# Parameters and acceptance criteria to verify the appropriateness of GMO certified reference materials

## 1. Rationale and Aim

The evaluation of the appropriateness of the certified reference material sample (CRM) including its certificate is performed according to Regulation (EU) No 503/2013 on applications for authorisation of genetically modified food and feed in accordance with Regulation (EC) No 1829/2003 of the European Parliament and of the Council and amending Commission Regulations (EC) No 641/2004 and (EC) No 1981/2006.

In particular, Annex III § 4 defines the general requirements under which the CRM shall be produced and the required competences of corresponding reference material producers. Reference is made to ISO Guide 34 (which has been transformed into the harmonised standard ISO 17034 in 2016) and to ISO/IEC 17025 for the assignment and verification of the property value of interest. The main characteristics of those internationally accepted technical provisions concern the material containers, the homogeneity testing, the stability testing, the batch characterisation, the final storage and the information required on the certificate for a CRM.

The appropriateness of the CRM will be verified by performing documentary and experimental checks. This document summarizes the parameters, acceptance criteria and thresholds set for each parameter verified by the EURL GMFF. The thresholds for the respective acceptance criteria are based on fit-for-purpose experiences in the application of state-of-the art analytical methods.

## 2. Documentary checks of the certificate

### 2.1. Certified value

A certified value expressed in g/kg or ng/µg should be provided. The certified value may either be expressed as a numerical value (x) or as larger than or as smaller than a numerical value (x).

The numerical value larger than or smaller than x is used when the certified value is based on the purity assessment of the raw material used for the production.

Acceptance criterion: a certified value expressed in g/kg or in ng/µg shall be provided.

## 2.2. Uncertainty statement

An uncertainty value estimated according to the ISO Guide to the Expression of Uncertainty in Measurement shall be provided.

Acceptance criterion: an uncertainty value is provided.

## 2.3. Sample intake

The minimum sample intake shall be specified, based on the homogeneity assessment of the material. It should not be larger than 1 g and preferably at a maximum of 200 mg for a powder.

Acceptance criterion: Sample intake is verified and is not exceeding 1 g.

## 2.4. Stability and shelf-life

The shelf-life of the CRM sample has to be stated.

Acceptance criterion: A shelf-life is specified on the certificate.

## 2.5. Homogeneity of the CRM powder

A key requirement for any CRM aliquoted into units (samples of a production batch) is the equivalence of the property values between units. Consequently, ISO Guide 34 (later converted into ISO 17034) requires RM producers to quantify the between-unit variation. This aspect should be covered in between-unit homogeneity studies. Quantification of between-unit inhomogeneity can be estimated by analysis of variance (ANOVA), which separates the between-unit variation (sbb) from the within-unit variation (swb). The latter is equivalent to the method intermediate precision, if the individual samples were representative of the whole unit.

The property values assessed in a homogeneity study have to relate the property relevant for the intended use of the CRM. The CRMs are used and indispensable for the calibration of the quantification methods based on quantitative PCR. The property value for the homogeneity study must therefore be defined as the copy number ratio between the event-specific targets and the taxon-specific reference targets. The consistency of this ratio must be demonstrated for a CRM. A claim that a material is homogenous because it is pure is insufficient because a "pure" material may be composed of heterogeneous tissues having different target gene ratios, which is typically seen in grain crops. Such "pure" but still heterogeneous material may deliver different calibration curves and create inconsistent quantification results. This homogeneity of the CRMs is particularly important for CRMs produced from heterozygous grain crops (e.g. maize, rice, …).

Acceptance criterion: the homogeneity of the CRM must have been correctly evaluated and reported on the certificate.

2.6. Breeding information and zygosity

The certificate has to provide information on the breeding of the GM line used to produce the CRM (male/female origin). This is required for CRMs produced from heterozygous crops only (e.g. maize, rice). For crops that exist with different ploidy status (oilseed rape, cotton, potato, etc.) also the ploidy status of the crop has to be provided, in particular informing about the copy numbers of the GM insert and the preferred taxon-specific reference target, or the expected ratio between them.

Acceptance criterion: Information about the zygosity, breeding origin and ploidy or expected copy number ratio provided on the certificate or in the certification report.

## 3. Experimental checks

3.1. Homogeneity of the genomic DNA CRM solution

The homogeneity of the material will be tested by digital PCR as described in 3.2.3.

3.2. Homogeneity of CRM powder

The homogeneity of the material must guarantee a homogeneous distribution of the plant tissues in the powder. This is particularly important for CRMs composed of a mixture of ground kernels, but also relevant for CRMs consisting of a single material that contains different numbers of copies of the GM target in different plant tissues. Additionally, a small particle size will allow a low sample intake for the DNA extraction and the further quantitative analysis by PCR.

3.2.1. Visual check for the presence of large particles sizes

The presence of large particles indicating an inhomogeneous material will be visually inspected and the sample will be photographed.

Acceptance criterion: absence of large particles that can be visually identified (e.g., absence of broken kernels among a fine powder)

3.2.2. Particle size analysis

In case large particles are visually identified (in 3.2.1), a manual sieve analysis or particle size analysis will be performed.

Acceptance criterion: Mass fraction >1000 µm is equal to 0 g or the particle volume size distribution value $X_{100,3} < 1000$ µm.

3.2.3. Digital PCR

A statement that the material is pure composed of only one material type and therefore homogenous is not sufficient as it is the homogeneity of the genetic modification that needs to be demonstrated.
For homozygous crops, where the target gene ratio do not vary within the seeds tissues, seed purity should suffice to justify sample homogeneity and additional ddPCR verifications will not been performed to assess the homogeneity.

For hemizygous crops, the verification will be performed by digital PCR (dPCR) on DNA extracted from the CRM in case of powder CRMs or on the DNA solution in case of CRMs consisting of already extracted genomic DNA. The dPCR measurements will be done by adapting the qPCR event-specific qPCR method that has been validated by (or submitted for validation) to the EURL GMFF into a digital PCR format. If the CRM has been produced at different content levels, the dPCR measurements will be performed on the CRM presenting the highest GM content. The observed relative standard deviation (RSD) of the measurement results under repeatability conditions depends upon the dPCR method repeatability but also on the homogeneity of the material. An RSD of the copy number ratio above 20 % is an indication that the CRM material is lacking homogeneity.

Acceptance criterion: The RSD of the event-specific/taxon-specific copy number ratio should not exceed 20 %[1].

3.3. Extractability of the DNA from the powder CRM

The amount of genomic DNA that can be extracted from a powder CRM may vary depending on the crop used, the particle size of the powder or the extraction method used. The genomic DNA will be extracted from the powder CRM by validated DNA extraction methods applied for plant tissues and quantified by a fluorimeter to guarantee that a minimum amount of genomic double stranded DNA (dsDNA) needed for the subsequent event-specific and taxon-specific reference systems can be extracted from the powder CRM.

Acceptance criterion: At least 5 µg dsDNA per sample intake can be extracted.

3.4. Amount of DNA in genomic DNA CRMs

For genomic DNA CRMs, the amount of DNA per CRM unit will be quantified in the same way as for the DNA extracted from powder CRM.

Acceptance criterion: At least 90 % of the DNA amount per CRM unit mentioned in the product description on the certificate is measured.

[1] This RSD value is a conservative acceptance criterion. RSD values of 5 to max 10% are generally reported for homogeneous CRMs tested by dPCR under repeatability conditions.