Verification of analytical methods for GMO testing when implementing interlaboratory validated methods

Guidance document from the European Network of GMO laboratories (ENGL)

Prepared by the ENGL working group on “Method Verification”
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The working group and its mandate

The working group on method verification was established on the basis of a mandate first given by the 1st CRL-GMFF (now EURL-GMFF) NRL workshop 28-29th of May 2009 and later adopted by the ENGL steering committee on 19-20th of November 2009. The working group has been chaired by Lotte Hougs, Danish Plant Directorate, (Pdir) Copenhagen, Denmark and Jana Zel, National Institute of Biology (NIB), Ljubljana, Slovenia. The other members of the working group have been: Chrystele Charles-Delobel, Joint Research Centre (JRC); Malcolm Burns, LGC, United Kingdom; Diana Charels, Joint Research Centre (JRC); Ilaria Ciabatti, Istituto Zooprofilattico Sperimentale delle Regioni Lazio e Toscana, Italy; Encarnacion Luque-Perez, Joint Research Centre (JRC); Joachim Mankertz, Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL), Germany; Marco Mazzara, Joint Research Centre (JRC); Frank Narendja, Umweltbundesamt, Austria; Martin Sandberg, NFA-National Food Administration, Uppsala, Sweden; Manuela Schulze, LAVES, Germany; Cristian Savini, Joint Research Centre (JRC); Ingrid Scholtens, RIKILT - Institute of Food Safety, The Netherlands and Thomas Weber, Joint Research Centre (JRC).

The mandate of the working group was as follows:

- **The scope of the working group is to make a guideline paper concerning Implementation/Adaption of pre-established validated methods in (National) control laboratories.**

- **The paper should give guidelines on how to introduce and verify a validated method in the laboratory in a way that easily can be copied and implemented in practice. The guidelines must reflect the demands in the “Definition of minimum performance requirements for analytical methods of GMO testing” version 13/10/2008. The guidelines are intended for laboratories under ISO 17025 accreditation by a broad range of national accreditation bodies. The “Flexible Scope” of accreditation must be considered in the guidelines. It should be expected that the laboratories are using a modular approach in their choice of methods. The guidelines must, if possible, discuss the impact of different instrumentation, PCR reaction mixes, primer concentrations, etc. The guidelines must be in line with other ENGL guidelines published in relation to GMO testing.**

- **The guidelines must give a number of examples on how to experimentally solve specific parts of the verification. The methods addressed will primarily be quantitative real time PCR methods validated by the EURL-GMFF. If possible, other real time PCR methods should be covered also.**
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Introduction

Regulation (EC) No 882/2004\(^{(3)}\) provides that official laboratories shall be accredited according to the ISO 17025\(^{(4)}\) standard. An ISO 17025 accreditation, under a fixed or flexible scope, implies that “the laboratory shall confirm that it can properly operate standard methods before introducing the tests or calibrations”. If the standard method changes, the confirmation shall be repeated (ISO 17025:2005 section 5.4.2).

In turn, still according to the same Regulation (EC) No 882/2004\(^{(3)}\) it is the task of the European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF) to provide national reference laboratories with details of analytical methods, including reference methods.

Note: in November 2010, the ENGL and the EURL GMFF have published a "Compendium of reference methods for GMO analysis" which includes 79 DNA-based detection methods that have been validated through a collaborative trial according to ISO5725 and/or the IUPAC protocol.

A new method evolves through a number of steps. After the initial development and optimisation phases, the developing laboratory performs an in-house validation on the method to ensure that the method is fit for the intended purpose. Before the method can be accepted as an International Standard, it needs to be validated in a number of laboratories\(^{(1,2)}\).

In the EU, method validation is an essential part of the process that regulates the introduction of new GMOs as food and/or feed into the market. The European Regulation laying down the methods of sampling and analysis for the official control of feed as regards the presence of genetically modified material for which an authorisation procedure is pending or the authorization of which has expired (so-called Low Level Presence (LLP) regulation) that was adopted in February 2011 also confirms that event-specific quantitative detection methods that are validated by the EU-RL GMFF need to be used.

When this inter-laboratory validation study is completed, the method is ready to be implemented in routine testing laboratories. When implementing the new method, the laboratory has to verify that the method can be used for its intended purpose (method verification). The scope of this document is to provide guidance on how to carry out the method verification.

In GMO detection laboratories qualitative and quantitative methods with different levels of specificity are used (e.g. genetic element-, construct-, or event-specific). For the detection of GMOs in food and feed products, the event-specific detection methods provided in applications for authorisation in the frame of Regulation (EC) No 1829/2003, are validated by the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL-GMFF) in collaboration with the ENGL. The validation is performed according to internationally recognized guidelines\(^{(1,2)}\) through collaborative studies. Data from the inter-laboratory validation process are evaluated according to the document “Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing”\(^{(5)}\) (MPR).

Despite the fact that several guidelines on method verification have been published\(^{(6,7)}\), no specific guidelines are available for GMO detection. Peer-reviewed papers have been published on verification of GMO detection methods in accredited laboratories\(^{(8,9,10)}\). The aim of this guidance document is to harmonise the in-house verification of inter-laboratory validated methods for the qualitative and quantitative detection of GMOs, including element-, construct-, and event-specific methods. Considering that the Polymerase Chain Reaction (PCR) is the method of choice in the EU for the
identification and quantification of GMOs, this document refers exclusively to real time PCR. However, if novel methods are subsequently developed that fulfill legal requirements, then this document will be amended accordingly.

