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

*Version 2*

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**Title:** Verification of analytical methods for GMO testing when implementing interlaboratory validated methods

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 of inter-laboratory validated methods for the qualitative and quantitative detection of GMOs. 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 fulfil legal requirements, then this document will be amended accordingly.

# Contents

## Executive summary

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 of inter-laboratory validated methods for the qualitative and quantitative detection of GMOs.

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 fulfil legal requirements, then this document will be amended accordingly.

This document provides the definitions of the parameters to be assessed by laboratories in a verification study and the related acceptance criteria. Moreover, indications and examples of experimental designs are also described.

## Scope of the report

The first working group on method verification was established on the basis of a mandate adopted by the ENGL Steering Committee on 19th - 20th of November 2009.

The working group was chaired by Lotte Hougs, Danish Veterinary and Food administration (DVFA) Ringsted, Denmark and Jana Žel, 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 Wageningen University & Research, The Netherlands and Thomas Weber, Joint Research Centre (JRC).

The mandate of the working group was to develop a guideline for the implementation of validated methods in control laboratories under ISO 17025:2005 accreditation taking into account the demands outlined in the ENGL guidelines and in particular the "Definition of minimum performance requirements for analytical methods of GMO testing" version 13/10/2008.

The resulting guideline 'Verification of analytical methods for GMO testing when implementing interlaboratory validated methods' was published in July 2011.

The 30th ENGL Steering Committee, held on 3rd - 4th of February 2016, established the working group on "Update of Method Verification Document" with the mandate of:

- Update the document to include verification criteria for techniques not currently covered.
- Align the document to the new version of the ENGL guideline "Definition of minimum performance requirements for analytical methods of GMO testing"[1].
- Review the terminology, also considering the new ISO 16577:2016[2].

The working group has been chaired by Lotte Hougs, Danish Veterinary and Food administration (FVST) Ringsted, Denmark. The other members of the working group have been Francesco Gatto, Joint Research Centre (JRC), European Commission; Ottmar Goerlich, Bavarian Health and Food Safety Authority, Germany; Lutz Grohmann, Federal Office of Consumer Protection and Food Safety (BVL), Germany; Kathrin Lieske, Federal Office of Consumer Protection and Food Safety (BVL), Germany; Marco Mazzara, Joint Research Centre (JRC), European Commission; Frank Narendja, Umweltbundesamt, Austria; Jaroslava Ovesná. Crop Research Institute, Czech Republic; Nina Papazova, Scientific Institute of Public Health (WIV-ISP), Belgium; Ingrid Scholtens, RIKILT Wageningen University & Research, The Netherlands and Jana Žel, National Institute of Biology (NIB), Slovenia.

# Introduction

A new analytical method evolves through a number of actions. After the initial development and optimisation phases, a laboratory performs an in-house validation on the method to ensure that the method is fit for the intended purpose during internal use. Before the method can be accepted as fit for use by several laboratories or as an International Standard, it needs to be validated by a number of laboratories[3,4]. When this inter-laboratory validation study is successfully completed, the method is ready to be implemented in routine testing laboratories. When implementing the new method in such a laboratory, it has to be verified there that the method can be used for its intended purpose.

Regulation (EU) No 625/2017[5] (repealing Regulation (EC) No 882/2004[6]) provides that official control laboratories shall be accredited according to the ISO/IEC 17025:2005[7] standard. Such an 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" and whenever "the standard method changes, the confirmation shall be repeated" (ISO/IEC 17025:2005, section 5.4.2).

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 and quantification 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[8] are validated by the European Union Reference Laboratory for Genetically Modified Food and Feed (EURL GMFF) in collaboration with the ENGL. These and other qualitative element-, construct- or event-specific methods are compiled in the GMOMETHODS database[91] according to Article 94 of Regulation (EU) No 625/2017 (and Article 32 of Regulation No. 882/2004). The method validation has to be performed according to internationally recognized guidelines[3,4] through collaborative studies. Data from the inter-laboratory validation of the methods are evaluated according to the document "Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing"[1] (MPR).

Despite the fact that several guidelines[1,10–13] and peer-reviewed papers[14–17] on in-house method validation have been published, no specific guidelines are available for the verification of GMO detection methods.

The aim of this document is to provide guidance and 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.

The principles of the modular approach[18] have been taken into account in this document, therefore this guidance refers to the verification of the PCR module and not the DNA extraction module (Figure 1). Nonetheless, indications on the evaluation of the suitability of the extracted DNA solutions are given to facilitate the verification exercise. The approach to independently assess the modules is already used for method validation[1,19], and allows laboratories to better suit the analytical procedures to different food and feed matrix materials.

The validation of methods for GMO detection as well as procedures for the calculation of the measurement uncertainty are not within the scope of this document.

Considering that the PCR is the method of choice in the EU for the identification and quantification of GMOs, this document refers exclusively to real-time PCR methods. If new methods, based on other technologies, will be developed that fulfil legal requirements, then this document will be amended accordingly.

[1] http://gmo-crl.jrc.ec.europa.eu/gmomethods/

# Terminology

*Amplification efficiency*[1]

The rate of amplification calculated from the slope of the standard curve obtained after a decadic semi-logarithmic plot of Cq values over the DNA copy numbers/quantity. The efficiency (in %) can be calculated by the following equation:

$$Efficiency = (10^{(-1/slope)}-1) \times 100$$

*Analytical sample*[20]

Sample prepared from the laboratory sample by grinding, if necessary, and homogenization (see also Fig. 1).

*Cq*[21]

The quantification cycle (Cq), also known as threshold cycle (Ct), is defined as the fractional cycle number at which the fluorescence generated by the amplification of a target DNA in a real time PCR experiment reaches a fixed threshold and so allows the quantification of the amount of target DNA.

*DNA extraction replicates (as used in this document)*

DNA extracted from different test portions from the same analytical sample.

*Dynamic range*

The range of concentrations over which the method provides a linear correlation between the measurement and the amount of the target, with an acceptable level of trueness and precision.

*Laboratory sample*[22]

Sample as received by the laboratory and intended for inspection or testing (see also Fig. 1).

*Limit of detection (LOD)*[1]

*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 for at least 95 % of the cases (samples) at the LOD, ensuring ≤ 5 % false negative results.*

*Limit of quantification (LOQ)*[1]

*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.*

*Multiplex PCR*[2]

PCR technique that employs multiple pairs of primers combined within a single reaction mixture to produce multiple amplicons.

*PCR replicates* (as used in this document)

PCR performed on the same DNA extraction replicate analysed in different reaction wells.

*Probability of detection (POD)*[1]

The probability of a positive (i.e., presence detected) analytical outcome for a qualitative method for a given matrix at a given concentration. It is estimated by the expected ratio of positive to negative results for the given matrix at the given analyte concentration.

