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Concordance Study: Methods of Quantifying Corn and Soybean Genomic DNA Intended for Real-Time Polymerase Chain Reaction Applications

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ABSTRACT: Quantitative real-time polymerase chain reaction (qPCR) is a technology commonly used for the detection and quantification of genetically engineered (GE) traits in grains and oilseeds. The method involves measuring copy numbers of taxon-specific, endogenous control genes exposed to the same manipulations as trait-specific target genes. Accurate DNA quantification is essential for successful and predictable results with qPCR. A systematic study of seven different DNA quantification methods, incorporating different chemistries and different instrumentation, were evaluated on corn and soy DNA that was extracted using two distinct extraction methods. A time course study showed that corn and soy DNA was stable under typical laboratory storage conditions. $Corn_{CTAB}$ and $corn_{Qiagen}$ DNA extracts produced statistically similar quantification values when measured by picogreen PG_{TD700}, $PG_{Lum20/20}$, $Hoescht_{TD700}$, and $Hoescht_{Lum20/20}$ methods, suggesting that these methods can be used interchangeably to quantify DNA in corn samples prior to initiation of qPCR. Soy_{Qiagen} provided greater stochastic measurement variability when quantification methods were compared, whereas soy_{CTAB} had statistically significant differences when a PG method was compared to a Hoescht method of DNA quantification. Finally, agarose gel electrophoresis data revealed more pronounced degradation for Qiagen-extracted DNA compared with CTAB extracts in both corn and soy. Consequently, C_t values generated by qPCR suggested that absolute copy numbers of PCR amplifiable targets were not concordant between Qiagen and CTAB DNA extracts. Understanding measurement uncertainty from component steps used in qPCR can contribute toward harmonizing methods for the detection of GE traits in grains and oilseeds.

KEYWORDS: *DNA quantification, corn, soy, quantitative PCR, Picogreen, Hoescht, A_{260}, genetically engineered traits*

INTRODUCTION

The responsible use of biotechnology provides a venue for academia, industry, and government organizations to resolve agricultural challenges and meet consumer needs into the 21st century.[1,2] Specific challenges are known to exist, however, in the management of genetically engineered (GE) products through commerce. For example, many countries that import grains into their country often regulate which types of GE crops are allowed into their markets and may require labeling when the GE content in the shipment is above a specified threshold level.[1,2,4] As grain moves from on-farm storage sites to export elevators, similar types and qualities are routinely commingled with one another into increasingly larger storage facilities (i.e., first grain is loaded onto a 10 ton truck → 50 ton railcar → 1000 ton river barge → Panamax ship carrying 50,000−100,000 tons of grain).[3] Diligence is taken to maintain comparable quality and purity, thereby retaining uniformity in graded types and market values. GE grains that have been deregulated in the United States are considered to be equivalent to conventional varieties, and the presence or absence of GE traits in lots is not a consideration in the grading of grain. Consequently, no extraordinary measures are taken to prevent comingling of GE grains with conventional varieties during handling and/or processing, unless specified in a contractual agreement, for example, organic grain or grain sold as "non-GE". Testing for the presence or absence of GE traits in grain lots is performed by testing laboratories to substantiate contractual agreements and demonstrate compliance with labeling and other regulatory requirements in export markets.[4] Real-time quantitative polymerase chain reaction (qPCR) is an internationally recognized analytical method used to detect GE traits in grains and oilseeds.[5−9] Accurate detection and quantification of DNA is a crucial step in a variety of biological applications and is especially relevant when used in conjunction with qPCR.[10,11] The target DNA for amplification by qPCR is a unique sequence found in the transgene construct that becomes integrated into the plant cell genome. Thus, highly purified genomic DNA is extracted and quantified prior to being dispensed into qPCRs. The main purpose of DNA extraction is to provide adequate amounts of intact DNA that can be used with qPCR, but will concurrently remove substances that might inhibit the PCR process.[12,13] Studies reveal that highly purified, intact DNA provides more predictable C_t values when using qPCR and, as a result, a higher level of confidence in analytical measurements.[14,15] A lesser degree of confidence in analytical measurements occurs as DNA becomes more degraded, if the amount of DNA introduced into the reaction is inadequate, or if DNA contains impurities that affect qPCR amplification efficiency.[14,16,17] Because PCR relies upon logarithmic amplification for quantification, slight variations in the initial copy number, in the early stages of the reaction process,

