Nested Multiplex Polymerase Chain Reaction for the Determination of DNA From Genetically Modified Corn and Soy Beans Using the Agilent 2100 Bioanalyzer

Application

Agriculture

Author

Mark Jensen
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610
USA

Abstract

This application note describes how the Agilent Technologies 2100 bioanalyzer and the DNA 500 LabChip can be used to detect polymerase chain reaction products corresponding to genetically modified elements and endogenous sequences in corn and soy beans. The DNA extraction protocol used in the preparation of polymerase chain reaction samples was characterized using the Protein 200 Plus LabChip.

Introduction

Six years after the introduction of genetically modified organisms (GMO), consumer concerns about the presence of such modified organisms in food remains an ongoing issue. There has been a continuous debate surrounding issues of how food products that contain GMO ingredients should be regulated and labeled. This debate has been further complicated by disagreements over how GMOs should be detected and the significance of the detected levels. In spite of these issues, the number of available transgenic events has continued to grow. At the current time there are 14 transgenic varieties of corn and soy beans that have been deregulated by the Animal and Plant Health Inspection Service of the United States Department of Agriculture (USDA).

Although enzyme immunoassay is an efficient means for detecting transgenic proteins in raw products, only DNA analysis has proved to be effective for the entire range of sample matrices from raw materials to highly processed foods. The polymerase chain reaction (PCR) has been widely accepted as a method for the detection of DNA from genetically modified ingredients such as soya or corn [1, 2]. PCR detection of GMOs can be done either as a screening test using endpoint PCR or as a quantitative test using real-time fluorescence detection of the PCR product.

Quantitation by real-time PCR is an expensive analysis requiring assay calibration for each sample lot and multiple replicates of each unknown sample. Typical service charges for a single analysis are between $150–$300. Since each transgenic event must be evaluated individually, the cost of rigorously testing an unknown food sample for all the possible current transgenic events is cost prohibitive.

These cost constraints make it necessary to screen samples for DNA components that are present in

most GMOs prior to any quantitative analysis. This type of screening analysis can be carried out with a commercially available PCR test kit, such as the Biosmart Allin 1.0 GMO Screening System from Promega (Madison, Wisconsin). This kit provides a protocol and reagents for a nested multiplex PCR assay for the detection of DNA from modified organisms containing the 35S promoter. This genetic element is derived from the cauliflower mosaic virus and is found in most transgenic crops. In addition to the 35S promoter, the multiplex PCR reaction also detects sequences for soya (lectin), corn (zein) and an internal positive control. These multiplex PCR products are all easily resolved and detected using the DNA 500 LabChip and the Agilent 2100 bioanalyzer.

Experimental

DNA Extraction

Three DNA extraction protocols were evaluated prior to the PCR analysis. These protocols were the DNeasy Plant Mini Kit from Qiagen N. V. (Venlo, Netherlands), the Wizard Genomic DNA Purification Kit from Promega (Madison, Wisconsin) and a cetyltrimethylammonium bromide (CTAB) precipitation procedure that was developed in-house. Detailed descriptions of the DNeasy and Wizard Genomic kit protocols can be found in the technical manuals that accompany these products. The CTAB protocol is described below.

CTAB Extraction protocol:

1. A 50-mg sample was added to a 1.0-mL aliquot of CTAB extraction buffer (0.055 M CTAB (cetyltrimethylammonium bromide), 0.1 M Tris, 1.4 M NaCl, 0.2 M disodium EDTA pH 8.0), heated at 65 °C for 15 min and then placed on ice.
2. The extract mixture was centrifuged at 15,000 g for 10 min to remove particulates.
3. The supernatant was removed and stored in a new microfuge tube at 4 °C. (Samples with high starch levels, for example corn, were treated with 0.2 µL of α-amylase and incubated at 37 °C for 30 min.)
4. The recovered supernatant was combined with an equal volume of chloroform mixed for 30 s and then centrifuged for 10 min at 15,000 g. The upper (aqueous) phase was transferred to a new tube.
5. Two volumes of the CTAB precipitation solution (0.014 M CTAB, 0.04 M NaCl) were added to aqueous extract and mixed.
6. The mixture was incubated at 25 °C for 30 min and then centrifuged for 10 min at 15,000 g. The supernatant was discarded.
7. The precipitate was redissolved by adding 250 µL of the 1.2 M NaCl and incubating 37 °C for 10 min. The mixture was centrifuged for 5 min at 15,000 g and the supernatant was transferred to a new tube.
8. One volume of isopropanol was added to the mixture and then the mixture was stored at 4 °C for at least 30 min.
9. The solution was centrifuged for 10 min at 15,000 g and then the supernatant was discarded.
10. The pellet was washed with 500 µL of cold 70% ethanol, the mixture was centrifuged for 5 min at 15,000 g, and then the ethanol solution was discarded.
11. After drying the tube, the DNA pellet was redissolved in 25 µL of Tris buffer (0.01 M Tris, 0.001 M EDTA pH 8.0).

