Application note

Quantifying soy DNA extracts for downstream GMO detection

NanoDrop Eight Spectrophotometer

Introduction

The controversy of grocery products containing genetically modified organisms (GMO) has heightened the demand for analytical testing in the food and beverage industry.[1,2] GMOs, or transgenic crops, contain DNA that was modified through bioengineering to introduce a desired trait that is not naturally occurring in the species.[1] Globally, soybeans stand as the most popular transgenic crop, where herbicide tolerance is the most desired trait, and pest resistance follows close behind.[3] Current legislation for regulating GMO-based products varies between countries, indicating the need for worldwide, reliable testing procedures.[2] Qualitative PCR is implemented first to screen for the presence or absence of common regulatory elements used in transgenic (GMO) crops. Quantitative PCR is then performed, detecting more specific targets and providing a quantitative analysis on the target copy number.[4] Completing a qualitative assessment prior to quantitative PCR saves time and resources by eliminating targets that were defined as absent. In this note, a qualitative assessment was performed to determine the presence or absence of common regulatory elements.

Transgenic crop production

The most common DNA construct regulatory elements that enable transgenic crop production are the cauliflower mosaic virus (CaMV) 35S promoter (P35S), the *Agrobacterium tumefaciens* nopaline synthase terminator (TNOS), and the figwort mosaic virus (FMV) 34S promoter (P34S).[5,6,7] The CaMV 35S RNA gene promoter sequence is well characterized and functions by facilitating transcription of the desired transgene.[5,8] The TNOS sequence contains the polyadenylation site that serves to terminate transcription.[6] The 34S promoter sequence from FMV shows a relatively high percent homology compared to the P35S sequence from CaMV, specifically in transcription initiation sites. Variations in the FMV enhancer sequence, along with the location relative to the promoter, may serve as drawbacks compared to CaMV.[7] However, designing a dual promoter vector with P35S and P34S has advantages for the efficient expression of two transgenes compared to incorporating one promoter in a bicistronic vector.[7,9]

There are two popular methods for producing transgenic crops with the designated DNA construct: particle bombardment (Figure 1A) and transformation via *Agrobacterium* (Figure 1B).[1] With the particle bombardment method, the DNA construct is bound to particles which are accelerated at high velocity into plant tissues or cells to the nucleus. The DNA construct releases from the particles and integrates into the plant DNA via homologous recombination.[10,11] After the bombardment, the cells are transferred to a selective media based on the chosen selection agent in the DNA construct, and the plant regenerates until it is a fully grown transgenic plant.[10,12] With the *Agrobacterium* method, *Agrobacterium* naturally infects plants and uses its own tumor-inducing (Ti) plasmid to integrate transfer DNA (T-DNA) into the plant genome.[10] When producing transgenic plants, the desired trait is inserted into the Ti plasmid, reintroduced to the *Agrobacterium*, and infects the plant. T-DNA containing the transgene integrates into the plant DNA, and cells are selected and regenerated as described with the particle bombardment method.[10,12]