The inter-laboratory validation of methods is not within the scope of this document.

The principles of the modular approach\(^{(11)}\) have been taken into account in this document.

Procedures for the calculation of measurement uncertainty are explained in the Guidance document on measurement uncertainty for GMO testing laboratories\(^{(12)}\) and will not be included further in this document.

**Terminology**

*Validation of method*
Validation is the confirmation by examination and provision of objective evidence that the particular requirements for a specific intended use are fulfilled (ISO 17025 section 5.4.5.1).

*Verification of method*
Verification is the confirmation, through the provision of objective evidence, that specified requirements have been fulfilled [ISO 9000:2000 section 3.8.4]. Verification that a laboratory can adequately operate a standard method requires that the laboratory provides objective evidence that the performance parameters specified in the test method have been met for the sample matrices to which the method is being applied. Most often, the critical requirements are the trueness (generally expressed in terms of bias\(^{(1)}\)) and the precision (generally accepted as repeatability and reproducibility) which are reflected in the measurement uncertainty. The objective evidence are the trueness and precision obtained from actual laboratory data\(^{(7)}\).

*Precision – Relative Repeatability Standard Deviation (RSDr)*
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.

*Laboratory sample (see also Fig. 1)*
Sample as prepared for sending to the laboratory and intended for inspection or testing \(^{(13)}\).

*Analytical sample*
Sample prepared from the laboratory sample by grinding, if necessary, and homogenisation\(^{(14)}\).

*Test portion*
Sample, as prepared for testing or analysis, the whole quantity being used for analyte extraction at one time\(^{(13)}\).

*Test result*
A test result is a Ct value or copy number originating from a PCR replicate

*DNA extraction replicates (as used in this document)*
DNA extracted from different test portions from the same analytical sample.

*PCR replicates (as used in this document)*
PCR performed on the same DNA extraction replicate analysed in different reaction wells.
**Working dilution**
The highest DNA concentration intended to be used in the subsequent PCR method.

**Limit of quantification (LOQ)**
LOQ is the lowest amount or concentration of analyte in a sample, which can be reliably quantified with an acceptable level of precision and trueness.

**Practical limit of quantification (practical LOQ)**
The practical LOQ is the lowest relative quantity of the target DNA that can be reliably quantified, given a known (determined/estimated) number of target taxon genome copies.

**Limit of detection (LOD)**
LOD is the lowest amount or concentration of analyte in a sample, which can be reliably detected but not necessarily quantified. Experimentally, methods should detect the presence of the analyte at least 95% of the times at the LOD, ensuring ≤5% false negative results.

**Practical limit of detection (practical LOD)**
The practical LOD is the lowest relative quantity of the target DNA that can be reliably (e.g. with ≥ 95% probability) detected, given a known (determined/estimated) number of target taxon genome copies.

**Specificity**
The property of the method to respond exclusively to the characteristic or the analyte of interest.

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

**Trueness**
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.

**Amplification efficiency**
The rate of PCR amplification that leads to a theoretical slope of -3.32 with an efficiency of 100% in each cycle. The efficiency (in %) can be calculated by the following equation:

\[
\text{Efficiency} = (10^{(-1/\text{slope})}-1)\times100
\]

**R² coefficient**
R² is the coefficient of determination, which is calculated as the square of the correlation coefficient (between the measured Ct-value and the logarithm of the concentration) of a standard curve obtained by linear regression analysis.

**Robustness**
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.
Figure 1: Illustration of replicates terminology

**Test portions**
Sample, as prepared for testing or analysis, the whole quantity being used for analyte extraction at one time.

**DNA extraction replicates**
DNA extracted from different test portions from the same analytical sample.

**PCR replicates**
PCR performed on the same DNA extraction replicate analysed in different wells.
General considerations

An accredited laboratory shall have a management system in place to provide objective evidence that the personnel is adequately qualified and regularly trained to perform the analysis (ISO/IEC 17025:2005 section 5.2). In addition, a metrology system shall ensure that the equipment used is periodically calibrated (ISO/IEC 17025:2005 section 5.5). When an inter-laboratory validated method is used by an accredited laboratory, the laboratory must, prior to its use, ensure that the chosen method shows performance characteristics similar to those assessed in the inter-laboratory study. The verification process must be documented and recorded in the quality system.

The laboratory must record the procedure used, the results obtained and a statement as to whether the method is fit for the intended use e.g.:
- Design and planning of the verification;
- Description of the method;
- Acceptance criteria and performance requirements, as decided by the laboratory;
- Test records;
- Approval of the method.

As inter-laboratory validated event-specific methods are assessed according to the acceptance criteria and performance requirements described in the document MPR(5). This document can be more generally used as a basis for assessing the results of a method in a verification process. The methods are available in the JRC reference report “Compendium of reference methods for GMO analysis”(15) as well as on: http://gmo-crl.jrc.ec.europa.eu/. The following sections describe the parameters to be studied for the verification of validated methods for the detection of GMOs. During the verification process, a laboratory should ensure compliance to the requirements described in the following standards: ISO 24276(13), ISO 21568(14), ISO 21570(16), ISO 21569(17) and ISO 21571(18).

