Street Address:
AOCS, 2710 S. Boulder Drive
Urbana, IL 61802-6996 USA
Phone: +1-217-359-2344;
Fax: +1-217-351-8091;
E-Mail: CRM@aocs.org; **Web:** www.aocs.org

Certified Reference Materials

AOCS 1012-C

Report of the certification process for

COT102

Cotton Certified Reference Materials

First Batch

OECD Unique Identifier SYN-IR1Ø2-7

Denise Williams
Technical Services Manager

Scott Bloomer
Technical Director

Version: 1.3 | Published: 31 October 2019

ACCREDITED CERT #3438.01 **ISO Guide 17034:2016**
A2LA Certificate 3438.01

Legal Notice

Neither AOCS nor any person acting on behalf of AOCS is responsible for the use which might be made of the following information.

AOCS Mission Statement

AOCS advances the science and technology of oils, fats, proteins, surfactants, and related materials, enriching the lives of people everywhere.

More information regarding AOCS is available at http://www.aocs.org

Table of Contents

Abstract

This report describes the preparation and certification of the Cotton AOCS CRM 1012-C 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. COT102 cotton powder was provided by Syngenta Crop Protection, LLC, and was prepared by grinding the bulk source at Syngenta Crop Protection, LLC. The COT102 cotton powder was then aliquoted and packaged under a nitrogen gas environment at Illinois Crop Improvement Association. The presence of COT102 in the cotton was verified using event-specific, qualitative real-time PCR analysis at Eurofins GeneScan, New Orleans, LA (an ISO 17025 accredited laboratory). The certified mass value of COT102 in COT102 cotton was based on the purity of the material and with 95% confidence, it is higher than 906.6 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-C was bottled. AOCS CRM 1012-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

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	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 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 quantified
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: COT102	Line of cotton, genetically engineered to confer resistance to certain lepidopteran insect pests

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-C 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 Processing and Particle Size Analyses

AOCS CRM 1012-C has been prepared by AOCS from homozygous cotton seed and Syngenta Crop Protection, LLC delivered 10 kg of COT102 cotton powder to AOCS. The COT102 cotton was packaged in 27-mL glass headspace vials and sealed under a nitrogen gas environment at the Illinois Crop Improvement Association.

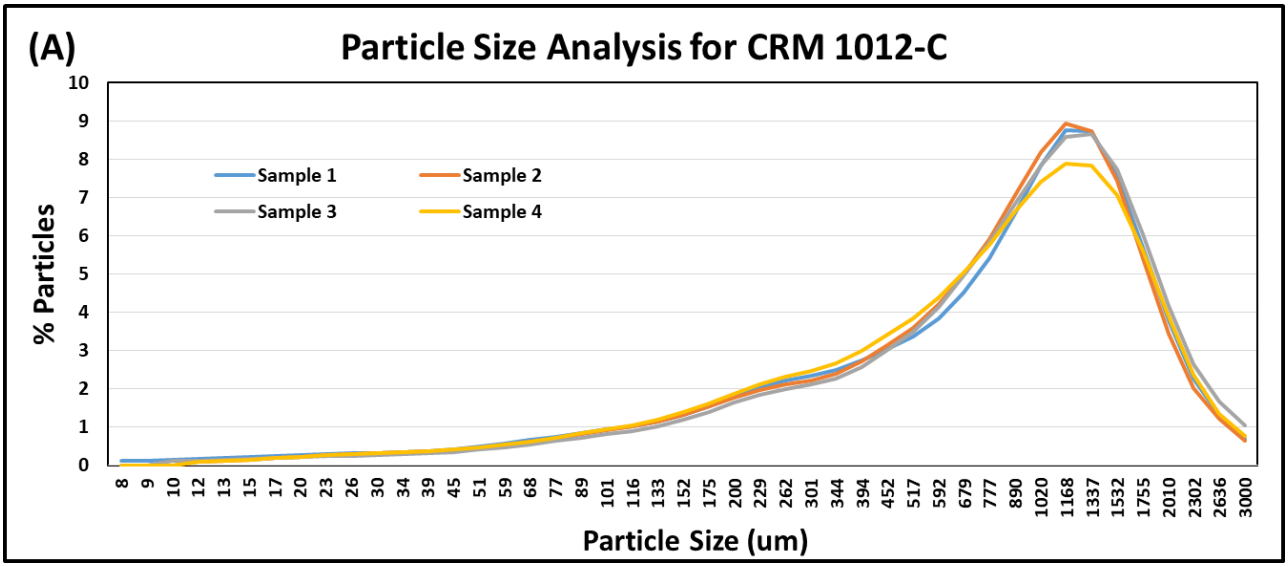
Once packaged, four randomly selected samples of AOCS CRM 1012-C cotton were subject to particle size analysis. Particle size analysis was conducted at AVEKA, Woodbury, Minnesota (an ISO 9001:2015 accredited laboratory) using a Horiba LA-950 particle size analyzer. For each sample, the particle size mean and range, and the percentage of particles below a given size was calculated. On average, the particle size of AOCS CRM 1012-C was 882.30 + 24.82 µm, and 90% of the particles (i.e. D90) were smaller than 1684.94 + 39.16 µm (Table 1)

