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Certified Reference Materials

AOCS 1114-C

Report of the certification process for

NP2391/NP2222 (MZHG0JG)

Maize Certified Reference Materials

First Batch

OECD Unique Identifier
SYN-ØØØJG-2

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Abstract

This report describes the preparation and certification of the maize AOCS CRM 1114-C produced by AOCS Technical Services in 2015. The CRMs have been prepared according to ISO Guides 30, 31, 34 and 35 and are intended to serve as control material for third party testing of maize for transformation events. NP2391/NP2222 (MZHG0JG) maize seed powder was provided by Syngenta Crop Protection, LLC and was prepared by grinding the bulk source at Syngenta Crop Protection, LLC. The NP2391/NP2222 (MZHG0JG) maize powder was then aliquoted and packaged under a nitrogen gas environment at Illinois Crop Improvement Association. The presence of NP2391/NP2222 (MZHG0JG) in the maize was verified using event-specific, qualitative real-time PCR analysis by Eurofins GeneScan, New Orleans, LA (an ISO 17025:2005 accredited laboratory). The certified mass value of MZHG0JG in NP2391/NP2222 (MZHG0JG) was based on the purity of the material and with 95% confidence, it is at least 759 g/kg. Homogeneity testing was performed at Eurofins GeneScan using qualitative real-time PCR prior to bottling, and quantitative real-time PCR after the AOCS CRM 1114-C was bottled. AOCS CRM 1114-C is available in 27-mL glass headspace vials. This CRM shall be stored dry in a sealed container at ambient or cooler conditions in the dark.

Acknowledgements

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Glossary

AOCS	American Oil Chemists' Society
Conventional Crop	Conventional counterpart means a related organism/variety, its components and/or products for which there is experience of establishing safety based on common use as food
Cycle threshold (Ct)	Number of PCR cycles required for the fluorescent signal to cross a threshold that exceeds background level
DNA	Deoxyribonucleic Acid is the linear, double-helix macromolecule that makes up the genetic material of most organisms
Detection Limit	Lowest level at which target DNA can be detected in a sample
EC	European Commission
Genome	The full set of genes and associated DNA characteristic of an organism
GMO	Genetically modified/engineered organism: an organism in which the genetic material has been changed through modern biotechnology in a way that does not occur naturally by multiplication and/or natural recombination
ISO	International Organisation for Standardisation
PCR	Polymerase Chain Reaction: technique used to deter mine whether a sample of plant tissue contains a particular DNA sequence. PCR relies on primer sets that bind to a particular target DNA sequence and a special DNA-copying enzyme (DNA polymerase) that exponentially amplifies the target sequence for identification and measurement

Qualitative PCR	PCR methods that determine the presence or absence of a specific target DNA sequence at a particular level of detection
Quantitation Limit	Lowest level at which the amount of target DNA sequence in a sample can be reliably quantitated
Quantitative PCR	PCR methods that estimate the relative amount of target DNA sequence in a mixture of DNA molecules
RSDr	Relative standard deviation
SD	Standard deviation
Trait:NP2391/NP2222 (MZHG0JG)	Line of maize, genetically engineered to confer tolerance to the herbicides glyphosate and glufosinate-ammonium

Introduction

Plant biotechnology is an extension of traditional plant breeding. It allows plant breeders to develop crops with specific traits including insect, disease, and herbicide resistance; processing advantages; and nutritional enhancement. An important component for identifying these new traits is a Certified Reference Material (CRM) created from leaf, seed, or grain containing the new trait as well as a CRM created from the conventionally bred matrix. The European Commission has mandated that from 18 April 2004, a method for detecting a new event derived from transgenic technology and Certified Reference Material must be available before the EC will consider authorization of a new crop derived from transgenic technology. Several nations outside Europe also require grain and ingredients to be labeled above a threshold level before accepting a shipment.

To meet the above regulatory requirements for GMO determination, AOCS CRM 1114-C was manufactured from maize seed according to ISO Guides 30, 31, 34 and 35 and in accordance with EC No 1829/2003, EC No 641/2004 and EC No 619/2011. This CRM is available from AOCS.

