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Certified Reference Materials

AOCS 0906-E2

Report of the certification process for

MON89034

Maize Certified Reference Material

Second Batch

OECD Unique ID MON-89Ø34-3

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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 0906-E2 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. The maize MON 89034 powder was provided by Monsanto Company, St. Louis, MO (hereinafter “Monsanto”). It was prepared by grinding the bulk seed at Monsanto. The certified value of AOCS 0906-E2 was based on the purity of the bulk seed material and with 95% confidence, the true value is ≥ 996 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 89034 in AOCS 0906-E2 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 0906-E2 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 Organization for Standardization
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 89034	Maize line that expresses two Bacillus thuringiensis toxins (cry1A.105 and cry2Ab2) introduced by mediated gene transfer techniques. The function is to protect the plant from lepidopteran insects, fall army worm (Spodoptera sp.), black cutworm (Agrotis ipsilon), European corn borer (Ostrinia nubilalis) and the corn ear worm (Helicoverpa zea).

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 0906-E2 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 89034 maize seeds used to prepare AOCS 0906-E2 were hemizygous through successive breeding generations, and the donor for the MON 89034 maize event was the male parent. Monsanto milled ~10 kg of MON 89034 maize seed. All of the seed powder was passed through a 500 µM mesh sieve. The seed powder was delivered to AOCS who contracted Illinois Crop Improvement Association 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 89034

The presence of the MON 89034 trait was assessed on 10 random vials of AOCS 0906-E2. AOCS used the Random Number Generator function of Microsoft Excel to select samples for verification of trait presence. Sample numbers that were randomly

selected were sent to Eurofins-GeneScan, New Orleans, LA (an ISO 17025 accredited laboratory) for event-specific, qualitative PCR analysis to verify the presence of MON 89034 in the samples (Table 1).

Table 1. Trait verification testing on AOCS 0906-E2 MON 89034 maize performed by Eurofins-GeneScan, New Orleans, LA (an ISO 17025 accredited laboratory).

AOCS 0906-E2 Sample	Trait MON 89034 Presence
Sample # 0486	Positive
Sample # 0379	Positive
Sample # 0151	Positive
Sample # 0406	Positive
Sample # 0051	Positive
Sample # 0268	Positive
Sample # 0126	Positive
Sample # 0295	Positive
Sample # 0263	Positive
Sample # 0276	Positive

Certified Value and Measurement Uncertainty

The genetic purity of the seed lot used to produce AOCS 0906-E2 was assessed by Monsanto. A total of 720 maize seeds were subjected to individual seed testing for the presence of MON 89034 by qualitative event-specific PCR. 720 of the 720 seeds tested positive for the presence of MON 89034.

Purity estimation was calculated using SeedCalc8 (Remund *et al*., 2008) and corresponds to the lower bound of the true % purity. The % purity in the sample was 100%, when 720 seeds were tested. Using a 95% confidence level, the true % purity of the MON 89034 seed lot was 99.6%. Consequently, with 95% confidence, the true value is ≥ 996 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 ($u_{bb,rel}$):

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

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

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 0906-E2 is +4 g/kg, -53 g/kg.

Homogeneity

The homogeneity of AOCS 0906-E2 is related to the purity of the seeds. 720 out of 720 seeds tested positive for the MON 89034 maize event by event-specific PCR. Based on the sample purity of 100%, as determined using SeedCalc8, the batch was expected to be homogenous.

To further confirm homogeneity, ten vials of AOCS 0906-E2 (randomly selected as described above) were provided by AOCS to Monsanto. Homogeneity was assessed using the MON 89034 specific quantitative PCR method (https://gmo-crl.jrc.ec.europa.eu/summaries/MON89034_validated_Method.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 89034 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 89034 target and copies of the *hmg* target.

The digital PCR data was used to evaluate the within-unit and between-unit homogeneity of AOCS 0906-E2 to ensure that the property value is valid within vials of CRM and between vials of CRM.

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 ($\mathrm{RSD_w}$), 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 AOCS 0906-E2.

CRM	RSD_w [%]	RSD_b [%]
AOCS 0906-E2	3.1	2.7

This confirms the homogeneity of AOCS 0906-E2.

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 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 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

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