*Practical limit of detection (practical LOD)*

The practical LOD is the lowest quantity of GMO, expressed as mass fraction or DNA copy number ratio, that can be reliably detected in a sample, when a known number of the taxon (ingredient) genome copies has been determined or estimated.

*Practical limit of quantification (practical LOQ)*

The practical LOQ is the lowest quantity of GMO, expressed as mass fraction or DNA copy number ratio, that can be reliably quantified in a sample, when a known number of taxon (ingredient) genome copies has been determined or estimated.

*Precision – Relative repeatability standard deviation ($RSD_r$)*[1]

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. *$RSD_r$ is calculated by dividing the repeatability standard deviation by the mean of results.*

*Repeatability standard deviation ($SD_r$)*[2]

Standard deviation of test results obtained under repeatability conditions.

*Relative repeatability standard deviation*[2]

See "Precision"

*$R^2$ coefficient*[1]

$R^2$ is the coefficient of determination, which is calculated as the square of the correlation coefficient (between the measured Cq-value and the decadic logarithm of the concentration) of a standard curve obtained by linear regression analysis.

*Robustness*[1]

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.

*Specificity*[1]

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

*Test portion*[22]

Sample, as prepared for testing or analysis, the whole quantity being used for analyte extraction at one time (see also Fig. 1).

*Test result*

A test result is a Cq value or copy number concentration originating from a PCR replicate.

*Trueness*[1]

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.

*Validation of method*[7]

Validation is the confirmation by examination and provision of objective evidence that the particular requirements for a specific intended use are fulfilled.

*Verification of method*[11]

Provision of objective evidence that a laboratory can adequately operate a method, achieving the performance requirements for the sample matrices to which the method is being applied.

*Working DNA concentration*

The highest DNA concentration intended to be used in PCR analysis.

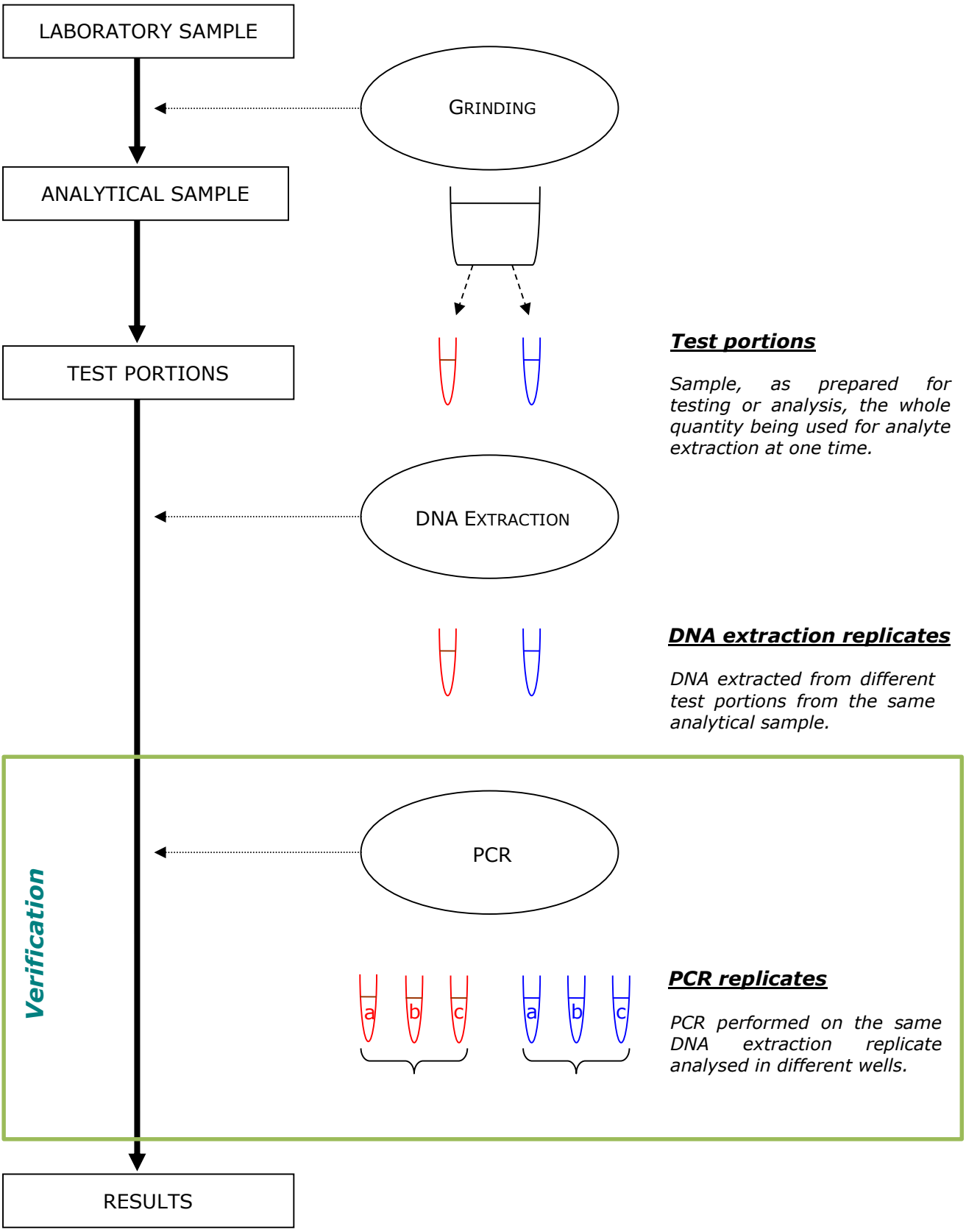



**Figure 1: Illustration of replicates terminology**

## 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 has to ensure that the chosen method shows, prior to its use, performance characteristics in the laboratory similar to those attributed in the inter-laboratory study. The verification process must be documented and recorded in the quality system.

The laboratory has to record the procedure used, the results obtained and a statement as to whether the method is fit for the intended use, i.e.:

- Design and planning of the verification;
- Description of the method;
- Acceptance criteria and performance requirements, as decided by the laboratory;
- Test records;
- Assessment of the method.

Inter-laboratory validated methods are assessed according to the acceptance criteria and performance requirements described in the document MPR[1]. This document can be more generally used also as a basis for assessing the performance results of a method in a verification process. The methods are available, e.g., in the GMOMETHODS database of the EURL GMFF[23]. 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: CEN/TS 15568:2006[20], ISO 24276:2006[22], ISO 21570:2005[24], ISO 21569:2005[25] and ISO 21571:2005[26].

As a matter of principle, a method should be implemented as validated in the inter-laboratory trial without introducing modifications. If single elements, like e.g. the brand of a 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 performance parameters should be experimentally assessed (e.g. specificity and robustness). Guidelines can be found in Woll *et al.*[27] and will also be published in the document "Guidelines on the update of GMO EURL GMFF validated methods" (in preparation).