Material Processing and Particle Size Analyses

The hemizygous NP2391/NP2222 (MZHG0JG) maize used in the preparation of AOCS CRM 1114-C resulted from the cross of female non-transgenic NP2391 and male NP2222 (MZHG0JG), and Syngenta Crop Protection, LLC delivered 10 kg NP2391/NP2222 (MZHG0JG) maize powder to AOCS. The NP2391/NP2222 (MZHG0JG) maize was packaged in 27-mL glass headspace vials and sealed under a nitrogen gas environment at the Illinois Crop Improvement Association.

Once packaged, four randomly selected samples of AOCS CRM 1114-C maize were subject to particle size analysis. Particle size analysis was conducted at AVEKA, Woodbury, Minnesota (an ISO 9001:2015 accredited laboratory) using a Horiba LA-950 particle size analyzer. For each sample, the particle size mean and range, and the percentage of particles below a given size was calculated. On average, the particle size

of AOCS CRM 1114-C was 616.66 + 11.21 µm, and 90% of the particles (i.e. D90) were smaller than 1362.42 + 62.70 µm (Table 1).

Table 1. Results of Particle Size Analyses of AOCS CRM 1114-C

	Sample 1 (µm)	Sample 2 (µm)	Sample 3 (µm)	Sample 4 (µm)	Average (µm)	Standard Deviation (µm)
Mean	627.33	601.04	621.02	617.25	**616.66**	**11.21**
Range	0.8 - 3000	0.8 - 2637	0.8 - 2637	0.8 - 3000	N/A[(a)]	N/A
D10 [(b)]	22.58	25.18	23.55	25.21	24.13	1.29
D50 [(b)]	505.54	534.20	545.13	512.74	524.40	18.42
D90 [(b)]	1445.70	1296.63	1339.95	1367.41	**1362.42**	**62.70**

[(a)] N/A = not applicable

[(b)] D10, D50 and D90 indicate that 10%, 50% or 90% of the particles, respectively, are smaller than size given in table, respectively

The particle size distribution for each of the samples analyzed is presented as a histogram, with discrete size bins up to 3000 µm (Figure 1). Figure 1-A represents the percentage of particles of a given size, and Figure 1-B represents the cumulative particle size distribution, which reflects the total percentage of particles smaller than a given size.