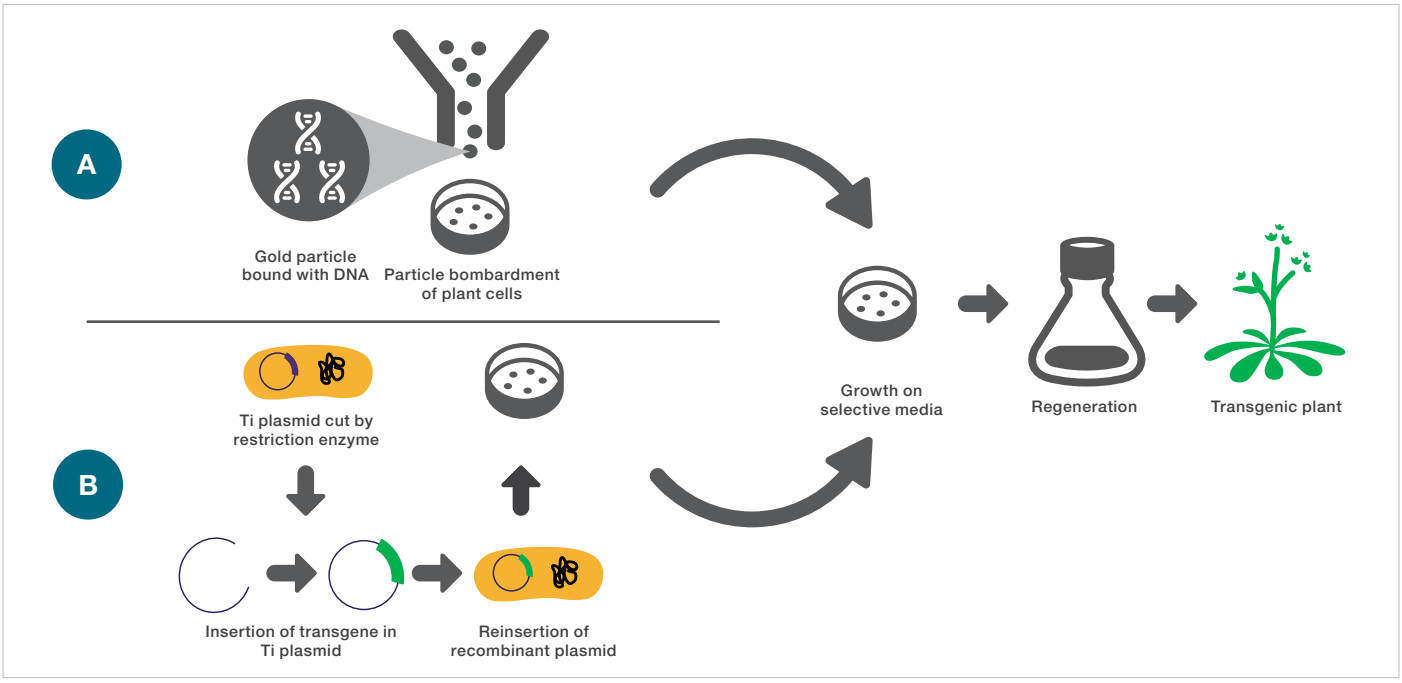

Figure 1: Transgenic crop production methods. A) Particle bombardment transformation. B) *Agrobacterium* transformation.

GMO detection via qPCR

GMO detection has become simpler through multiplex qPCR assays where the targets are P35S/CaMV, TNOS/A. *tumefaciens*, and P34S/FMV. Because these regulatory elements are naturally found in their corresponding virus or bacteria, it is common to experience false GMO-positive reactions if the plant has been naturally infected.[8] Multiplex qPCR allows simultaneous detection of the regulatory elements and the virus or bacteria to eliminate false-positive results.

PCR is a sensitive assay that requires template DNA to be a specific amount and optimal purity to ensure a successful and reproducible reaction.[13] To determine the quantity and quality of template DNA, the Thermo Scientific™ NanoDrop™ Eight Microvolume UV-Vis Spectrophotometer calculates concentration and identifies contaminants from nucleic acid extractions. Through chemometric algorithms, the Thermo Scientific™ Acclaro™ Sample Intelligence Technology provides valuable information on sample contaminants, improving necessary sample clean-up efficiencies (Figure 2). To further simplify sample processing upstream of PCR, the NanoDrop Eight instrument includes eight microvolume sample pedestals for high-throughput protocols.

Common PCR inhibitors are residual extraction materials such as protein and phenol,[13] which are identified with the Acclaro technology built into the NanoDrop Eight software. When extracting nucleic acids from food sources, proteins are abundant and frequently co-extracted if proper extraction technique is not followed. Contaminating proteins are known to inhibit the DNA polymerase in PCR, reducing PCR efficiency.[14] Identifying a contaminant with the NanoDrop Eight software allows scientists to make simple modifications to extraction protocols without the need for extensive troubleshooting when PCR efficiency is poor.

