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Certified Reference Materials

AOCS 1012-A

Report of the certification process for

Non-modified

Cotton Certified Reference Materials

First Batch

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Abstract

This report describes the preparation and certification of the cotton AOCS CRM 1012-A produced by AOCS Technical Services in 2011. 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 cotton for transformation events. Non-modified cotton powder (Breeding line: Coker 312) was provided by Syngenta Crop Protection, LLC and was prepared by grinding the bulk source at Syngenta Crop Protection, LLC. The non-modified cotton powder was then aliquoted and packaged under a nitrogen gas environment at Illinois Crop Improvement Association. The absence of COT102 in the cotton was verified using event-specific, qualitative real-time PCR analysis by Eurofins GeneScan, New Orleans, LA (an ISO 17025 accredited laboratory). The certified mass value of non-modified cotton AOCS CRM 1012-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 AOCS CRM 1012-A was bottled. AOCS CRM 1012-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

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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 CRM 1012-A was manufactured from cotton 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 10 kg of non-modified cotton to AOCS for the production of AOCS CRM 1012-A. The non-modified cotton was processed, 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 1012-A cotton 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. On average, the particle size of AOCS CRM 1012-A was 637.48 + 50.17 µm, and 90% of the particles (i.e. D90) were smaller than 1412.47 + 116.77 µm (Table 1).

.Table 1. Results of Particle Size Analyses of AOCS CRM 1012-A						
	Sample 1 (µm)	**Sample 2 (µm)**	**Sample 3 (µm)**	**Sample 4 (µm)**	**Average (µm)**	**Standard Deviation (µm)**
Mean	659.94	677.80	564.56	647.63	**637.48**	**50.17**
Range	0.5 - 3000	0.7 -3000	0.7 - 2636	0.6 - 3000	N/A[(a)]	N/A
D10 [(b)]	26.49	22.93	22.09	20.65	23.04	2.48
D50 [(b)]	562.54	554.29	478.62	548.92	536.10	38.72
D90 [(b)]	1441.49	1527.78	1249.89	1430.73	**1412.47**	**116.77**

(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.

