**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 1208-A3

Report of the certification process for

NP2222 (MIR162)/NP2391

Maize Certified Reference Materials

Third Batch

OECD Unique Identifier
SYN-IR162-4

Denise Williams
Technical Services Manager

Scott Bloomer
Technical Director

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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 Certified Reference Material (CRM) AOCS 1208-A3 produced by AOCS Technical Services in 2019. The CRMs have been prepared at AOCS according to ISO 17034:2016 and are intended to serve as control material for third party testing of maize for transformation events. NP2222 (MIR162)/NP2391 maize seed powder was provided by Syngenta Crop Protection, LLC and was prepared by grinding the bulk source at AVEKA, Inc., Woodbury, MN (an ISO 9001:2015 accredited facility). The NP2222 (MIR162)/NP2391 maize seed powder was then aliquoted and packaged under a nitrogen gas environment at Illinois Crop Improvement Association (an ISO 17025:2017 accredited facility). The certified mass value of Event MIR162 in NP2222 (MIR162)/NP2391 maize was based on seed purity and with 95% confidence, is at least 993 g/kg. Homogeneity testing was performed at Eurofins-GeneScan New Orleans, LA (an ISO 17025:2005 accredited laboratory) using quantitative real-time PCR after the CRM AOCS 1208-A3 was bottled. Homogeneity results indicated that CRM AOCS 1208-A3 is homogenous and were used to verify the presence of the Event MIR162 in this CRM. CRM AOCS 1208-A3 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, E. Pearce Smith, and Daniel Thompson, 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 |
| Cycle threshold (Ct) | Number of PCR cycles required for the fluorescent signal to cross a threshold that exceeds background level |
| Conventional Crop | A related organism/variety, its components and/or products for which there is experience of establishing safety based on common use as food |
| 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 quantitated |
| 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: NP2222 (MIR162)/NP2391 | Line of maize, genetically engineered to be resistant certain lepidoperan 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, CRM AOCS 1208-A3 was manufactured from maize seed according to ISO 17034:2016 and in accordance with EC No 1829/2003. The CRM is available from AOCS.

## Material Processing and Particle Size Analyses

The hemizygous NP2222 (MIR162)/NP2391 maize seed used in the preparation of CRM AOCS 1208-A3 resulted from the cross of female NP2222 (MIR162) and male non-transgenic NP2391. The NP2222 (MIR162)/NP2391 maize seed was first milled and analyzed for particle size distribution at AVEKA, Inc., Woodbury, Minnesota (an ISO 9001:2015 accredited facility).

Bulk seed received by AVEKA, Inc from Syngenta Crop Protection, LLC was milled in a Fitzmill cryogenic hammermill using first a 690 µm screen. To further reduce particle size, this ground material was milled again under the same conditions using a 510 µm screen. The material was blended in a Patterson-Kelley V-blender, and after homogenization six samples taken at random were subject to particle size analyses using a Horiba LA-950 Light Scattering Particle 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 CRM AOCS 1208-A3 was 104.18 $\pm$ 5.23 µm, and 90%

of the particles were smaller than 230.71 ± 11.98 µm (Table 1).

**Table 1. Results of Particle Size Analyses of CRM AOCS 1208-A3 Conducted by AVEKA**

| | Sample 1 (µm) | Sample 2 (µm) | Sample 3 (µm) | Sample 4 (µm) | Sample 5 (µm) | Sample 6 (µm) | Average (µm) | Standard Deviation (µm) |
|---|---|---|---|---|---|---|---|---|
| **Mean** | 102.89 | 100.91 | 96.23 | 109.58 | 105.87 | 109.59 | **104.18** | **5.23** |
| **Range** | 0.77-394 | 0.77-344 | 0.77-344 | 0.77-394 | 0.77-394 | 0.77-394 | N/A[(a)] | N/A |
| **D10** [(b)] | 12.97 | 15.11 | 14.39 | 14.57 | 9.19 | 13.89 | 13.35 | 2.16 |
| **D50** [(b)] | 81.83 | 79.96 | 79.84 | 93.07 | 83.34 | 91.76 | 84.97 | 5.93 |
| **D90** [(b)] | 231.34 | 224.48 | 209.85 | 238.87 | 237.63 | 242.08 | **230.71** | **11.98** |

(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 the x-axis showing discrete size bins up to 394.24 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. For all samples analyzed, 100% of particles were ≤ 394.24 µm.