As a matter of principle, a method should be implemented as validated in inter-laboratory trial, without introducing modifications. If single elements like e.g. the brand of ready-to-use reaction mix or Taq polymerase, the PCR reaction volume, the primer and probe concentrations, and/or PCR cycling parameters are modified, additional parameters should be experimentally assessed (e.g. specificity and robustness).

When the method is only qualitative, or employed as qualitative determination, it is not necessary to verify the LOQ, trueness or precision.

DNA extraction and purification

The DNA isolation method should provide DNA of suitable quality and quantity for subsequent analysis. Although this document focuses on the verification of PCR methods, the evaluation of DNA extraction methods is a crucial step, as the quality and quantity of DNA extracted may significantly affect the final result.

Methods for DNA extraction can be inter-laboratory validated methods, single-laboratory validated methods, standardised methods (e.g. ISO) and/or other available methods.

Procedure: If the DNA extraction method was previously validated, the DNA extraction is carried out at least twice (three times recommended) each time on 2 test portions, if possible on different days and with different operators. The DNA extractions have to meet the acceptance criteria for DNA
concentration and quality (e.g. by controlling amplification efficiency and presence of inhibitors by real-time PCR, see annex 2).

DNA extraction methods applied to one matrix may not be suitable for other matrices. This procedure may need to be carried out on different matrices. For the verification of a DNA extraction method the tested matrix does not necessarily have to contain GMO.

DNA concentration

Procedure: The concentration of the DNA extracts should be measured by the method applied to the routine samples.

Acceptance criterion: In the verification process, when a DNA extraction method is applied to the same matrix of the validation study, the yield should be comparable to the results obtained in that study. The method should provide DNA in an appropriate yield for the intended analysis (at least enough to meet the desired practical LOD/LOQ).

If a DNA extraction method does not give an appropriate yield for the intended analysis on a particular matrix, the practical LOD will be affected (Annex 1).

Absence of inhibitors in the DNA extracts

Procedure: Each DNA extraction replicate is diluted to a working dilution (e.g. 40 ng/μL). From this working dilution, a dilution series of e.g. four points are analysed using real-time PCR (at least two PCR replicates per dilution). A calibration curve is obtained from these four dilutions.

The preferred PCR assay for the inhibition test is the reference gene assay (e.g. the taxon-specific reference system). The total DNA amount in the working dilution should be at least the same as the total DNA amount intended to be used in the verification process and in routine analysis (e.g. the DNA amount indicated in the PCR protocol of the taxon-specific reference system).

Acceptance criterion: The average difference (ΔCt) between the measured Ct value and the extrapolated Ct value of the working dilution should be < 0.5 [(Measured Ct – extrapolated Ct) < 0.5] and the slope of the inhibition curve should lie between -3.6 and -3.1. An example for an inhibition assay is given in Annex 2.

If the extracted DNA contains inhibitors the DNA has to be further purified or diluted to the level where no inhibition of PCR reaction is observed, before it is used for real time PCR.

Specificity

Specificity should already have been investigated in the context of method validation. Therefore, the specificity does not need to be re-investigated in a verification study. In the case of screening methods targeting genetic elements common to several GM events, the method should be tested in-house (if this validation data are not available) for cross-reactivity with new GMO events placed on the market after the method has been taken into use. This can only be done when positive control materials are available for the new GMO events.
Dynamic range, $R^2$ Coefficient, and Amplification Efficiency

**Procedure**: Dynamic range, $R^2$ coefficient, and amplification efficiency are verified simultaneously from standard curves when testing other parameters, such as trueness and precision. The average values of at least two standard curves should be taken. (See Table 1 for details).

**Acceptance criterion for dynamic range**: The dynamic range must cover the values corresponding to the expected use. This can be expressed as GMO % or copy number range. 
**Example**: 0.09 % and 4.5 % for a 0.9 % GMO target concentration or 50 and 2500 genome copies if the target is 500 copies.

**Acceptance criterion for amplification efficiency**: For both, qualitative and quantitative methods, the average value of the slope of the standard curve shall be in the range of $-3.6 \leq \text{slope} \leq -3.1^{(5)}$, corresponding to an amplification efficiency of 90 - 110 %.

**Acceptance criterion for $R^2$ coefficient**: the average value of $R^2$ shall be $\geq 0.98$.

Trueness

**Procedure**: The trueness should be determined at a level close to the level set in legislation (e.g. 0.9 % threshold), or according to the intended use of the method, and additionally at a level close to the LOQ. The trueness can be measured using Certified Reference Material(s) (CRM), with at least two concentrations (e.g. 0.1 % and 1 %) and if possible a third one at the upper end of the dynamic range (e.g. 5 %). Alternatively, a reference sample (for example 1 %) can be made from a higher percentage CRM. Annex 3 provides a guideline for the preparation of such a reference sample.

Testing conditions (reaction volume, PCR machine, etc.) should be the same as during routine testing of samples. At least results from 16 PCR replicates should be obtained. Examples for possible test designs are shown in Table 1 and Figure 2 and 3.