The verification process is usually conducted on a certified reference material (CRM). If CRMs are not available, other GM positive materials can be used, such as Proficiency Test samples or routine samples. A CRM certified for a specific event, can be used for element- or construct-specific method verification, if the event contains the element or construct, even when the CRM is not certified for the element or construct.

## DNA extraction and purification

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. The DNA isolation method should be assessed on a range of representative materials and provide DNA of suitable quality and quantity for subsequent analysis.

*Procedure*: The DNA extraction method should be applied to the same material as for the validation study as well as to representative samples expected to be analysed. Even if the DNA extraction method was previously validated on a particular matrix, the DNA extraction shall be carried out at least twice (three times recommended) on 2 independent test portions, if possible on different days and with different operators. The extracted DNA has to meet the acceptance criteria for DNA concentration and quality[1] (e.g. by controlling amplification efficiency and presence of inhibitors by real-time PCR).

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 DNA concentration can be determined by using fluorimetric or spectrophotometric techniques. It is recommended to use the same technique in the verification study as foreseen for the analyses of samples since the quantification of DNA could be affected by the method used[28].

*Acceptance criterion*: The method should provide DNA in an appropriate yield for the intended analysis (at least enough to meet the desired practical LOD/LOQ). Where applicable, the yield should be comparable to the results obtained in the validation study.

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).

Purity of DNA extracts

The isolation of the DNA may lead to the co-purification of substances that inhibit the PCR reaction resulting in the absence or a lower rate of amplification. In the first case, false negative results may be obtained or, as in the second case, the quantification of the analyte can be underestimated.

Therefore, the laboratory needs to verify that the DNA extraction procedure guarantees the removal of such inhibitors.

*Procedure:*

The presence or absence of PCR inhibitors can be verified by testing different dilutions prepared from a DNA solution so that the more the DNA solution is diluted, the less is the concentration of inhibitors.

Two or more dilution levels should be tested with a validated taxon-specific reference system (*e.g.* lectin for soybean DNA) with the first dilution level representing the 'working DNA concentration', *i.e.* the total DNA amount per reaction intended to be used in the verification process and in routine analysis.

After the completion of the amplification, the Cq value from the more concentrated DNA solution is compared to the Cq values of the other concentration levels and to the theoretical value computed by assuming the absence of PCR inhibitors.

Example 1: Each DNA extraction replicate is diluted to at least two different concentrations and analysed by using a taxon-specific assay. Then the difference between the average Cq from the most diluted and more concentrated portions is calculated (ΔCq) and compared to the theoretical ΔCq.

*Acceptance criterion*:

Example 1: The theoretical ΔCq for a 1:4 dilution is 2.0. The difference between the measured ΔCq value and the theoretical ΔCq (2.0) value of the sample should be <0.5.

Annex 2 describes in more details an alternative inhibition assay and the calculation sheet is given as electronic supplementary material.

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 of a particular assay 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, if the conditions of the assay (*e.g.* primers/probe concentration; annealing temperature; fluorescent dye) are unchanged.

Data regarding specificity can be retrieved from the validation report or peer review articles, or from databases[9,29–31]. If these data are not available or cannot be retrieved, the method should be tested in-house.

The method should be tested regarding responsiveness towards new GMO events that contain the target sequence. This can only be done when the respective positive control materials are available for the new GMO events.

One has to consider that matrix reference materials are only certified for the presence or absence of a particular GM event and not for the absence or presence of other GM events that could be present as trace constituents.

For procedure and details see the MPR[1].

## Dynamic range, $R^2$ Coefficient and Amplification Efficiency

The dynamic range must cover the values expected for the specific application. Within the dynamic range, the standard curves should meet the acceptance criteria for the amplification efficiency and the $R^2$.

*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).

*Example*: Dynamic range from 0.09 % (m/m) to 4.5 % (m/m) for a 0.9 % (m/m) GMO target concentration, or 50 to 2500 copies/reaction if the target is 500 copies/reaction.

*Acceptance criterion for amplification efficiency*: The average value of the slope of the standard curve shall be in the range of -3.6 ≤ slope ≤ -3.1, corresponding to an amplification efficiency of 90 - 110 %[1].

*Acceptance criterion for $R^2$ coefficient*: the average value of $R^2$ shall be ≥ 0.98[1].

## Trueness

*Procedure:* The trueness should be determined at a content level close to the level set in legislation (*e.g.* threshold 0.9 % m/m), or according to the intended use of the method, and, if appropriate, additionally at a level close to the LOQ. The trueness can be assessed by using CRM. Usually two concentrations (*e.g.* 0.1 % and 1 % m/m) and, if possible, a third one at the upper end of the dynamic range (*e.g.* 5 % m/m) should be investigated. Alternatively, a reference sample could be prepared, preferably from a higher concentrated CRM. Annex 3 provides a guideline for the preparation of such a reference sample.

The analytical procedure used including reaction volume, PCR instrument, etc. should be the same as during routine testing of samples. Results from at least 16 PCR replicates should be evaluated. Examples for possible test designs are shown in Table 1 and Figures 2 and 3.

Annex 4 provides guidelines for the calculation of the 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 sufficiently characterized proficiency test material can be employed. However, the assigned value of the PT material shall be a reference value independently established outside the PT exercise, *i.e.* the GMO content established by a 'consensus value from participants' results' are not suitable for the estimation of the trueness.

The laboratory result from a proficiency testing (PT) exercise may also be used under the conditions that a sufficiently characterized proficiency test material has been employed (see above) and that the standard deviation for PT assessment had been properly chosen.

*Acceptance criterion*: The trueness of the own measurement results is within ± 25 % of the accepted reference value or a Z-score within the range of 2 and -2 has been obtained in a proficiency test using a sufficiently characterized proficiency test material.

## Relative Repeatability Standard Deviation ($RSD_r$)

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

The analytical procedure used should be the same as during routine testing of samples. At least 16 single test results should be evaluated. Examples for possible test designs are shown in Table 1 and Figures 2 and 3.

Annex 4 provides guidelines for the calculation of standard deviation and $RSD_r$ of GMO-content of related and unrelated real-time PCR replicates.

*Acceptance criterion*: The $RSD_r$ should be ≤ 25 %, over the dynamic range of the method.

Note: For verification of methods intended to be used under Regulation (EU) No 619/2011[32], the laboratory should demonstrate a relative repeatability standard deviation ≤ 25 % established on samples containing 0.1 % GM related to mass fraction of GM material (see specific guidance[33]).

## Estimation of the Limit of Quantification (LOQ)

An LOQ can be determined for a ratio, i.e. the mass fraction or DNA copy number ratio, as well as for the number of measurable DNA copies.

*Procedure for Relative LOQ ($LOQ_{rel}$)*: the laboratory should demonstrate the capacity in reliably quantifying a sample at the 0.1 % m/m corresponding to the minimum concentration level tested in validation[1]. The experimental assessments are described in the sections for trueness and precision.