The soya reference standards were used in the evaluation of the DNA extraction because soya contains a high level of protein that can be accurately tracked through each step of the extraction procedure. Although the soya protein is not problematic for the PCR assay, it serves as a useful indicator of overall DNA purity. Protein levels in the final DNA extracts were determined by a protein measurement using the Protein 200 Plus LabChip and the Agilent 2100 bioanalyzer. Absorbance measurements were made with the ND-1000 spectrophotometer from NanoDrop Technologies, Inc. (Wilmington, Delaware).

PCR Protocol

Samples used in the GMO analysis consisted of Institute for Reference Materials and Measurements (IRMM) Certified Reference GMO Soy and GMO Corn, as well as commercial corn meal and soya powder. The PCR analysis was carried out using the Biosmart Allin 1.0 GMO Screening System from Promega (Madison, Wisconsin). A modified PCR cycling protocol was developed for use with the PTC 200 Peltier Thermal Cycler from MJ Research, Inc. (Waltham, Massachusetts). The PCR protocol is described below.

Multiplex PCR Protocol

1. Dilute the DNA sample to an initial concentration of 5–50 ng/µL. (This corresponds to an optical density, (OD), at 260 nm of 0.1–1.0 for a 1-cm cell.)
2. For the first stage of the multiplex PCR combine the following per reaction:

   17-µL 2X Qiagen Multiplex PCR Master Mix

   27-µL Allin Mix 1

   5-µL Internal control

   1-µL Extracted DNA
3. Vortex the solution.
4. Perform first stage of PCR amplification using the following cycling program.

Step	Time	Temperature (°C)
1	15 min	95
2	15 s	95
3	60 s	55
4	30 s	72
5	Repeat steps 2–4 an additional 39 times	
6	3 min	72

5. For the second stage of the Multiplex PCR combine the following per reaction

   24.5-µL 2X Qiagen Multiplex PCR Master Mix

   24.5-µL Allin Mix 2

   1.0-µL Product from first stage PCR
6. Perform second stage of PCR amplification using the following cycling program.

Step	Time	Temperature (°C)
1	15 min	95
2	15 s	95
3	60 s	57
4	30 s	72
5	Repeat steps 2–4 an additional 39 times	
6	3 min	72

Analysis of PCR Products

The PCR products were analyzed using the DNA 500 LabChip and the Agilent 2100 bioanalyzer. For comparison, the same PCR products were also characterized by gel electrophoresis in a 4% NuSieve 3:1 Plus Gel. The electrophoretic separation conditions were 150 V for 60 min in a 1X Tris-borate-EDTA (TBE) buffer.

Results

DNA Extraction

DNA extraction protocols were evaluated for both yield and purity of DNA. Protein levels in the DNA extracts were determined using the Protein 200 Plus LabChip [3]. The results of this comparison are summarized in Table 1.

Table 1. Summary of DNA Extraction

Method	Yield (%)	DNA/Protein w/w	A_{260}/A_{280}
CTAB	0.008	2.7	1.82
DNeasy	0.004	2.5	1.89
Wizard Genomic	0.008	0.3	1.62

The CTAB and Wizard Genomic protocols showed the highest levels of DNA recovery with final DNA yields of 0.008% of the initial sample weight. The DNA/protein ratio of 0.3 found in the Wizard DNA extract indicated that this extract still contained a high level of protein. The corresponding ratio for CTAB was 2.7, indicating that the protein content of the CTAB extract was only 1/9 of the Wizard protocol.