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translate into large differences in the final analytical result.[1,5] Obtaining minimally degraded and highly purified DNA is a laudable goal for PCR applications, but it is not always achievable because of a multitude of factors.[14,15] Analytical methods using qPCR strive to introduce equal amounts of DNA (and, consequently, equal copy numbers of an endogenous control gene) into individual reaction wells based upon a specified DNA quantification method. Several DNA extraction and quantification methods exist, and several papers have been published describing the advantages/disadvantages of each.[18] Two commonly used methods of DNA isolation from plant material are (1) a hexadecyltrimethylammonium bromide (CTAB) detergent extraction procedure and (2) a silica gel column whereby DNA binds/elutes on the basis of varying salt concentrations. A plethora of other methods have been described in the literature for extracting and quantifying genomic DNA.[10,14,18] Specific methods offer both advantages and disadvantages over other methods. Some methods are simple to perform but lack sensitivity, whereas others are highly specific but have associated human health risks. DNA quantification by spectrophotometry measures absorbance at a specified wavelength of 260 nm.[19] Although this method is relatively simple and straightforward, it cannot discern between double-stranded DNA (dsDNA) and other biological compounds that, during an extraction process, copurify with dsDNA. Impurities that can copurify with dsDNA include RNA, single-stranded DNA, and nucleotides, which all absorb at 260 nm.[20] Consequently, spectrophotometry can lead to erroneously high quantification measurements and, as a result, fewer copies of target DNA introduced into qPCR reactions.[14,21] Fluorescent dyes are generally more sensitive than spectrophotometry, with a reported lower detection limit of 25 pg/mL compared with 150 ng/mL for spectrophotometry.[22,23] However, pH, salts, detergents, and organic solvents can affect the accuracy in measuring DNA content with fluorescent dyes.[22,24] Picogreen is a popular fluorescent dye used for DNA quantification.[22] It produces responses to an excitation wavelength at approximately 480 nm and yields an emission wavelength near 520 nm.[22,23] This wavelength emission makes Picogreen compatible for use with most commercially available thermal cyclers currently in use. DNA is quantified by comparing the fluorescence intensity of a test sample to the fluorescence intensity of reference standards that are incorporated into the method during each analysis. Hoescht dyes are excited by ultraviolet light at around 346 nm and emit blue/cyan fluorescence light around an emission maximum at 460 nm.[23] Real-time PCR instruments detect excitation emissions outside this 460 nm value and, thus, Hoescht is not a compatible dye for use with most commercially available real-time PCR instruments. Hoescht dyes require high salt concentrations to distinguish dsDNA from contaminating RNA, and Hoescht is adenine thymine-selective, whereas Picogreen is not.[25] Other fluorescent dyes, such as ethidium bromide, bind dsDNA through intercalation, but the compound is somewhat toxic and a known mutagen.[20,23] Ethidium bromide also has an extremely narrow range for optimal dye/DNA ratio allowing for accurate quantification.[22,26] The influence that different DNA extraction methods have on DNA quantification and, ultimately, qPCR has not been extensively investigated. This study utilized two uniquely different DNA extraction methods for corn and soybeans, in conjunction with different DNA quantification procedures, to evaluate concordance and variability between DNA quantification methods. The seven methods included Picogreen and Hoescht fluorescent DNA binding dyes read on both TD 700 and Luminometer 20/20 instruments, a Picogreen DNA binding dye read on an ABI 7500 instrument, conventional absorbance at A_{260} using spectrophotometry, and ethidium bromide staining of DNA with agarose gel electrophoresis. Corn and soy genomic DNA, extracted using two different protocols, was evaluated for stability over time and quantified using a combination of fluorescent dyes and instruments. A statistical analysis of variance was used on the data to identify whether differences in measurements could be declared between extraction procedures, dyes, and instrumentation.

MATERIALS AND METHODS

Safety. Ethidium bromide is a known human carcinogen, and proper precautions should be utilized during the use and disposal of this reagent.

Sample Sources. Well-characterized, non-GE (event-free) corn was kindly provided by Pioneer Hi-Bred (Johnston, IA, USA). Non-GE (event-free) soybean was purchased from Battleground Industries, LLC (Brookston, IN, USA). Separate 50 g samples of whole kernel corn and soybean were each ground cryogenically to the consistency of fine flour using liquid nitrogen and a highly controlled environment with a SPEX Certi Prep 6800 Freezer Mill (Metuchen, NJ, USA). These sample sources have been characterized by GIPSA and by laboratories that participate in the USDA-GIPSA proficiency program. All maize and soy samples were stored at 4 °C until used for the extraction procedure. Zygosity traits of individual seeds were not characterized for either Pioneer corn or Battleground soybean cultivars.

Time Course Study. Triplicate DNA extractions were performed on finely ground corn and soybean samples using either a Qiagen extraction kit (silica resin technique) or a CTAB lysis and purification approach, as described below. Specifically, the time course study was designed to evaluate (1) variability in measurements associated with a specific binding dye over time (i.e., Picogreen (PG) or Hoescht), (2) similarities of measurements between binding dyes (i.e., comparing PG with Hoescht), (3) concordance between different instruments (TD 700 vs Luminometer 20/20), and (4) whether statistically significant differences exist between different methods of DNA quantification.