*Procedure for Absolute LOQ ($LOQ_{abs}$)*: A dilution series of known amounts of copies per reaction is tested in at least 10 PCR replicates (e.g. 80, 60, 40, 20, 10, 5 copies and 1 copy per reaction). Then the $RSD_r$ is calculated for each dilution level. The $LOQ_{abs}$ is estimated as the last dilution level where the $RSD_r$ of the measurements is below 25 %. Please note that the standard curve of the method should include the $LOQ_{abs}$.

The probability distribution suggests that analysis at 1 copy per reaction should give approximately 30 % of negative results. In order to verify that the target copies per reaction of the dilution series are approximately correct, at least 1/10 of the replicates should provide negative results for analysis at 1 copy per reaction.

*Acceptance criterion*: MPR Acceptance criterion: The LOQ should be ≤ the lowest amount of copies per reaction or ratio included in the dynamic range.

## Estimation of the Limit of Detection (LOD)

The LOD can be determined for a ratio, i.e. mass fraction or DNA copy number ratio, as well as for the number of measurable DNA copies.

To estimate the LOD of a method with 95 % confidence it is necessary to analyse at least 60 PCR replicates for each tested concentration[34,35]. As this may not be feasible, a pragmatic approach based on a lower number of replicates could be followed for the verification of the LOD. This approach allows an approximate estimation of the LOD.

*Procedure for Relative LOD ($LOD_{rel}$):* a reference material of low GM content can be measured in e.g. 10 PCR replicates and if all replicates are positive, this infers that the $LOD_{rel}$ is below or equal to this content level. If needed, a reference material at a specific level could be prepared as described in Annex 3.

*Acceptance criterion*: The $LOD_{rel}$ should be in line with the validation data and/or the MPR document (< 0.045 % m/m with a 95 % confidence level)[1].

*Procedure for Absolute LOD ($LOD_{abs}$)*: Dilution series representing the range above and below the expected $LOD_{abs}$, based on prior knowledge of the $LOD_{abs}$ performance of that method, are tested in e.g. 10 PCR replicates for each concentration level. The lowest concentration where all replicates are positive is the estimated $LOD_{abs}$. As for the $LOQ_{abs}$, the correctness of the dilution series tested could be ascertained by the results observed for the analysis of the sample at 1 copy per reaction (see above $LOQ_{abs}$). Please note that an $LOD_{abs}$ cannot be lower than 3 copies per reaction[36].

A similar approach is based on modelling of the probability of detection (POD). This procedure is used for the assessment of the variability of the measured number of copies around $LOD_{abs}$[13]. The repeatability standard deviations are compared to the theoretical values resulting from the Poisson model. A web-service[2] allows computation of the $LOD_{abs}$ and its confidence interval and of a mean POD curve with the corresponding 95 % confidence range[36].

Examples for possible test designs are given in Table 2.

*Acceptance criterion*: The LOD should be in line with the validation data and/or the MPR document (< 25 copies with a level of confidence of 95 %)[1].

[2] Web Service 'Validation of qualitative PCR methods within a single laboratory'. https://quodata.de/content/validation-qualitative-pcr-methods-single-laboratory

Note: The practical LOD ($LOD_{prac}$) is out of the scope of this document because it is not a part of the verification of the method. Nevertheless, Annex 1 shows the relation between copy number and $LOD_{prac}$.

*Additional LOD acceptance criteria for multiplex qualitative PCR modules*

The $LOD_{asym}$ for each module of a multiplex PCR method is determined by testing its analyte target at low amount or concentration (corresponding or close to the absolute LOD, i.e. not more than 25 copies/reaction) in the presence of increasing amounts of the other target(s) which are amplified in parallel by the other module(s) in the multiplex assay. The amount of other target(s) should not exceed 20,000 copies/reaction.

The $LOD_{asym}$ is expressed in absolute copies/reaction and is given for the corresponding amount of the other target(s) used in the tests.

*Procedure:* The $LOD_{asym}$ should already have been investigated in the context of method validation. For the verification at least one of the most critical combinations, according to the validation data, should be tested. It is recommended to test the analyte target at low amount (close to the absolute LOD, but not below) in the presence of high amount of the other target(s), e.g., not more than 25 copies/reaction of the target sequence in the presence of target DNA amplified in parallel by the other PCR module(s). The amount of other target(s) should not exceed 20,000 copies/reaction.

*Acceptance criterion*: The $LOD_{asym}$ should be in line with validation data.

## Robustness

Robustness should have been investigated already 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.

**Table 1. Example of practical settings for the verification of a quantitative real-time PCR method**

| | |
|---|---|
| 1. Preliminary test to define appropriate DNA amount | Optional: Test at least 3 target amounts in the range of 200 ng – 0.1 ng per reaction (dependent on plant species)[37].<br>For example: 200 ng soybean DNA corresponds to 176,991 soybean haploid genome copies with one haploid genome assumed to correspond to 1.13 pg of soybean genomic DNA[38]. This means that in standard curves made from e.g. 10 % (m/m) GMO soybean certified reference material 200 ng DNA corresponds to 176,991 copies of the endogenous gene target (if single copy) and to 17,699 copies of the GMO target (under the assumption that the material is homozygous for the GM locus), whereas 1 ng DNA corresponds to 885 copies of the endogenous gene target and 88 copies of the GMO target. |
| 2. Dynamic range, $R^2$ coefficient, and amplification efficiency | The method should only be used within its dynamic range.<br>Example 1: Two calibration curves (minimum requirements)<br>5 calibration points with 3 PCR replicates each (i.e., triplicates). All slopes shall be in the range of -3.6 ≤ slope ≤ -3.1 and all $R^2$ values should be ≥ 0.98.<br>Example 2: Four calibration curves<br>5 calibration points with 2 PCR replicates each (i.e., duplicates); average of the 4 slopes and $R^2$ are used to verify the acceptance. |
| 3. Trueness, $RSD_r$ | At least 2 GM content levels (one around labelling threshold and one around LOQ, a third recommended to the upper part of the dynamic range).<br>Example 1: Two DNA extraction replicates per GM level, 2 PCR replicates per extraction/plate, 4 plates resulting in 16 test results and 8 GM-content estimations per GM level* (Fig. 2).<br>Example 2: Two DNA extraction replicates per GM level, 4 PCR replicates per extraction/plate, 2 plates resulting in 16 test results and 4 GM-content estimations per GM level* (Fig. 3). |
| 4. LOQ, LOD | LOQ: 10 PCR replicates at a low content (e.g. 80, 60, 40, 20 copies and 1 copy/reaction). LOQ is the lowest content of a series where the $RSD_r$ of the copy number measurements is below 25 %.<br>If method is also used qualitatively:<br>LOD: 10 PCR replicates at a low content (e.g. 20, 10, 5 copies and 1 copy per reaction). LOD is then the lowest content 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.

