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Certified Reference Materials

AOCS 1012-C2

Report of the certification process for

COT102

Cotton

Second Batch

OECD Unique Identifier SYN-IR1Ø2-7

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Abstract

This report describes the preparation and certification of the cotton CRM AOCS 1012-C2 produced by AOCS Technical Services in 2020. The CRMs have been prepared according to ISO 17034:2016 and are intended to serve as control material for third party testing of cotton for transformation events. COT102 cotton powder was provided by Syngenta Crop Protection, LLC, and was prepared by grinding the bulk source at AVEKA, Inc., Woodbury, MN (an ISO 9001-accredited facility). The COT102 cotton powder was then aliquoted and packaged under a nitrogen gas environment at Illinois Crop Improvement Association (an ISO 17025-accredited facility). The certified mass value of Event COT102 in COT102 cotton was based on seed purity of the material and with 95% confidence, it is higher than 883 g/kg. Homogeneity testing was performed at Eurofins GeneScan, New Orleans, LA (an ISO 17025-accredited laboratory) using quantitative real-time PCR after the CRM AOCS 1012-C2 was bottled. Homogeneity results indicated that CRM AOCS 1012-C2 is homogenous and were used to verify the presence of the Event COT102 in this CRM. CRM AOCS 1012-C2 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 Kristina Burgin, 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, and E. Pearce Smith, Eurofins GeneScan for event-specific, real-time PCR analysis including the provision of information on running the analyses and interpreting the results.

Glossary

AOCS	American Oil Chemists' Society
Conventional Crop	Crop variety with no history of modern biotechnology and is produced through plant-breeding techniques that rely on selecting and mating parent plants possessing promising traits and repeatedly selecting for superior performance among their offspring
DNA	Deoxyribonucleic Acid
Detection Limit	Lowest level at which target DNA can be detected in a sample and be reliably tested by PCR methods. It is typically expressed as a percentage: the ratio of the number of modern biotechnology derived genomes to the number of crop genomes times 100 percent
EC	European Commission
GMO	Organism that has had genetic sequences modified using molecular-level techniques
ISO	International Organization for Standardization
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 zero in on a particular target DNA sequence and a special DNA-copying enzyme (DNA polymerase) that makes enough copies of 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 quantified. It is typically expressed as the ration of the number of transgenic genomes to the number of crop genomes times 100 percent
Quantitative PCR	PCR methods that estimate the relative amount of target DNA sequence in a mixture of DNA molecules
Trait: COT102	Resistance to lepidopteran insects

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 1012-C2 was manufactured from cotton seed according to ISO 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 Processing and Particle Size Analyses

CRM AOCS 1012-C2 has been prepared by AOCS from homozygous cotton seed. The COT102 cotton seed was first milled and analyzed for particle size distribution at AVEKA, Inc. Woodbury, Minnesota (an ISO 9001-accredited facility).

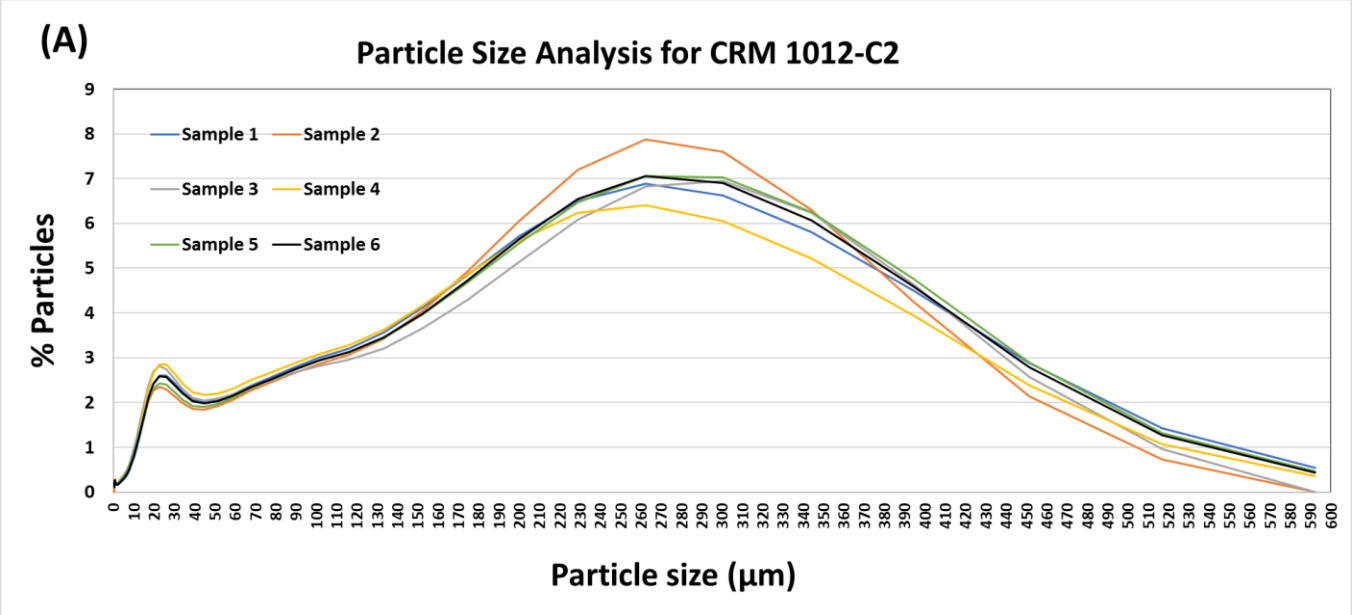
Bulk seed received by AVEKA, Inc. from Syngenta Crop Protection, LLC was milled in a Fitzpatrick cryogenic hammermill using a 510 µm screen. The material was blended in a Patterson-Kelley V-blender, and after homogenization, six samples taken at random were subject to particle size analyses using a Horiba LA-950 Light Scattering Particle Analyzer. For each sample, the particle size mean and range, and the percentage of particles below a given size was calculated (Table 1). On average, the particle size of CRM AOCS 1012-C2 was 152.05 + 4.97 µm, and 90% of the particles (i.e. D90) were smaller than 332.60 + 7.25 µm