DNA Extractions. DNA was isolated from approximately 1.0 g aliquots of finely ground corn or soy starting material using either hexadecyltrimethylammonium bromide (CTAB) (Sigma-Aldrich, St. Louis, MO, USA) or commercially available Qiagen (Valencia, CA, USA) extraction protocol. For the Qiagen extraction protocol, the manufacturer's instructions were followed with only minor modifications. Briefly, each sample was transferred to a 15 mL centrifuge tube. Five milliliters of buffer AP1 (preheated to 65 °C) and 10 μL of RNaseA stock solution (Fermentas, Hanover, MD, USA) was added to the sample and vortexed rigorously. The mixture was then incubated for 10 min at 65 °C, inverting the tube at least three times during the incubation period. Next, 1.8 mL of buffer AP2 was added to the lysate. The sample was vortexed and then incubated for 10 min on ice. The mixture was centrifuged at 5000*g* for 5 min in a Beckman Allegra 6R centrifuge (Beckman, Ramsey, MN, USA), with a swing-out type rotor, at room temperature. The supernatant was decanted into the QIA-shredder maxi spin column and centrifuged again, at 5000*g* for 5 min at room temperature. The supernatant was transferred, without disturbing the pellet, to a new 50 mL conical centrifuge tube. Approximately 7.5 mL or 1.5 volumes of buffer AP3/E was then added directly to the lysate and mixed immediately by vortexing. Corn/soy genomic DNA was bound to a solid matrix once the sample was applied to the DNeasy maxi spin column and centrifuged at 5000*g* for 5 min. The corn/soy genomic DNA was further purified upon addition of 12 mL of 0.5× Tris−EDTA (TE), pH 8.0, buffer and centrifuged a second time for 10 min at 5000*g*, drying the membrane. The DNeasy spin column was transferred to a new 50 mL conical tube. Five

Table 1. Primers and Probe Sequences for the Taxon-Specific Endogenous Control Reference Genes Used in This Study

reference system name	endogenous control reference gene primer/probe sequence	amplicon (bp)
starch synthase (SSIIb-3)	forward: 5′-CCA ATC CTT TGA CAT CTG CTC C-3′ reverse: 5′-GAT CAG CTT TGG GTC CGG A-3′ probe: 5′-FAM-AGC AAA GTC AGA GCG CTG CAA TGC A-TAMRA-3′	114
lectin	forward: 5′-GCC CTC TAC TCC ACC CCC A-3′ reverse: 5′-GCC CAT CTG CAA GCC TTT TT-3′ probe: 5′-FAM-AGC TTC GCC GCT TCC TTC AAC TTC AC-TAMRA-3′	120

hundred microliters of 0.5× TE buffer, pH 8.0 (preheated to 65 °C), was added directly onto the DNeasy maxi spin column membrane and incubated for 5 min at room temperature. The column was centrifuged for 5 min at 5000*g*, which caused purified corn/soybean genomic DNA to be eluted off the membrane. To maximize recovery, a second 500 μL aliquot of 0.5× TE buffer, pH 8.0, was added to the column. The column was centrifuged again for a final elution. No other additional steps were performed prior to DNA quantification.

With the CTAB extraction protocol, DNA was isolated from approximately a 1.0 g aliquot of finely ground corn/soy prepared as described above.[27,28] Corn/soy flour was incubated in 7.0 mL of 1× CTAB extraction buffer (20 g/L CTAB, 1.4 M NaCl, 0.1 M Tris, 20 mM Na_2EDTA, pH 8.0) and 3.0 mL of water at 65 °C to lyse the cells. Following cellular lysis, an extended 60 min treatment with 50 μL of RNase A at 37 °C (Fermentas), followed by a 200 μL proteinase K (Sigma Aldrich) digest, was performed to divest the DNA product of contaminating RNA, polysaccharides, proteins, and other biological materials. The resulting digests were extracted with chloroform twice to eliminate PCR-inhibiting polysaccharides and polyphenols and were incubated in 1× CTAB precipitation buffer (5 g/L CTAB, 0.04 M NaCl, pH 8.0) at 25 °C overnight to allow selective precipitation of DNA. After precipitation, the samples were resolubilized into 175 μL of 0.5× TE (5 mM Tris and 0.5 mM EDTA) buffer and treated with 10 μL of RNase A at 37 °C for 1 h. An equal volume (175 μL) of 2.4 M NaCl was added to each sample followed by a chloroform extraction, and then the sample was ethanol-precipitated overnight at −20 °C using twice the volume (700 μL) of 100% EtOH (Sigma-Aldrich). After overnight precipitation, the samples were washed with 500 μL of 70% EtOH and dried in a vacuum microfuge (Eppendorf, Westbury, NY, USA). The resulting DNA pellets were dissolved in 100 μL of 0.5× TE, pH 8.0, buffer. The expected yield from maize or soy flour was generally 200−500 μg of DNA from 1.0 g of starting material. Typically, DNA stock samples were solubilized in 100 μL of 0.5× TE buffer, pH 8.0, at a concentration of 200−300 ng/μL and stored at 4 °C until further use.

