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Certified Reference Materials

AOCS 0407-A

Report of the certification process for

Non-modified

Maize Certified Reference Materials

First Batch

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Abstract

This report describes the preparation and certification of the non-modified maize CRM AOCS 0407-A produced by AOCS Technical Services in 2006. 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. Non-modified maize seed was provided by Syngenta Crop Protection, LLC. The non-modified maize was prepared by grinding the bulk source according to maize processing protocols by Texas A&M University and was then aliquoted and packaged under a nitrogen gas environment at Illinois Crop Improvement Association. The absence of the GA21, MIR604, MIR162 and MZHG0JG events in the maize was verified using event-specific, qualitative PCR analysis by Eurofins-GeneScan, Metairie, LA (an ISO 17025:2005 accredited laboratory). The certified value of non-modified CRM AOCS 0407-A was based on the absence of genetically modified impurities and with 95% confidence, is lower than 1 g/kg. Homogeneity testing was performed at Eurofins GeneScan using qualitative real-time PCR prior to bottling, and quantitative real-time PCR after the CRM AOCS 0407-A was bottled. CRM AOCS 0407-A 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.

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Glossary

AOCS	American Oil Chemists' Society
Conventional Crop	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 determine 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

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, CRM AOCS 0407-A was manufactured from maize seed according to ISO Guides 30, 31, 34 and 35 and in accordance with EC No 1829/2003. The CRM is available from AOCS.

Material Preparation and Particle Size Analyses

Syngenta Crop Protection, LLC delivered non-modified maize seed to AOCS for the production of CRM AOCS 0407-A. The non-modified maize was ground at the Texas A&M Food Protein Research and Development Center using a hammer mill fitted with a 1/8 inch screen. The maize flour was then 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 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 certain size was calculated (Table 1). On average, the particle size of CRM AOCS 0407-A was 250.84 + 5.12 µm, and 90% of the particles (i.e. D90) were smaller than 521.40 + 5.81 µm.

Table 1. Results of Particle Size Analyses of CRM AOCS 0407-A						
	Sample 1 (µm)	**Sample 2 (µm)**	**Sample 3 (µm)**	**Sample 4 (µm)**	**Average (µm)**	**Standard Deviation (µm)**
Mean	254.17	246.95	246.02	256.23	**250.84**	**5.12**
Range	0.8-1020	0.8-1020	0.8-1020	0.8-1020	N/A [(a)]	N/A
D10 [(b)]	28.43	27.06	26.97	28.32	27.70	0.79
D50 [(b)]	220.76	209.22	208.62	221.78	215.10	7.15
D90 [(b)]	523.67	517.69	515.75	528.50	**521.40**	**5.81**

[(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

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.