Table 1. Results of Particle Size Analyses of CRM AOCS 1012-C2 Conducted by AVEKA								
	Sample 1 (µm)	**Sample 2 (µm)**	**Sample 3 (µm)**	**Sample 4 (µm)**	**Sample 5 (µm)**	**Sample 6 (µm)**	**Average (µm)**	**Standard Deviation (µm)**
Mean	155.41	152.75	147.51	144.48	156.93	155.23	152.05	4.97
Range	0.51-592.39	0.584-517.2	0.51-517.2	0.51-592.39	0.51-592.39	0.51-592.39	N/A[a]	N/A
D10 [b]	15.12	13.52	12.95	14.04	13.87	14.62	14.02	0.77
D50 [b]	132.19	138.18	118.99	116.06	135.16	133.07	128.94	9.13
D90 [b]	338.98	323.59	330.84	324.84	340.05	337.27	332.60	7.25

[a] N/A = not applicable

[b] D10, D50 and D90 indicate that 10%, 50% or 90% of the particles, respectively, are smaller than largest 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 600 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. For all samples analyzed, 100% of particles were ≤ 592.39 µm.



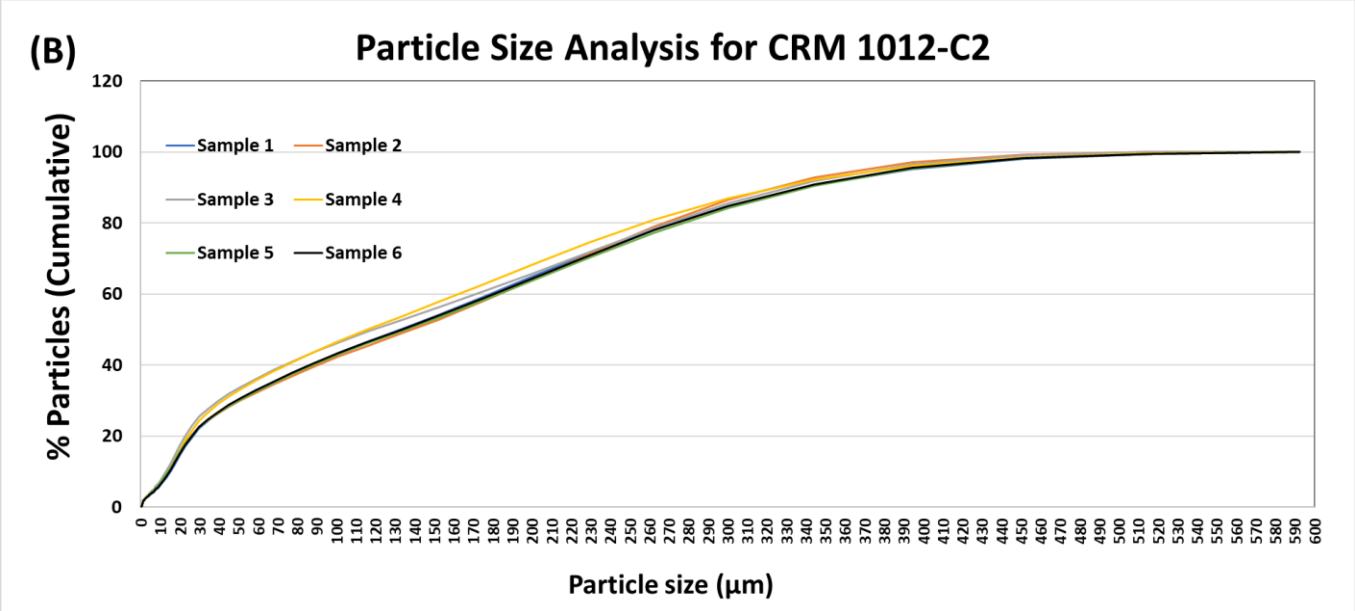


Figure 1. Particle size distribution plots. (A) Percentage of particles of a given size. (B) Cumulative distribution of particle sizes

