EUROPEAN COMMISSION
JOINT RESEARCH CENTRE

Institute for Health and Consumer Protection
Molecular Biology and Genomics

**TECHNICAL GUIDANCE DOCUMENT FROM THE EUROPEAN UNION REFERENCE LABORATORY
FOR GENETICALLY MODIFIED FOOD AND FEED
ON THE IMPLEMENTATION OF COMMISSION REGULATION (EU) NO 619/2011**

1.   Background

The recently adopted Commission Regulation (EU) No 619/2011[1] lays down the methods for sampling and analysis for the official control of feed as regards to presence of genetically modified material for which an authorisation procedure is pending or the authorisation of which has expired (the so-called LLP, Low Level Presence Regulation). The requirements of this regulation are technically demanding because they set a non-compliance limit of 0.1% GMO (mass fraction).

The European Union Reference Laboratory for Genetically Modified Food and Feed (EU-RL GMFF) plays a crucial role in the implementation of this regulation because only methods validated by the EU-RL that show a RSDr value of maximum 25% at the level of 0.1 % related to mass fraction of GM material can be used on GMOs falling under that regulation.

The aim of this document is to explain how this Regulation will affect the process of validation and how the laboratories need to operate under that Regulation.

This document may vary with time and therefore comments are welcome at:
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2.   Validation of a method of analysis supplied within Regulation (EC) No 1829/2003 for application at low level presence

Article 20 of the Regulation (EC) No. 1829/2003 mandates the validation of methods of analysis for GM food and feed to be marketed. The validation of such methods has to be concluded before a decision is taken on the authorisation.

The European Network of GMO Laboratories sets rules for minimum acceptance criteria for analytical methods of GM testing.These established that the RSDr % should be equal or below 25% over the whole dynamic range[2] to demonstrate the ability of the method to provide adequate control measurements.

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[1] OJ L 116 OF 25.6.2011

[2] Definition of minimum performance requirements for analytical methods of GMO testing, ENGL, 2008,
     http://gmo-crl.jrc.ec.europa.eu/guidancedocs.htm

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RSDr is *in mathematical terms* the ratio between the standard deviation and the mean, times 100 (RSDr = (σ / μ) x 100) and is calculated differently *within a laboratory* and *within a ring-trial*[3].

Article 1 of the LLP Regulation (EU) No 619/2011 sets the following definitions:

(1) '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;

2) 'Minimum Required Performance Limit (MRPL)': the lowest amount or concentration of analyte in a sample that has to be reliably detected and confirmed by official laboratories;

In addition, paragraph 14 of Regulation (EU) No 619/2011 states that "it is appropriate to set as a Minimum Required Performance Limit (MRPL) the lowest level of GM material which is considered by the EU-RL for the validation of quantitative methods. This level corresponds to 0.1 % related to mass fraction of GM material in feed and is the lowest level where results are satisfactorily reproducible between official laboratories when appropriate sampling protocols and methods of analysis for measuring feed samples are applied."

Annex II B of Regulation (EU) No 619/2011 also confirms that official laboratories shall ensure that, considering the whole analytical method starting with the treatment of the laboratory sample of feed, they are in a position to carry out the analysis at the level of 0.1 % related to mass fraction of GM material in feed with an adequate precision (RSDr less than or equal to 25 %).

In the frame of implementation of Regulation (EU) No 619/2011, the following will apply:

– The EU-RL GMFF accepts only methods where the applicant shows that the RSDr at the level of 0.1 % related to mass fraction of GM material is ≤ 25%; this value will be published in the validation reports.
– The EU-RL GMFF will determine in-house the RSDr at the level of 0.1 % related to mass fraction of GM material by running 15 replicates and will publish the data obtained in the validation report.
– Following a ring-trial, the EU-RL GMFF calculates again the RSDr, this time according to ISO standard 5725. This value has been and will continue to be published in the validation reports.
– In order to be fit for the purpose of meeting the requirements of the LLP regulation, all RSDr values mentioned above have to be below 25%.

According to the provisions of ISO 17025:2005 section 5.4.2, the official laboratory shall provide evidence that it can properly run a reference method by meeting the described performance parameters using actual laboratory data[4]. In particular, the RSDr of the method shall not exceed 25% at the GMO concentration of 0.1% expressed in percentage of mass.

