Certified Reference Materials

AOCS 0306-E3

Report of the certification process for

LLCotton25

Cotton Certified Reference Materials

Third Batch

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AOCS advances the science and technology of oils, fats, surfactants and related materials, enriching the lives of people everywhere.

More information regarding AOCS is available at http://www.aocs.org
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Abstract

This report describes the preparation and certification of the cotton CRM AOCS 0306-E3 produced by AOCS Technical Services in 2015. The CRMs have been prepared according to ISO Guides 30, 31, 34 and 35 and are intended to serve as control material for third party testing of cotton for transformation events. The presence of LLCotton25 in the cotton was verified using event-specific, qualitative PCR analysis by Eurofins-GeneScan, New Orleans, LA (an ISO 17025 Accredited laboratory). AOCS 0306-E3 is available in 0.5 ml skirted screw-cap self-sealing tubes. The cotton LLCotton25 DNA was extracted from clean leaves by BASF Agricultural Solutions Seed US LLC. The leaf DNA extract sample shall be stored dry in a sealed container at +4 °C 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 Benoit Maes and Ray Shillito, BASF Agricultural Solutions Seed US LLC, for offering AOCS the opportunity to manufacture and distribute these products; to Heather Waxdahl, SGS Midwest Seed Services for packaging the samples; and to Frank Spiegelhalter, Greg Ditta, E. Pearce Smith, and Daniel Thompson, Eurofins-GeneScan for event-specific, qualitative PCR analysis including the provision of information on running the analyses and interpreting the results.
## Glossary

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>AOCS</td>
<td>American Oil Chemists’ Society</td>
</tr>
<tr>
<td>Conventional Crop</td>
<td>A related organism/variety, its components and/or products for which there is experience of establishing safety based on common use as food</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid is the linear, double-helix macromolecule that makes up the genetic material of most organisms</td>
</tr>
<tr>
<td>Detection Limit</td>
<td>Lowest level at which target DNA can be detected in a sample.</td>
</tr>
<tr>
<td>EC</td>
<td>European Commission</td>
</tr>
<tr>
<td>Genome</td>
<td>The full set of genes and associated DNA characteristic of an organism</td>
</tr>
<tr>
<td>GMO</td>
<td>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.</td>
</tr>
<tr>
<td>ISO</td>
<td>International Organisation for Standardisation</td>
</tr>
<tr>
<td>PCR</td>
<td>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</td>
</tr>
</tbody>
</table>
(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

Trait: LLCotton25  Phosphinothricin (PPT) herbicide tolerance, specifically glufosinate ammonium
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 0306-E3 was manufactured from cotton according to ISO Guides 30, 31, 34 and 35 and in accordance with EC No 1829/2003. The CRM is available from AOCS.

Materials and Methods

BASF Agricultural Solutions Seed US LLC prepared the bulk material by taking source leaf material from plants which had been tested individually using several quality standards and were grown from seeds harvested from plants that had themselves passed the same criteria. Plants not meeting the quality standards were removed and destroyed. Leaf material was harvested from the plants which met the quality standards and frozen immediately and stored at -70 °C. The genomic DNA was extracted from leaves of one or more plants according to CTAB-based (Doyle JJ and Doyle JL, 1987) protocol. The integrity and concentration of the genomic DNA was determined by electrophoresis in a 1.0% agarose gel and ethidium bromide-staining and compared to lambda molecular weight standards by digital imaging quantification. The concentration measurement was
done in triplicate, repeated in three different gels. No indications for physical degradation were apparent and the DNA migrated at positions higher than 40 Kb.

BASF Agricultural Solutions Seed US LLC delivered 1.5 mg of LLCotton25 cotton DNA to AOCS. Five (5) working samples of DNA, 10 μg each, were prepared from the composite and sent to Eurofins-GeneScan, New Orleans, LA (an ISO 17025 Accredited laboratory) for event-specific, qualitative PCR analysis to screen for the presence of the intended event, LLCotton25. This testing was for presence confirmation as well as homogeneity purposes.

The leaf used to manufacture the LLCotton25 materials was shown to contain the LLCotton25 event as well as the absence of LL117, LLCSIRO, 2mEPSPS, NPTII, and Cry1Ab by PCR protocols at BASF Agricultural Solutions Seed US LLC. The LLCotton25 cotton leaf DNA was packaged by SGS-Midwest Seed Services in sterile, 0.5 ml skirted screw-cap self-sealing tubes in aliquots of 10 μg.

AOCS used the Random Number Generator function of Microsoft Excel to select samples for verification of gene presence, homogeneity, and to rule out degradation during packaging. Sample numbers AOCS 0306-E3: 2, 5, 31, 81, and 118 were sent to Eurofins-GeneScan, New Orleans, LA (an ISO 17025 Accredited laboratory) for event-specific, qualitative PCR analysis to screen for LLCotton25 presence in the samples.

**Stability**

Stability of these CRMs has been listed as 1 year from the introduction date. The materials were sealed and stored in the dark at 4 °C, therefore not exposed to air and are expected to be stable for longer than the estimated expiration date. The stability of the leaf DNA extract material will be reevaluated annually. If the samples still test positive for the presence of the trait, the certificates will be extended.
Results and Discussion

Sample Homogeneity
The PCR data for the LLCotton25 homogeneity samples is presented in Table 1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>LLCotton25 Presence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogeneity Sample 1</td>
<td>Positive</td>
</tr>
<tr>
<td>Homogeneity Sample 2</td>
<td>Positive</td>
</tr>
<tr>
<td>Homogeneity Sample 3</td>
<td>Positive</td>
</tr>
<tr>
<td>Homogeneity Sample 4</td>
<td>Positive</td>
</tr>
<tr>
<td>Homogeneity Sample 5</td>
<td>Positive</td>
</tr>
</tbody>
</table>
Prepared Sample Verification
After the bulk material was packaged, five (5) samples were identified by the Microsoft Excel Random Number Generator and sent to Eurofins-GeneScan, New Orleans, LA (an ISO 17025 Accredited laboratory) for event-specific, qualitative PCR analysis. These results are presented in Table 2. This data confirms the presence of the LLCotton25 gene after the packaging of AOCS 0306-E3. These results are consistent with the homogeneity data presented in Table 1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>LLCotton25 Presence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOCS 0306-E3 2</td>
<td>Positive</td>
</tr>
<tr>
<td>AOCS 0306-E3 5</td>
<td>Positive</td>
</tr>
<tr>
<td>AOCS 0306-E3 31</td>
<td>Positive</td>
</tr>
<tr>
<td>AOCS 0306-E3 81</td>
<td>Positive</td>
</tr>
<tr>
<td>AOCS 0306-E3 118</td>
<td>Positive</td>
</tr>
</tbody>
</table>
References

Center for Environmental Risk Assessment GM Database
http://www.cera-gmc.org/?action=gm_crop_database

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
http://www.gmotesting.com

ISO Guide 30:1992 (E/F), Reference Materials – Selected Terms and Definitions

ISO Guide 31:2000 (E), Reference Materials- Contents of Certificates, Labels and
Accompanying Documentation

ISO Guide 34:2009 (E), Reference Materials – General Requirements for the
Competence of Reference Material Producers

Assessment of Homogeneity and Stability

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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