Note: If all 10 replicates for the 1 copy/reaction dilution are positive, the DNA content should be reassessed, because it is probably higher than expected.

**Table 2. Example of practical setting for the verification of a qualitative real-time PCR method.**

| | |
|---|---|
| 1. Dynamic range, $R^2$ coefficient, and amplification efficiency | Optional: for examples see Table 1.2 |
| 2. LOD | Example 1: Test 10 PCR replicates around the expected $LOD_{abs}$ (e.g. a serial dilution with 20, 10, 5, 3 copies and 1 copy/reaction)*. The LOD is the lowest content in the series where all replicates are positive.<br><br>Example 2: Test six dilution levels (e.g. 20, 10, 5, 2, 1, 0.1 copies/reaction) and 12 PCR replicates per level[13]. On basis of modelling a POD curve the $LOD_{abs}$ at the 95 % confidence interval is computed (see https://quodata.de/content/validation-qualitative-pcr-methods-single-laboratory)[36].<br><br>Example 3: Test 60 replicates at the concentration of the expected $LOD_{abs}$. The LOD at this concentration is verified if at least 59 replicates are positive[34]. This test relies on the knowledge and correctness of the DNA concentration (copies of the target sequence per volume solution measured). |
| 3. Specificity | Optional if already assessed by validation study.<br><br>Example for *in silico* evaluation: Verify the specificity of the method by use of the JRC GMO-Matrix interface[30]. Select the taxon(s) or specific GMO(s) and the PCR method to be tested and run the *in silico* simulation. The result of the simulation is a list of GMO events for which amplification by the method is predicted or not predicted.<br><br>Example for experimental evaluation: Reference materials available for new GMO events that contain the target sequence are tested in duplicate using at least 100 copies/reaction of target DNA per PCR[1]. |
| 4. $LOD_{asym}$ (Only for multiplex methods) | At least, one of the most critical combinations according to the validation data should be tested.<br><br>10 PCR replicates at a low amount of the target sequence (corresponding or close to the absolute LOD*) in the presence of high amount of another target(s) (e.g. 25 copies/reaction of one target sequence in the presence of a background of the other target(s) summed at the level of 20,000 copies/reaction). |