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[3] For most statistical analyses the preferred measure of variation is the *variance.* A disadvantage of the variance is that it is measured in the *square* of the units used and for many purposes, it is more convenient to express the variation in the original units by taking the square root of the variance; this is called the *standard deviation.* In a validation study, according to ISO 5725-2, the variance used in this context is the repeatability variance.

[4] ALACC guide "How to Meet ISO 17025 Requirements for Method Verification". AOAC Intl. 2007

Each individual lab should calculate U for each method, initially based on the RSDr obtained for CRMs and subsequently on data from routine analysis. A minimum of 10 data to calculate the RSDr from an estimated 0.1% CRM-derived sample can be obtained in various ways but a valuable option could be to perform five extractions and two quantitative analyses on each extract. In this way an estimation of the variability due to DNA extraction is partially included.

When the value of RSDr supersedes 25%, the conditions of the Regulations are not met and the laboratory shall not apply the method for routine analysis. When the value is smaller than or equal to 25%, than this one shall be used to calculate U and the associated acceptance/rejection level. In practice, this should be verified on each individual method for the GMOs falling under Regulation (EU) No 619/2011.

According to Regulation (EU) No 619/2011, the measurement uncertainty shall be considered for the whole analytical method.

The analytical procedure in GMO analysis based on real-time PCR consists of two steps (see Reg. (EC) No 641/2004):

- The DNA extraction step:
  The ENGL document on "Definition of minimum performance requirements for analytical methods of GMO testing" and the ENGL document on method verification[5] explain that DNA extracts should pass 'quality criteria' to access the qPCR step.
- The Quantitative PCR (qPCR):
  This step is fully covered by the Method Acceptance and Method Performance documents

The EU-RL, together with ENGL, has analysed its own historical validation data and has consulted a number of peer-reviewed papers such as:

− MacArthur, Roy, Feinberg, Max and Yves Bertheau (2010). Construction of Measurement Uncertainty Profiles for Quantitative Analysis of Genetically Modified Organisms Based on Interlaboratory Validation Data. Journal of AOAC International, Vol. 93 (3), p 1046 – 1056.
− Takashi, Kodama, Yasunori Kurosawa, Kazumi Kitta, and Shigehiro Naito. (2010). Tendency for Interlaboratory Precision in the GMO Analysis Method Based on Real-Time PCR. Journal of AOAC International vol. 93 (2) p 734 – 749.

This analysis allowed the conclusions: (1) that the criteria set in Regulation (EU) No 619/2011 are scientifically justifiable and (2) that the contribution to RSDr from the DNA extraction step would be negligibly small or considerably smaller than that of the PCR quantification step.

However, more specific data analysis is needed and the EU-RL will continue to provide further information.

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[5]   See "Verification of Analytical Methods for GMO Testing when Implementing Interlaboratory Validated Methods" at:
    http://gmo-crl.jrc.ec.europa.eu/doc/ENGL%20MV%20WG%20Report%20July%202011.pdf

3. **Calculation of the expanded measurement uncertainty**

Paragraph 16 of Regulation (EU) No 619/2011 states that "Measurement uncertainty should be determined by each official laboratory and confirmed as described in the guidance document on Measurement Uncertainty for GMO testing laboratories developed by the Joint Research Centre of the Commission (JRC)".

In addition Annex IIB of Regulation (EU) No 619/2011 further confirms that :

− To ensure a level of confidence of approximately 95 %, the outcome of the analysis shall be reported as x +/− U whereby x is the analytical result for one measured transformation event and U is the appropriate expanded measurement uncertainty.
− U shall be specified by the official laboratory for the whole analytical method and confirmed as described in the guidance document on Measurement Uncertainty for GMO testing laboratories developed by JRC.
− A feed material, feed additive or, in the case of compound feed each of the feed material and feed additive of which it is composed shall be considered as non-compliant with Regulation (EC) No 1829/2003 when the analytical result (x) for one measured transformation event minus the expanded measurement uncertainty (U) equals or exceeds the level of 0.1 % related to mass fraction of GM material.

In the frame of implementation of Regulation (EU) No 619/2011, the following will apply:

The expanded measurement uncertainty U is obtained by multiplying the standard uncertainty $u(x)$ by a coverage factor k:

$$U = k * u(x) \tag{1}$$

The standard uncertainty $u(x)$ corresponds to the relative standard deviation of test results obtained by the laboratory under repeatability conditions (RSDr). The coverage factor k is a function of the number of replicates and can be approximated to 2 when the number of test replicates is at least 10 (see footnote[6] for reference).