Annex 4 provides guidelines for the calculation of mean, standard deviation and relative repeatability standard deviation of GMO-content of related and unrelated real time PCR replicates.

If CRMs for estimating the trueness are not available, a proficiency test material can be used or a Z-score in a proficiency test can provide an indication of the trueness of the method.

**Acceptance criterion**: The trueness shall be within $\pm 25 \%$ of the accepted reference value or a Z-score within the range of 2 and -2 should be obtained.

Relative Repeatability Standard Deviation (RSDr)

**Procedure**: Repeatability can be determined in a similar way as described under Trueness. It is calculated from PCR replicates run under repeatability conditions (see Terminology). Repeatability should be available for all tested GM-levels.

Testing conditions (reaction volume, PCR machine, etc.) should be the same as during routine testing of samples. At least 16 single test results should be obtained. Examples for possible test designs are shown in Table 1 and Figure 2 and 3.
Annex 4 provides guidelines for the calculation of standard deviation and relative repeatability standard deviation of GMO-content of related and unrelated real time PCR replicates.

**Acceptance criterion:** The relative repeatability standard deviation should be $\leq 25\%$, over the dynamic range of the method.

### Estimation of the Limit of Quantification (LOQ)

Relative LOQ (in %) and/or absolute LOQ (in copy numbers) can be determined.

**Procedure for Relative LOQ (LOQ\textsubscript{rel}):** A positive control material of e.g. 0.1 % can be analysed in 10 PCR replicates of the GM target and 10 replicates of the reference gene target. The RSD of the LOQ\textsubscript{rel} should be below 25 %. To establish the true LOQ\textsubscript{rel}, it will be necessary to make dilutions with a lower GM percentage.

**Procedure for Absolute LOQ (LOQ\textsubscript{abs}):** A dilution series of a known amount of a positive control material of e.g. 1 % can be measured in 10 PCR replicates (e.g. 80, 60, 40, 20, 10, 5 and 1 copies). The LOQ\textsubscript{abs} can be estimated as the last dilution in a series where the RSD of the measurements is below 25 %. The standard curve should cover the value of LOQ\textsubscript{abs}.

The probability distribution suggests that 1 copy should give approximately 30% negative results. Therefore, as a requirement to verify that the copy numbers of the dilution series are approximately correct, at least one replicate has to be negative for the 1 copy dilution.

The mean of the 10 PCR replicates may be a part of the standard curve as long as the standard curve still meets the acceptance criterion for $R^2$ and slope/efficiency. The rest of the standard curve only needs to have 2 PCR replicates per point.

**Acceptance criterion:** whenever validation data are available, the LOQ should be in line with (or better than) those data.

### Estimation of Limit of Detection (LOD)

Relative LOD (in %) and/or absolute LOD (in copy number) can be determined.

**Procedure for Relative LOD (LOD\textsubscript{rel}):** a positive control material of low GM concentration can be measured in 10 PCR replicates and if all replicates are positive, this infers that the LOD\textsubscript{rel} is below or equal to the positive control material level.

**Procedure for Absolute LOD (LOD\textsubscript{abs}):** to calculate the LOD\textsubscript{abs} of a method with 95% confidence it is necessary to analyse 60 PCR replicates for each tested concentration. As this may not be a practical approach, it is proposed to calculate the false negative rate on a lower number of replicates e.g. 10 replicates. The false negative rate has to be below 5% (i.e. all 10 PCR replicates have to be positive). This approach allows an approximate estimation of the LOD\textsubscript{abs}.

The definition of false negative rate is the probability that a known positive test sample is classified as negative by the method. An increase in the false negative rate is observed when the amount of analyte approaches the LOD of the method. The dilution series should be chosen so that it represents an interval above and below the expected LOD\textsubscript{abs} based on prior knowledge of the performance of that method (e.g. 20, 10, 5, and 1 copies). The lowest concentration where all 10 replicates are positive is the estimated LOD\textsubscript{abs}. The probability distribution suggest that 1 copy should give approximately 30% negatives. Therefore, as a requirement to verify that the copy numbers of the dilution series are
approximately correct, at least one replicate has to be negative for the 1 copy dilution. See also Table 1 for details.

Acceptance criterion: whenever validation data are available the LOD should be in line with those data. The practical LOD (LOD\textsubscript{prac}) is out of the scope of this document because it is sample and not method dependent. However, the LOD\textsubscript{prac} should be determined for each individual sample tested\textsuperscript{(13)}.

**Robustness**
Robustness should have been investigated previously during method development/optimisation, before the method was subjected to a collaborative trial. Therefore, the robustness does not need to be re-evaluated in a verification study.

