Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing

European Network of GMO Laboratories (ENGL)

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INTRODUCTION

The scope of this European Network of Genetically Modified Organism Laboratories (ENGL) document is to provide recommendations on how methods for genetically modified organism (GMO) analysis shall be evaluated and validated by the Community Reference Laboratory for Genetically Modified Food and Feed (CRL-GMFF) in the context of Commission Regulation (EC) No.1829/2003[1)].

There is synergy between recommendations made within this document and those of the Codex Alimentarius Commission[2)].

Reliable analytical methods are required for compliance with national and international regulations in all areas of analysis[3)]. It is internationally recognised that a laboratory must take appropriate measures to ensure that it is capable of providing and does provide data of the required quality. Such measures include:

- using validated methods of analysis;
- using internal quality control procedures;
- participating in proficiency testing schemes; and
- becoming accredited to an International Standard, normally ISO/IEC 17025[4)].

Method validation is therefore an essential component of the measures that a laboratory should implement to allow it to produce reliable analytical data. In some sectors, most notably in the analysis of food, the requirement for methods that have been "fully validated" is prescribed by legislation[5)]. 'Full' validation for an analytical method is usually taken to comprise an examination of the characteristics of the method in an inter-laboratory method performance study (also known as a collaborative study or collaborative trial). Internationally accepted protocols have been established for the 'full' validation of a method of analysis by a collaborative trial, most notably the International Harmonised Protocol[6)] and the ISO procedure[7)]. These protocols/standards require a minimum number of laboratories and test materials to be included in the collaborative trial to validate fully the analytical method.

As laid down in the Annex of Commission Regulation (EC) No. 1829/2003[1)] as amended by Commission Regulation (EC) No 1981/2006[8)], and Annex 1 of Commission Regulation (EC) No. 641/2004[9)] the CRL-GMFF is responsible for the evaluation and validation of methods of analysis in the context of GMO authorisation under Commission Regulation (EC) No. 1829/2003[1)]. The CRL-GMFF is assisted by the ENGL.

The ENGL recommends that the assessment, by the CRL-GMFF, of methods submitted by the applicants as part of the GMO authorisation process is undertaken in two distinct phases:

Phase 1 - evaluation of the method performance data submitted by the applicant as part of the official dossier; and

Phase 2 - evaluation of method performance data following a full validation study by collaborative trial.

At present this document refers to polymerase chain reaction (PCR) based analytical methods for the determination and quantification of GMOs. However, if novel methods (e.g. those using technologies other than PCR) are subsequently developed that fulfil legal requirements then this document will be amended accordingly.

The ENGL recommends that applicants, where possible, utilise already published/validated protocols (e.g. DNA extraction method, reference genes, etc….) within methods supplied as part of the official GMO authorisation process.

In cases of submission of an already fully validated method(s) for which single lines have been previously authorised under Commission Regulation (EC) No. 1829/2003[1)], the assessment of such method(s) is performed according to parameters described in Annex 1.

PHASE ONE - METHOD ACCEPTANCE CRITERIA

This section defines method performance criteria, hereafter “acceptance criteria” to be met before a method is considered ready to enter the full validation process (i.e. Phase 2).

The applicant shall supply the CRL-GMFF with:

- evidence that the submitted method fulfils the general principle conditions provided in Annex 1 of Commission Regulation (EC) No. 641/2004[9)]. The method shall be event-specific and thus must only be functional with the GMO or GM based product considered and shall not be functional if applied to other events already authorised; otherwise the method cannot be applied for unequivocal detection/identification/quantification; and
- evidence that the submitted method meets the acceptance criteria indicated below. Such evidence will include supporting experimental data together with an indication of the reference values and experimental design chosen by the applicant during method testing and optimisation.

Applicability

Definition: The description of analytes, matrices and concentrations to which the method can be applied.

Acceptance Criterion: The applicability statement should provide information on the scope of the method and include data for the indices listed below for the product/s for which the application is submitted. The description should also include warnings to known interferences by other analytes, or inapplicability to certain matrices and situations.

