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## **Certified Reference Materials**

### **AOCS 0216-A**

Report of the certification process for

MON 87403

Maize Certified Reference Materials

First Batch

OECD Unique ID MON-874Ø3-1

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ISO 17034:2016  
A2LA Certificate 3438.01

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## Abstract

This report describes the preparation and certification of the maize CRM AOCS 0216-A produced by AOCS Technical Services in 2019. The CRMs have been prepared according to ISO 17034:2016 and are intended to serve as control material for third party testing of maize for transformation events. Coarsely processed seed of MON 87403 was provided by Monsanto Company, St. Louis, MO (hereinafter “Monsanto”). The coarsely ground MON 87403 seed powder was further milled by grinding the bulk source according to maize processing protocols at Texas A&M University. The certified value of AOCS 0216-A was based on the purity of the bulk seed material and with 95% confidence, the true value is  $\geq 991$  g/kg. The powder was aliquoted and bottled in 27-mL glass headspace vials and sealed under a nitrogen gas environment at Illinois Crop Improvement Association. The presence of MON 87403 in the maize was verified using event-specific, qualitative PCR analysis by Eurofins-GeneScan, New Orleans, LA (an ISO 17025 accredited laboratory). Homogeneity was verified on random vials of AOCS 0216-A using digital PCR analysis by Monsanto. CRM samples should be stored in a dry, sealed container at ambient or cooler conditions in the dark.

## **Acknowledgements**

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## Glossary

AOCS	American Oil Chemists' Society
Conventional Crop	Crop variety with no history of transgenic technology and is produced through traditional 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 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
ISO	International Organisation for Standardisation
GMO	Organism that has had genetic sequences modified using molecular-level techniques
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 reproducible.
Quantitative PCR	PCR methods that estimate the relative amount of target DNA sequence in a mixture of DNA molecules
Trait: MON 87403	Through introduction of the ATHB17 gene, MON 87403 has increased ear biomass at an early reproductive phase (R1) compared to conventional control maize.

## **Introduction**

Plant genetic modification 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 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 authorizing acceptance 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 0216-A was manufactured from maize according to ISO 17034:2016 and in accordance with EC No 1829/2003. The CRM is available from AOCS.

## **Material Processing**

MON 87403 maize seeds used to prepare AOCS 0216-A were hemizygous through successive breeding generations, and the donor for the MON 87403 maize event was the female parent. Monsanto coarsely processed ~15 kg of MON 87403 maize seed, and delivered the seed powder to AOCS. The coarsely ground MON 87403 seed powder was further milled by Texas A&M University according to industry standard maize processing procedures. Illinois Crop Improvement Association was contracted for packaging the samples. The powder was aliquoted and bottled in 27-mL glass headspace vials and sealed under a nitrogen gas environment.

## **Trait Verification to Certify Presence of MON 87403**

Prior to packaging, bulk seed powder 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 (an ISO 17025 Accredited laboratory) were used to verify the presence of MON 87403 (Table 1).

**Table 1. Trait verification testing on random composite samples of MON 87403 maize performed by Eurofins-GeneScan on bulk material provided by Monsanto**

Sample	MON 87403 Presence
Composite Sample 1	Positive
Composite Sample 2	Positive
Composite Sample 3	Positive
Composite Sample 4	Positive
Composite Sample 5	Positive

After the bulk material was packaged, the presence of the MON 87403 trait was assessed on 5 random vials of AOCS 0216-A. AOCS used the Random Number Generator function of Microsoft Excel to select samples for verification of trait presence and to rule out degradation during packaging. AOCS 0216-A sample numbers 520, 544, 658, 771, and 933 were sent to Eurofins-GeneScan, New Orleans, LA (an ISO 17025 Accredited laboratory) for MON 87403 event-specific, qualitative PCR analysis (Table 2). This data confirms the presence of the MON 87403 in vials of AOCS 0216-A.

**Table 2. Trait verification testing on AOCS 0216-A MON 87403 maize performed by Eurofins-GeneScan, New Orleans, LA (an ISO 17025 accredited laboratory).**

Sample	MON87403 Presence
AOCS0216-A 520	Positive
AOCS 0216-A 544	Positive
AOCS 0216-A 658	Positive
AOCS0216-A 771	Positive
AOCS 0216-A 933	Positive

## Certified Value and Measurement Uncertainty

The genetic purity of the seed lot used to produce AOCS 0216-A was assessed by Monsanto. A total of 719 maize seeds were subjected to individual seed testing for the presence of MON 87403 by qualitative event-specific PCR. 717 of the 719 seeds tested positive for the presence of MON 87403.

