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Certified Reference Materials

AOCS 0917-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 maize CRM AOCS 0917-A produced by AOCS Technical Services in 2017. The CRMs have been prepared according to ISO Guide 17034:2016 and are intended to serve as control material for third party testing of maize for transformation events. Non-modified maize powder (Breeding line: NP2222/NP2391) was provided by Syngenta Crop Protection, LLC and was prepared by grinding the bulk source at Syngenta Crop Protection, LLC. The non-modified maize powder was then aliquoted and packaged under a nitrogen gas environment at Illinois Crop Improvement Association. The absence of the MIR162 and MIR604 events in the maize was verified using event-specific, qualitative PCR analysis by Eurofins-GeneScan, New Orleans, LA (an ISO 17025 Accredited laboratory). The certified mass value of non-modified CRM 0917-A was based on the absence of genetically modified impurities (i.e. Adventitious Presence) and with 95% confidence, the Adventitious Presence 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 0917-A was bottled. AOCS 0917-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.

Acknowledgements

The authors would like to express sincere appreciation and gratitude to several individuals and their companies for support and guidance throughout this project. Thanks go to Monique Nesbitt, Syngenta Crop Protection, LLC, for offering AOCS the opportunity to manufacture and distribute these products; to Sandra Harrison and Charlie Drennan at Illinois Crop Improvement Association for packaging the samples; and to Frank Spiegelhalter, Greg Ditta, E. Pearce Smith, and Daniel Thompson, Eurofins-GeneScan for event-specific, PCR analysis including the provision of information on running the analyses and interpreting the results.

Glossary

AOCS	American Oil Chemists' Society
Cycle threshold (Ct)	Number of PCR cycles required for the fluorescent signal to cross a threshold that exceeds background level
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
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, AOCS 0917-A was manufactured from maize seed according to ISO Guide 17034:2016 and in accordance with EC No 1829/2003, EC No 641/2004 and EC No 619/2011. This CRM is available from AOCS.

Material Preparation and Particle Size Analyses

Syngenta Crop Protection, LLC delivered non-modified maize to AOCS for the production of CRM 0917-A. The non-modified maize was aliquoted and packaged in 27-ml glass headspace vials and sealed under a nitrogen gas environment.

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 0917-A was 278.05 $\pm$19.48 µm, and 90% of the particles were smaller than 659.41 $\pm$ 70.83 µm.

Table 1. Results of Particle Size Analyses of CRM 0917-A						
	Sample 1 (µm)	**Sample 2 (µm)**	**Sample 3 (µm)**	**Sample 4 (µm)**	**Average (µm)**	**Standard Deviation (µm)**
Mean	288.36	272.26	253.56	298.01	**278.05**	**19.48**
Range	0.9-1337	0.8-1668	0.8-890	0.8-1532	N/A[a]	N/A
D10[b]	15.62	15.53	15.66	15.67	15.62	0.07
D50[b]	215.77	206.39	208.82	190.40	205.34	10.73
D90[b]	676.48	634.63	579.26	747.29	**659.41**	**70.83**

[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.