*consider also $LOD_{asym}$ from validation of the method

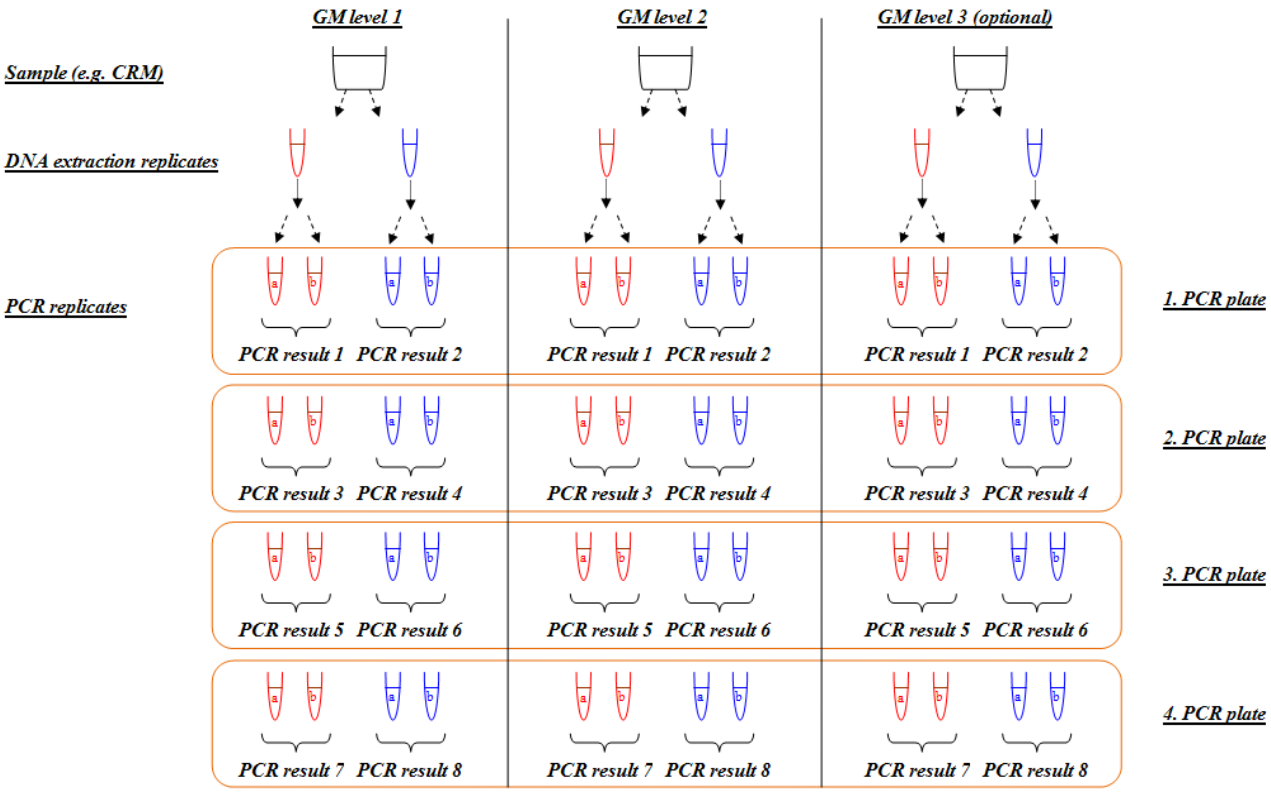



**Figure 2: Experimental design for Trueness/Precision (example 1)**

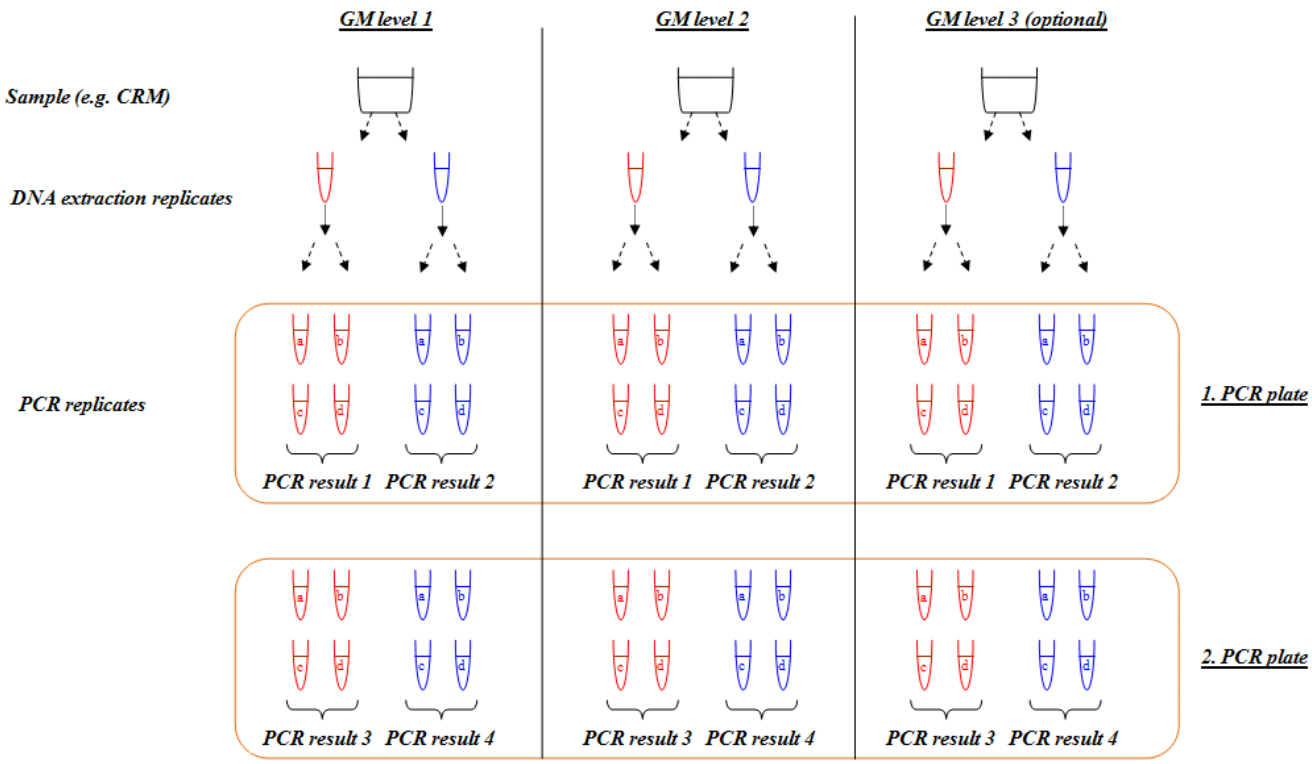



**Figure 3: Experimental design for Trueness/Precision (example 2)**

# Annex 1: Effect of DNA content on the practical LOD

As shown in Table 3, 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 3). The practical LOD should be calculated for each individual sample[22].

**Table 3: Example of the effect of DNA content on the practical LOD**

| Copies of taxon specific gene | Absolute LOD (copies of GMO target) | Practical LOD (%) |
|---|---|---|
| 100,000 | 10 | 0.01 |
| 10,000 | 10 | 0.1 |
| 1000 | 10 | 1 |

# 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 ($Mg^{2+}$).

DNA extraction procedures should 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 and on the extraction procedure applied. Plant material may have secondary metabolites such as polyphenols, oils and polysaccharides which can form complexes with DNA strands and 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 $R^2$) 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 Cq of a non-inhibited undiluted sample with its measured Cq, 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 dilution (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 DNA concentration). From this first sample, a four-fold dilution series is prepared (from 1:4 to 1:256). To assess the presence of inhibitors, the Cq 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 Cq value of the 'undiluted' sample extrapolated from the linear regression is compared with the Cq 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 ($R^2$) is equal to or above 0.98; and the difference between the measured Cq and the extrapolated Cq value ($\Delta$Cq) is below 0.5.

The calculation sheet[3] for the evaluation of the absence of PCR inhibitors is available as electronic supplementary material (http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm).

Cq values from two PCR replicates from each dilution are necessary for running this test.

[3] This file was made available for educational purposes only. You may download this file and use it freely. The ENGL, JRC and the authors shall not be liable for any loss, damage etc. resulting from its use.

Evaluation of DNA quality

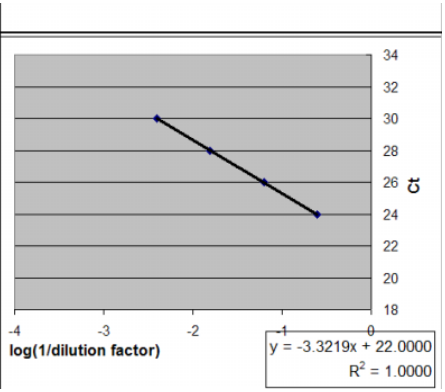
In the figures below the wording 'working DNA concentration' in the blue cell reflects the term 'undiluted sample'.

Example A: Acceptable DNA quality: all criteria met

**Sample code :**

| Dilution factor | | Measured Ct | Ct Mean | ΔCt | Expected ΔCt | Logarithm |
|---|---|---|---|---|---|---|
| 1 : | 4 | 24 | | | | -0.6021 |
| | | 24 | 24.00 | 2.00 | 2 | -0.6021 |
| 1 : | 16 | 26 | | | | -1.2041 |
| | | 26 | 26.00 | 2.00 | 2 | -1.2041 |
| 1 : | 64 | 28 | | | | |
| | | 28 | 28.00 | 2.00 | 2 | -1.8062 |
| 1 : | 256 | 30 | | | | -2.4082 |
| | | 30 | 30.00 | 2.00 | 2 | -2.4082 |

| | Measured Ct | Ct Mean | Extrapol. Ct | Extrapol. - Mean Ct | |
|---|---|---|---|---|---|
| Working dilution | 22 | | | | |
| | 22 | 22.00 | 22.00 | 0.00 | Ok |

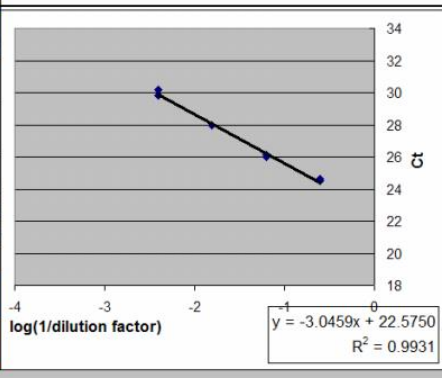



Example B: DNA inhibited. Even though the ΔCq does not exceed the limit of 0.5 (although it is close to this value) the underperforming DNA quality is demonstrated by the delay in reaction onset (Cq) for the undiluted sample and the 1:4 diluted sample. The latter affects the slope expressed by the serial dilution which appears flatter than acceptable (-3.0).