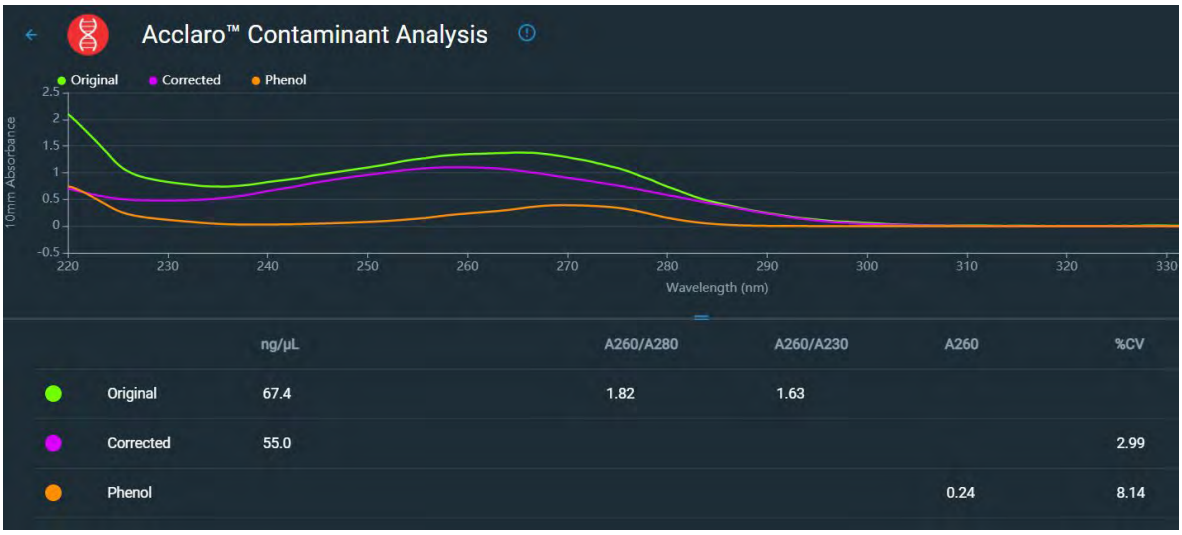

Figure 2: The Acclaro technology built into the NanoDrop Eight operating software. Phenol was identified as a contaminant, and its absorbance contribution was reported. A corrected DNA concentration was calculated, and the corresponding spectrum is displayed in purple.

Experimental procedures

DNA extraction with CTAB

Soy DNA was isolated from commercial tofu, soy milk, edamame, and soybeans with a modified protocol from Doyle and Doyle (1987) using the ionic detergent cetyltrimethylammonium bromide (CTAB). A stock of CTAB extraction buffer was made by adding 1.4 M NaCl and 20 mM EDTA to CTAB buffer (Promega, MC1411). Soybeans were soaked in dH_2O overnight. A 700 µL aliquot of CTAB extraction buffer was added to 200 mg of tofu, edamame, and soybeans and 200 µL of soy milk. Solid samples were pulverized twice via sonication for 30 seconds at 40% amplitude using a Branson Sonifier SFX150. All samples were incubated at 65°C for one hour with occasional vortexing and centrifuged at 16,000 x g for 5 minutes. One volume phenol:chloroform:isoamyl alcohol (25:24:1 v/v) (Invitrogen, 15593031) was added to the supernatant, and the sample was vortexed and centrifuged at 16,000 x g for 5 minutes. The aqueous layer was treated with 10 µL RNase A (Thermo Scientific, EN0531) at 37°C for 30 minutes. One volume chloroform:isoamyl alcohol (24:1 v/v) (Sigma, 25666) was subsequently added, vortexed, and centrifuged at 16,000 x g for 5 minutes. The aqueous layer was mixed with 2/3 volume isopropanol and 0.08 volume sodium acetate and incubated for one hour at room temperature to precipitate DNA. The DNA was pelleted by centrifuging at 16,000 x g for 5 minutes, and the pellet was washed with ice-cold 70% ethanol and centrifuged twice, removing the supernatant after each spin. The pellet was dried at room temperature for 10 minutes and dissolved in 50 µL Tris-EDTA (TE) pH 8.0. Samples were measured in replicates of four using the eight-channel mode on the NanoDrop Eight instrument.

qPCR with GMO screening kit

The Thermo Scientific™ TaqMan™ GMO Screening Kit was designed to test for the presence or absence of GMOs in DNA extracts from food and feed samples. The kit includes a positive control and the necessary primers and probes for multiplex reactions targeting: P35S/CaMV, TNOS/A. *tumefaciens*, and P34S/FMV, as well as an endogenous plant control and internal positive control for identifying PCR inhibition. Tofu, soy milk, edamame, and soybean DNA samples were diluted to 20 ng/µL prior to loading on the qPCR plate. The qPCR was performed with an Applied Biosystems™ QuantStudio™ 6 Pro Real-Time PCR System with the cycling conditions outlined in the TaqMan GMO Screening Kit procedure. Results were analyzed using the Presence Absence Analysis qPCR application on the Thermo Fisher Connect Platform.