DNA Quantifications. DNA was quantified using a fluorometric assay with Luminometer 20/20 and TD 700 fluorometric instruments (Turner Biosystems Inc., Sunnyvale, CA, USA) in conjunction with both the Quant-iT PicoGreen (PG) reagent kit (Invitrogen/Molecular Probes, Eugene, OR, USA) and Hoescht dye (Acros Organics, Geel, Belgium). The PG reagent binds dsDNA with high specificity. Stock DNA samples were diluted between 1:1000 and 1:2000 with 0.5× TE buffer to a target concentration of 30−250 pg/μL. The PG reagent was prepared according to the manufacturer's protocol; the diluted stock DNA test and reference samples were mixed 1:1 with PG reagent to a final volume of 200 μL to produce 1:2000−1:4000 final dilutions and compared with fluorometric measurements from a standard curve. A calibration curve was generated from λ phage DNA, supplied by the manufacturer (Molecular Probes) at a stock concentration of 100 ng/μL and diluted to 250, 125, 62.5, 31.3, and 0.0 pg/μL with 0.5× TE buffer. The mean of DNA quantification values from both the PG and Hoescht dye methods provided an "accepted" concentration for a specified sample. Stock DNA samples were diluted to a working stock concentration of 20 ng/μL.

For this study, DNA was also quantified using an Applied Biosystems 7500 (ABI) thermal cycler and an Agilent spectrophotometer model 8453 (Agilent, Foster City, CA, USA). With the ABI 7500 method, 25 μL of 2× DNA standards, prepared exactly as described for calibrants used for quantification by the Luminometer20/20 or TD 700 methods, was used to generate a standard curve by plotting concentration against raw fluorescence values generated by the ABI 7500 instrument. Aliquots of λ DNA standards and corn/soy test samples were diluted 1:1 with 0.5× TE containing PG reagent into 96-well reaction plates to a final volume of 50 μL/well. The 96-well reaction plate was placed into the ABI thermal cycler, and the reaction was allowed to incubate at 26 °C for 32 s and then at 27 °C for 32 s prior to collection of a fluorescence reading. Triplicate readings from individual wells were measured for reference standards and test samples. The means of triplicate fluorescence measurements at each concentration were plotted. A linear coefficient of correlation value of 0.999 was typically obtained. Test samples were diluted accordingly so that measurements were in the linear range of the assay. With the UV spectrophotometer assay, absorbances at 260 and 280 nm were used.[20] The instrument was checked for absorbance accuracy in assaying nucleic acids by using a diluted preparation of λ phage DNA from the PicoGreen Quant-iT kit (Invitrogen/Molecular Probes, Carlsbad, CA, USA); the instrument was capable of accurately and precisely quantifying DNA at concentrations that would give absorbance units (AU) readings of ≥0.1 AU. The standards and unknowns were prepared as 1:25 dilutions of the stock DNA extracts and assayed twice to confirm that repeatable values of ≥0.1 AU could be obtained. The DNA concentrations were computed using the molar absorption coefficient of DNA and an appropriate dilution factor.[29] The average of the values was used as the "accepted" A_{260} quantification value for respective samples; typical A_{260}/A_{280} ratios ranged from 1.8 to 2.0 for corn and from 1.4 to 2.0 for soy.

A time course study was undertaken to evaluate DNA stability and the resulting variability associated with different DNA quantification methods. In addition, time course data were used to assess concordance between different fluorescent dyes and different instruments used for DNA quantification. For the time course study, corn and soy DNA samples were stored over a 5 month period in a 0.5× TE buffer at a constant 4 °C temperature, except during quantification when samples reached ambient temperatures.

Gel Electrophoresis. The integrity of DNA extracts was determined by electrophoresis in an 0.8% agarose gel, stained with ethidium bromide.[20] After DNA quantification, the equivalent of 1 μL of stock DNA (based on a mean PG quantification value) was loaded into individual lanes as neat. Also, stock samples were all diluted to equivalent concentrations of 20 ng/μL. Five microliter aliquots (corresponding to 100 ng of DNA each) were loaded into individual lanes to compare band intensities of the samples. Lambda *Hin*d III (Sigma) was supplied in aqueous 1× TE solution. The presence of an intact ≥23 kb band, with minimal degradation, indicated intact corn or soy genomic DNA with minimal RNA contamination. Lambda DNA (Invitrogen) was serially diluted at 200, 100, 50, and 25 ng of DNA per well. These standards were used to compare band intensities with test samples and to obtain an estimate of DNA concentration.

Quantitative Real-Time PCR. The method as described consists of a trait-specific, real-time quantitative *Taqman* PCR procedure for the content of corn-specific starch synthase IIb-3 (SSIIb-3) and soybean-specific lectin endogenous control genes.[6,11] Each method employs gene-specific primers and a sequence-specific 6-carboxyfluorescein/carboxytetramethylrhodamine (FAM/TAMRA) probe that are described in Table 1. The qPCR reactions as described amplify their respective cultivar-specific endogenous control genes. All reactions