Table 1. Results of Particle Size Analyses of AOCS CRM 1012-C						
	Sample 1 (µm)	**Sample 2 (µm)**	**Sample 3 (µm)**	**Sample 4 (µm)**	**Average (µm)**	**Standard Deviation (µm)**
Mean	874.58	870.75	919.04	864.83	**882.30**	**24.82**
Range	8 - 3000	12 - 3000	10 - 3000	12 - 3000	N/A[(a)]	N/A
D10 [(b)]	131.95	147.95	160.08	143.65	145.91	11.62
D50 [(b)]	830.35	824.81	861.70	790.77	826.91	29.05
D90 [(b)]	1675.73	1640.87	1735.61	1687.54	**1684.94**	**39.16**

(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.



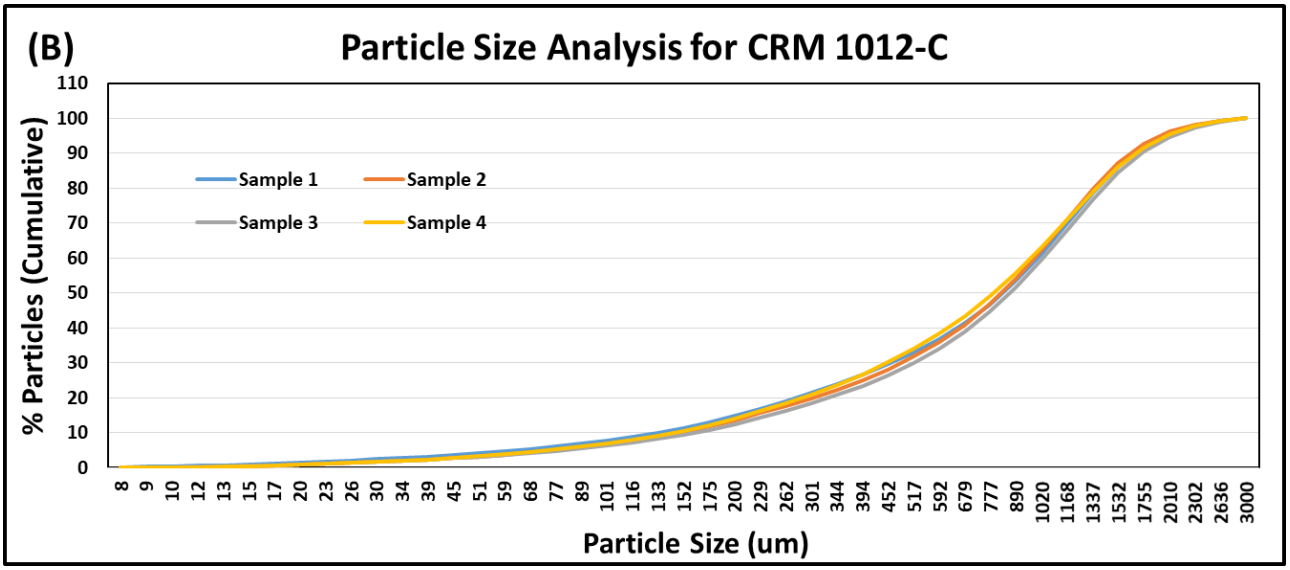


Figure 1. Particle size distribution plots. (A) Percentage of particles of a given size. (B) Cumulative distribution of particle sizes

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 presence, homogeneity, and to rule out degradation during packaging. A total of ten (10) AOCS CRM 1012-C samples (5, 44, 118, 121, 193, 312, 326, 359, 466 and 492) 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. This data confirms the presence of COT102 after the packaging of AOCS 1012-C.