Practicability

Definition: The ease of operations, the feasibility and efficiency of implementation, the associated unitary costs (e.g. cost/sample) of the method.

Acceptance criteria: The method should generally be practicable in line with other methods for a similar purpose. More specifically the method is deemed unacceptable, unless suitable justification is supplied, if:

- it requires a new type of apparatus (not generally available) or expensive equipment; or
- the resources required to perform the method (time, workload, reagents, costs) are considerably higher than the resources required to perform other methods for similar purpose.

Other practicability considerations may also deem the method impracticable.

DNA Extraction and Purification

The aim of a DNA extraction procedure is to provide DNA of suitable quality for subsequent analysis. DNA quality depends on the average length, structural integrity and chemical purity of the extracted DNA.

It is recognised that highly fragmented DNA and co-extracted impurities of a DNA preparation may hinder the correct process of detecting and quantifying genetically modified DNA. Food and feed made of various ingredients may exert a matrix effect, depending on the DNA extraction method employed, and impair the sensitivity of the following analytical approach. For this purpose, critical steps of DNA extraction and purification should be clearly highlighted in the technical documentation accompanying a method and acceptance criteria are established to allow objective determination of PCR quality of DNA extracts which can be considered suitable for subsequent detection experiments (e.g. qualitative and/or quantitative PCR).

DNA extraction procedures should result in repeatable recovery, fragmentation profile, concentration and PCR quality of DNA extracts. As such, it is recommended to process the given DNA extraction protocol on different days (e.g. 3 days) with an adequate number of test portions (e.g. 6 per day).

In agreement with international guidelines (e.g. EN ISO 21571[10)], EN ISO 24276[11)]) the following criteria are used to assess method performance.

a) DNA concentration

Definition: amount of an analyte per unit volume of solution

Acceptance criterion: The DNA extraction method employed shall be appropriate to obtain the quantity of nucleic acid required for the subsequent analysis. The DNA concentration measured as weight of the analyte/volume of solution should be higher than the working concentration described in the protocol of the detection method.

Example: if the RT-PCR protocol indicates 40 ng/µL as the DNA concentration of the DNA solution to be added to the master-mix, the concentration of the DNA extract should be > 40 ng/µL.

b) DNA fragmentation state

Definition: Breakage of genomic (high molecular weight) DNA into smaller DNA fragments

Acceptance criterion: For quantitative (real time-based) analysis, the molecular weight of the extracted DNA sample should be at least higher than the amplicon size produced by

the event specific and the taxon specific reference systems as established by comparison with a reference nucleic acid marker.

For qualitative analysis, in case of DNA suspensions to be used in qualitative analysis, the presence of a certain proportion of DNA molecules of molecular weight lower than the amplicon size produced by the method may be considered acceptable.

c) Purity of DNA extracts

Definition: the absence of co-extracted compounds in a DNA sample impairing the efficiency of the PCR reactions and leading to a delay in the onset of the exponential phase of the amplification profile

Acceptance criterion: The difference (ΔCt) average between the measured Ct value and the extrapolated Ct value of the first diluted sample of the inhibition test should be <0.5. [(measured Ct – extrapolated Ct)] <0.5 and the slope of the inhibition curve should be within -3.6 and -3.1.

The preferred PCR assay for the inhibition test is the internal control assay (e.g. the taxon specific reference system). The total DNA amount in the first sample of the dilution series should be not less than the total DNA amount used in the submitted method (e.g. the DNA amount indicated in the PCR protocol of the taxon specific reference system).

Specificity

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

Acceptance Criterion: The method should not produce amplification signals with target sequences different for the target sequence for which the method was developed. This should be demonstrated by similarity searches against databases (e.g. EMBL, GenBank, Patent, etc.) and with empirical results from testing the method with non-target transgenic events and non-transgenic material.

For detection of specific GM events, the target sequence shall be event specific.