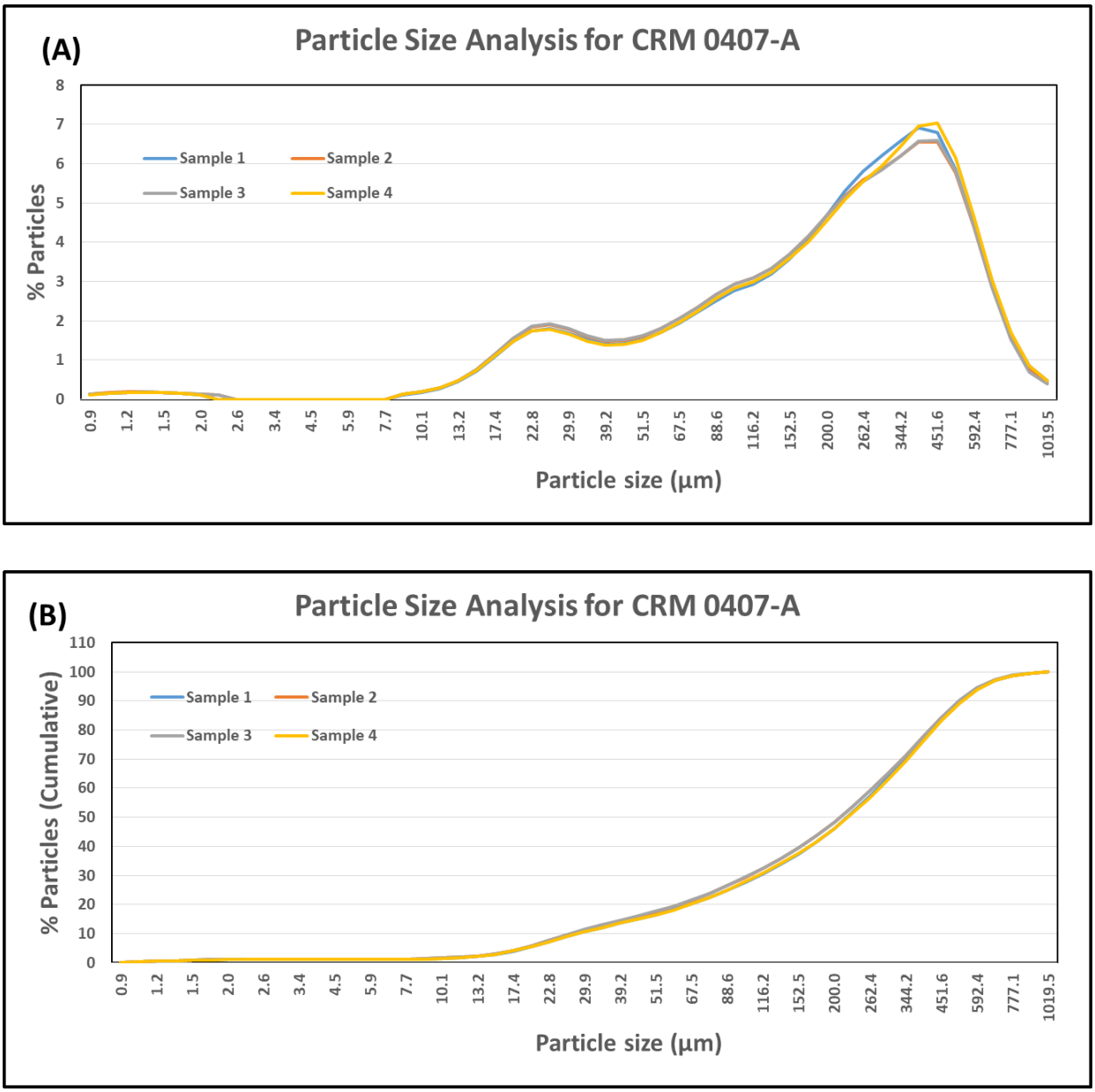

Figure 1. Particle size distribution plots. (A) Percentage of particles of a given size. (B) Cumulative distribution of particle sizes

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 absence, homogeneity, and to rule out degradation during packaging. A total of ten (10) randomly selected CRM AOCS 0407-A samples (24, 67, 198, 287, 346, 605, 764, 789, 950 and

998) were sent to Eurofins-GeneScan, Metairie, LA for event-specific, qualitative PCR analysis to screen for GA21, MIR604, MIR162 and MZHG0JG presence in the samples. These results are presented in Table 2. These data confirms the absence of the GA21, MIR604, MIR162 and MZHG0JG events after the packaging of CRM AOCS 0407-A.

Table 2. Results for the verification of CRM AOCS 0407-A non-modified maize as tested by Eurofins GeneScan using event-specific, qualitative PCR analysis

Sample Number	GA21, MIR604, MIR162 and MZHG0JG Presence
AOCS 0407-A 24	Negative
AOCS 0407-A 67	Negative
AOCS 0407-A 198	Negative
AOCS 0407-A 287	Negative
AOCS 0407-A 346	Negative
AOCS 0407-A 605	Negative
AOCS 0407-A 764	Negative
AOCS 0407-A 789	Negative
AOCS 0407-A 950	Negative
AOCS 0407-A 998	Negative

Certified Value and Measurement Uncertainty

The genetic purity of the seed lot used to produce CRM AOCS 0407-A was assessed by Syngenta Crop Protection, LLC. A total of 3000 seeds (10 pools of 300 seeds/pool) of maize breeding line NP2391/NP2673 were subjected to seed testing for the presence of GA21, MIR604, MIR162 and MZHG0JG maize by real-time PCR. None of the NP2391/NP2673 maize seed pools tested positive for GA21, MIR604, MIR162 and MZHG0JG maize.

Purity estimation in non-modified maize was calculated using SeedCalc8 (Remund *et al*., 2008). The % impurity in the sample was 0% when 3000 seeds were tested. Based on the upper bound of true % purity and with 95% confidence, true certified value is < 1 g/kg.

The measurement uncertainty (U_{CRM}) is the expanded uncertainty with a coverage factor of 1.65 and a confidence level of 95%. It is obtained by combining the uncertainties from the purity assessment ($u_{char,rel}$) and the homogeneity assessment ($u_{bb,rel}$):

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

$$U_{CRM} = 1.65 \times u_{CRM,rel} \times \boldsymbol{purity\ estimation} \times 1000\ g/kg$$

The expanded measurement uncertainty for this CRM is 0.5 g/kg. It should be noted that the purity estimation (**in bold**) does not apply to non-genetically modified materials.

Homogeneity Testing

The non-modified maize material used for the production of CRM AOCS 0407-A is highly pure with respect to the absence of genetic impurities (see Certified Value and Measurement Uncertainty section) and was expected to be homogenous. To further confirm homogeneity, the ground material was analytically assessed before and after bottling using real-time PCR.

