# ASEAN GUIDELINE ON GMO METHOD VALIDATION AND VERIFICATION

## CONTENTS

## 1. SCOPE

This document was prepared to provide ASEAN GMO testing laboratories with a guideline on validation and verification of GMO detection methods. This guideline serves to provide the minimal requirements necessary when developing GMO detection methods for DNA extraction, purification, qualitative and quantitative PCR.

## 2. TERMINOLOGY

**a)** ***Working DNA concentration***: DNA concentration used in subsequent PCR analyses.

**b)** ***DNA concentration***: Amount of DNA per volume unit of DNA solution

**c)** ***DNA yield***: Total amount of DNA in the extract

**d)** ***Dynamic Range***: The range of concentrations over which the protocol performs in a linear manner with an acceptable level of trueness and precision.

**e)** ***Certified Reference Material***: reference material accompanied by a certificate, one or more of whose property values are certified by a technically valid procedure, accompanied by or traceable to a certificate or other documentation which is issued by a certifying body

**f)** ***Test portion:*** Test sample, as prepared for testing or analysis, the whole quantity being used for analyte extraction at one time.

**g)** ***Limit of detection (LOD)***: The practical LOD is the lowest relative quantity of the target DNA that can be detected, given a known number of target taxon genome copies. For the calculation of the copy numbers, based on the molecular mass of the respective species genome, the genome sizes should be used.

**h)** ***Limit of quantification (LOQ):*** The practical LOQ is the lowest relative quantity of the target DNA that can reliably be quantified, given a known number of target taxon genome copies. For the calculation of the copy numbers, based on the molecular mass of the respective species genome, the genome sizes should be used.

**i)** ***Specificity:*** property of a method to respond exclusively to the characteristic or analyte under investigation

**j)** ***Sensitivity:*** change in the response divided by the corresponding change in the concentration of a standard (calibration) curve

**k)** ***Accuracy:*** closeness of agreement between a test result and the accepted reference value.

**l)** ***Trueness:*** closeness of agreement between the average value obtained from a large series of test results and an accepted reference value

**m)** ***Precision:*** closeness of agreement between independent test results obtained under stipulated conditions.

**n)** ***Repeatability:*** precision under repeatability conditions

**o)** ***Reproducibility:*** precision under reproducibility conditions

**p)** ***Repeatability conditions:*** conditions where independent 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.

**q)** ***Reproducibility conditions:*** conditions where test results are obtained with the same method on identical test items in different laboratories with different operators using different equipment.

## 3. DNA EXTRACTION AND PURIFICATION

The aim of DNA extraction is to provide suitable quality and quantity of DNA for downstream analysis. DNA quality depends on the length, structural integrity, and physical-chemical purity of the extracted DNA [1]. The same DNA extraction method should be used on the validation study material as well as the representative samples expected to be analysed. As part of the DNA extraction assessment, the given DNA extraction protocol is recommended to be carried out at least twice on 2 independent test portions, processed on different days and if possible with different operators [2]. This is to obtain suitable data for the evaluation of the DNA extraction protocol.

The acceptance criteria for the DNA concentration, DNA yield, DNA structural integrity, and purity should be verified on the same working DNA concentration. Since DNA extraction methods applied to one matrix may not be suitable for other matrices, the DNA extraction criteria should be assessed on a range of representative materials, in line with the intended scope of the protocol. At least two aliquots should be taken from the homogenised laboratory sample as test portions for DNA extraction and subsequent analysis.

The following minimum criteria are used to assess the performance of a DNA extraction module in line with international guidelines [3,4].

### 3.1. DNA concentration

DNA concentration can be determined by using spectrophotometric or fluorometric techniques. It is recommended to use the same technique in validation study as for analyses of samples since quantification of DNA could be affected by the method used [5].

### 3.2. DNA yield

The yield should be adequate in quantity for subsequent downstream analyses. The extraction protocol should provide similar yields for both GM and non-GM material of the same matrix.