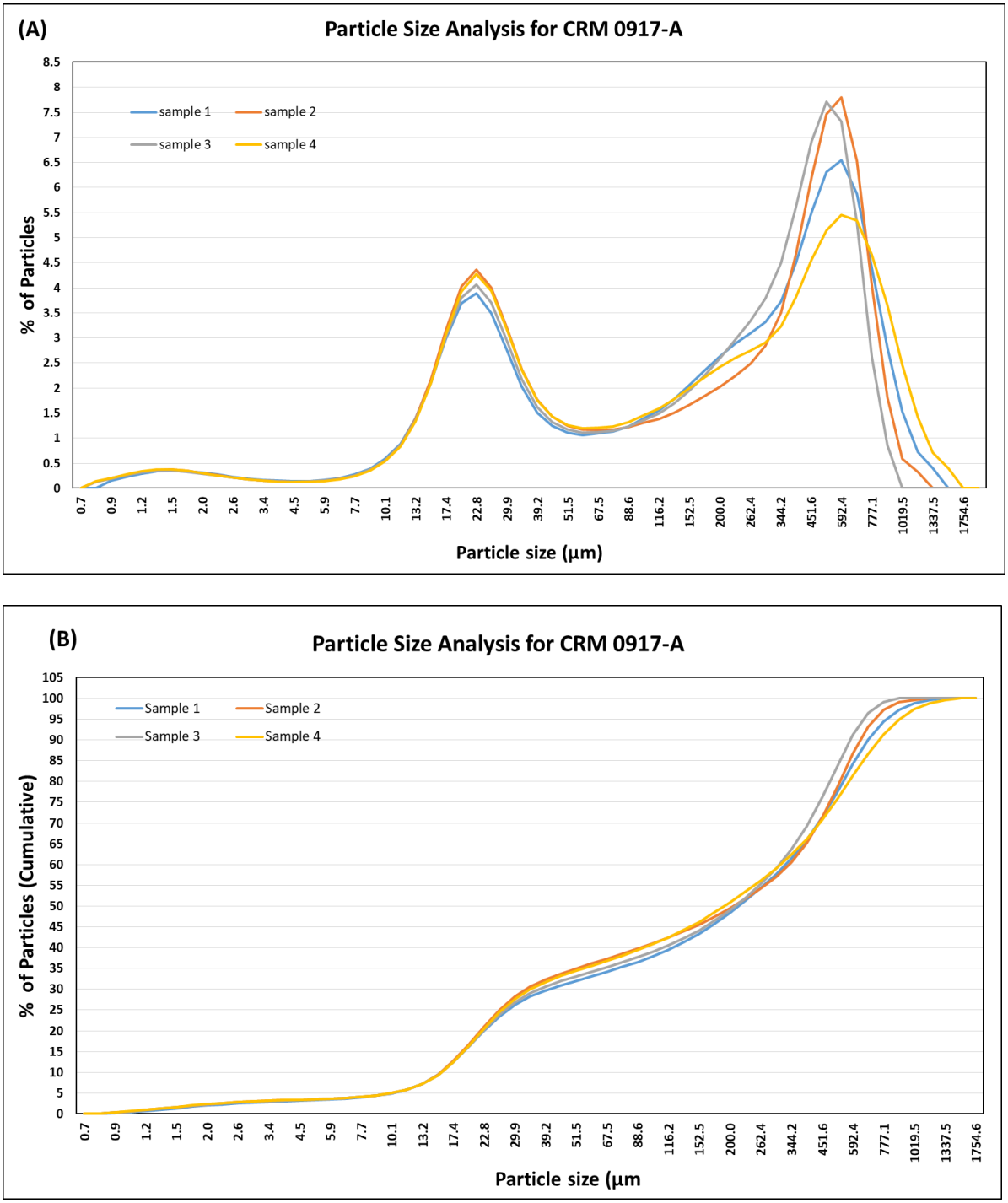

Figure 1. Particle size distribution plots. (A) Percentage of particles of a given size. (B) Cumulative distribution of particle sizes

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, and to rule out degradation during packaging. A total of five (5) AOCS 0917-A samples (4, 37, 56, 91 and 132) were sent to Eurofins-GeneScan, New Orleans, LA for event-specific, qualitative PCR analysis to screen for MIR162 and MIR604 presence in the samples. These results are presented in Table 2. These data confirm the absence of the MIR162 and MIR604 events after the packaging of AOCS 0917-A.

Table 2. Results for the verification of AOCS 0917-A non-modified maize samples as tested by Eurofins-GeneScan, New Orleans, LA with event-specific, qualitative PCR analysis.

Sample	MIR162 and MIR604 Presence
AOCS 0917-A 4	Negative
AOCS 0917-A 37	Negative
AOCS 0917-A 56	Negative
AOCS 0917-A 91	Negative
AOCS 0917-A 132	Negative

Certified Value Assignment

The presence of genetically modified (GM) impurities (also known as Adventitious Presence) was assessed in the non-genetically modified material used to produce 0917-A. A total of 3000 seeds (10 pools of 300 seeds/pool) of maize breeding line NP2222/NP2391 were evaluated by real-time PCR at Syngenta Crop Protection, LLC. The analysis was conducted to determine the Adventitious Presence of commercial events where testing methodology is available, and regulated corn events under development at Syngenta.