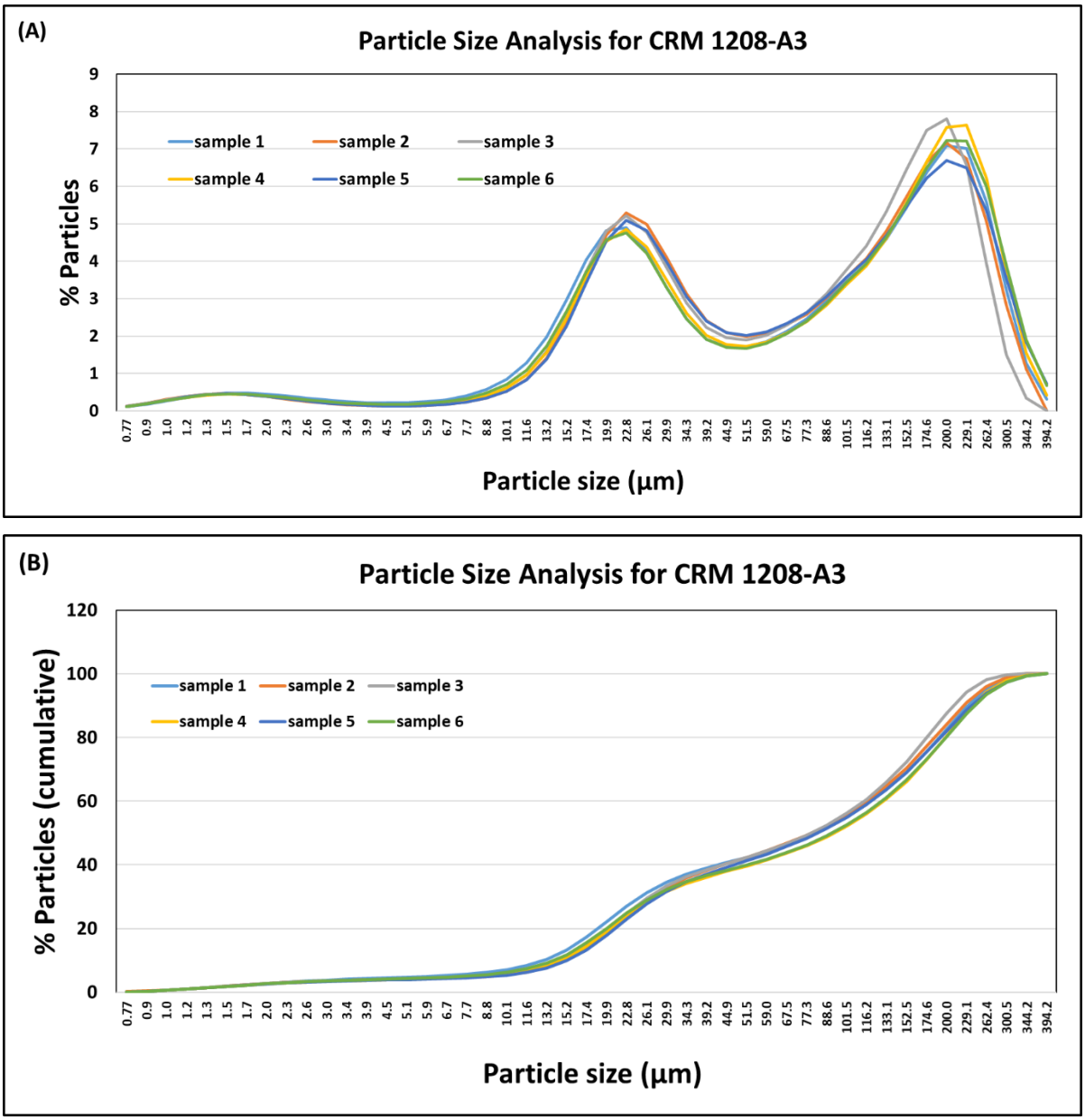


**Figure 1**. Particle size distribution plots. (A) Percentage of particles of a given size. (B) Cumulative distribution of particle sizes.