The A_{260}/A_{280} ratio is also a useful indicator of DNA purity, with high purity DNA having an absorbance ratio of 1.8–1.9. A comparison of the A_{260}/A_{280} ratios for the CTAB and Wizard extracts, 1.82 and 1.62 respectively, confirmed the higher DNA purity in the CTAB process. The DNeasy extraction process produced DNA of comparable quality to the CTAB process, but at a significantly lower yield. Since the CTAB extraction protocol gave the highest yield of high purity DNA, this protocol was used to prepare the DNA extracts used in the PCR analysis.

Analysis of PCR Products

The Biosmart Allin 1.0 GMO Screening System is a multiplex PCR kit that is capable of generating four PCR products. These PCR products include the following:

Base pairs (bp)	Product
118	Soy lectin gene
150	35S Promoter
217	Internal control (corn zein sequence added to the PCR mix)
278	Corn zein gene

All four of these products can be found in either the positive or the negative control reactions using the Biosmart GMO Screening System. The DNA 500 LabChip has ample separation to resolve all of these PCR products. This resolution is illustrated in Figure 1, which shows a composite of the bioanalyzer electropherograms for the positive and negative controls.

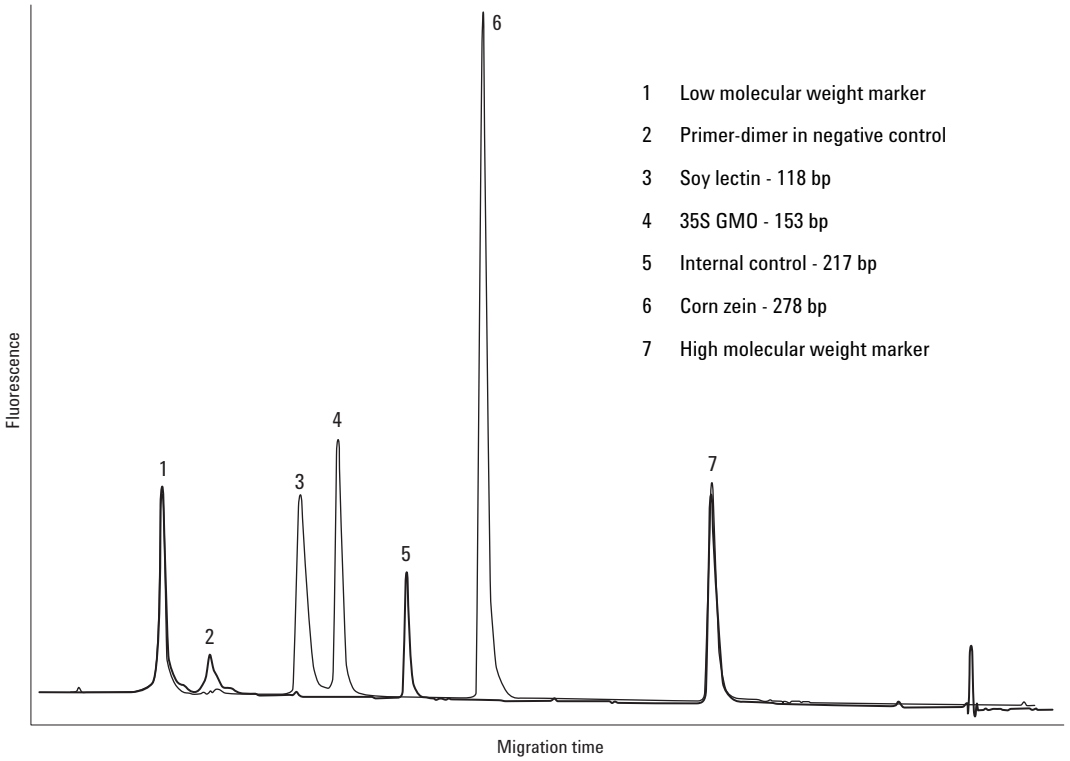

Figure 1. Composite of bioanalyzer electropherograms for positive and negative controls.

The PCR analysis was carried out on corn and soya sample sets. The corn sample set consisted of IRMM Certified Reference MON810™ corn standards at the following levels: 0% MON810, 0.1% MON810, 0.5% MON810, 1.0% MON810, 2.0% MON810, and 5.0% MON810, and commercial corn meal. The soya sample set was made of IRMM Certified Reference Roundup Ready® soya samples at the following levels: 0% Roundup Ready, 0.1% Roundup Ready, 0.5% Roundup Ready, 1.0% Roundup Ready, 2.0% Roundup Ready, and 5.0% Roundup Ready and commercial soya powder. Both sample sets contained a positive control with 0.5% each Bt176 corn and Roundup Ready soya, and deionized water as a negative control.