### 3.3. Purity of DNA extracts

The purity of DNA extracts is indicated by the absence of PCR inhibitors which may lead to false negative results or underestimation of the analyte. Pure DNA will have a 260/280 absorbance ratio of about 1.8. Types of PCR inhibitors commonly found in samples include polyphenols, oils, polysaccharides, salts, dyes, etc. It is necessary the laboratory verifies that the DNA extraction procedure employed has removed most or all inhibitors present. The presence or absence of inhibitors can be verified by testing the DNA sample in different dilution series, so that the more the DNA is diluted, the less the concentration of the inhibitors.

### 3.4. DNA integrity

The minimum size of most of the DNA fragments in the sample should be larger than the size of the PCR amplicon. This can be checked by carrying out a gel electrophoresis using a reference nucleic acid size marker. The DNA extraction protocol used should not drastically affect the structural integrity of the DNA. This is not applicable for highly processed food with low DNA concentration in the sample.

## 4. QUALITATIVE PCR

### 4.1. Specificity

Specificity testing is carried out during method development and validation stages and need not be carried out for verification study. For validation of a PCR protocol, the PCR should only produce products with the target sequence for which the protocol was developed. This can be demonstrated by similarity searches against databases (e.g. GenBank, Patent, EMBL, etc.) and experimentally testing the protocol against non-target transgenic events, non-transgenic material, and target material. Tests should be conducted with approximately 2500 copies of non-target DNA and at least 100 copies of target DNA.

Event-specific methods should only detect the targeted GM event while element- or construct-specific methods should produce only target sequence products.

The absence of allelic variation and copy number variation should be demonstrated experimentally for taxon-specific detection methods. Taxon-specific methods for quantitative analysis should target a single DNA copy per allele within the taxon. The variability of Cq values in amplification should not exceed 1 Cq.

For multiplex qualitative PCR protocols, it is recommended to check using gel electrophoresis or melting point analysis that the primers do not produce additional amplicons other than the expected ones.

When using certified reference materials for specificity testing, the potential for obtaining unexpected positive results should be considered as these reference samples are usually only characterised with respect to the presence/absence of a limited set of targets.

### 4.2. Limit of Detection (LOD)

Limit of detection (LOD) is the lowest amount of concentration or amount of analyte in a sample that can be reliably detected, but not necessarily quantified. The LOD can be determined for a ratio (e.g., mass fraction or DNA copy number) as well as the number of measurable DNA copies. For statistically evaluating data obtained

during method validation with 95% Confidence interval (CI), it may be necessary to analyse at least 12 PCR replicates for each concentration with all positive results [10].

For verification however, a more pragmatic approach can be used by measuring a number of replicates (e.g., 10 replicates) of low GM content reference material (e.g. a serial dilution with 20, 10, 5, 3 copies, and 1 copy/reaction). If all are positive, it can be inferred that the LOD is below or equal to this GM level.

### 4.3. Robustness

A method is tested for robustness to measure its capacity to remain unaffected by small deliberate changes in the experimental conditions stated in the protocol. The following factors should be tested:

- Different thermal cyclers (brands and models)
- Master mix concentrations
- Total reaction volume
- Forward and reverse primers concentration
- Probe concentrations
- Different annealing temperatures

For qualitative protocols tested:

- All replicates should give positive results
- The target amount /concentration to be tested should be 3 times the LOD

## 5. QUANTITATIVE PCR

### 5.1. Relative Repeatability Standard Deviation ($RSD_r$) (Precision)

The relative standard deviations of test results which are obtained using the same method, laboratory, operator and equipment on identical test items within short intervals of time. The relative repeatability SD should be ≤25% over the whole dynamic range of the PCR protocol for GM assay and taxon-specific assays. The RSDr ≤25% should be established by the laboratory at the minimum level of GM

material allowed as stated in the country's legislation [8]. Estimates of the repeatability should be obtained on a sufficient number of test results, for example at least 15 [9].

### 5.2. Limit of Quantification (LOQ)

The LOQ is the lowest amount or concentration included in the dynamic range that can be reliably quantified with an acceptable level of trueness and precision. The LOQ can be determined either using mass fraction or copy number. This should be obtained on a sufficient number of test results (recommended at least 15) similar to the requirements set for the assessment of RSDr. Thus, assessment of LOQ and RSDr can be carried out simultaneously.