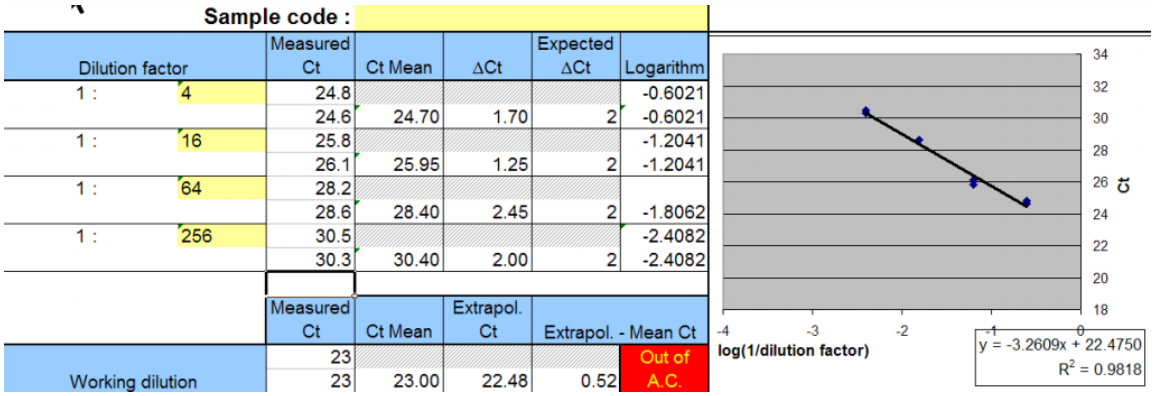
**Sample code :**

| Dilution factor | | Measured Ct | Ct Mean | ΔCt | Expected ΔCt | Logarithm |
|---|---|---|---|---|---|---|
| 1 : | 4 | 24.5 | | | | -0.6021 |
| | | 24.6 | 24.55 | 1.55 | 2 | -0.6021 |
| 1 : | 16 | 26 | | | | -1.2041 |
| | | 26.1 | 26.05 | 1.50 | 2 | -1.2041 |
| 1 : | 64 | 28.1 | | | | |
| | | 28 | 28.05 | 2.00 | 2 | -1.8062 |
| 1 : | 256 | 30.2 | | | | -2.4082 |
| | | 29.8 | 30.00 | 1.95 | 2 | -2.4082 |

| | Measured Ct | Ct Mean | Extrapol. Ct | Extrapol. - Mean Ct | |
|---|---|---|---|---|---|
| Working dilution | 23 | | | | |
| | 23 | 23.00 | 22.58 | 0.43 | Out of A.C. |




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 Cq 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 ΔCq demonstrated co-extraction of compounds inhibiting DNA amplification.

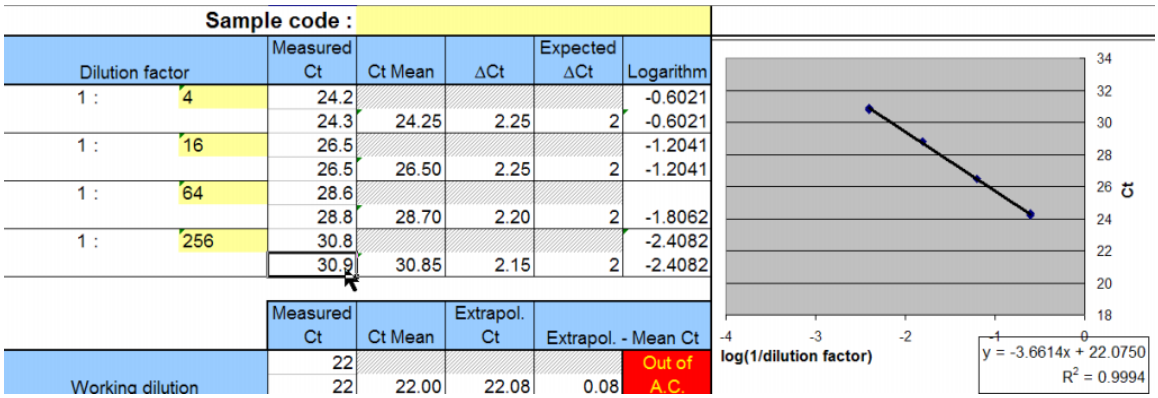


| Sample code : | | | | | | |
|---|---|---|---|---|---|---|
| Dilution factor | | Measured Ct | Ct Mean | ΔCt | Expected ΔCt | Logarithm |
| 1 : | 4 | 24.8 | | | | -0.6021 |
| | | 24.6 | 24.70 | 1.70 | 2 | -0.6021 |
| 1 : | 16 | 25.8 | | | | -1.2041 |
| | | 26.1 | 25.95 | 1.25 | 2 | -1.2041 |
| 1 : | 64 | 28.2 | | | | |
| | | 28.6 | 28.40 | 2.45 | 2 | -1.8062 |
| 1 : | 256 | 30.5 | | | | -2.4082 |
| | | 30.3 | 30.40 | 2.00 | 2 | -2.4082 |

| | Measured Ct | Ct Mean | Extrapol. Ct | Extrapol. - Mean Ct | |
|---|---|---|---|---|---|
| | 23 | | | | Out of A.C. |
| Working dilution | 23 | 23.00 | 22.48 | 0.52 | |



Example D: In this example, the slope of the regression line is out of the acceptance criteria (-3.66). However, contrary to example B, there is no delay in Cq onset for the undiluted sample and in the first samples of the dilution series. The 'ΔCq' column shows the measured difference in Cq 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. 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*.



| Sample code : | | | | | | |
|---|---|---|---|---|---|---|
| Dilution factor | | Measured Ct | Ct Mean | ΔCt | Expected ΔCt | Logarithm |
| 1 : | 4 | 24.2 | | | | -0.6021 |
| | | 24.3 | 24.25 | 2.25 | 2 | -0.6021 |
| 1 : | 16 | 26.5 | | | | -1.2041 |
| | | 26.5 | 26.50 | 2.25 | 2 | -1.2041 |
| 1 : | 64 | 28.6 | | | | |
| | | 28.8 | 28.70 | 2.20 | 2 | -1.8062 |
| 1 : | 256 | 30.8 | | | | -2.4082 |
| | | 30.9 | 30.85 | 2.15 | 2 | -2.4082 |

| | Measured Ct | Ct Mean | Extrapol. Ct | Extrapol. - Mean Ct | |
|---|---|---|---|---|---|
| | 22 | | | | Out of A.C. |
| Working dilution | 22 | 22.00 | 22.08 | 0.08 | |

# 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:

$$X = \left(\frac{A}{B}\right)(Y - 1) + 1$$

*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 100 % GM to 10 % GM = 10x

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): 10,000 copies/5 µL

B (from DNA B): 8000 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: (10,000/8000)*(10-1)+1=((10,000/8000)*9) +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.

To prepare further dilutions:

**1 % GM** can be made by diluting 1:10 the prepared 10 % solution with the 0 % solution.

**0.1 % GM** can be made by diluting 1:10 the prepared 1 % solution with the 0 % solution.

The DNA concentration of a 10 times diluted sample is close to the concentration of the original 0 % DNA preparation and this concentration can be used in the calculation of further dilutions.

The trueness of the mixtures can be analysed using the 100 % mixture for standard curve and analysing 3 samples in triplicates on 3 times.