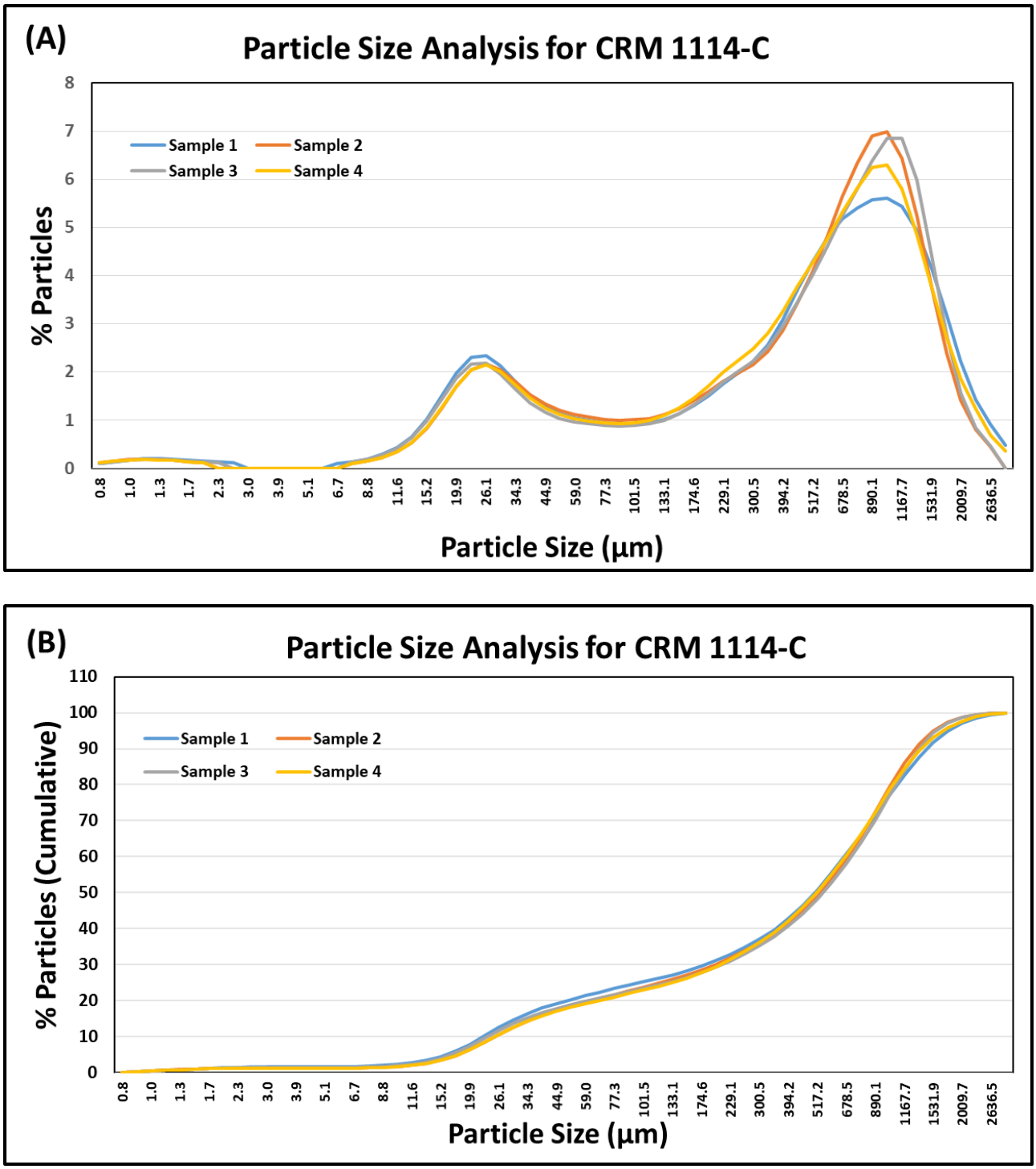

Figure 1. Particle size distribution plots. (A) Percentage of particles of a given size. (B) Cumulative distribution of particle sizes.

Trait Verification

After the bulk material was packaged, AOCS used the random number generator function of Microsoft Excel to select samples for verification of trait presence, homogeneity, and to rule out degradation during packaging. A total of ten (10) AOCS CRM 1114-C samples (32, 39, 139, 161, 420, 489, 597, 608, 632 and 676) were sent to Eurofins GeneScan, New Orleans, LA for event-specific, qualitative real-time PCR analysis to screen for NP2391/NP2222 (MZHG0JG) presence in the samples. These results are presented in Table 2. These data confirm the presence of MZHG0JG after the packaging of AOCS 1114-C.

Table 2. Results for the verification of AOCS CRM 1114-C NP2391/NP2222 (MZHG0JG) maize as tested by Eurofins GeneScan, New Orleans, LA with MZHG0JG event-specific, qualitative real-time PCR analysis.

Sample	MZHG0JG Presence
AOCS 1114-C 32	Positive
AOCS 1114-C 39	Positive
AOCS 1114-C 139	Positive
AOCS 1114-C 161	Positive
AOCS 1114-C 420	Positive
AOCS 1114-C 489	Positive
AOCS 1114-C 597	Positive
AOCS 1114-C 608	Positive
AOCS 1114-C 632	Positive
AOCS 1114-C 676	Positive

Certified Value and Measurement Uncertainty

The genetic purity is based on the presence of MZHG0JG in NP2391/NP2222 (MZHG0JG) maize and was assessed by Syngenta Crop Protection, LLC. A total of 398 NP2391/NP2222 (MZHG0JG) maize seeds were evaluated by qualitative, MZHG0JG-specific real time PCR. The results showed that 390 of the 398 seeds tested (98.0%) were positive for the presence of MZHG0JG.