For taxon specific target sequences (target sequence), the absence of allelic and copy-number variation across a globally representative and diverse sample of the species variety shall be demonstrated. Allelic and/or copy-number variation in other lines shall be reported if such variation is known by the applicant. The specificity of the target sequence shall be verified by *in silico* studies against publicly available sequence databases (e.g. EMBL, GenBank, etc.) and experimentally by demonstrating the absence of amplification products when the target sequence specific assay is applied to individual PCRs of pure genomic DNA of a representative sample of the closest relatives to the target taxa as well as of the most important food crops.

Dynamic Range

Definition: The range of concentrations over which the method performs in a linear manner with an acceptable level of trueness and precision.

Acceptance Criterion: The dynamic range of the method should include the 1/10 and at least 5 times the target concentration. Target concentration is intended as the threshold relevant for legislative requirements. The range of the standard curve(s) for real-time PCR should allow testing of blind samples throughout the entire dynamic range, including the lower (10%) and upper (500%) ends.

Example: 0.09% and 4.5% for a 0.9% GMO concentration or 50 and 2500 genome copies if the target is 500 copies.

Trueness

Definition: The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

Acceptance Criterion: The trueness shall be within ±25% of the accepted reference value over the whole dynamic range.

Amplification Efficiency

Definition: The rate of amplification that leads to a theoretical slope of –3.32 with an efficiency of 100% in each cycle. The efficiency of the reaction can be calculated by the following equation:

$$Efficiency = 10^{\left(\frac{-1}{slope}\right)} - 1$$

Acceptance Criterion: The average value of the slope of the standard curve shall be in the range of (-3.1 ≥ slope ≥ -3.6)

R^2 Coefficient

Definition: The R^2 coefficient is the correlation coefficient of a standard curve obtained by linear regression analysis.

Acceptance Criterion: The average value of R^2 shall be ≥0.98.

Precision - Relative Repeatability Standard Deviation (RSD_r)

Definition: The relative 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.

Acceptance Criterion: The relative repeatability standard deviation should be ≤25% over the whole dynamic range of the method.

Note: Estimates of repeatability submitted by the applicant should be obtained on a sufficient number of test results, at least 15, as indicated in ISO 5725-3[7)].

Limit of Quantification (LOQ)

Definition: The limit of quantification is the lowest amount or concentration of analyte in a sample that can be reliably quantified with an acceptable level of precision and accuracy.

Acceptance Criterion: LOQ should be less than 1/10th of the value of the target concentration with an RSD_r ≤25%. Target concentration should be intended as the threshold relevant for legislative requirements.

Example: For a 0.9% nominal value LOQ <0.09%.

Limit of Detection (LOD)

Definition: The limit of detection is the lowest amount or concentration of analyte in a sample, which can be reliably detected, but not necessarily quantified, as demonstrated by single-laboratory validation.

Acceptance Criterion: LOD should be less than 1/20th of the target concentration. Experimentally, quantitative methods should detect the presence of the analyte at least

95% of the time at the LOD, ensuring ≤5% false negative results. Target concentration should be intended as the threshold relevant for legislative requirements.

Example: For a 0.9% nominal value LOD <0.045%.

Robustness

Definition: The robustness of a method is a measure of its capacity to remain unaffected by small, but deliberate deviations from the experimental conditions described in the procedure.

Note: 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 shall 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 concentrations.

Acceptance Criterion: The response of an assay with respect to these small changes shall not deviate more than ±30%.

Alternatively, robustness can be demonstrated through the application of formal robustness tests using factorial designs such as those published by Plackett Burman[12)] or Youden[13)].

PHASE 2 - METHOD PERFORMANCE REQUIREMENTS

The purpose of a collaborative trial is to verify the transferability and performance of a method among laboratories, according to the principles of either the IUPAC harmonised Protocol[6)] or ISO 5725[7)]. This section sets and describes the criteria according to which the results of collaborative trials are evaluated.

Precision - Relative Reproducibility Standard Deviation (RSD_R)

Definition: The relative 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. Reproducibility standard deviation describes the inter-laboratory variation.

Acceptance Criterion: The relative reproducibility standard deviation RSD_R should be <35% over the whole dynamic range. However, at concentrations <0.2% then RSD_R values <50% are deemed acceptable.

