

Use of Certified Reference Materials for the quantification of GMO in DNA copy number ratio

This application note provides guidance on the correct use of European Reference Materials certified for their GM (genetically modified) copy number fraction of a specific GM event. The details given below refer particularly to the use of the CRMs ERM-BF413d and ERM-AD413 and the upcoming CRMs certified for copy number ratio.

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INTRODUCTION

The JRC-IRMM has recently developed two new types of GMO Certified Reference Materials (CRMs) which permit the correct implementation of the Recommendation (EC) 787/2004 [1] using a metrologically traceable system. This note gives instructions on how the new CRMs should be applied.

CHARACTERISTICS OF THE NEW GMO CRMs

A. New GMO matrix CRMs

The certified values are based on two different measurement units. Besides a certified value for the mass fraction of a specific genetic modification event (see application note 4) the new CRM is certified for the DNA copy number ratio. This parameter, expressed in %, is calculated according to:

$$\text{DNA copy number ratio [\%]} = \frac{\text{GM DNA copy number [cp]}}{\text{Target taxon-specific DNA copy number [cp]}} \times 100$$

The certification is based on QRT-PCR (quantitative real-time PCR) measurements. These measurements are calibrated by using a dedicated plasmid DNA calibrant that is certified to contain one copy of both the GM and the taxon-specific target sequence per plasmid (see part B).

Therefore, the GM maize DNA copy number ratio is directly related to the GM event analysed. Moreover, in the example below, the CRM ERM-BF413d should only be used in conjunction with the plasmid calibrant ERM-AD413 and the event-specific MON 810 detection method [2]. The certified MON 810 DNA copy number ratio (0.57 %) is different from the certified mass fraction (10.0 g/kg or 1.0 %) as the certified MON 810 copy number ratio takes into account the zygosity, ploidy and endoreduplication status of the seeds used to produce this material.

The matrix CRM is intended for the quality control of analytical procedures including the DNA extraction and purification as well as the PCR measurement steps for a particular GM event.

B. New GMO plasmid CRMs

The certified calibrants contain a defined DNA fragment specific for a genetic modification as well as a defined DNA fragment specific for the taxon analysed. That plasmid contains a 170 base pair (bp) fragment of the MON 810 5' *plant-P35S* junction and a 351 bp fragment of the maize endogenous high mobility group gene (*hmg*).

The certified values are the number of cloned GM and the number of taxon-specific DNA fragment per plasmid respectively. The number ratio between those two DNA fragments is provided as an indicative value obtained by duplex and simplex real-time PCR.

USE OF THE GMO PLASMID CALIBRANT

The calibrant has to be used in conjunction with a defined QRT-PCR method [1].

Each calibrant is delivered on dry-ice in a closed plastic tube and should be kept at -20 °C until use. The contents should be first thawed, then mixed and finally opened and diluted under a laminar flow to reduce the risk of contamination. Each tube contains approximately 2×10^6 copies (cp) of plasmid per μL and the recommended starting volume for the dilution series is 50 μL . The dilution protocol stated on the certificate should be followed. The dilution buffer is not provided.

The dilution series should always be prepared fresh and any excess discarded in closed tubes. The dilution series is used to prepare two calibration curves (CCs) (one CC for the transgene and one CC for the taxon-specific gene) each having 5 points, each point measured in triplicate in the PCR reaction (see example). One tube provides enough calibrant to prepare 10 CCs for both targets which means that a total number of 100 to 250 samples can be quantified with one tube. The recommended sample intake for QRT-PCR is 5 μL of template DNA per PCR well.

Measured fluorescence threshold values (C_t values) must be plotted against the theoretical number of copies of both fragments to generate two CCs. These CCs are used for the

quantification of the GM target relative to the taxon-specific target in an unknown sample. The results can then be calculated as the ratio of both targets and expressed in percentage according to the Recommendation (EC) 787/2004. An internal quantity control (QC) PCR can be made by calculating the average ratio of measured Ct

values for the taxon-specific and transgenic targets for the calibration points corresponding to 2000 cp/μL. That ratio should be in agreement with 1.04 % (expanded uncertainty 0.06 %) for a simplex PCR as indicated in the certification report (see also ERM Application Note 1).

EXAMPLE

Genomic DNA extracted from an unknown sample and from ERM-BF413d used as QC material are analysed by QRT-PCR using ERM-AD413 as calibrant. Ct values are obtained for the ERM-AD413 calibrant after amplification of the *hmg* and MON 810 fragments (**Table 1**). Average Ct values for the unknown sample, 32.76 and 25.44 are obtained for the amplification of MON 810 and *hmg* fragments, respectively. For the QC material, average Ct values of 31.22 and 22.20 are obtained for the MON 810 and *hmg* fragments, respectively.