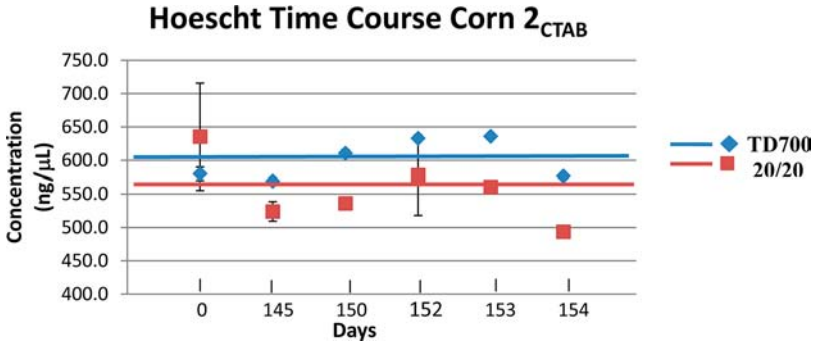

Figure 1. Representative scatter plot for time course study. Genomic DNA was extracted from finely ground corn and soy flour in triplicate using CTAB and Qiagen extraction procedures as described under Materials and Methods. Samples were quantified using Picogreen and Hoescht fluorescent dyes along with TD 700 and Luminometer 20/20 instruments. A representative scatter plot for data generated using the Hoescht dye with TD 700 instrument (method 3) and Luminometer instrument (method 4) is shown over a 154 day time course for corn-2 sample extracted with CTAB. Error bars represent ±1 standard deviation from the mean of triplicate measurements on each sample by both instruments. The data revealed no positive or negative correlation over time, suggesting that DNA samples were stable under the storage conditions of the time study (i.e., no significant amounts of DNA degradation occurred) and that variability in DNA quantification was attributable to random errors inherent to the specified method.

Table 2. Time Course Study: Average Values from DNA Quantification Methods with Different Dyes and Instrumentation (All Values Are Expressed as Nanograms per Microliter)

	Picogreen				Hoescht			
	TD 700 method 1		Lum 20/20 method 2		TD 700 method 3		Lum 20/20 method 4	
sample	range	mean ± SD	range	mean ± SD	range	mean ± SD	range	mean ± SD
corn-1_{CTAB} [a]	423−554	515.3 ± 48.0	407−548	505.9 ± 51.2	523−580	546.7 ± 20.4	474−598	532.3 ± 42.2
corn-2_{CTAB}	517−632	574.9 ± 42.9	506−614	554.2 ± 40.0	569−636	600.9 ± 29.7	494−635	554.6 ± 49.2
corn-3_{CTAB}	504−549	523.7 ± 19.4	492−524	506.5 ± 13.0	505−563	535.2 ± 27.0	461−553	508.3 ± 17.1
$corn_{1-3CTAB}$	$\bar{x}_{PGR}$ = 530.1 ± 28.3				$\bar{x}_{Hoescht}$ = 546.3 ± 31.0			
corn-1_{Qiagen} [b]	12−13	12.6 ± 0.4	12−13	12.6 ± 0.3	12−13	12.9 ± 0.2	11−14	12.6 ± 0.4
corn-2_{Qiagen}	12−13	12.8 ± 0.6	12−14	12.8 ± 0.6	12−14	13.1 ± 0.9	10−14	12.7 ± 1.6
corn-3_{Qiagen}	7−8	7.7 ± 0.6	7−8	7.7 ± 0.5	7−8	7.8 ± 0.5	6−9	7.9 ± 0.9
$corn_{1-3Qiagen}$	$\bar{x}_{PGR}$ = 11.0 ± 2.6				$\bar{x}_{Hoescht}$ = 11.1 ± 2.7			
soy-1_{CTAB} [a]	50−55	51.6 ± 2.2	49−56	50.8 ± 2.7	70−76	72.4 ± 2.7	67−76	71.7 ± 3.4
soy-2_{CTAB}	102−116	109.7 ± 6.2	99−115	106.6 ± 5.9	143−159	147.9 ± 6.7	117−153	128.2 ± 14.2
soy-3_{CTAB}	78−84	80.2 ± 2.9	75−87	78.4 ± 5.1	99−107	103.0 ± 2.7	95−114	105.5 ± 6.8
$soy_{1-3CTAB}$	$\bar{x}_{PGR}$ = 79.6 ± 25.5				$\bar{x}_{Hoescht}$ = 104.8 ± 30.2			
soy-1_{Qiagen} [b]	32−43	37.6 ± 3.6	33−41	35.6 ± 3.5	25−28	26.8 ± 1.4	25−29	27.3 ± 1.5
soy-2_{Qiagen}	23−29	25.8 ± 2.1	22−28	25.1 ± 2.0	24−27	25.7 ± 1.1	25−27	25.7 ± 0.8
soy-3_{Qiagen}	29−36	32.1 ± 2.6	29−42	33.1 ± 4.9	28−31	29.5 ± 1.0	28−32	30.2 ± 1.3
$soy_{1-3Qiagen}$	$\bar{x}_{PGR}$ = 31.6 ± 5.1				$\bar{x}_{Hoescht}$ = 27.5 ± 1.9			

[a]CTAB extracts were dissolved into 100 µL total volume of 0.5× TE. [b]Qiagen samples were dissolved into 100 µL total volume of 0.5× TE.