Trueness

Definition: The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value. The measure of trueness is usually expressed in terms of bias.

Acceptance Criterion: The trueness should be within ±25% of the accepted reference value over the whole dynamic range.

REFERENCES

1) Commission Regulation (EC) No 1829/2003 of the European Parliament and Council of 22 September 2003 on genetically modified food and feed.

2) FAO-WHO. 2005. Codex Alimentarius Commission. Codex committee of methods of analysis and sampling: consideration of the methods for the detection and identification of foods derived from biotechnology. General approach and criteria for the methods. 26th Session, Hungary.

3) Thompson, M., S.L.R. Ellison, R. Wood. 2002. Harmonised guidelines for single-laboratory validation of methods of analysis. Pure Appl. Chem.74: 835-855.

4) International Standard (ISO) 17025, 2005. General requirements for the competence of testing and calibration laboratories. International Organisation for Standardisation, Genéve, Swizerland.

5) Commission Regulation (EC) No 882/2004 of the European Parliament and Council of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules.

6) IUPAC Protocol for the Design, Conduct and Interpretation of Method Performance Studies, Pure & Appl. Chem., 67, 331-343, 1995.

7) International Standard (ISO) 5725. 1994. Accuracy (trueness and precision) of measurement methods and results. International Organization for Standardisation, Genéve, Swizerland.

8) Commission Regulation (EC) No 1981/2006 of 22 December 2006 on detailed rules for the implementation of Article 32 of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the Community reference laboratory for genetically modified organisms.

9) Commission Regulation (EC) No 641/2004 of 6 April 2004 on detailed rules for the implementation of Regulation (EC) No 1829/2003 of the European Parliament and of the Council as regards the application for the authorisation of new genetically modified food and feed, the notification of existing products and adventitious or technically unavoidable presence of genetically modified material which has benefited from a favourable risk evaluation.

10) EN ISO 21571, Foodstuffs – Method of Analysis for the detection of genetically modified organisms and derived products – nucleic acid extraction, 2005.

11) EN ISO 24276, Foodstuffs - Nucleic acid based methods of analysis for the detection of genetically modified organisms and derived products - General requirements and definitions, 2006.

12) R.L. Plackett and J.P. Burman, "The Design of Optimum Multifactorial Experiments", Biometrika 33 (4), pp. 305-25, June 1946.

13) Statistical Manual of the AOAC, W.J. Youdens and E.H. Steiner, 1987.

ANNEX 1 – METHOD VERIFICATION CRITERIA

This Annex sets and describes the criteria to be met when an already fully-validated method is considered ready to enter the CRL-GMFF validation process. These criteria, hereafter "*Method Verification Criteria"*, are used to evaluate if the performance of a method is sufficiently satisfactory in order to proceed with the CRL-GMFF method verification process.

As part of the official dossier, the applicant shall provide:

- evidence that the submitted method fulfils the general principle conditions provided in Annex 1 of Commission Regulation (EC) No 641/2004[9)]: the method should detect and quantify the specific GM event in a product; it should be event specific and applicable to the notified (or otherwise relevant as determined by the CRL-GMFF) material as well as to the samples of the food and feed, and their control samples;
- evidence that the submitted method meets the acceptance criteria indicated below. Such evidence will include the supporting experimental data and all the necessary information, together with indication of the reference values and experimental design chosen by the applicant during method testing and optimisation; and
- reference to previous application(s) including details and relevant information from the detection method to be referred to.

In this section the characteristics and their definition used for method evaluation refer to those described and defined within Phase 1.

For the assessment of the following characteristics the applicant should refer to previous application(s) for which the detection method has been positively evaluated:

Applicability

Practicability

Specificity

Limit of Detection (LOD)

Robustness

The following characteristics shall be evaluated by the applicant against the samples of the food and feed, and control samples related to the new application. The evaluation shall be performed as described in sections dealing with Phase 1:

Dynamic Range

Trueness

Amplification Efficiency and R^2 Coefficient

Repeatability Standard Deviation (RSD_r)

Limit of Quantification (LOQ)