### 5.3. Trueness

Trueness refers to the closeness of agreement between the mean value obtained from a large number of test results and the true or accepted reference value. Trueness should be determined at a level close to the level set in legislation, or according to the intended use of the method. This can be assessed using CRMs at two concentrations (e.g. 0.1% and 1% m/m) and if possible, a third at the upper end of dynamic range (e.g. 5% m/m). Alternatively, a reference sample could be prepared from a higher concentration CRM.

If CRMs are not available, a sufficiently characterised proficiency test (PT) material can be used. However, the assigned value of the PT material should have been independently established outside the PT exercise (consensus value from participants' results are not suitable for trueness estimation).

The procedure and instrumentation used should be the same as during routine testing of samples. Results of at least 16 PCR replicates should be evaluated.

The trueness should fall within ±25% of the accepted reference value over the whole dynamic range of the protocols or a Z-score within the range of +2 and -2 has been obtained if using proficiency testing material.

### 5.4. Dynamic Range

The dynamic range should cover the values (either in % GMO or copy number range) corresponding to the expected use. This is established by preparing a standard curve tested on a minimum of four concentration levels evenly distributed at least in duplicate (recommended triplicate). For combined real-time PCR protocols (i.e. GM assay and taxon-specific assay) the dynamic range expressed in relative concentration should be between 0.09% and 4.5%.

### 5.5. Amplification Efficiency

Amplification efficiency is the amplification rate calculated from the slope of the standard curve plot of Cq values over the DNA copy number/quantity. The average value of the standard curves should be in the range of -3.1 ≤ slope ≥-3.6, corresponding to amplification efficiencies of 110% to 90%. The amplification efficiency for each protocol should be assessed by at least 5 individual runs with the standard curve covering the entire dynamic range. The efficiency of the reaction can be calculated by the following equation:

$$Efficiency\ (\%) = \left(10^{\left(\frac{-1}{slope}\right)} - 1\right) x\ 100$$

A theoretical amplification efficiency of 100% is a slope of -3.2.

### 5.6. $R^2$ Coefficient

$R^2$ is the coefficient of determination which is used to analyse how differences in one variable can be explained by a difference in a second variable (measured Cq value and the logarithm of the copy numbers/DNA quantity of a standard curve obtained by linear regression analysis). The $R^2$ values of the standard curves should be ≥0.98. $R^2$ should be assessed for each method by at least 5 individual runs. The standard curve should cover the whole dynamic range.

### 5.7. Robustness

The factors tested for robustness in quantitative testing is similar to those tested in qualitative testing in para 4.3.

For quantitative protocols:

- The combination of changes RSDr and trueness should not exceed 30% (based on the accepted criterion of ≤25%).
- The target amount/concentration to be tested should be at the LOQ

## 6. REFERENCES

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[4] ISO21571:2005, Foodstuffs-Methods of analysis for the detection of genetically modified organisms and derived products- Nucleic acid extraction, Int. Organ. Stand. (2005).

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[9] ISO 5725-3:1994, Technical Corrigendum 1: Accuracy (trueness and precision) of measurement methods and results - Part 3: Intermediate measures of the precision of a standard measurement method, Int. Organ. Stand. (1994).

[10] Guidelines for the single-laboratory validation of qualitative real-time PCR methods. Bundesamt Verbraucherschutz und Lebensmittelsicherheit (BVL). 2016.

## 7. ADDITIONAL READING MATERIALS

3.1 ISO/IEC 17043:2010: Conformity assessment – General requirements for proficiency testing.

3.2 ISO/IEC 17025:2017: General requirements for the competence of testing and calibration laboratories.

3.3 Protocol for Proficiency Testing Schemes, Version 5, September 2016. Fapas, Fera Science Ltd (Fera), Part 1- Common Principles.

3.4 Protocol for Proficiency Testing Schemes, Version 5, April 2017. Fapas, Fera Science Ltd (Fera), Part 4 – Fapas® GM Scheme (GeMMA).