**Relevant samples**
Laboratories would like to remain informed of methods for new GMOs becoming available on the market. However, at the time of the method verification usually only CRMs are available. Therefore, the verification process is usually conducted on CRMs. If these are not available, other GM positive material can be used. It can also be useful to additionally test a routine sample alone or spiked with reference material.
Table 1. Example of practical settings for the verification of a quantitative real time PCR method.

| 1. Optional: Preliminary test to define appropriate DNA concentrations | Test at least 3 target concentrations in the range of 300 ng – 0.1 ng (dependent on plant species)\(^{19}\).  
E.g. 300 ng Maize DNA corresponds to approx. 110 000 endogenes gene copies whereas 0.1 ng corresponds to approximately 37 copies. |
|---|---|
| 2. Dynamic range, R\(^2\) coefficient, and amplification efficiency | Example 1: 2 calibration curves minimum requirements  
5 calibration points with 3 PCR replicates each (triplicates)  
All slopes shall be in the range of -3.6 ≤ slope ≤ -3.1 and all R\(^2\) values should be ≥ 0.98.  
Example 2: 4 calibration curves;  
5 calibration points with 2 PCR replicates each (duplicates)  
average of 4 slopes and R\(^2\) are used to verify the acceptance.  
Example 3: 2 calibration curves;  
8 calibration points in 5 PCR replicates (pentaplicates) also covering the low concentrations for LOD and LOQ. Average of the part above LOQ for slope and R\(^2\) are used to verify the acceptance. |
| 3. Trueness, Precision, RSD\(_r\) | At least 2 GM levels (one around labeling threshold and one around LOQ, a third recommended to the upper part of the dynamic range)  
Example 1: 2 DNA extraction replicates per GM level, 2 PCR replicates per extraction/plate, 4 plates resulting in 16 test results and 8 GM-estimations per GM level* (Fig. 2)  
Example 2: 2 DNA extraction replicates per GM level, 4 PCR replicates per extraction/plate, 2 plates resulting in 16 test results and 4 GM-estimations per GM level* (Fig. 3)  
For estimation of the intermediate precision PCR runs are carried out at least on two different days by the same operator or, if possible, by an additional operator. |
| 4. LOQ, LOD | LOQ: 10 PCR replicates at a low concentration (e.g. 80, 60, 40, 20, 10, 5, and 1 copies). LOQ is the lowest concentration of a series where RSD of the copy number measurements are below 25% and the point is covered by the standard curve.  
LOD: 10 PCR replicates at a low concentration (e.g. 20, 10, 5, and 1 copies). LOD is then the lowest concentration in a series where all replicates are positive. |

*If based on experience, the laboratory can prove that the repeatability among two experienced operators is the same as the repeatability among repetitions of one person, it is not necessary to have the repetition done by another operator.
Note: It may be feasible to assess some of the parameters simultaneously in Table 1.

Note: For a single assay the standard curve and the samples have to be on the same plate. Two assays (e.g. endogene and transgene assay) can be performed on two different plates using the same dilutions of the samples and having a standard curve on each plate.

![Experimental design for Trueness/Precision (example 1)](image)

**Figure 2: Experimental design for Trueness/Precision (example 1)**
Figure 3: Experimental design for Trueness/Precision (example 2)
Annex 1

Effect of DNA content on the practical LOD

As shown in Table 2, in a 0.1% GM sample there are 1000 times more copies of the target-taxon specific sequence than of the GMO target. This implies that for an absolute LOD of the method of 10 copies, it is necessary to load in PCR 10,000 copies of the taxon-specific sequence to have a practical LOD of 0.1%. If the absolute LOD is 10 copies and 100,000 copies are loaded in the PCR reaction, then the practical LOD is 0.01% (see Table 2). The practical LOD should be calculated for each individual sample.13

Table 2: Example of the effect of DNA content on the practical LOD

<table>
<thead>
<tr>
<th>Copies of taxon specific gene</th>
<th>Absolute LOD (copies of GMO target)</th>
<th>Practical LOD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100,000</td>
<td>10</td>
<td>0.01</td>
</tr>
<tr>
<td>10,000</td>
<td>10</td>
<td>0.1</td>
</tr>
<tr>
<td>1000</td>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

Annex 2

Evaluation of DNA- extraction method (Inhibition test)

Background
Substances known to inhibit the PCR reaction components affect the efficiency of target DNA amplification by interacting with the DNA template, by interfering with the DNA polymerase activity or decreasing the efficiency of enzymatic cofactors (Mg²⁺). DNA extraction procedures eliminate or reduce considerably the amount of PCR-inhibiting substances. However, the final amount of inhibitors in a sample depends very much on the sample nature. The more the sample has been processed, the more likely the DNA will be fragmented. Plant DNA can also be affected by the presence of secondary metabolites such as polyphenols, oils and polysaccharides which can form complexes with DNA strands. Inhibitors can be added by the DNA isolation procedure: KCl and NaCl, ionic detergents, ethanol, isopropanol and phenol among others.

Different strategies can be adopted to test DNA preparations for presence of PCR inhibitory compounds. This Annex illustrates the application of the ENGL acceptance criteria to evaluate reaction efficiency (slope and R²) of serially diluted samples from an undiluted source with the ability to test for presence of PCR inhibitors in the undiluted sample intended for the PCR analyses.

Basically the inhibition depends on the concentration of the inhibitors. When DNA is diluted, the effect of inhibitors is often reduced or eliminated at lower DNA concentrations. Evaluation of the reaction efficiency on the diluted series and comparison of the theoretical Ct (threshold cycle) of a non-inhibited undiluted sample with its measured Ct, discloses information for the assessment of DNA quality for PCR applications. In case only the highest DNA concentration shows inhibition a lower DNA concentration can be used for quantification, but this will affect the practical LOD and LOQ.