were performed in separate wells in a simplex reaction system. *Taq* DNA polymerase (Applied Biosystems, Foster City, CA; *TaqMan* 2× Universal PCR Master Mix containing passive reference ROX, kit 4304437, including $MgCl_2$, dATP, dCTP, dGTP, dUTP, Amperase, and AmpliTaq Gold) and primers/probes were dissolved in sterile 0.5× TE to a concentration of 10 µM. The master mix contained *Taq* DNA polymerase at 1×, primers at 400 nM, and probe at 200 nM final concentrations. Nuclease-free water was added to adjust the final volume to 25 µL per reaction. qPCR products were measured during each cycle by means of a target-specific oligonucleotide probe, labeled with two fluorescent dyes: 6-FAM as a reporter dye at the 5′ end and TAMRA as a quencher dye at the 3′ end. All qPCR reactions were performed on an ABI 7500 instrument (Applied Biosystems). Samples were heated to 95 °C for 10 min (activation of *Taq* DNA polymerase), cooled to 60 °C for 60 s (annealing/extension), and heated to 95 °C for 15 s (denaturation). Annealing/extension and denaturation steps were repeated for a total of 45 cycles.

DNA extracts quantified by the PG method were diluted to 20 ng/µL each. A 5 µL aliquot (corresponding to 100 ng of DNA) was delivered individually into a 96-well plate (Applied Biosystems), and each qPCR was performed in either duplicate or triplicate for each analytical sample. Wells containing the appropriate master mix amplified specifically for the SSIIb-3 or lectin gene. qPCR efficiency was measured by generating a standard curve and starting with 100 ng of corn DNA and serially diluting to 50, 25, 12.5, and 6.25 ng of DNA in 0.5× TE. The level corresponding to 100 ng was defined as 100% endogenous control, and the remaining diluted samples were defined accordingly.

RESULTS

Time Course Study. Individual test samples were quantified in triplicate with PG and Hoescht fluorescent dyes that were simultaneously evaluated with both TD 700 and Luminometer 20/20 instruments. A representative scatter plot

for quantitative measurements obtained from $corn_{2CTAB}$ sample, obtained over a 154 day period using a Hoescht fluorescent dye and TD 700/Luminometer 20/20 instruments, are shown in Figure 1. Scatter plot data revealed relatively negligible correlation over time, suggesting that $corn_{1-3CTAB}$, $corn_{1-3Qiagen}$, $soy_{1-3CTAB}$, and $soy_{1-3Qiagen}$ DNA samples, for each extraction method, were all stable during the incubation period of this study. Next, average values of each triplicate measurement, collected along the time course continuum, were assembled and compiled into a master worksheet as summarized in Table 2. Range, mean, and standard deviation values were determined for each quantification method used in this study (i.e., PG_{TD700}, $PG_{Lum20/20}$, $Hoescht_{TD700}$, $Hoescht_{Lum20/20}$) with each extraction method (i.e., $corn_{1-3CTAB}$, $corn_{1-3Qiagen}$, $soy_{1-3CTAB}$, $soy_{1-3Qiagen}$). When extraction procedures were compared, using a statistical nonparametric approach, the data revealed much higher yields of DNA recovery using CTAB, as compared with Qiagen ($p < 0.0001$), and more pronounced absolute differences in corn extracts compared to soy. These observations have been reported by others.[18,30,31] From this experimental design, an array of data was generated that allowed for evaluation of different quantification methods commonly used to detect the presence of GE traits by qPCR in grains and oilseeds. With DNA extracted samples from $corn_{1-3CTAB}$, mean data revealed no consistent statistically significant differences in quantification values when the method utilized PG/Hoescht fluorescent dyes and either a TD 700 or a Luminometer 20/20 instrument ($\bar{x}_{PGR(1-3CTAB)} = 530.1 \pm 28.3$; $\bar{x}_{Hoescht(1-3CTAB)} = 546.3 \pm 31.0$) and a practical 1:1 ratio. A similar 1:1 ratio was observed for mean data generated by $corn_{1-3Qiagen}$ DNA ($\bar{x}_{PGR(1-3Qiagen)} = 11.0 \pm 2.6$; $\bar{x}_{Hoescht(1-3Qiagen)} = 11.1 \pm 2.7$). As expected, the ratios of $PG_{TD700}/PG_{Lum20/20}$, $Hoescht_{TD700}/Hoescht_{Lum20/20}$, $PG_{TD700}/Hoescht_{TD700}$, and $PG_{Lum20/20}/Hoescht_{Lum20/20}$ were also approximately 1:1 for any given $corn_{CTAB}$ or $corn_{Qiagen}$ sample. These data indicated reasonably good concordance between Hoescht and PG fluorescent binding dyes with quantification methods as described in this study for both $corn_{CTAB}$ and $corn_{Qiagen}$ DNA. Furthermore, the data suggested that no systematic errors contributed to the differences in measurements, and the observed differences were attributable to method variability inherent within the respective procedures.

