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Certified Reference Materials

AOCS 0822-B

Report of the certification process for

DP-910521-2

Maize

First Batch

OECD Unique ID DP-91Ø521-2

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ISO 17034:2016
A2LA Certificate 3438.01

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Contents

Abstract

This report describes the preparation and certification of the maize CRM AOCS 0822-B produced by AOCS Technical Services in 2022. 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 DP-910521-2 powder was provided by Corteva Agriscience, Johnston, IA. It was prepared by grinding the bulk seed at Corteva Agriscience. The certified value of AOCS 0822-B was based on the purity of the bulk seed material and with 95% confidence, the true value is ≥ 969 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 DP-910521-2 and the homogeneity in AOCS 0822-B was verified using event-specific, qualitative and quantitative PCR analysis by Eurofins-GeneScan, New Orleans, LA (an ISO 17025-accredited laboratory). CRM samples should be stored in a dry, sealed container at ambient or cooler conditions in the dark.

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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: DP-910521-2	Resistance to the insect lepidopteran and the herbicide glufosinate

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 0822-B was manufactured from maize according to ISO 17034:2016 and in accordance with EC No 1829/2003, EC No 641/2004 and EC No 619/2011. The CRM is available from AOCS.

Material Processing

DP-910521-2 maize seeds used to prepare AOCS 0822-B were homozygous through successive breeding generations, and the donor for the DP-910521-2 maize event was the male parent to create hemizygous seed. Corteva Agriscience milled ~4 kg of DP-910521-2 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 DP-910521-2

The presence of the DP-910521-2 trait was assessed on 10 vials of AOCS 0822-B. Sample numbers that were 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 DP-910521-2 in the samples (Table 1).

Table 1. Trait verification testing on AOCS 0822-B DP-910521-2 maize performed by Eurofins-GeneScan, New Orleans, LA (an ISO 17025-accredited laboratory).

AOCS 0822-B Sample	Trait DP-910521-2 Presence
Sample # 7	Positive
Sample # 96	Positive
Sample # 133	Positive
Sample # 148	Positive
Sample # 194	Positive
Sample # 221	Positive
Sample # 269	Positive
Sample # 295	Positive
Sample # 312	Positive
Sample # 348	Positive

Certified Value and Measurement Uncertainty

The genetic purity of the seed lot used to produce AOCS 0822-B was assessed by Corteva Agriscience. A total of 336 maize seeds were subjected to individual seed testing for the presence of DP-910521-2 by qualitative event-specific PCR. 336 of the 336 seeds tested positive for the presence of DP-910521-2.

Purity estimation was calculated using SeedCalc8 (Remund *et al*., 2008) and corresponded to the lower bound of true % purity. The % purity in the sample was 100%, when 336 seeds were tested. Using a 95% confidence level, the true % purity of the DP-910521-2 seed lot was at least 96.9%. Consequently, with 95% confidence, the true value is ≥ 969 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 AOCS 0822-B is -31 g/kg.

Homogeneity

The homogeneity of AOCS 0822-B is related to the purity of the seeds. 336 out of 336 seeds tested positive for the DP-910521-2 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 0822-B (randomly selected as described above) were provided by AOCS to Eurofins-GeneScan, New Orleans, LA (an ISO 17025-accredited laboratory). Homogeneity was assessed using the DP-910521-2 specific quantitative PCR method (Corteva Agriscience method). For each of the 10 CRM vials analyzed, there were 2 independent DNA extractions. Each DNA extraction was subject to 3 PCR replicates. The data produced from these PCR reactions provided the numeric copies of DP-910521-2 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 DP-910521-2 target and copies of the *hmg* target.

The PCR data was used to evaluate the within-unit and between-unit homogeneity of AOCS 0822-B 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 with-in relative standard deviation (RSD_w) and between-unit relative standard deviation (RSD_b) are both ≤ 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 ($\mathrm{RSD_w}$), 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 0822-B.

CRM	RSD_w [%]	RSD_b [%]	$u^*_{bu,rel}$ [%]
AOCS 0822-B	7.6	n.c.[1]	3.7

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

This confirms the homogeneity of AOCS 0822-B.

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

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/

International Seed Testing Association, International Rules of Seed Testing: Seed Science and Technology Rules, 2012

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

ISO 17034:2016, General Requirements for the Competence of Reference Material Producers

Linsinger, T., Pauwels, J., van der Veen, A. *et al.* Homogeneity and stability of reference materials. *Accred Qual Assur* 6**,** 20–25 (2001). doi.org/10.1007/s007690000261

Regulation (EC) No 1829/2003 of the European Parliament and of the Council of 22 September 2003 on genetically modified food and feed 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/services-header/tools/statistics-committee/statistical-tools-seed-testing.html