Figure 2 shows the electrophoretic gel separation and a gel-like bioanalyzer image for all of the samples listed above. The PCR products for the corn samples are shown in Figures 2A and 2C. Figures 2B and 2D show the soya results.

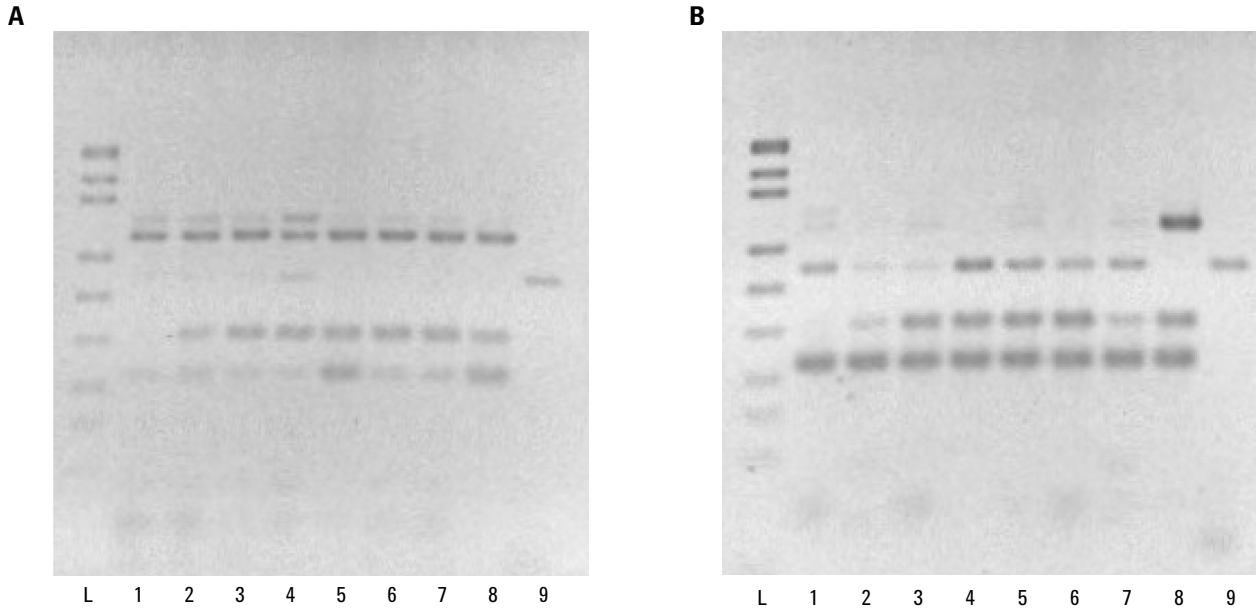

Figure 2A and 2B. 4% NuSieve 3:1 Plus Gel, 150 V for 60 min.