In practice, a feed shall be considered as non compliant with Regulation (EC) No 1829/2003 when the analytical result (x) minus the expanded measurement uncertainty (U) equals or exceeds the level of 0.1 % related to mass fraction of GM material.:

$$x - U \geq 0.1\%$$
or
$$x \geq 0.1\% + U \tag{2}$$

In practice, assuming the maximum RSDr % allowed of 25% and an applied coverage factor of 2, the rejection criterion for low level presence of unauthorized GMO in feed concerns any GMO concentration larger than 0.15%, since:

$$0.1\% + U = 0.1\% + (50\% * 0.1\%) = 0.15\% \tag{3}$$

Each individual lab should calculate U for each method; the 0.15% indicated above cannot be used as a *de facto* rejection level.

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[6] From James Rohlf, Robert R. Sokal (1995) Statistical Tables, W.H. Freeman

4. Expression of results in mass/mass % versus cp/cp %

Article 3 of Regulation (EU) No 619/2011 states that "The information accompanying the certified reference material shall include information on the breeding of the plant which has been used for the production of the certified reference material and on the zygosity of the insert(s). The certified value of the GMO content shall be given in mass fraction and, where available, in copy number per haploid genome equivalent."

In the frame of implementation of Regulation (EU) No 619/2011, the EU-RL will apply the same criterion for the control samples it receives to carry out its assessment.

In GM quantification experiments, the value of the reported GM % in a sample depends on the calibrant used. The calibrants determine the measurement unit of the results. The following rule applies:

> Measurement results calibrated with a known mass fraction (i.e. a CRM), lead to measurement results expressed in mass fraction, likewise the calibration with a calibrant of a known copy number ratio (i.e. a CRM and/or a dual target plasmid), leads to measurement results expressed in copy number ratios.

The Certified Reference Materials (CRMs) frequently used for calibration are in general certified for their GM *mass fraction* (i.e. m/m %). Where CRMs are certified for their mass fraction and the DNA copy number ratio (cp/cp %), the latter is obtained through a collaborative trial using plasmid DNA as a calibrant.

When results are expressed as GM-DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes[7], they shall be either reanalysed with an appropriate calibrant for mass fraction measurements or converted into mass fractions by taking the associated additional uncertainty into consideration. Some guidance concerning the uncertainty associated to the uncertainty related to the conversion from one measurement unit into the other can be found elsewhere[8].

Annex IIB of Regulation (EU) No 619/2011 further confirms that "When results are primarily expressed as GM-DNA copy numbers in relation to target taxon specific DNA copy numbers calculated in terms of haploid genomes, they shall be converted into mass fraction in accordance with the information provided in each validation report of the EU-RL."

The conversion of the measurement results from mass fraction into copy numbers or *vice versa* is possible but includes an uncertainty. In order to minimise the uncertainty, matrix-based certified reference materials should ideally consist of 100% (mass/mass) of homozygous GM material.

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[7]   Laboratories should be able to express their results either in mass percentage or in percentage of copy numbers provided they have used adequate calibrants for the results to be expressed in the corresponding unit. Evidently, in the test reports, reference should be given to the unit of measurement used and the reference material used.

[8]   ERM application note 4: Use of certified reference materials for the quantification of GMO in food and feed, available at
http://www.erm-crm.org/ERM_products/application_notes/application_note_4/Pages/index.aspx

In the frame of implementation of Regulation (EU) No 619/2011, the following will apply:

Crops, hemizygous for the GM-insert (e.g. hemizygous GM-maize)

The possible variability related to the *biological* composition of the sample is well documented for maize which can be homozygous or heterozygous with the GM part deriving from the male or the female parent. Such *biological variability* derives from the nature and composition of the test sample and not from the analytical measurement.

In hybrid maize, this variability ranges approximately between 40 % (in case of a hybrid derived from a male GM and a female non-GM) and 60% (in case of a hybrid derived from a female GM and a male non-GM). The EU-RL will verify this relationship by digital PCR and publish it in the validation report.

Considering that the maize commodity market consists mainly of hybrid maize, the proposed conversion factor for heterozygous single inserts in maize is:

> **GM % in DNA copy number ratio= 50% [GM% in mass fraction]**

Note: If a mass fraction certified CRM is used which is based on homozygous maize, this equation should not be applied.

Crops, homozygous for the insert (e.g. homozygous GM soya)

Proposed conversion factor for homozygous single inserts in soya:

> **GM % in DNA copy number ratio= 100% [GM% in mass fraction]**