However, in certain cases inhibitor compounds attached to DNA fragments may not be eliminated by sample dilution, thus resulting in less DNA copies available for amplification than expected from the nominal DNA concentration in a sample.
Procedure

DNA quality (relative absence of PCR inhibitors) can be demonstrated by analysing two PCR replicates using four points of a four-fold serial dilutions (1:4, 1:16, 1:64 and 1:256) of each DNA extraction replicate (inhibition runs) using the taxon-specific reference system. The DNA extract is first brought to a level corresponding to the highest DNA concentration intended to be used in the subsequent PCR method, the so called ‘undiluted’ sample (working dilution). From this first sample, a four-fold dilution series is prepared (from 1:4 to 1:256). To assess the presence of inhibitors, the Ct values of the four serially diluted samples are plotted against the logarithm of the dilution factor and an equation is calculated by linear regression. The Ct value of the ‘undiluted’ sample extrapolated from the linear regression is compared with the Ct measured from the same sample. To accept DNA extracts three conditions should be met: the slope of the regression line must be between -3.6 and -3.1; the coefficient of determination (R²) is equal to or above 0.98; and the difference between the measured Ct and the extrapolated Ct value (ΔCt) is below 0.5.

The Ct values from two PCR replicates from each dilution are needed for the calculation sheet illustrated below.

Evaluation of DNA quality

In the figures below the wording ‘working dilution’ in the blue cell reflects the term ‘undiluted sample’.

Example A: Acceptable DNA quality: all criteria met

Example B: DNA inhibited. Even though the ΔCt does not exceed the limit of 0.5 (although it is close to this value) the underperforming DNA quality is demonstrated by the delay in reaction onset (Cts) for the undiluted sample and the 1:4 diluted sample. The latter affects the slope expressed by the serial dilution which appears flatter than acceptable (-3.0).
**Example C:** DNA inhibited. This is another occurrence of low quality associated with the DNA extract. The slope of the dilution series is within the acceptance range, however, the extrapolated Ct for the undiluted sample (22.48), based on the four-point straight line should be lower than measured (23). This indicates a delay in onset for the undiluted sample which is less evident on the subsequent diluted sample 1:4. Therefore, while the slope of the linear regression falls within the range -3.6 to -3.1, the ΔCt demonstrated co-extraction of compounds inhibiting DNA amplification.

**Example D:** In this example, the slope of the regression line is out of the ENGL acceptance criteria (-3.66). However, contrary to example B, there is no delay in Ct onset for the undiluted sample and in the first samples of the dilution series. The ‘ΔCt’ column shows the measured difference in Ct values between subsequent samples of the dilution series. These values are always greater than the expected value of 2 for a reaction with 100% efficiency. Basically, the dilution series behaves as if less DNA than calculated was present in the diluted samples. Therefore, we do not expect to classify this sample as affected by the presence of inhibitory compounds. However, if technical mistakes are ruled out (pipetting errors) and no other reasons are clearly identified, the possibility of inhibitors attached to DNA targets should not be discarded *a priori.*
Annex 3

Production of intermediate concentrations of positive material

Some of the reference materials are only available in one or a few limited GM concentrations. It may be necessary to mix the positive material with non GM material to produce other GM concentrations e.g. for determining the relative LOQ and LOD.

This can be done by measuring the content of the reference gene for the GM positive and a GM negative DNA preparation on the same plate with the same standard curve. Following this the dilution factor for the two DNA preparations can be calculated using the following formula:

\[ X = \frac{A}{B} \times (Y-1) + 1 \]

- \( X \) = the practical dilution factor (how much the GM material has to be diluted compensated for difference in concentration)
- \( A \) = copy number of reference gene for the GM positive DNA preparation
- \( B \) = copy number of the reference gene for the GM negative DNA preparation
- \( Y \) = the theoretical dilution factor e.g. from 10 % GM to 0.1 % GM = 100x

Example:
DNA A = 100% GMO,
DNA B = 0%
5 µL DNA is added per PCR well for A and B

Quantification as unknown sample on reference gene calibration curve

Result:
A (from DNA A): 10 copies/5 µL
B (from DNA B): 8 copies/5 µL

To make 10 % GMO from 100 % GM corresponds to 10 times dilution (theoretically \( Y = 10 \)).

\[ X \] has to be used like \( Y \) in calculating the volumes to be mixed. If \( X=12.25 \), then

practical dilution factor \( X \): \( \left( \frac{10}{8} \right) \times (10-1) + 1 = \left( \frac{10}{8} \right) \times 9 + 1 = 90/8 + 1 = 11.25 + 1 = 12.25 \), so 1 µl A has to be mixed with 11.25 µl B.

After adding together the two DNA preparations, the DNA solution has to be mixed thoroughly.
The trueness of the mixtures can be analysed using the 100% mixture for standard curve and analysing 3 samples in triplicates on 3 different days.
Annex 4

Estimation of the mean, standard deviation and relative repeatability standard deviation of GM-content from real-time PCR

The correct calculation of GM content and its standard deviation from PCR assays is in most experimental designs a two-step process combining mean values that are calculated in two different ways. The procedure outlined in detail below starts from the measured values of the copy numbers of target and reference genes. From these test results, an estimate of GM-content and a standard deviation is calculated. Most experimental procedures provide several such values (for example, from runs on the same or different plates) of GM content and standard deviation. These estimates can, if needed and appropriate, be combined in a standard way, for example by taking the arithmetic mean in case of the GM-content.