Statistically different values were declared on both $soy_{1-3CTAB}$ and $soy_{1-3Qiagen}$ DNA extracted samples when Hoescht versus PG fluorescent dyes were compared($p < 0.001$). A greater absolute difference was observed for quantitative values generated on $soy_{1-3CTAB}$ Hoescht ($\bar{x} = 104.8 \pm 30.2$) compared with $soy_{1-3CTAB}$ PG ($\bar{x} = 79.6 \pm 25.5$), whereas a smaller absolute and relative difference was observed for quantitative values generated on $soy_{1-3Qiagen}$ Hoescht ($\bar{x} = 27.5 \pm 1.9$) versus $soy_{1-3Qiagen}$ PG ($\bar{x} = 31.6 \pm 5.1$). Collectively, the data suggested that the observed differences for $soy_{1-3CTAB}$ were attributable to Hoescht dye binding to DNA with a different efficiency compared with PG or that the presence of impurities in the DNA extraction method affected the fluorescence chemistry of the Hoescht dye differently, compared with PG. An approximate 25% greater quantitative measurement was observed for $soy_{1-3CTAB}$ when a Hoescht fluorescent dye quantitative method was compared to PG. Whereas the ratio of Hoescht/PG binding dyes provided statistically significant differences between the methods regardless of instrumentation, no practical statistically significant differences were declared when in the comparison of different instruments with the same binding dye (i.e., comparing $soy_{1-3CTAB}$ DNA samples using PG_{TD700} with $PG_{Lum20/20}$ or $Hoescht_{TD700}$ with $Hoescht_{Lum20/20}$). These preliminary data suggested that factors that are contributing differences to analytical measurements for the soy_{CTAB} DNA extraction method are less significant than for the soy_{Qiagen} DNA extraction method, but these data should be interpreted with trepidation based on repeatability data as described in Table 3 of this manuscript.

Concordance Studies of DNA Quantification with Different Methods. $Corn_{1-3Qiagen}$, $corn_{1-3CTAB}$, $soy_{1-3Qiagen}$, and $soy_{1-3CTAB}$ DNA extracts were exhausted in the time course study; thus, new extracts of DNA samples labeled $corn_{4-6CTAB}$, $corn_{4-6Qiagen}$, $soy_{4-6CTAB}$, and $soy_{4-6Qiagen}$ were prepared as described under Materials and Methods. Stock DNA extracts were diluted to an appropriate concentration and quantified using seven different methods. PG, Hoescht, and ethidium bromide fluorescent dyes were utilized for specified methods that were compatible with appropriate instrumentation. DNA samples were concurrently quantified on Turner TD 700 and Luminometer 20/20 instruments (PG and Hoescht dyes) as previously described, but in addition, samples were quantified using an ABI 7500 thermal cycler method (PG dye) and an Agilent 8453 spectrophotometer (measuring absorbance at A_{260}) and by gel electrophoresis (ethidium bromide staining). The results of these DNA quantification methods are summarized in Table 3, whereas gel electrophoresis data are presented in Figure 2. Results revealed that PG and Hoescht methods of DNA quantification using TD 700 and Luminometer 20/20 for $corn_{CTAB}$, $corn_{Qiagen}$, and soy_{CTAB} were repeatable and generated observations statistically similar to values as described (statistics data not shown). $Soy_{4-6Qiagen}$ provided quantitative values that were more disparate compared with $soy_{1-3Qiagen}$ values. With sample set $soy_{4-6Qiagen}$ DNA, using the Hoescht dye quantitative method, consistently higher quantitative measurements were observed when compared with PG ($p < 0.0001$). Quantitative measurements generated a PG/Hoescht ratio of 1:1.2 using the TD 700 and 1:1.3 using the Luminometer 20/20 instrument. Interestingly, the ratio of PG/Hoescht for $soy_{4-6Qiagen}$ DNA extracts was of a smaller magnitude compared to the ratio of PG/Hoescht for $soy_{1-3CTAB}$ or $soy_{4-6CTAB}$ DNA extracts (consistently 1:1.4 with both TD 700 and Luminometer 20/20 instruments). These data suggested that challenges exist in the obtainment of highly repeatable and predictable quantitative measurements from soy_{Qiagen} extracted DNA samples and that soy_{CTAB} extracted DNA samples generate statistically significant discordant values when when comparing PG with Hoescht dyes (see Table 3).