The statistical purity of NP2391/NP2222 (MZHG0JG) maize was calculated using SeedCalc8 (Remund *et al*., 2008) and corresponds to the lower bound of true % purity. Using a 95% confidence level, the true % purity of NP2391/NP2222 (MZHG0JG) maize is at least (75.9%) Consequently, with 95% confidence, the true value of NP2391/NP2222 (MZHG0JG) maize is ≥ 759 g/kg.

The measurement uncertainty (U_{CRM}) is the expanded uncertainty with a coverage factor of 2 and a confidence level of 95%. It is obtained by combining the uncertainties from the purity assessment ($u_{char,rel}$), the homogeneity assessment ($u_{bb,rel}$), the transport stability assessment ($u_{sts,rel}$) and the long-term stability assessment ($u_{lts,rel}$):

$$u_{CRM,rel} = \sqrt{{u_{char,rel}}^2 + {u_{bb,rel}}^2 + {u_{sts,rel}}^2 + {u_{lts,rel}}^2}$$

$$U_{CRM} = 2 \times u_{CRM,rel} \times 1000\ g/kg$$

When using an asymmetric uncertainty, the reported measurement uncertainty is truncated on the right side such that the value does not exceed 1000 g/kg. Consequently, the expanded measurement uncertainty for this CRM is +20 g/kg, -221 g/kg.

Homogeneity Testing

The material used for the production of AOCS CRM 1114-C, NP2391/NP2222 (MZHG0JG), is 96.4% pure (see Certified Value and Measurement Uncertainty section) and is expected to be homogenous. To further confirm homogeneity, the ground material was analytically assessed before and after bottling using real-time PCR.

Primary samples were taken from randomly selected areas and depths to form a 3-kg composite sample in accordance with the International Seed Testing Association's (ISTA) Seed Science and Technology Rules for batches up to 500 kg, ten (10) working samples of 100 g each were prepared from the composite sample and sent to Eurofins GeneScan, New Orleans, LA (an ISO 17025:2005 accredited laboratory) for event-specific, qualitative real-time PCR analysis. The analyses performed by Eurofins GeneScan, New Orleans, LA were used to assess the homogeneity of the seed lot based on the trait presence A summary of the qualitative PCR results for the NP2391/NP2222 (MZHG0JG) homogeneity samples is presented in Table 3.

Table 3. Results of the qualitative homogeneity testing performed by Eurofins GeneScan, New Orleans, LA on the NP2391/NP2222 (MZHG0JG) bulk material provided by Syngenta Crop Protection, LLC

Sample	MZHG0JG Presence
Homogeneity Sample 1	Positive
Homogeneity Sample 2	Positive
Homogeneity Sample 3	Positive
Homogeneity Sample 4	Positive
Homogeneity Sample 5	Positive
Homogeneity Sample 6	Positive
Homogeneity Sample 7	Positive
Homogeneity Sample 8	Positive
Homogeneity Sample 9	Positive
Homogeneity Sample 10	Positive

In addition, homogeneity was also assessed after bottling of AOCS CRM 1114-C using a MZHG0JG specific, quantitative real-time PCR method (http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-04-16-VR.pdf). A total of 10 samples were analyzed, and for each sample, 2 independent DNA extractions and quantifications were performed at Eurofins Genescan using a test portion of 1 gram. Extracted DNA was checked for integrity by gel-electrophoresis and quantified prior to using it in quantitative real-time PCR. For each of the DNA extracts, all PCR reactions were done in triplicate.