Estimation of GM-content from copy numbers of target and reference genes

Two assays are required to estimate the percentage of GM-DNA from samples using real-time PCR: one assay is used to detect the copy number of the GM target DNA sequence (X), the other is used to determine the copy number of the endogenous reference gene DNA sequence (Y). The estimate of the percentage of GM content is obtained using the following ratio of

\[ \text{%GM} = \frac{\text{target DNA copy number}}{\text{reference DNA copy number}} \times 100 = \frac{X}{Y} \times 100. \]  

Both X and Y are random variables\(^1\). It is standard practice to run assays for the target and the reference genes in duplicates, triplicates etc. This results in 2, 3, etc. test results for the GM target DNA sequence and 2, 3, etc. test results for the reference gene DNA target sequence, and what is required is the calculation of an average GM-percentage from these two sets of test results. There are unfortunately no exact formula for the mean and variance of the ratio of random variables, but approximations do exist\(^2\). The mean, denoted by \( E[\cdot] \), and the variance of a ratio of independent random variables are approximated by

\[ E\left[ \frac{X}{Y} \right] \approx \bar{x} + \frac{\bar{x}}{\bar{y}} Var(y) \]  

and

\[ Var\left[ \frac{X}{Y} \right] \approx \left( \frac{\bar{x}}{\bar{y}} \right)^2 \left( \frac{Var(x)}{\bar{x}^2} + \frac{Var(y)}{\bar{y}^2} \right), \]  

where \( \bar{x} \) is the arithmetic mean of the target GM DNA copy numbers and \( \bar{y} \) is the arithmetic mean of the reference DNA copy numbers.

These approximations assume that there is no correlation between X and Y. The standard deviation is given by \( sd[X/Y] = \sqrt{Var[X/Y]} \). Relative repeatability standard deviation \( RSD_r \) is calculated at the end of the procedure from the component standard deviations; the details of how to calculate \( RSD_r \) are outlined in the examples below.

All these calculation can be implemented in Excel.

\(^1\) A random variable can be thought of as a quantity whose value is not fixed, but which can take on different values according to a probability distribution (which may be determined by e.g. measurement uncertainty). It is common to use the capital letter for a random variable (i.e. \( X \) for target DNA concentration) and small letters for an actual value, i.e. a measurement result.
Examples:
In the examples, we use $x$ for the copy number of the GM target gene and $y$ for the copy number of the reference gene. These examples correspond to the examples given in Table 1 and demonstrate in detail the calculations needed for one plate and then describe how the calculation results from several plates are combined.

Example 1: Two DNA-extractions, for each extraction both GM target and reference gene are tested in two PCR replicates on four plates
This design provides two GM-estimates and standard deviations for each plate and thus eight GM-estimates ($GM_{1,8}$) and eight standard deviations ($sd_{1,8}$) in total. Each of these eight GM-estimates and standard deviations is derived using equations (1) and (2) from two test results each of the target gene copy number and the reference gene copy number. If the mean of all eight GM-estimates is taken, this average value depends on 16 test results of the target gene copy number and 16 test results of the reference gene copy number; this also applies of course to the combined standard deviation.

Extraction 1:

<table>
<thead>
<tr>
<th>GM target gene</th>
<th>Reference gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ct copy number</td>
<td>Ct copy number</td>
</tr>
<tr>
<td>24.41</td>
<td>16119</td>
</tr>
<tr>
<td>24.61</td>
<td>13954</td>
</tr>
<tr>
<td>21.30</td>
<td>156758</td>
</tr>
<tr>
<td>21.18</td>
<td>171196</td>
</tr>
</tbody>
</table>

Thus, $\bar{x} = 15036.97$, $\bar{y} = 163977$, $\text{Var}(x) = 2343612.5$ and $\text{Var}(y) = 104227922$. Putting the appropriate values into equation (2) gives a mean $GM_1$ of 0.092 or 9.2%; using equation (3) and taking the square root gives a standard deviation $sd_1$ of 0.010943.

Extraction 2:

<table>
<thead>
<tr>
<th>GM Target gene</th>
<th>Reference gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ct copy number</td>
<td>Ct copy number</td>
</tr>
<tr>
<td>25.50</td>
<td>13405</td>
</tr>
<tr>
<td>25.44</td>
<td>14000</td>
</tr>
<tr>
<td>21.10</td>
<td>172089</td>
</tr>
<tr>
<td>21.19</td>
<td>160907</td>
</tr>
</tbody>
</table>

Here, $\bar{x} = 17027.5$, $\bar{y} = 166498$, $\text{Var}(x) = 177012.5$ and $\text{Var}(y) = 62518562$. With the appropriate values, equation (2) gives a mean $GM_2$ of 0.082 or 8.2%; using equation (3) and taking the square root gives a standard deviation $sd_2$ of 0.004654.

Combining the plates
This entire procedure is repeated on four different plates, giving in addition to $GM_1$, $GM_2$, $sd_1$ and $sd_2$ the means $GM_3$, $GM_4$, ..., $GM_8$ and the standard deviations $sd_3$, $sd_4$, ..., $sd_8$.