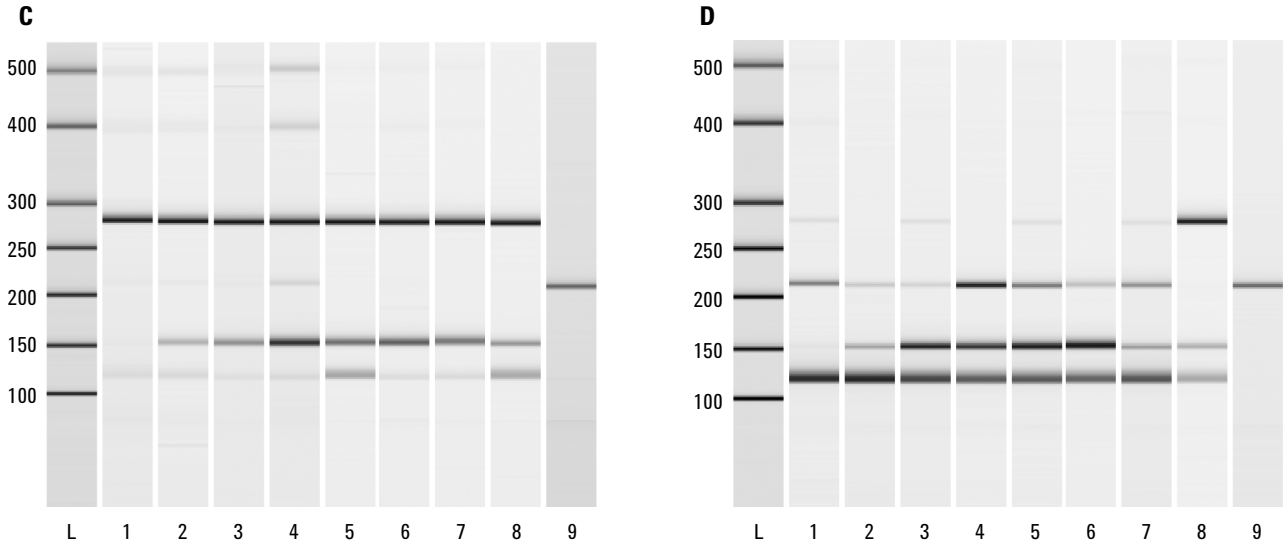

Figure 2C and 2D. Gel-like bioanalyzer image.

Figure 2. 2A (gel) and 2C (bioanalyzer)
L) Molecular weight ladder: 501, 489, 404, 353, 242, 190, 147, 110, 89, 67, 34, 34, and 26 bps **1**) 0% MON810 corn, **2**) 0.1% MON810 corn, **3**) 0.5% MON810 corn, **4**) 1.0% MON810 corn, **5**) 2.0% MON810 corn, **6**) 5.0% MON810 corn, **7**) Commercial corn meal, **8**) Allin positive control (0.5% Bt 176 maize and 0.5% Roundup Ready soybean), and **9**) Negative control (deionized water).

2B (gel) and 2D (bioanalyzer)
L) Molecular weight ladder, **1**) 0% Roundup Ready soya, **2**) 0.1% Roundup Ready soya, **3**) 0.5% Roundup Ready soya, **4**) 1.0% Roundup Ready soya, **5**) 2.0% Roundup Ready soya, **6**) 5.0% Roundup Ready soya, **7**) Commercial soya powder, **8**) Allin positive control (0.5% Bt 176 maize and 0.5% Roundup Ready soybean), and **9**) Negative control (deionized water).

Comparison of Gel and Bioanalyzer Response

The bioanalyzer DNA 500 LabChip clearly shows superior resolution and uniformity of band location compared to the 4% NuSieve gels. The enhanced reproducibility of band location in the gel-like bioanalyzer image is readily apparent in a visual comparison of Figures 2C and 2D to Figures 2A and 2B. Both the 4% gel and the bioanalyzer have sufficient sensitivity to visualize the 35S PCR product at the minimum corn and soy reference standard levels of 0.1% GMO.

A comparison of the initial 35S level in the sample to the amount of 35S PCR product shows that the 35S GMO PCR band increases as the GMO content increases. The PCR response in both corn and soy samples appears to saturate before the maximum GMO standard level of 5% is reached. This effect can be clearly demonstrated using the bioanalyzer's ability to measure PCR product concentrations. Tables 2 and 3 show the concentrations of the corn, soy, and 35S PCR products. For the corn samples, the PCR product response saturates at 1.0% GMO. In the soy samples, the saturation occurs at 0.5% GMO.

Table 2. GMO Corn Response - Corn and 35S PCR Products

Sample	278 bp-Corn amplicon (ng/µL)	153 bp-35S amplicon (ng/µL)	Ratio of 35S/ corn amplicons
0% MON810 Corn	2.5	0.03	0.01
0.1% MON810 Corn	3.4	1.2	0.4
0.5% MON810 Corn	6.2	3.8	0.6
1.0% MON810 Corn	3.5	4.3	1.2
2.0% MON810 Corn	5.2	4.4	0.9
5.0% MON810 Corn	4.7	4.7	1.0
Corn meal	6	5.2	0.9