Prior to bottling, 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 10 g each were prepared from the composite sample and sent to Eurofins-GeneScan, Metairie, LA (an ISO 17025:2005 accredited laboratory) for event-specific, qualitative PCR analysis. The analyses performed by Eurofins-GeneScan, Metairie, LA were used to assess the trait absence and homogeneity of the lot. A summary of the qualitative PCR results for the non-modified maize homogeneity samples is presented in Table 3.

Table 3. Homogeneity testing prior to bottling using event-specific qualitative real-time PCR on the non-modified maize bulk material provided by Syngenta Crop Protection, LLC

Sample Number	GA21, MIR604, MIR162 and MZHG0JG Presence
Homogeneity Sample 1	Negative
Homogeneity Sample 2	Negative
Homogeneity Sample 3	Negative
Homogeneity Sample 4	Negative
Homogeneity Sample 5	Negative
Homogeneity Sample 6	Negative
Homogeneity Sample 7	Negative
Homogeneity Sample 8	Negative
Homogeneity Sample 9	Negative
Homogeneity Sample 10	Negative

In addition, homogeneity was also assessed after bottling of CRM AOCS 0407-A and was based on the quantification of the endogenous *adh1* maize gene using real-time PCR

(http://gmo-crl.jrc.ec.europa.eu/gmomethods/docs/GA21_Validation_Report+Protocol[1].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 for quantitative real-time PCR. For each of the DNA extracts, all PCR reactions were done in triplicate. The absence of events GA21 and MIR162 in CRM AOCS 0407-A was assessed using either GA21-specific (http://gmo-crl.jrc.ec.europa.eu/gmomethods/docs/GA21_Validation_Report+Protocol[1].pdf). or MIR162-specific (https://gmo-crl.jrc.ec.europa.eu/summaries/MIR162_validated_Method.pdf), quantitative real-time PCR method.

The cycle threshold (Ct) value for the *adh1* gene and for event GA21 and MIR162 was used to calculate the number of copies (cp#) for either target. Subsequently, the ratio between event copy number and *adh1* copy number (cp#/*adh1* cp#) was calculated and used to estimate the 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 CRM AOCS 0407-A.		
CRM	RSD_w [%]	RSD_b [%]
AOCS 0407-A	Below LOQ	Below LOQ

The CRM will be determined to be homogeneous if the within-unit relative standard deviation ($\mathrm{RSD_w}$) and between-unit relative standard deviation ($\mathrm{RSD_b}$) are both ≤ 20%. No amplification was observed from non-modified CRM AOCS 0407-A DNA extracts when using a PCR method specific to Events GA21 and MIR162. Based on the quantitative real-time PCR analyses conducted, it was concluded that CRM AOCS 0407-A is homogenous (Table 4) because all results are below LOQ. These results are in agreement with homogeneity results from qualitative real-time PCR analyses and with the purity estimate for material non-modified 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. Therefore, only the influence of time need be investigated.

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 year from the introduction 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 reevaluated at time of expiration. If the samples are determined to be stable, the certificates will be extended.

References

AVEKA; 2045 Wooddale Drive. Woodbury, MN 55125; Telephone : +1 651 730 1729; https://www.aveka.com/

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/

Illinois Crop Improvement Association, 3105 Research Road, Champaign, IL 61826;Telephone: +1 217 359 4053 Fax: +1 217 359 4075; https://www.ilcrop.com/

Texas A&M University; Food Protein Research and Development Center;373 Olsen Blvd; College Station, TX 77845, USA; Telephone: +1 979 862 2262 Fax: +1 979 845 2744; https://perdc.tamu.edu/

ISO 9001:2015, Quality Management Systems – Requirements

ISO 17025:2005 and ISO 17025:2017, General Requirements for the Competence of Testing and Calibration Laboratories

ISO Guide 17034:2016, General Requirements for the Competence of Reference Material Producers

ISO Guide 30:1992 (E/F), Reference Materials – Selected Terms and Definitions

ISO Guide 31:2000 (E), Reference Materials- Contents of Certificates, Labels and Accompanying Documentation

ISO Guide 34:2009 (E), Reference Materials – General Requirements for the Competence of Reference Material Producers

ISO Guide 35:2006 (E) Reference Materials – Guidance for Characterization and Assessment of Homogeneity and Stability

International Seed Testing Association, International Rules of Seed Testing: Seed Science and Technology Rules, 2012

Linsinger, T., Pauwels, J., van der Veen, A. *et al.* Homogeneity and stability of reference materials. *Accred Qual Assur* **6,** 20–25 (2001). https://doi.org/10.1007/s007690000261

Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed (https://eur-lex.europa.eu/legal- content/EN/TXT/PDF/?uri=CELEX:32003R1829&from=en)

Remund K, Simpson R, Laffont J-L, Wright D, and Gregoire S. Seedcalc8. 2008. (https://www.seedtest.org/en/statistical-tools-for-seed-testing-_content---1--3449--1102.html)