Flow devices for implementation of the technique within their laboratory as long as the method is performed according to the manufacturer's instructions. Each lateral flow device is an individual stand-alone unit, capable of performing to the standards described in the product package insert according to the quality assurance scheme of the provider. For ELISA methods, validation should be carried out to ensure that the method performs as expected in the individual laboratory.

29. In order to establish an on-site procedure for threshold testing, the threshold level should first be established. To establish that the lateral flow device is able to differentiate between samples containing target protein above or below the threshold, both a negative reference and a threshold reference containing a known proportion of matrix with target protein should be assayed concurrently. The negative reference is a sample of the test matrix known to contain none of the protein analyte and is assayed to demonstrate that the method can distinguish between zero and the threshold level. A sufficient number of these samples (e.g. USDA, 2004) are run to ensure that assay sensitivity is adequate to determine whether the level in the test sample is greater or less than the threshold level. During routine testing of bulk commodity samples, the lateral flow devices would typically be used without running the concurrent negative and threshold reference samples.

VALIDATION OF A QUALITATIVE PROTEIN-BASED METHOD

30. 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

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

32. 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. In the case of an ELISA method (for example) it should show reasonable precision and low bias, and should be shown to be repeatable with a reasonable RSD_R. The criteria for determination of the Limit of Detection (LOD) should be established for the method. The LOD of the assay can be determined by absorbance readings on a number of extracts known not to contain the target protein (zero dose replicates), or experimentally using samples with known amounts of the target protein (Keith et al., 1983). In addition it should be possible to establish a standard curve using the specific protein in the matrix that is being tested. For new matrices, a sample of each matrix fortified with the specific protein can be used to establish the efficiency of recovery and used to estimate the Limit of Quantification (LOQ) (Keith et al., 1983). Proving extractability of a protein is difficult and this can be established by repeated extractions (for relatively stable proteins), or by use of incurred samples with well known characteristics.

33. For determination of 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

34. The practicability of the method should be demonstrated.

35. The same types of control samples, and criteria for acceptance/rejection of the result can be used as for qualitative PCR methods. The LOD is expressed as 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). However, lateral flow strip tests are generally applied at test concentrations that are at least two fold (or more) above the LOD.

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