Table 3. GMO Soya Response - Soya and 35S PCR Products

Sample	118 bp-Soya amplicon (ng/µL)	153 bp-35S amplicon (ng/µL)	Ratio of 35S/ soya amplicons
0% Roundup Ready soy	5.1	0.1	0.02
0.1% Roundup Ready soy	5.4	0.8	0.15
0.5% Roundup Ready soy	4.9	3.4	0.7
1.0% Roundup Ready soy	5.3	4.0	0.8
2.0% Roundup Ready soy	5.1	4.4	0.9
5.0% Roundup Ready soy	4.8	5.0	1.0
Soya powder	4	0.9	0.2
Positive control	4.8	2.2	0.5

Although concentrations of PCR products can be determined quite accurately, some care must be exercised in the interpretation of these quantitative results. Under conditions where a correlation can be seen between the GMO content and the concentration of GMO amplicon, for example, GMO <0.5%, a rough estimate of GMO concentration can be made. However, such an estimate is only reliable if the same PCR master mix and thermocycler are used for all the amplification reactions. In addition, sample and calibration standard matrices must also be highly similar.

For the Biosmart GMO Screening System, quantitative conclusions cannot be made at concentrations >0.5% GMO because the PCR response is saturated. Once the PCR product concentrations have reached this level, small differences in amplicon concentrations are not useful in making quantitative estimates. An example of this can be seen in Table 3. The 0.5% GMO soy reference has a 35S/soy amplicon ratio of 0.7. In the positive control, where the sample has 0.5% GMO soy and 0.5% GMO corn, the corresponding ratio is only 0.5. Since the positive control contains at least twice as much 35S GMO marker as the 0.5% soy reference, this ratio would be expected to be greater than 0.7. It is difficult to explain why the ratio is lower in the positive control. This behavior could be the result of the complex reaction kinetics in a multiplex nested PCR assay or may simply reflect the imprecision in endpoint PCR amplicon concentrations.

PCR Product Composition

The internal control in the Biosmart Allin 1.0 GMO Screening System is a 217 bp corn sequence that uses the same primer sequences as the corn PCR product. According to the manufacturer, when high levels of corn DNA are present, competition for primer may result in the loss of the internal control PCR product. An examination of the corn PCR products in Figure 2C shows this effect in that the 217 bp is either absent or visible only at trace levels. However, in the soy PCR assay in Figure 2D, the 217 bp fragment can easily be seen in all the samples except the positive control that contains corn DNA.

In the corn sample set, a weak band can be seen at around 120 bp. This suggests that during the DNA isolation step, trace amounts of soya DNA were introduced into the corn samples. Likewise, the presence of a band at 280 bp in some of the soy samples indicates low levels of corn DNA were present in several of the soy samples. It is not surprising that trace levels of cross contamination should be apparent in a nested PCR assay. Since all of the samples undergo a net 80 cycles of amplification, even a few copies of soy or corn DNA will be sufficient to produce a detectable PCR product.

In PCR assays using a large number of amplification cycles, it is not uncommon for amplicons of similar sequence to cross hybridize. In the case of the Biosmart Allin 1.0 GMO Screening System, both the corn PCR product and the internal control share a region of common DNA sequence. When these two amplicons cross-hybridize, the resulting product has both single-stranded and double-stranded regions. These structures, known as heteroduplexes, have substantially lower mobility than a corresponding double-stranded structure. The relative mobility shift depends on such factors as gel composition, ionic strength, and gel temperature [4]. In Figure 2, PCR products that are larger than the 278 bp corn amplicon are observed. These bands occur at about 320 bps in the 4% gel and at 400 and 500 bps in the bioanalyzer electropherogram. Cross hybridization of the corn and the internal control amplicons is probably responsible for these products.

Conclusion

This application note described the use of the Agilent 2100 bioanalyzer with the Protein 200 Plus and DNA 500 LabChip Kits in the evaluation of sample preparation and the analysis of multiplex PCR products. The Protein 200 Plus was used to determine which DNA extraction procedure was most effective in removing residual protein. The DNA 500 LabChip was used to characterize the Biosmart Allin 1.0 GMO Screening System, a nested multiplex PCR assay for the genetically modified corn and soy beans. Resolution and sensitivity in these assays was sufficient to identify all of the targeted multiplex PCR amplicons and to differentiate these targets from PCR artifacts. Sensitivity of the assay was sufficient to detect GMO content even at the minimum GMO standard level of 0.1% in both corn and soy reference standards.

References

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