Bulk, NP2222 (MIR162)/NP2391 maize seed powder for the production of CRM AOCS 1208-A3 was delivered to AOCS and it was then aliquoted and packaged in 27-mL glass headspace vials and sealed under a nitrogen gas environment at the Illinois Crop Improvement Association (an ISO 17025:2017 accredited facility).

## Certified Value and Measurement Uncertainty Assignment

The genetic purity based on the presence of Event MIR162 in NP2222 (MIR162)/NP2391 maize was assessed by Syngenta Crop Protection, LLC. A total of 400 NP2222 (MIR162)/NP2391 maize seeds were evaluated by qualitative MIR162-specific real time PCR. The results showed that 400 of the 400 seeds tested (100%) were positive for the presence of Event MIR162.

The statistical seed purity of NP2222 (MIR162)/NP2391 maize was calculated using SeedCalc8 (Remund *et al*., 2008) and corresponded to the lower bound of true % purity. Using a 95% confidence level, the true % purity of NP2222 (MIR162)/NP2391 maize seed is at least 99.3 %. Consequently, with 95% confidence, the true value is ≥ 993 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 number of 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 this CRM is +7 g/kg, -126 g/kg.

## Homogeneity Testing

The material used for the production of CRM AOCS 1208-A3, NP2222 (MIR162)/NP2391 maize, is 99.3% pure and is expected to be homogenous. After NP2222 (MIR162)/NP2391 maize seed was ground and bottled as described above, ten samples

of CRM AOCS 1208-A3 were randomly selected using the Microsoft Excel Random Number Generator function and were sent to Eurofins-GeneScan, New Orleans, LA (an ISO 17025:2005 accredited laboratory) for homogeneity testing using quantitative real-time PCR.

Homogeneity was assessed after bottling of AOCS 1208-A3 CRM using a MIR162-specific, quantitative real-time PCR method (http://gmo-crl.jrc.ec.europa.eu/summaries/MIR162_validated_Method.pdf). A total of 10 samples 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 the endogenous *adh1* maize gene and for Event MIR162 were used to calculate the number of copies (cp#) for either target. Subsequently, the ratio between Event MIR162 copy number and *adh1* copy number (MIR162 cp#/*adh1* cp#) was calculated and used to estimate within-unit relative standard deviation ($\mathrm{RSD_w}$) and between-unit relative standard deviation ($\mathrm{RSD_b}$). 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 1208-A3.** | | |
|---|---|---|
| CRM | $RSD_w$ [%] | $RSD_b$ [%] |
| AOCS 1208-A3 | 9.1 | 6.3 |

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 ≤ 20%. Based on the quantitative real-time PCR analyses conducted, it was concluded that CRM AOCS 1208-A3 is homogenous (Table 2). These results are in agreement with homogeneity results from qualitative real-time PCR analyses and with the purity estimate for material MIR162 maize calculated in the Certified Value and Measurement Uncertainty section above.

## Trait Verification

The presence of the NP2222 (MIR162)/NP2391 trait was assessed in the same ten CRM AOCS 1208-A3 samples that were analyzed for homogeneity using MIR162-specific quantitative PCR analysis. Quantitative results were converted to qualitative data, and the results are presented in Table 3. In all instances Event MIR162 was present.

| **Table 3. Qualitative results for the verification of CRM AOCS 1208-A3 as tested by Eurofins-GeneScan with MIR162-specific, quantitative PCR analysis.** | |
|---|---|
| **Sample** | **Event MIR162 Presence** |
| AOCS 1208-A3 11 | Positive |
| AOCS 1208-A3 42 | Positive |

| | |
|---|---|
| AOCS 1208-A3 85 | Positive |
| AOCS 1208-A3 107 | Positive |
| AOCS 1208-A3 153 | Positive |
| AOCS 1208-A3 160 | Positive |
| AOCS 1208-A3 163 | Positive |
| AOCS 1208-A3 230 | Positive |
| AOCS 1208-A3 340 | Positive |
| AOCS 1208-A3 406 | Positive |

## 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

AVEKA; Inc 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 9001:2015, Quality Management Systems – Requirements

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

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

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)

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