Bulk COT102 cotton powder for the production of CRM AOCS 1012-C2 was delivered to AOCS and it was then aliquoted and packaged in 27-ml glass headspace vials and sealed under a nitrogen gas environment at the Illinois Crop Improvement Association (an ISO 17025-accredited facility).

Certified Value and Measurement Uncertainty

The genetic purity based on the presence of Event COT102 in COT102 cotton was assessed by Syngenta Crop Protection, LLC. A total of 433 COT102 cotton seeds were evaluated by qualitative, COT102-specific real-time PCR. The results showed that 433 of the 433 seeds tested (100 %) were positive for the presence of Event COT102.

The statistical purity of Event COT102 in COT102 cotton was calculated using SeedCalc8 (Remund *et al*., 2008) and was based on the lower bound of true % purity. Using a 95% confidence level, the true % purity of COT102 cotton was at least 88.3%. Consequently, with 95% confidence, the true value is ≥ 883 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 ($u_{lts,rel}$):

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

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

Consequently, the expanded measurement uncertainty for this CRM is -117 g/kg.

Homogeneity Testing

The material used for the production of CRM AOCS 1012-C2, COT102 cotton, is 100 % pure and is expected to be homogenous. After COT102 cotton seed was ground and bottled as described above, ten samples of CRM AOCS 1012-C2 were randomly selected using the Microsoft Excel Random Number Generator function and were sent to Eurofins-GeneScan, New Orleans, LA (an ISO 17025-accredited laboratory) for homogeneity testing using quantitative real-time PCR.

Homogeneity was assessed after bottling of CRM AOCS 1012-C2 using a COT102-specific, quantitative, real-time PCR method (gmo-crl.jrc.ec.europa.eu/method-validation/details/all/2041/COT102). A total of 10 samples of CRM AOCS 1012-C2 cotton

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 *sah7* cotton gene and for Event COT102 were used to calculate the number of copies (cp#) for either target. Subsequently, the ratio between Event COT102 copy number and *sah7* copy number (COT102 cp#/*sah7* 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 2. The within-unit relative standard deviation (RSD_w), and the between-unit relative standard deviation (RSD_b) for vials of CRM AOCS 1012-C2.

CRM	RSD_w [%]	RSD_b [%]	$u^*_{bb,rel}$ [%]
AOCS 1012-C2	12.7	n.c.[1]	5.7

[1]n.c: RSD_b cannot be calculated as $\mathrm{MS_{between}} < \mathrm{MS_{within}}$. In this situation, maximum hidden inhomogeneity ($u^*_{bb,rel}$) is provided as an alternative.

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%. Based on the quantitative real-time PCR analyses conducted, it was concluded that CRM AOCS 1012-C2 is homogenous (Table 2). These results are in agreement with the

purity estimate for material COT102 calculated in the Certified Value and Measurement Uncertainty section above.

Trait Verification

The presence of the COT102 trait was assessed in the same ten CRM AOCS 1012-C2 samples that were analyzed for homogeneity using COT102-specific quantitative PCR analysis. Quantitative results were converted to qualitative data, and the results are presented in Table 3. In all instances Event COT102 was present.

Table 3. Qualitative results for the verification of CRM AOCS 1012-C2 as tested by Eurofins GeneScan with a COT102-specific, quantitative PCR analysis

Sample	Event COT102 Presence
AOCS 1012-C2 5	Positive
AOCS 1012-C2 33	Positive
AOCS 1012-C2 83	Positive
AOCS 1012-C2 124	Positive
AOCS 1012-C2 174	Positive
AOCS 1012-C2 230	Positive
AOCS 1012-C2 286	Positive
AOCS 1012-C2 300	Positive
AOCS 1012-C2 348	Positive
AOCS 1012-C2 418	Positive

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 °C (fridge), 25 °C (ambient), 60 °C (oven)) for 4 different durations (0, 1, 2, and 4 weeks). The study concluded that samples are stable at 4 °C (fridge) and 25 °C (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 °C (ambient) and the stability of the sample is monitored as long as the samples is available. The storage temperate studied is 25 °C 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 °C (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 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; 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 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; www.ilcrop.com/

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