Application Note 4

European Reference Materials

Use of Certified Reference Materials for the quantification of GMO in food and feed

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This application note provides information and guidance on the correct use of JRC's Reference Materials certified for their GM (genetically modified) mass fraction of a specific GM event. It explains how the measurement system for GMO quantification in the EU context is set up.

INTRODUCTION

In the EU genetically modified organisms (GMOs) need to be authorised before they are allowed to be placed on the European market. Regulation (EC) No 1830/2003 demands that food and feed products containing more than 0.9 % of GMOs are labelled. According to Regulation (EU) No 619/2011 feed may contain 0.1 (m/m) % of a GMO for which an authorisation process is pending, or for which the authorisation in the EU has expired. For the implementation of EU legislation quantification of GMOs in food/feed products has to be performed in a reliable manner. Therefore, the authorisation decision for each GMO, published by the EC, specifies the method of detection and the certified reference material (CRM) which set up the measurement system for quantification. The official CRM is used to calibrate the event-specific quantitative PCR reference method validated by the European Reference Laboratory for GM Food and Feed (EU-RL GMFF).

GMO CRM CHARACTERISTICS

The certified values of all GMO CRMs from JRC are based on the masses of dried genetically modified plant material (in most cases seed powder) and/or dried non-genetically modified plant material. For CRMs requiring the mixing of GM and non-GM materials, the masses are corrected for their water content and the purity estimates during certification. The GM mass fraction is calculated as:

$$\frac{corrected\ mass\ GM}{corrected\ mass\ GM\ +\ corrected\ mass\ nonGM}$$

Each GMO CRM is certified for the mass fraction of a specific GM event (stated on the certificate). The CRM can only be used to quantify this event and the corresponding blank material (non-GM material) can only be used to prove the absence of this event below the threshold given on the certificate.

For each GMO CRM the recommended storage conditions are provided on the certificate. The RM producer cannot be held responsible for changes that happen during storage of the material at the customer's premises, especially of opened samples. If a GMO CRM is used multiple times, water uptake of the hygroscopic materials should be minimised by closing the bottle immediately after taking a sample.

GMO QUANTIFICATION METHOD

Quantitative Polymerase Chain Reaction (qPCR) is commonly used to quantify the GM content in food and feed samples. This DNA-based quantification technique measures the ratio between transgenic deoxyribonucleic acid (DNA), i.e. derived from the genetic modification, and endogenous DNA, which is specific for the biological species.

The GMO CRMs released by the JRC are intended to be used in conjunction with the event-specific quantitative PCR reference method validated by the EU-RL GMFF. Quantitative PCR detection methods submitted and validated under the provision of Regulation (EC) No 1829/2003 are accessible for the public via the homepage of the EU-RL GMFF (http://gmo-crl.jrc.ec.europa.eu/StatusOfDossiers.aspx). If the CRMs are used in screening assays, the user has to take into account that the non-GMO CRM may create positive signals with the screening assay while they are true negatives for the event-specific method. As a consequence positive screening signals need to be interpreted with care.

During the preparation of GMO CRMs, special care is taken to ensure that the GM and non-GM powders are similar with respect to their particle size distribution. This is particularly important as the amount of extractable DNA correlates with the particle size. Different DNA extraction efficiencies of the GM and non-GM powder would influence the GM content measured by qPCR. Furthermore, only DNA extraction methods which were validated to fulfil the requirements for GMO quantification and which proved to work with different varieties of the same species should be used. During certification the GM mass fraction of the CRM is verified using an event-specific qPCR method. If different DNA extractability has been observed this information is provided.

The genetic composition of different parts of the seeds of monocotyledons (e.g. maize endosperm, seed coat and embryo) differs, and the value of the DNA ratio in wholemeal flour produced from whole grains differs from flour produced from refined grains (only containing the endosperm). The JRC uses whole seed flours for its GMO CRMs.

All GMO CRMs from the JRC are gravimetrically produced using pure non-GM and GM plant materials. They are certified for the GM mass fraction supporting the implementation of the mass fraction thresholds set in the corresponding EU legislation for food and feed. Most of the GMO CRMs are intended to be used as calibrants for qPCR measurements.

The measurement system for the quantification of GMOs in the EU legal context is established by the official CRM, specified in the authorisation decision, and the EU-RL validated measurement method, therefore no correction needs to be applied. Likewise, commutability, which is a crucial characteristic for reference materials if different measurement methods are applied, does not have to be considered here.

GMO CRM CODE

Each GMO CRM has an individual code which consists of a combination of letters and numbers. The General format is ERM-BF123xy. The "ERM®" specifies the brand (protected trademark for European Reference Material) and the BF specifies that this is a plant CRM certified for the GM content. The following three numbers are specific for each particular event. The x is a small letter ranging from a to g and added behind the event code to indicate the mass fraction level (i.e. ERM-BF412a = nominal 0 g/kg, ERM-BF412b = nominal 1000 g/kg, ERM-BF412c = nominal 1 g/kg, ERM-BF412d = 10 g/kg, nominal ERM-BF412e = 100 g/kg). The y is a small letter ranging from k to z and indicates the second, third etc. CRM production series. The first production series comes without this additional letter y.
In most cases five different concentration levels are produced per set: nominal 0 and 100 % and the mixtures 0.1, 1, 10 (m/m) %. Colour-coded caps are used to facilitate the identification of the different mass fraction levels: nominal 0 g/kg = silver, nominal 1000 g/kg = black, nominal 1 g/kg = gold, nominal 10 g/kg = red, nominal 100 g/kg = brown.

