Certified Reference Materials

AOCS 0112-A

Report of the certification process for

SYHT0H2

Soybean Certified Reference Materials

First Batch

OECD Unique Identifier
SYN-000H2-5

Denise Williams
Technical Services Manager

Scott Bloomer
Technical Director

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Abstract

This report describes the preparation and certification of the soybean CRM AOCS 0112-A produced by AOCS Technical Services in 2012. 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 soybean for transformation events. SYHT0H2 soybean powder was provided by Syngenta Crop Protection, LLC. The SYHT0H2 soybean powder was prepared by grinding the bulk seed at Syngenta Crop Protection, LLC. The SYHT0H2 soybean powder was then aliquoted and packaged under a nitrogen gas environment at Illinois Crop Improvement Association. The presence of SYHT0H2 in the soybean was verified using event-specific, qualitative PCR analysis by Eurofins GeneScan, New Orleans, LA (an ISO 17025 accredited laboratory). The certified mass value was based on the genetic purity of the material and with 95% confidence, the true SYHT0H2 soybean mass fraction of the material is above 991.1 g/kg. Homogeneity testing was performed at Eurofins using qualitative real-time PCR prior to bottling, and quantitative real-time PCR after CRM 0112-A was bottled. AOCS 0112-A 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

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOCS</td>
<td>American Oil Chemists’ Society</td>
</tr>
<tr>
<td>Cycle threshold (Ct)</td>
<td>Number of PCR cycles required for the fluorescent signal to cross a threshold that exceeds background level</td>
</tr>
<tr>
<td>Conventional Crop</td>
<td>Conventional counterpart means 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

**RSDr**  
Relative standard deviation

**SD**  
Standard deviation

**Trait: SYHT0H2**  
Confers tolerance to mesotrione and glufosinate herbicides
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, AOCS 0112-A was manufactured from soybean seed according to ISO Guides 30, 31, 34 and 35 and in accordance with EC No 1829/2003. The CRM is available from AOCS.

Material Preparation and Particle Size Analyses

AOCS 0112-A has been prepared by AOCS from homozygous soybean seed, and Syngenta Crop Protection, LLC delivered 10 kg SYHT0H2 soybean flour to AOCS. The SYHT0H2 soybean was processed, and subsequently packaged in 27-mL glass headspace vials and sealed under a nitrogen gas environment at Illinois Crop Improvement Association.

Once packaged, four randomly selected samples were subject to particle size analysis. Particle size analysis was conducted at AVEKA, Woodbury, Minnesota, (an ISO 9001:2015 accredited laboratory) using a Horiba LA-950 particle size 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 0112-A was 886.75 + 38.30 μm, and 90% of the particles (i.e. D90) were smaller than 1735.02 + 60.57 μm (Table 1)
The particle size distribution for each of the samples analyzed is presented as a histogram, with discrete size bins up to 3000 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.

### Table 1. Results of Particle Size Analyses of CRM 0112-A

<table>
<thead>
<tr>
<th>Sample 1 (µm)</th>
<th>Sample 2 (µm)</th>
<th>Sample 3 (µm)</th>
<th>Sample 4 (µm)</th>
<th>Average (µm)</th>
<th>Standard Deviation (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>907.18</td>
<td>917.68</td>
<td>831.84</td>
<td>890.29</td>
<td>886.75</td>
</tr>
<tr>
<td>Range</td>
<td>5.1-3000</td>
<td>3.5-3000</td>
<td>6.7-3000</td>
<td>3.4-3000</td>
<td>N/A(a)</td>
</tr>
<tr>
<td>D10(b)</td>
<td>67.42</td>
<td>72.58</td>
<td>83.58</td>
<td>69.63</td>
<td>73.30</td>
</tr>
<tr>
<td>D50(b)</td>
<td>880.36</td>
<td>908.30</td>
<td>739.62</td>
<td>897.91</td>
<td>856.54</td>
</tr>
<tr>
<td>D90(b)</td>
<td>1781.50</td>
<td>1790.75</td>
<td>1699.98</td>
<td>1667.85</td>
<td>1735.02</td>
</tr>
</tbody>
</table>

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

**Trait Verification**

After the bulk material was packaged, AOCS used the random number generator function of Microsoft Excel to select samples for verification of trait presence, homogeneity and to rule out degradation during packaging. A total of ten (10) AOCS 0112-A samples (55, 90,
184, 216, 217, 268, 376, 422, 435, and 454) were sent to Eurofins GeneScan, New Orleans, LA for event-specific, qualitative PCR analysis to screen for SYHT0H2 presence in the samples. These results are presented in Table 2. These data confirm the presence of SYHT0H2 after the packaging of AOCS 0112-A.

<table>
<thead>
<tr>
<th>Sample</th>
<th>SYHT0H2 Presence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AOCS 0112-A 55</td>
<td>Positive</td>
</tr>
<tr>
<td>AOCS 0112-A 90</td>
<td>Positive</td>
</tr>
<tr>
<td>AOCS 0112-A 184</td>
<td>Positive</td>
</tr>
<tr>
<td>AOCS 0112-A 216</td>
<td>Positive</td>
</tr>
<tr>
<td>AOCS 0112-A 217</td>
<td>Positive</td>
</tr>
<tr>
<td>AOCS 0112-A 268</td>
<td>Positive</td>
</tr>
<tr>
<td>AOCS 0112-A 376</td>
<td>Positive</td>
</tr>
<tr>
<td>AOCS 0112-A 422</td>
<td>Positive</td>
</tr>
<tr>
<td>AOCS 0112-A 435</td>
<td>Positive</td>
</tr>
<tr>
<td>AOCS 0112-A 454</td>
<td>Positive</td>
</tr>
</tbody>
</table>