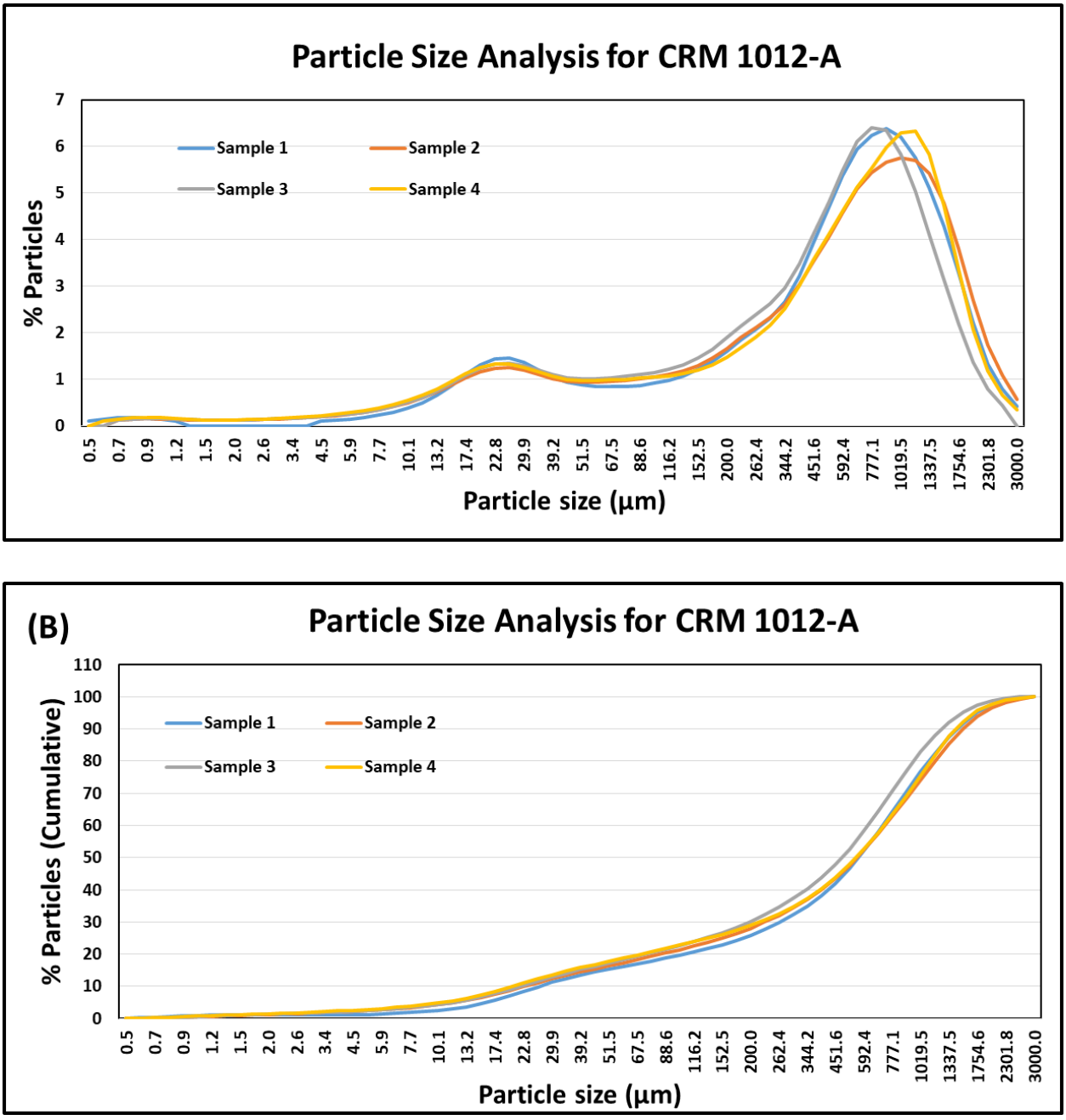

Figure 1. Particle size distribution plots. (A) Percentage of particles of a given size. (B) Cumulative distribution of particle sizes

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) AOCS CRM 1012-A samples (61, 70, 79, 87, 139, 148, 236, 334, 386 and 416) were sent to Eurofins GeneScan, New Orleans, LA for event-specific, qualitative real-time PCR analysis to screen for COT102 presence in the samples. These results are presented in Table 2. These data confirm the absence of COT102 after the packaging of AOCS 1012-A.

Table 2. Results for the verification of 1012-A non-modified maize as tested by Eurofins GeneScan with a COT102 event-specific, qualitative PCR analysis

Sample	COT102 Presence
AOCS 1012-A 61	Negative
AOCS 1012-A 70	Negative
AOCS 1012-A 79	Negative
AOCS 1012-A 87	Negative
AOCS 1012-A 139	Negative
AOCS 1012-A 148	Negative
AOCS 1012-A 236	Negative
AOCS 1012-A 334	Negative
AOCS 1012-A 386	Negative
AOCS 1012-A 416	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 AOCS CRM 1012-A. A total of 3000 seeds (30 pools of 100 seeds/pool) of non-modified cotton breeding line Coker 312 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 when applicable, regulated cotton

events under development at Syngenta. None of the non-modified Coker 312 cotton seed pools tested positive for the GM events evaluated.

The percentage of Adventitious Presence in non-modified Coker 312 cotton 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 Coker 312 cotton mass fraction of the material is below 1 g/Kg.

Homogeneity Testing

The non-modified maize material used for the production of AOCS CRM 1012-A is highly pure with respect to the absence of genetic impurities (see Certified Value Assignment 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, New Orleans, LA (an ISO 17025 accredited laboratory) for event-specific, qualitative real-time 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 cotton homogeneity samples is presented in Table 3.

Table 3. Homogeneity testing prior to bottling using COT102 event-specific qualitative real-time PCR on the non-modified maize bulk material provided by Syngenta Crop Protection, LLC	
Sample	**COT102 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 AOCS CRM 1012-A and it was based on the quantification of the endogenous *sah7* cotton gene using real-time PCR (http://www.detection-methods.com/product/vipcot/). 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 absence of event COT102 in AOCS CRM 1012-A was assessed using a COT102-specific quantitative real-time PCR method (http://www.detection-methods.com/product/vipcot/). As expected, no amplification was observed when using PCR methods specific to the COT102 event. For each of the DNA extracts, the cycle threshold (Ct) value for the *sah7* gene was used to calculate *sah7* copy number. The

sah7 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 39645 to 53839 *sah7* copies, and the associated within-sample SD ranged from 903.65 to 9102.16. Except for one sample where RSDr was 22.76%, for all other samples RSDr ranged from 1.68% to 8.39% (Table 4). The between-sample estimates were 44432, 3804.4 and 8.56% for average *sah7* copy number, SD and RSDr, respectively (Table 4). The 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 sample)
s = within-sample SD

Table 4. Homogeneity testing for AOCS CRM 1012-A (copy numbers calculated using *sah7*-specific, quantitative real-time PCR method)

	Average	SD	RSDr (%)
Within-sample [(a)]	39645 - 53839	903.65 - 9102.16	1.68 - 22.76
Between-sample	44432	3804.4[(b)]	8.56

(a) Within-sample estimates are given as a range of values calculated for each of the 10 sample 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 1012-A is homogenous. These results are in agreement with homogeneity results from qualitative real-time PCR analyses and from the purity estimate for material COT102 cotton calculated in the Certified Value Assignment section above.

Stability

Stability of these CRMs has been listed as 1 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 still test negative the presence of the intended events, 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/

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