The overall mean of the sample $\overline{GM}$ can then be calculated by taking the arithmetic mean of $GM_1$ - $GM_8$, i.e. $\overline{GM} = \frac{\sum_{i=1}^{8} GM_i}{8}$. Using $n$ as the number of replicates per extraction ($n = 2$ in this example) and $k$ as the number of separate standard deviations to be pooled ($k = 8$ here), the standard deviation of the overall mean $sd_{GM} = \sqrt{\frac{\sum_{i=1}^{8} (n_i - 1)sd_i^2}{\sum_{i=1}^{8} n_i - k}}$; the term in the denominator is $16 – 8 = 8$ in this example. The relative repeatability standard deviation $RSD_r = \frac{sd_{GM}}{\overline{GM}} 100$. 

20
**Example 2:** Two DNA-extractions, both GM target and reference gene are tested in four PCR-replicates on two plates

This design provides two GM-estimates and standard deviations for each plate and thus four GM-estimates (GM₁−₄) and four standard deviations (sd₁−₄) in total. Each of these four GM-estimates and standard deviations is derived using equations (1) and (2) from four test results each of the target gene copy number and the reference gene copy number. If the mean of all four GM-estimates is taken, the average value depends – as in example 1 above – on 16 test results of the target gene copy number and 16 test results of the reference gene copy number; this also applies of course to the combined standard deviation.

**Extraction 1:**

<table>
<thead>
<tr>
<th></th>
<th>GM target gene</th>
<th>Reference gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ct copy number</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24.41</td>
<td>16119</td>
<td>21.30</td>
</tr>
<tr>
<td>24.61</td>
<td>13954</td>
<td>21.18</td>
</tr>
<tr>
<td>25.50</td>
<td>13405</td>
<td>21.10</td>
</tr>
<tr>
<td>25.44</td>
<td>14000</td>
<td>21.19</td>
</tr>
</tbody>
</table>

Here, \( \bar{x} = 14369.5 \), \( \bar{y} = 165237.5 \), \( \text{Var}(x) = 1433394 \) and \( \text{Var}(y) = 57700642 \). Applying equation (2) gives a mean \( GM_1 \) of 0.087 or 8.7%; using equation (3) and taking the square root gives a standard deviation \( sd_1 \) of 0.00828.

**Extraction 2:**

<table>
<thead>
<tr>
<th></th>
<th>GM target gene</th>
<th>Reference gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ct copy number</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26.21</td>
<td>14826.97</td>
<td>21.09</td>
</tr>
<tr>
<td>26.30</td>
<td>13885.92</td>
<td>21.09</td>
</tr>
<tr>
<td>26.38</td>
<td>13099.69</td>
<td>21.20</td>
</tr>
<tr>
<td>26.20</td>
<td>14935.39</td>
<td>21.25</td>
</tr>
</tbody>
</table>

Here, \( \bar{x} = 14187 \), \( \bar{y} = 157308 \), \( \text{Var}(x) = 747515 \) and \( \text{Var}(y) = 89272181 \). Applying equation (2) gives a mean \( GM_2 \) of 0.091 or 9.1%; using equation (3) and taking the square root gives a standard deviation \( sd_2 \) of 0.0077.

**Combining the plates**

This entire procedure is repeated on two different plates, giving in addition to \( GM_1 \), \( GM_2 \), \( sd_1 \) and \( sd_2 \) the means \( GM_3 \) and \( GM_4 \) and the standard deviations \( sd_3 \) and \( sd_4 \).

The overall mean of the sample \( \overline{GM} \) can then be calculated by taking the arithmetic mean of \( GM_1 - GM_8 \), i.e. \( \overline{GM} = \frac{\sum_{i=1}^{4} GM_i}{4} \). Using \( n \) as the number of replicates per extraction (\( n = 4 \) in this example) and \( k \) as the number of separate standard deviations to be pooled (\( k = 4 \) here), the standard deviation of the overall mean \( sd_{GM} = \sqrt{\frac{\sum_{i=1}^{4} (n_i - 1)sd_i^2}{\sum_{i=1}^{4} n_i - k}} \); the term in the denominator is \( 16 - 4 = 12 \) in this example. The relative repeatability standard deviation \( RSD_r = \frac{sd_{GM}}{\overline{GM}} \times 100 \).
References
Abstract
In the EU, method validation is an essential part of the process that regulates the introduction of new GMOs as food and/or feed into the market. When the inter-laboratory validation study is completed, the method is ready to be implemented in routine testing laboratories. When implementing the new method, the laboratory has to verify that the method can be used for its intended purpose (method verification). The scope of this document is to provide guidance on how to carry out the method verification. Despite the fact that several guidelines on method verification have been published, no specific guidelines are available for GMO detection. The aim of this guidance document is to harmonise the in-house verification of inter-laboratory validated methods for the qualitative and quantitative detection of GMOs, including element-, construct-, and event-specific methods. Considering that the Polymerase Chain Reaction (PCR) is the method of choice in the EU for the identification and quantification of GMOs, this document refers exclusively to real time PCR. However, if novel methods are subsequently developed that fulfill legal requirements, then this document will be amended accordingly.
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