The slopes and Y-intercepts of the two calibration curves must be determined to calculate the MON 810 content in both samples assuming a straight line as the best-fit model function. The built-in modules of Microsoft®Excel or any other available calibration/determination software can be used to calculate the slopes and Y-intercepts.

The slopes (*b*) of both linear regression lines can be calculated using the formula: $b = \frac{\sum (\log(x) - \log(\bar{x})) (y - \bar{y})}{\sum (\log(x) - \log(\bar{x}))^2}$ where, *x*

represents the number of copies of the fragment amplified and *y* represents the corresponding Ct value. The slopes of the calibration curves of the *hmg* and the MON 810 fragments given in **Table 1** are respectively -3.25 and -3.32. The Y-intercepts (*a*) of the regression lines are calculated using the formula: $a = \bar{y} - b\bar{x}$ when $\log(x) = 0$. In our example, the *a*-values for the *hmg* and MON 810 regression lines are 39.26 and 40.93, respectively. Those values represent the theoretical Ct values corresponding to 1 cp of both fragments. The slope serves to calculate the PCR efficiency (ϵ) with the formula: $\epsilon = (10^{-1/b} - 1) * 100$. In our example, the PCR efficiencies were equal to 99.7 % and 103.1 % for the amplification of the MON 810 and *hmg* fragments, respectively. The number of MON 810 copies (*cp*) present in the

unknown sample is calculated as: $cp_{MON810} = 10^{\left(\frac{Ct_{MON810} - a_{MON810}}{b_{MON810}}\right)}$, where Ct_{MON810} , a_{MON810} and b_{MON810} are the Ct values, the Y-intercept and the slope obtained for MON 810 amplification. The same calculation is done to determine the

number of copies of the *hmg* fragment with the formula: $cp_{hmg} = 10^{\left(\frac{Ct_{hmg} - a_{hmg}}{b_{hmg}}\right)}$. In our example, the estimated average copy numbers of MON810 and *hmg* fragments present in the unknown sample is 289 and 17878 cp, respectively. The MON 810 content of the unknown sample expressed in percentage is therefore equal to: $\frac{289}{17878} cp_{MON810} * 100 = 1.62 \%$. The

same calculation is made for the QC material containing: $\frac{841}{177513} cp_{MON810} * 100 = 0.47 \%$ of MON 810. Taking into account the

uncertainty associated with the ERM-BF413d and the uncertainty of the measurement (see ERM Application Note 1), it can be verified if the measured value agrees with the certified values of ERM-BF413d. In the example given above the ratio of the average Ct values of 1.05 obtained for 10000 cp (5 μL of ERM-AD413 at 2000 cp/μL) is in agreement with the indicative value of 1.04 ± 0.06 reported on the ERM-AD413 certificate, meaning that this GM quantification was under control.

[1] European Commission Recommendation (EC) N° 787/2004 of 4.10.2004 on technical guidance for sampling and detection of genetically modified organisms and material produced from genetically modified organisms as or in products in the context of Regulation (EC) No 1830/2003. Off. J. Eur. Union L 348 (2004) 18-26

[2] ISO 21570:2005 Foodstuffs - Methods of analysis for the detection of genetically modified organisms and derived products - Quantitative nucleic based methods. Annex D2 Event-specific method for the relative quantitation of maize line MON 810 DNA using real-time PCR. 93-99.

† Cross-contamination or unspecific amplification should be suspected if the Ct values for the NTC differs from the number of PCR cycles performed.

Total number of cp of the MON 810 fragment	Ct values measured			average Ct
	replicate 1	replicate 2	replicate 3	
50000	25.30	25.19	25.15	25.21
10000	27.57	27.62	27.66	27.62
1000	31.19	30.89	31.14	31.07
100	33.99	35.12	34.80	34.64
25	35.79	35.67	36.37	35.95
NTC	45.00	45.00	45.00	45.00
Total number of cp of the <i>hmg</i> fragment				
500000	20.76	20.67	20.63	20.69
100000	22.92	23.00	23.04	22.99
10000	26.39	26.36	26.33	26.36
5000	27.31	27.29	27.37	27.32
1000	29.38	29.19	29.57	29.38
NTC	45.00	45.00	45.00	45.00

Table 1: Example of experimental Ct values obtained using the ERM-AD413 as calibrant. NTC[†] = no template control.