Purity estimation was calculated using SeedCalc8 (Remund *et al.*, 2008) and the Certified Value corresponds to the lower bound of true % purity. The % purity in the sample was 99.7% when 719 seeds were tested. Using a 95% confidence level, the true % purity of the MON 87403 seed lot was 99.1%. Consequently, with 95% confidence, the true value is  $\geq 991$  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 ( $u_{char,rel}$ ) and the homogeneity assessment ( $u_{bb,rel}$ ):

$$u_{CRM,rel} = \sqrt{u_{char,rel}^2 + u_{bb,rel}^2}$$
$$U_{CRM} = 2 \times u_{CRM,rel} \times \text{purity estimation} \times 1000 \text{ g/kg}$$

Purity estimation is based on the actual number of positive seeds detected per seeds tested. When using an asymmetric uncertainty, the reported measurement uncertainty is truncated on the right side such that the value does not exceed 1000 g/kg. Consequently, the expanded measurement uncertainty for AOCS 0216-A is +9 g/kg, -22 g/kg.

## Homogeneity

The homogeneity of AOCS 0216-A is related to the purity of the seeds. 717 out of 719 seeds tested positive for the MON 87403 maize event. Based on the sample purity of 99.7%, as determined using SeedCalc8, the batch was considered to be homogeneous.

To further confirm homogeneity, ten vials of AOCS 0216-A (randomly selected as described above) were provided by AOCS to Monsanto. Homogeneity was assessed

using the MON 87403 specific quantitative PCR method (<https://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-03-12-VM.pdf>) that was adapted for digital PCR (dPCR), which has the advantage over qPCR of quantifying targets without the need for calibration curves. For each of the 10 CRM vials analyzed, there were 2 independent DNA extractions. Each DNA extraction was subject to 3 dPCR replicates. The data produced from these dPCR reactions provided the numeric copies of MON 87403 and the numeric copies of *hmg*, a maize specific endogenous reference gene. The property value assessed here is defined as the ratio between copies of the MON 87403 target and copies of the *hmg* target.

The digital PCR data was used to evaluate the within-unit and between-unit homogeneity of AOCS 0216-A to ensure that the property value is valid within vials of CRM and between vials of CRM. The CRM will be determined to be homogeneous if the within-unit relative standard deviation ( $RSD_w$ ) and between-unit relative standard deviation ( $RSD_b$ ) are both  $\leq 20\%$ .

Quantification of between-unit (vial/sample) inhomogeneity was undertaken by analysis of variance (ANOVA), which separates the between-unit variation from the within-unit variation. Preliminary analysis showed that there is no significant variation between the two DNA extractions within each vial, so the DNA extraction effect was not considered in the analysis. That is, all replicates for each vial were treated as independent observations regardless of which DNA extraction they were from.

Within-unit relative standard deviation ( $RSD_w$ ), between-unit relative standard deviation ( $RSD_b$ ) were calculated as:

$$\text{Within-unit RSD: } RSD_w = \frac{\sqrt{MS_{within}}}{\bar{y}}$$

$$\text{Between-unit RSD: } RSD_b = \frac{\sqrt{\frac{MS_{between} - MS_{within}}{n}}}{\bar{y}}$$

where,

$MS_{within}$	within-unit mean square from an ANOVA
$MS_{between}$	between-unit mean square from an ANOVA
$\bar{y}$	mean of all results of the homogeneity study

$n$  mean number of replicates per unit (5.9 for MON 87403)

**Table 2. The within-unit relative standard deviation (RSD<sub>w</sub>), and the between-unit relative standard deviation (RSD<sub>b</sub>) for vials of AOCS 0216-A.**

CRM	RSD <sub>w</sub> [%]	RSD <sub>b</sub> [%]	u* <sub>bu,rel</sub> [%]
AOCS 0216-A	2.3	n.c. <sup>1</sup>	1.0

<sup>1</sup>n.c: RSD<sub>b</sub> cannot be calculated as MS<sub>between</sub> < MS<sub>within</sub>. In this situation, maximum hidden inhomogeneity (u\*<sub>bu,rel</sub>) is provided as an alternative

This confirms the homogeneity of AOCS 0216-A.

## 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 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

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; <http://www.ilcrop.com/index.htm>

ISO 17034:2016 (E) General requirements for the competence of reference material producers

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

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%3A32003R1829&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>

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