### Certified Value Assignment

The genetic purity based on the presence of SYHT0H2 in SYHT0H2 soybean was assessed by Syngenta Crop Protection, LLC. A total of 370 SYHT0H2 soybean seeds were evaluated by qualitative SYHT0H2-specific real time PCR. The results showed that 370 of the 370 seeds tested (100%) were positive for the presence of SYHT0H2.
The statistical purity of SYHT0H2 soybean was calculated using SeedCalc8 (Remund et al., 2008) and was based on the lower bound of true % purity using a 95% confidence level. Based on this analysis, the statistical purity of SYHT0H2 soybean was (99.19%), and it was used to assign a certified mass value of 991.9 g/kg to this CRM. The associated uncertainty was based on the desired confidence level, and consequently, with 95% confidence, the true SYHT0H2 soybean mass fraction of the material is above 991.9 g/kg.

**Homogeneity Testing**

The material used for the production of CRM 0112-A is 99.19% pure (see Certified Value Assignment section) and was expected to be homogenous. However, to further confirm homogeneity, the ground material was analytically assessed before and after bottling using real-time PCR.

Primary samples were taken from randomly selected areas and depths to form a 3-kg composite sample in accordance with the International Seed Testing Association's (ISTA) Seed Science and Technology Rules for batches up to 500 kg, ten (10) working samples of 100 g each were prepared from the composite sample and sent to Eurofins GeneScan, New Orleans, LA (an ISO 17025 accredited laboratory) for event-specific, qualitative PCR analysis. The analyses performed by Eurofins GeneScan, Metairie, LA were used to assess the trait presence and homogeneity of the lot. A summary of the qualitative PCR results for the SYHT0H2 homogeneity samples is presented in Table 3.
Table 3. Results of the qualitative homogeneity testing performed by Eurofins GeneScan, New Orleans, LA on the SYHT0H2 bulk material provided by Syngenta Crop Protection, LLC

<table>
<thead>
<tr>
<th>Sample</th>
<th>SYHT0H2 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>
<tr>
<td>Homogeneity Sample 6</td>
<td>Positive</td>
</tr>
<tr>
<td>Homogeneity Sample 7</td>
<td>Positive</td>
</tr>
<tr>
<td>Homogeneity Sample 8</td>
<td>Positive</td>
</tr>
<tr>
<td>Homogeneity Sample 9</td>
<td>Positive</td>
</tr>
<tr>
<td>Homogeneity Sample 10</td>
<td>Positive</td>
</tr>
</tbody>
</table>

In addition, homogeneity was also assessed after bottling of 0112-A CRM using a SYHT0H2 specific, quantitative real-time PCR method (http://gmo-crl.jrc.ec.europa.eu/summaries/EURL-VL-04-12-VP.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 2 grams. 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 an endogenous lectin soybean gene and for event SYHT0H2 were used to calculate the number of copies (cp#) for either target. Subsequently, the ratio between event SYHT0H2 copy number and lectin copy number
(SYHT0H2 cp#/lectin cp#) was calculated and used to estimate within- and between-sample average, standard deviation (SD), and relative standard deviation (RSDr). The within-sample averages ranged from 0.62-0.88, the within-sample SD ranged from 0.005-0.14, and the within-sample RSDr ranged from 0.77% -19.91%. The between-sample estimates for average, SD and RSDr were 0.78, 0.08 and 9.66, respectively (Table 4). Between-sample SD was calculated according to the following formula:

$$s_{pooled} = \sqrt{\frac{(n_1-1)s_1^2 + (n_2-1)s_2^2 + ... + (n_k-1)s_k^2}{n_1 + n_2 + ... + n_k - k}}$$

- $n = 2$ (number of tests per sample)
- $k = 10$ (number of samples)
- $s =$ within-sample standard deviation

**Table 4. Homogeneity testing for CRM 0112-A using the SYHT0H2-event specific, quantitative real-time PCR method (estimates based SYHT0H2 cp#/lectin cp#)**

<table>
<thead>
<tr>
<th></th>
<th>Average</th>
<th>SD</th>
<th>RSDr (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Within-sample</strong> (a)</td>
<td>0.62-0.88</td>
<td>0.005-0.14</td>
<td>0.77-19.91</td>
</tr>
<tr>
<td><strong>Between-sample</strong></td>
<td>0.78</td>
<td>0.08 (b)</td>
<td>9.66</td>
</tr>
</tbody>
</table>

(a) Within-sample estimates are given as a range of values calculated for each of the 10 samples assessed. Average and SD estimates are based on independent measurements from two DNA extracts.

(b) The SD is a pooled SD calculated as described in this section.

Based on the quantitative real-time PCR analyses conducted, it was concluded that CRM 0112-A is homogenous. These results are in agreement with homogeneity results from qualitative real-time PCR analyses and from the purity estimate for the SYHT0H2 material calculated in the Certified Value Assignment section above.

**Stability**

Stability of these CRMs has been listed as 1 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 still test positive for the presence of the intended trait, the certificates will be extended.
References

Biosafety Clearing House Living Modified Organism (LMO) Registry
http://bch.cbd.int/database/lmo-registry/

Eurofins-GeneScan; 2315 N Causeway Blvd, Suite 200, Metairie, LA 70001; 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 Guide 30:1992 (E/F), Reference Materials – Selected Terms and Definitions