The cycle threshold (Ct) values for an endogenous *adh1* maize gene and for event MZHG0JG were used to calculate the number of copies (cp#) for either target. Subsequently, the ratio between event MZHG0JG copy number and *adh1* copy number (MZHG0JG cp#/*adh1* cp#) was calculated and used to estimate within-unit relative standard deviation ($\mathrm{RSD_w}$) and between-unit relative standard deviation ($\mathrm{RSD_b}$). Within-unit relative standard deviation ($\mathrm{RSD_w}$), between-unit relative standard deviation ($\mathrm{RSD_b}$) were calculated as:

Within-unit RSD: $RSD_w = \frac{\sqrt{MS_{within}}}{\bar{y}}$

Between-unit RSD: $RSD_b = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}}$

where,

$\mathrm{MS_{within}}$ within-unit mean square from an ANOVA
$\mathrm{MS_{between}}$ between-unit mean square from an ANOVA
$\bar{y}$ mean of all results of the homogeneity study
n mean number of replicates per unit

Table 4. The within-unit relative standard deviation (RSD_w) and the between-unit relative standard deviation (RSD_b) for vials of AOCS CRM 1114-C.

CRM	RSD_w [%]	RSD_b [%]
AOCS 1114-C	13.0	10.9

The CRM will be determined to be homogeneous if the within-unit relative standard deviation (RSD_w) and between-unit relative standard deviation (RSD_b) are both ≤ 20%. Based on the quantitative real-time PCR analyses conducted, it was concluded that AOCS CRM 1114-C is homogenous (Table 4). These results are in agreement with homogeneity results from qualitative real-time PCR analyses and with the purity estimate for material NP2391/NP2222 (MZHG0JG) maize calculated in the Certified Value and Measurement Uncertainty section above.

Stability

Time, temperature and light are regarded as the most relevant influences on the stability of CRM (Linsinger et al., 2001). The influence of light is mitigated by shipping and storing the vials in boxes, thus minimizing the possibility of degradation due to light. The influence of temperature is mitigated by storing the vials in a temperature-controlled room, and shipping vials at ambient temperature.

The effect of temperature and time are investigated.

A transport (short-term) stability study is conducted to assess the stability of maize CRM during transport. The temperature and time conditions in the study cover the typical conditions and the not so rare situations. The outcome of the study is considered transferable to other CRMs of similar property. Samples were subject to 3 different temperatures (4 ℃ (fridge), 25 ℃ (ambient), 60 ℃ (oven)) for 4 different durations (0, 1, 2, and 4 weeks). The study concluded that samples are stable at 4 ℃ (fridge) and 25 ℃ (ambient) for 4 weeks. The estimated uncertainty contribution from transport (short-term) stability is 1.0%.

A long-term stability study is conducted to assess the stability of maize CRM during storage. Samples are stored at 25 ℃ (ambient) and the stability of the sample is monitored as long as the samples is available. The storage temperature study is 25 ℃ and the length of time to be studied is 10 years. The outcome of the study is considered transferable to other CRMs of similar property. In the initial 1-year stability study, samples were subject the storage condition for 4 different durations (0, 1, 3, 6 and 12 months).

The study concluded that samples are stable at 25 ℃ (ambient) for 12 months. The estimated uncertainty contribution from long-term stability is 0.42%.

CRM stability over time will be analyzed by repeating the homogeneity study described above at a chosen shelf life of approximately every 24 months. The 24-month shelf life of CRM is chosen because the influence of analytical variation can be reduced by increasing the length of the stability study (Linsinger, et al., 2001).

The initial ratio between the number of copies of the GM event and the number of copies of the endogenous reference gene from the homogeneity study will establish the base line for the stability study. The ratio at each 24-month interval will be compared to the ratio established in the homogeneity study. The CRM will be determined to be stable if the variability of the ratios, determined as relative standard deviation (RSD) is ≤ 20%.

Stability of these CRMs has been listed as 2 years from the certification date. The materials were processed and are stored at ambient temperature, under nitrogen gas, in 27 -mL glass headspace vials. These materials are expected to be stable for longer than the estimated expiration date. The stability of the powder material will be re-evaluated at time of expiration. If the samples are determined to be stable, the certificates will be extended.

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Biosafety Clearing House Living Modified Organism (LMO) Registry
http://bch.cbd.int/database/lmo-registry/

Eurofins GeneScan; 2219 Lakeshore Drive, Suite 400, New Orleans, LA 70122; Telephone: +1 504 297 4330 Toll Free: +1 866 535 2730 Fax: +1 504 297 4335 https://www.eurofinsus.com/food-testing/testing-services/gmo/

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