Each CRM production series has been processed using different plant materials (e.g. seeds lots). Additionally the particle size distribution may differ despite all efforts to keep it constant between the older and the newer series. Therefore, the calibration lines established with any new CRM series may differ from the calibration lines established with the old series and a new quality control chart should be started when a CRM from a new series is used for the first time. Likewise it is advised to change for all applications, including calibration and quality control, from using the old series to the new series. For traceability reasons the specific CRM used for calibration should always be specified.

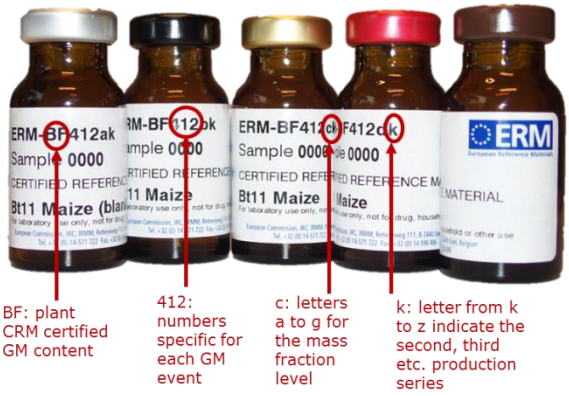

Figure 1: Set of GMO CRMs

SPECIFIC CERTIFICATION ASPECTS

For the majority of the GMO CRMs also a pure non-GMO powder and a pure GMO powder is available. The non-GMO is certified to contain 'less than' a given mass fraction of GMO based on the limit of detection (LOD) of the method used for characterisation. The pure GMO powder is certified to contain 'more than' a given mass fraction, based on the number of seeds tested and statistical evaluation. When used for calibration the laboratory needs to ensure to use the certified values (see example).

A few GMO CRMs are certified for their identity (i.e. ERM-BF421b). Such CRMs are intended to be used as positive control samples in e.g. screening exercises. They should be used to confirm the presence (or absence) of a specific GMO event but are not suitable for the quantification of GMOs.

Some GMO CRMs have been certified with an asymmetric uncertainty range. If such a CRM is used for bias control (see ERM Application Note 1), the ‘plus uncertainty’ has to be used in the case that the average measurement result exceeds the certified value and the ‘minus uncertainty’ has to be used in the case that the average measurement result is lower than the certified value.

A few GMO CRMs of a specific concentration level have been certified, additionally to the GM mass fraction, for their GM DNA copy number ratio (i.e. ERM-BF413e, ERM-BF415e, ERM-BF425c, ERM-BF427c) and a corresponding plasmid based calibrant has been made available (i.e. ERM-AD413, ERM-AD415, ERM-AD425, ERM-AD427). If used for legal compliance testing in the EU, the measurement results have to be converted from DNA copy number ratios into mass fractions. Note, that the EU-RL GMFF provides established conversion factors for this purpose.

EXAMPLE ON THE USE OF PURE GMO CRMS AND THEIR UNCERTAINTIES

The following information can be found on the certificates of analysis for ERM-BF412ak and ERM-BF412bk (https://crm.jrc.ec.europa.eu/):

	Bt11 maize mass fraction [1]	
	Certified value [g/kg]	Uncertainty [g/kg] [4]
ERM-BF412ak	< 0.12 [2]	-
ERM-BF412bk	> 970 [3]	-

1) Genetically modified maize with the unique identifier SYN-BTØ11-1.

2) The certified reference material has been produced from conventional, non-modified maize seeds. No contamination was detected in this material when using an event-specific quantitative polymerase chain reaction assay targeting the Bt11 maize event. The limit of detection (LOD) was 0.12 g/kg. With 95 % confidence, the true Bt11 maize mass fraction of the material is below 0.12 g/kg. The certified value is traceable to the International System of Units (SI).

3) This certified reference material was produced from genetically modified Bt11 maize seeds. The certified value is based on the genetic purity of the maize powder with regard to Bt11 maize. In total 209 seeds were tested individually for the presence of the Bt11 maize event of which 207 seeds tested positive. With 95 % confidence, the true Bt11 maize mass fraction of the material is above 970 g/kg. The certified value is traceable to the International System of Units (SI).

4) The uncertainty is the expanded uncertainty with a coverage factor $k = 2$ corresponding to a level of confidence of about 95 %, estimated in accordance with ISO/IEC Guide 98-3, Guide to the Expression of Uncertainty in Measurement (GUM:1995), ISO, 2008.

Based on the above information, it can be concluded that CRM ERM-BF412ak is certified to contain < 0.12 g Bt11/kg and that no contamination was detected in the powder material. With 95 % confidence the true Bt11 maize mass fraction of the material is below 0.12 g/kg. Therefore, for further calculations, including the set-up of calibration curves, the value 0 g/kg should be used.

CRM ERM-BF412bk was certified to contain > 970 g/kg. 207 of 209 seeds tested, were found positive for the presence of the Bt11 maize event. With 95 % confidence, the true Bt11 maize mass fraction of the material is above 970 g/kg. For further calculations, when setting up a calibration curve for this GM, the estimated genetic purity of 990 g/kg should be used. Note that for pure GMO powder CRMs in which no contamination was found, 1000 g/kg should be used.

In all cases, the uncertainty to be used is calculated by the interval between the numerical value and the given limit divided by √3. For example:
ERM-BF412ak: Value used is 0.0 g/kg; the uncertainty is 0.12 g/kg – 0 g/kg)/ √3 = 0.07 g/kg
ERM-BF412bk: The value used, based on the genetic purity, is 207 / 209 * 1000 = 990 g/kg. The "minus uncertainty" is 990-970) / √3 = 11 .5 g/kg. The "plus uncertainty" is 10 g/kg as the possible GM content cannot exceed 1000 g/kg.