None of the NP2222/NP2391maize seed pools tested positive for the GM events evaluated.

The percentage of Adventitious Presence in non-modified NP2222/NP2391 maize was calculated using SeedCalc8 (Remund *et al*., 2008) and was based on the upper bound of true % purity using a 95% confidence level. Based on this analysis, the Adventitious Presence is less than 0.1% and this value was used to assign a certified mass value of 1 g/Kg to this CRM. The associated uncertainty was based on the desired confidence level, and consequently, with 95% confidence, the Adventitious Presence in NP2222/NP2391 maize mass fraction of the material is below 1 g/Kg.

Homogeneity Testing

The non-modified maize material used for the production of CRM 0917-A is highly pure with respect to the absence of genetic impurities (see Certified Value Assignment section) and was expected to be homogenous. However, 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. Five (5) working samples of 10 g each were prepared from the composite sample and sent to Eurofins-GeneScan, New Orleans, LA (an ISO 17025 Accredited laboratory) for event-specific, qualitative PCR analysis. The analyses performed by Eurofins-GeneScan, New Orleans, 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. Results of the qualitative homogeneity testing performed by Eurofins-GeneScan, New Orleans, LA on the non-modified maize bulk material 0917-A provided by Syngenta Crop Protection, LLC	
Sample	**MIR162 or MIR604 Presence**
Homogeneity Sample 1	Negative
Homogeneity Sample 2	Negative
Homogeneity Sample 3	Negative
Homogeneity Sample 4	Negative
Homogeneity Sample 5	Negative

In addition, homogeneity was also assessed after bottling of 0917-A CRM and was based on the quantification of the endogenous *adh1* maize gene using real-time PCR (http://gmo-crl.jrc.ec.europa.eu/summaries/MIR162_validated_Method.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 2 grams. 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 absence of event MIR162 or MIR604 in 0917-A was assessed using a MIR162-specific or a MIR604-specific, quantitative real-time PCR methods (http://gmo-crl.jrc.ec.europa.eu/summaries/MIR162_validated_Method.pdf, http://publications.jrc.ec.europa.eu/repository/bitstream/JRC84134/2013_10_18%20final%20jrc84134.pdf).

As expected, no amplification was observed when using PCR methods specific to either MIR162 or MIR604 events. For each of the DNA extracts, the cycle threshold (Ct) value for the *adh1* gene was used to calculate *adh1* copy number. The *adh1* copy numbers were used to estimate within- and between-sample average, standard deviation (SD), and relative standard deviation (RSDr). The within-sample average ranged from 2763-4315 *adh1* copies, and the associated within-sample SD and RSDr ranged from 2-462, and from 0.06%-13.8%, respectively (Table 4). The between-sample estimates were 3235, 260 and 8.04% for average *adh1* copy number, SD and RSDr, respectively (Table 4). It should be

noted that between-sample SD was calculated according to the following formula.

$$s_{\text{pooled}} = \sqrt{\frac{(n_1-1)s_1^{\,2} + (n_2-1)s_2^{\,2} + \ldots + (n_k-1)s_k^{\,2}}{n_1 + n_2 + \ldots + n_k - k}}$$

n = 2 (number of tests per sample)

k = 10 (number of samples)

s = within-sample standard deviation

Table 4. Homogeneity testing for CRM 0917-A
(copy numbers calculated using *ahd1*-specific, quantitative real-time PCR method)

	Average	SD	RSDr (%)
Within-sample [(a)]	2763-4315	2-462	0.06%-13.8%
Between-sample	3235	260 [(b)]	8.04%

(a) Within-sample estimates are given as a range of values calculated for each of the 10 samples assessed. Average and SD estimates are based on independent measurements from two DNA extracts.

(b) The SD is a pooled SD calculated as described in this section.

Based on the quantitative real-time PCR analyses conducted, it was concluded that CRM 0917-A is homogenous. These results are in agreement with homogeneity results from qualitative real-time PCR analyses and from the purity estimate for material NP2222/NP2391 calculated in the Certified Value Assignment section above.

Stability

Stability of these CRMs has been listed as 1 year 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 reevaluated a time of expiration. If the samples still test negative the presence of the intended events, the certificates will be extended.

References

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

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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/

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