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Certified Reference Materials

AOCS 0523-B

Report of the certification process for

KWS20-1 Sugar Beet

Certified Reference Material

First Batch

OECD Unique ID KB-KWS2Ø1-6

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ACCREDITED CERT #3438.01 ISO 17034:2016
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Abstract

This report describes the preparation and certification of the sugar beet Certified Reference Material AOCS 0523-B produced by AOCS Technical Services in 2023. The CRM has been prepared according to ISO 17034:2016 and is intended to serve as control material for third party testing of sugar beet for transformation events. The sugar beet KWS20-1 powder was provided by KWS SAAT SE & Co. KGaA. It was prepared by grinding the bulk seed at IBRL at University of Illinois. The certified value of AOCS 0523- B was based on the purity of the material and with 95% confidence, the true value is ≥ 938 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 homogeneity of KWS20-1 in AOCS 0523-B was verified on random vials of AOCS 0523-B using quantitative PCR analysis by Eurofins GeneScan GmbH, Freiburg, Germany. 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 Counterpart	Conventional counterpart (to a genetically modified organism) means a non-genetically modified genotype with a genetic background as close as possible to the genetically modified plant.
DNA	Deoxyribonucleic Acid is the linear, double-helix macromolecule that makes up the genetic material of most organisms.
EC	European Commission.
GMO	Genetically modified 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 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.
Quantitative PCR	PCR methods that estimate the relative amount of target DNA sequence in a mixture of DNA molecules.

Trait in KWS20-1	Herbicide tolerance to dicamba, glufosinate and glyphosate

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 (CRM) created from leaf, seed, or grain containing the new trait as well as a CRM created from the conventional counterpart. The European Commission has mandated that from 18 April 2004, a method for detecting a new event derived from transgenic technology and CRM 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 0523-B was manufactured from sugar beet according to ISO 17034:2016 and in accordance with EC No 1829/2003. The CRM is available from AOCS.

Material Processing

The KWS20-1 sugar beet seed used to prepare AOCS 0523-B is hemizygous hybrid seed produced by crossing a non-genetically modified line as female donor and a homozygous KWS20-1 line as male donor. IBRL at University of Illinois milled ~ 10 kg of KWS20-1 sugar beet seed. All of the seed powder was passed through a 710 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 KWS20-1

The presence of the KWS20-1 trait was assessed on 10 random vials of AOCS 0523-B. 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 GmbH, Freiburg, Germany (an ISO 17025 accredited laboratory)

for event-specific quantitative PCR analysis to verify the presence of KWS20-1 in the samples (Table 1).

Table 1. Trait verification testing on AOCS 0523-B KWS20-1 sugar beet performed by Eurofins GeneScan GmbH, Freiburg, Germany (an ISO 17025 accredited laboratory).

AOCS 0523-B Sample	Trait KWS20-1 Presence
Sample # 57	Positive
Sample # 142	Positive
Sample # 224	Positive
Sample # 376	Positive
Sample # 455	Positive
Sample # 550	Positive
Sample # 663	Positive
Sample # 718	Positive
Sample # 751	Positive
Sample # 849	Positive

Certified Value and Measurement Uncertainty

The genetic purity of the seed lot used to produce AOCS 0523-B was assessed by Eurofins GeneScan GmbH, Freiburg, Germany (an ISO 17025 accredited laboratory). A total of 96 sugar beet seeds were germinated, and one leaf was collected from each of the 96 plants and subjected to individual leaf punch testing for the presence of KWS20-1 by event-specific qualitative PCR. 96 of the 96 leaves tested positive for the presence of KWS20-1.

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 96 leaves were tested. Using a 95% confidence level, the true % purity of the

KWS20-1 seed lot was at least 93.80%. Consequently, with 95% confidence, the true value is ≥ 938 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 0523-B is -62 g/kg.

Homogeneity

The homogeneity of AOCS 0523-B is related to the purity of the seeds. 96 out of 96 leaves from the seeds tested positive for the KWS20-1 sugar beet event by event-specific qualitative 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 0523-B (randomly selected as described above) were provided by AOCS to Eurofins GeneScan GmbH, Freiburg, Germany (an ISO 17025 accredited laboratory). Homogeneity was assessed using the KWS20-1 event-specific quantitative PCR method (Kørner, 2023). For each of the 10 CRM vials analyzed, there were 2 independent DNA extractions. Each DNA extraction was subject to 3 quantitative PCR replicates. The data produced from these quantitative PCR reactions provided the numeric copies of KWS20-1 and the numeric copies of glutamine synthetase (GS), a sugar beet endogenous reference gene. The property value assessed here is defined as the ratio between copies of the KWS20-1 target and copies of the glutamine synthetase (GS) target.

The quantitative PCR data was used to evaluate the within-unit and between-unit homogeneity of AOCS 0523-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 0523-B.

CRM	RSD_w [%]	RSD_b [%]	$u^*_{bu,rel}$ [%]
AOCS 0523-B	5.8	n.c.[1]	2.8

[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 0523-B.

Stability

Time, temperature, and light are regarded as the most relevant influences on the stability of a 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 GmbH, Engresserstraße 4, D-79108 Freiburg, Germany Telephone: +49 761 6400 4011 Online: https://www.eurofinsus.de/food-analysis

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

Linsinger T.P.J., Pauwels J., van der Veen A.M.H., Schimmel H., and Lamberty A. 2001. Homogeneity and stability of reference materials. *Accred. Qual. Assur.* 6:20-25.

Kørner C. 2023 Report on the Validation of a Quantitative Event-specific, Real-time PCR System for the Detection of KWS20-1 Sugar beet.

Remund K., Simpson R., Laffont J-L., Wright D., and Gregoire S. Seedcalc8. 2008. https://www.seedtest.org/api/rm/928M856R576687N/seedcalc8.zip

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