Results

Following the CTAB extraction method, DNA from tofu, soy milk, edamame, and soybeans was checked for concentration and purity with the NanoDrop Eight Spectrophotometer (Figure 3). The DNA was extracted in a high yield; all samples were close to 100 ng/µL. All samples exhibited excellent purity, evidenced by the A_{260}/A_{280} purity ratios falling between 1.85 and 1.90 and the absence of any Acclaro technology warnings. Incubating at cold temperatures during the isopropanol precipitation step was found to precipitate excess salts; thus, incubating at room temperature is recommended. Some DNA extraction protocols include RNase treatment after resuspending DNA in buffer post-precipitation, thus requiring a second DNA precipitation step.[15] The RNase treatment in this experiment was performed in between chloroform phase separations to remove contaminating enzymatic material and to prevent DNA loss from a second precipitation.

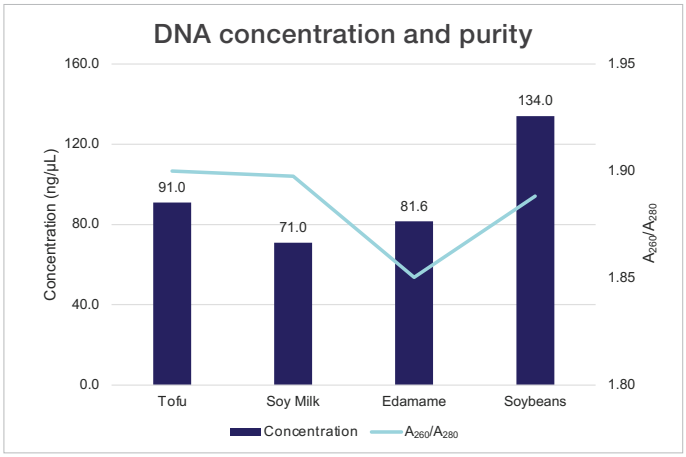

Figure 3: DNA concentration (ng/µL) and A_{260}/A_{280} purity ratio reported by the NanoDrop Eight instrument for DNA extracted using the CTAB method. Data displayed are averaged from four replicate measurements.

The results of the presence-absence qPCR are outlined in Table 1. All samples tested positive for the plant endogenous control as well as the internal positive control (IPC), indicating the samples were of plant origin and PCR inhibitors were not present. Tofu and soy milk DNA were negative for the presence of any transgenic material and natural infection from CaMV, A. *tumefaciens*, and FMV. The edamame and soybean DNA tested positive for P34S and FMV, indicating the natural presence of FMV and no transgenic material specific to this assay. The soybean DNA also tested positive for P35S and CaMV, indicating the natural presence of CaMV and no transgenic material specific to this assay.

Table 1: Results from the Presence Absence Analysis qPCR application on the Thermo Fisher Connect Platform after performing qPCR with the TaqMan GMO Screening Kit.

Duplex Assay	Component	Tofu	Soy milk	Edamame	Soybeans
P35S/CaMV	P35S	–	–	–	+
	CaMV	–	–	–	+
TNOS/A. *tumefaciens*	TNOS	–	–	–	–
	A. tumefaciens	–	–	–	–
P34S/FMV	P34S	–	–	+	+
	FMV	–	–	+	+
Plant/IPC	Plant	+	+	+	+
	IPC	+	+	+	+

For the edamame and soybeans, the next step would entail a qPCR relative quantification curve using a soy-specific endogenous control to further confirm these findings and quantify target copy numbers.[4] When performing quantification curves, it is crucial that samples are pure, which can be a challenge for highly processed food samples.[14] A slight change in the quantification cycle due to the presence of PCR inhibitors or an inaccurate template amount has a considerable effect on target quantification. Implementing the NanoDrop Eight instrument prior to performing the quantification curve will instill confidence in qPCR results.

Conclusions

When utilizing qPCR for GMO detection, data reliability is a top priority as food and beverage products must comply with government regulations on transgenic crops. Preparing DNA for analysis by qPCR requires excellent purity and the concentration to be known. With the NanoDrop Eight spectrophotometer, DNA quality and quantity of eight microvolume samples are determined simultaneously in less than twenty seconds. The Acclaro technology provides important sample contaminant information, ensuring a successful extraction protocol and subsequent qPCR run.

NanoDrop Eight Microvolume UV-Vis Spectrophotometer

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