# 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

$$\%GM = \frac{target\ DNA\ copy\ number}{reference\ DNA\ copy\ number} 100 = \frac{X}{Y} 100 \qquad (1)$$

Both *X* and *Y* are random variables. 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[39]. The mean, denoted by $E[\frac{X}{Y}]$, and the variance of a ratio of independent random variables are approximated by

$$E\left[\frac{X}{Y}\right] \approx \frac{\bar{x}}{\bar{y}} + \frac{\bar{x}}{\bar{y}^3} Var(y) \qquad (2)$$

And

$$Var\left[\frac{X}{Y}\right] \approx \left(\frac{\bar{x}}{\bar{y}}\right)^2 \left(\frac{Var(x)}{\bar{x}^2} + \frac{Var(y)}{\bar{y}^2}\right) \qquad (3)$$

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.

**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 ($GM_{1\text{-}8}$) and eight standard deviations ($sd_{1\text{-}8}$) 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:

| **GM target gene** | | **Reference gene** | |
|---|---|---|---|
| **Cq** | **copy number** | **Cq** | **copy number** |
| 24.41 | 16,119 | 21.30 | 156,758 |
| 24.61 | 13,954 | 21.18 | 171,196 |

Thus, $\bar{x} = 15036.97$, $\bar{y} = 163977$, Var(*x*) = 2343612.5 and Var(*y*) = 104,227,922. Putting the appropriate values into equation (2) gives a mean $GM_1$ of 0.092 or 9.2 %; using equation (3) with the above values and taking the square root gives a standard deviation $sd_1$ of 0.010943.

Extraction 2:

| **GM Target gene** | | **Reference gene** | |
|---|---|---|---|
| **Cq** | **copy number** | **Cq** | **copy number** |
| 25.50 | 13,405 | 21.10 | 172,089 |
| 25.44 | 14,000 | 21.19 | 160,907 |

Here, $\bar{x} = 13702.5$, $\bar{y} = 166498$, Var(*x*) = 177012.5 and 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. $\overline{GM} = \sum_{i=1}^{8} GM_i \Big/ 8$ . 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 $sd_{GM} = \sqrt{\sum_{i=1}^{8}(n_i - 1)sd_i^2 \Big/ \left(\sum_{i=1}^{8} n_i - k\right)}$; the term in the denominator is 16 – 8 = 8 in this example. The relative repeatability standard deviation $RSD_r = \frac{sd_{GM}}{\overline{GM}}100$.

***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 ($GM_{1\text{-}4}$) and four standard deviations ($sd_{1\text{-}4}$) 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:

| **GM target gene** | | **Reference gene** | |
|---|---|---|---|
| **Cq** | **copy number** | **Cq** | **copy number** |
| 24.41 | 16,119 | 21.30 | 156,758 |
| 24.61 | 13,954 | 21.18 | 171,196 |
| 25.50 | 13,405 | 21.10 | 172,089 |
| 25.44 | 14,000 | 21.19 | 160,907 |

Here, $\bar{x} = 14369.5$, $\bar{y} = 165237.5$, Var($x$) = 1433394 and Var($y$) = 57,700,642. 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:

| **GM target gene** | | **Reference gene** | |
|---|---|---|---|
| **Cq** | **copy number** | **Cq** | **copy number** |
| 26.21 | 14,826.97 | 21.09 | 165,248 |
| 26.30 | 13,885.92 | 21.09 | 165,248 |
| 26.38 | 13,099.69 | 21.20 | 152,168 |
| 26.20 | 14,935.39 | 21.25 | 146,569 |

Here, $\bar{x} = 14187$, $\bar{y} = 157308$, Var($x$) = 747,515 and Var($y$) = 89,272,181. 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. $\overline{GM} = \sum_{i=1}^{4} GM_i \Big/ 4$ . 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 $sd_{GM} = \sqrt{\sum_{i=1}^{4}(n_i - 1)sd_i^2 \Big/ \left(\sum_{i=1}^{4} n_i - k\right)}$ ; the term in the denominator is 16 – 4 = 12 in this example. The relative repeatability standard deviation $RSD_r = \frac{sd_{GM}}{\overline{GM}} 100$ .

***Excel files for calculation of verification data***

Two Excel files for verification of quantitative real time PCR methods are available for download.

In the Excel file '*Method Verification Calculations RIKILT WUR.xlsx*'[4] (Supplemental Material 2) the Example 2 from Annex 4 can be found on the sheet named '*Verification Doc example 2*'. The Excel sheets with names '*E R2 slope LOD'* and '*bias RSDr*' contain an example with actual cotton validation data. In two identical plates are pipetted (see also Table 1): duplicate standard curves of 5 points (verification of Efficiency, $R^2$, slope); 20, 10, 5, 1 GMO copies/reaction in 5-fold (verification of LOD); 50 ng 1 % and 0.1 % GMO reference material from 2 DNA extractions in 4-fold (verification of bias and $RSD_r$); DNA extraction control 2-fold; negative PCR control, water 2-fold.

In the Excel file *'Method Verification Calculations_Documentation BVL.xlsx'*[d] (Supplemental Material 3) the Excel sheet '*report*' contains all relevant data for the verification: e.g. information of the used material (e.g. species, target sequence/haploid genome copies, % GMO, DNA concentration of samples), PCR volume, template volume, equipment; Cq-values of PCR runs (Plate A-C); overview and evaluation of the acceptance criteria. The file includes Excel sheets for preparation of dilution series (Plate A-C); preparation of dilution of samples with GM level 1, GM level 2 and samples for specificity test; plate layout, preparation of reaction mix and cycling program (Plate A-C).

Plate A: 3 calibration points with 3 PCR replicates (level A-C; e.g. 2.500, 500, 100 GMO copies) and 6 dilution levels with 10 PCR replicates around the expected $LOD_{abs}$ (level D-I; e.g. 60, 40, 20, 10, 5, 1 GMO copies). Plate B+C (two identical plates): triplicate standard curve of 5 points (verification of efficiency and $R^2$ coefficient); two GM level with 2 DNA extraction replicates and 4 PCR replicates per extraction (verification of trueness, precision and $RSD_r$); PCR control reactions; new GMO events can be tested for specificity.

[4] These files were made available for educational purposes only. You may download these files and use them freely. The ENGL, JRC and the authors shall not be liable for any loss, damage etc. resulting from its use.

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## List of abbreviations and definitions

CRL-GMFF: Community Reference Laboratory for Genetically Modified Food and Feed

CRM: Certified Reference Materials

ENGL: European Network of GMO Laboratories

EURL GMFF: European Union Reference Laboratory for Genetically Modified Food and Feed

GMO: Genetically Modified Organisms

LOD: Limit of Detection

LOQ: Limit of Quantification

MPR: Minimum Performance Requirements

NRL: National Reference Laboratory

PCR: Polymerase Chain Reaction

PT: Proficiency Test

WG: Working Group

POD: Probability of Detection

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