Table 2. Results for the verification of AOCS 1012-C as tested by Eurofins GeneScan with a COT102 event-specific, qualitative PCR method

Sample	COT102 Presence
AOCS 1012-C 5	Positive
AOCS 1012-C 44	Positive
AOCS 1012-C 118	Positive
AOCS 1012-C 121	Positive
AOCS 1012-C 193	Positive
AOCS 1012-C 312	Positive
AOCS 1012-C 326	Positive
AOCS 1012-C 359	Positive
AOCS 1012-C 466	Positive
AOCS 1012-C 492	Positive

Certified Value Assignment

The genetic purity based on the presence of event COT102 in COT102 cotton was assessed by Syngenta Crop Protection, LLC. A total of 381 COT102 cotton seeds were evaluated by qualitative, COT102-specific real-time PCR. The results showed that 355 of the 381 seeds tested (93.18%) were positive for the presence of 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. Based on this analysis, the statistical purity of COT102 cotton was 90.66% and it was used to assign a certified mass value of 906.6 g/kg to this CRM. The associated uncertainty was based in the desired confidence level, and consequently, with 95% confidence, the true COT102 cotton mass fraction of the material is above 906.6 g/kg.

Homogeneity Testing

The material used for the production of AOCS CRM 1012-C, COT102 cotton, is 90.66 % pure (see Certified Value Assignment 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 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 presence and homogeneity of the lot. A summary of the qualitative PCR results for the COT102 homogeneity samples is presented in Table 3.

Table 3. Homogeneity testing prior to bottling using COT102 event-specific qualitative real-time PCR on the COT102 cotton bulk material provided by Syngenta Crop Protection, LLC	
Sample	**COT102 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 1012-C using a COT102 specific, quantitative real-time PCR method (http://www.detection-methods.com/product/vipcot/). A total of 10 samples of AOCS CRM 1012-C 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 within- and between-sample average, standard deviation (SD), and relative standard deviation (RSDr). For one sample (sample 10), the two independent COT102 cp#/ *sah7* cp# estimates differed by almost 50% (0.45 and 0.82). This was unexpected, and consequently two analyses were conducted (with and without sample 10).

When all ten samples were analyzed, the within-sample averages ranged from 0.63 to 1.00, the within-sample SD ranged from 0.019 to 0.29, and the within-sample RSDr ranged from 2.36% to 42.11%. RSDr estimates for six of the samples were below 20%. The between-sample estimates were 0.81, 0.18 and 22.54% for average, SD and RSDr, respectively (Table 4). When nine samples were analyzed (i.e. excluding sample 10), the within-sample averages ranged from 0.68 to 1.00, the within-sample SD ranged from 0.019 to 0.29, and the within-sample RSDr ranged from 2.36% to 31.19%. RSDr estimates for six of the samples were below 20%. The between-sample estimates were 0.83, 0.16 and 19.53% for average, 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 samples)

s = within-sample SD

Table 4. Homogeneity testing for AOCS CRM 1012-C using the COT102-event specific, quantitative real-time PCR method (estimates based on COT102 cp#/*sah7* cp#)

		Average	SD	RSDr (%)
Analyses with 10 samples	**Within-sample [a]**	0.63 - 1.00	0.019 - 0.29	2.36 - 42.11
	Between-sample	0.81	0.18[b]	22.54
Analyses with 9 samples	**Within-sample [a]**	0.68 - 1.00	0.019 - 0.29	2.36 - 31.19
	Between-sample	0.83	0.16[b]	19.53

(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.

The within-sample variability observed in some vials was unexpected because the material used for the production of AOCS CRM 1012-C is highly pure with respect to the presence of event COT102, and DNA integrity was checked prior to conducting PCR analysis. The source of the observed variability is likely due to unintended errors such as pipetting errors or presence of inhibitors on DNA extracts, and not due to the lack of homogeneity of COT102 event in this material. To further investigate the between-sample homogeneity of AOCS CRM 1012-C, means from all ten samples were compared using a Student's t-test and they were not significantly different (α=0.05). Consequently, it was concluded that AOCS CRM 1012-C is homogenous.

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 positive for the presence of the intended trait, 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/

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

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)