An ABI 7500 thermal cycler was programmed to detect fluorescence using a PG dye and, consequently, concordance measurements between different instruments with $corn_{4-6CTAB}$, $corn_{4-6Qiagen}$, $soy_{4-6CTAB}$, and $soy_{4-6Qiagen}$ extracted DNA samples. This experimental approach allowed for comparison of quantitative methods using a $PG_{ABI7500}$ instrument with other aforementioned methods including PG_{TD700}, $PG_{Lum20/20}$, $Hoescht_{TD700}$, and $Hoescht_{Lum20/20}$. Mean values generated by $PG_{ABI7500}$ showed consistently lower measurements compared with PG_{TD700} and $PG_{Lum20/20}$ on $corn_{4-6CTAB}$, $corn_{4-6Qiagen}$, $soy_{4-6CTAB}$, and $soy_{4-6Qiagen}$ DNA extracts. $PG_{ABI7500}$ measurements compared with the other dye and reader combinations as described in Table 3 were declared to be significantly different in most instances for $corn_{Qiagen}$ and $corn_{CTAB}$ DNA extracts (statistical data not shown). The general trend of the data set suggested that the $PG_{ABI7500}$ method provided closer agreement

Table 3. Range, Mean, and Standard Deviation Values Generated on DNA Yields for Corn and Soy Samples Using Six Different Methods of DNA Quantification

	Picogreen				Hoescht				Picogreen		A_{260}	
	TD 700 method 1		20/20 method 2		TD 700 method 3		20/20 method 4		ABI 7500 method 5		Agilent 8453 method 6	
sample	range	mean ± SD	range	mean ± SD	range	mean ± SD	range	mean ± SD	range	mean ± SD	range	mean ± SD
corn-4_{CTAB}	108–114	111.0 ± 2.4	111–117	113.9 ± 2.5	107–118	113.3 ± 5.6	107–117	113.1 ± 4.4	94–104	98.1 ± 3.7	135–138	137.0 ± 1.5
corn-5_{CTAB}	138–154	145.7 ± 6.3	141–157	149.0 ± 6.6	147–164	155.8 ± 7.6	142–159	151.9 ± 6.5	119–132	127.1 ± 4.3	184–245	215.0 ± 28.9
corn-6_{CTAB}	200–224	208.0 ± 8.3	202–226	210.5 ± 8.7	211–238	226.3 ± 11.9	221–264	234.3 ± 15.6	174–204	193.6 ± 11.7	294–363	320.6 ± 32.4
corn-4_{Qiagen}	43–45	44.0 ± 0.63	43–48	46.4 ± 1.7	47–50	48.2 ± 1.2	47–52	49.5 ± 1.9	34–52	44.9 ± 9.5	181–200	195.0 ± 9.5
corn-5_{Qiagen}	49–55	52.3 ± 2.3	52–58	55.4 ± 2.7	58–60	58.6 ± 0.89	59–68	61.4 ± 3.5	41–54	46.1 ± 4.3	265–316	290.0 ± 38.2
corn-6_{Qiagen}	55–64	59.5 ± 3.7	57–64	62.1 ± 2.9	65–67	66.0 ± 0.89	66–71	68.1 ± 1.7	46–58	51.0 ± 4.3	333–385	363.0 ± 27.1
soy-4_{CTAB}	54–60	56.7 ± 2.8	54–61	58.0 ± 2.4	77–85	81.0 ± 3.4	76–92	83.1 ± 5.9	44–59	48.5 ± 5.5	97–154	128.7 ± 28.1
soy-5_{CTAB}	151–165	157.8 ± 6.2	156–173	162.4 ± 7.1	213–235	223.5 ± 10.9	216–235	225.5 ± 9.1	124–150	136.9 ± 10.4	505–521	513.0 ± 8.0
soy-6_{CTAB}	61–66	63.7 ± 2.6	61–67	64.8 ± 2.4	86–98	91.5 ± 5.8	85–97	90.9 ± 4.5	55–62	58.5 ± 2.8	150–180	165.3 ± 15.2
soy-4_{Qiagen}	47–52	50.0 ± 2.1	51–54	52.1 ± 1.2	58–63	60.7 ± 2.3	60–70	65.9 ± 4.4	34–48	39.4 ± 5.6	765–807	789.0 ± 28.1
soy-5_{Qiagen}	50–55	52.3 ± 2.3	52–56	53.7 ± 1.5	61–66	64.2 ± 2.2	66–70	67.4 ± 2.3	36–51	40.0 ± 5.0	796–844	812.3 ± 27.7
soy-6_{Qiagen}	50–60	55.2 ± 4.4	50–55	54.3 ± 2.3	64–70	67.7 ± 2.1	67–71	68.2 ± 1.7	32–51	38.3 ± 6.6	725–844	783.3 ± 59.5

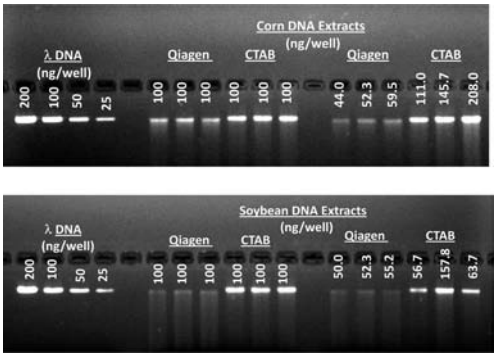

Figure 2. Gel electrophoresis based method of DNA quantification using ethidium bromide dye (method 7). Lambda standards were serially diluted to 200, 100, 50, and 25 ng/mL and loaded into individual wells. Genomic DNA was extracted from finely ground corn and soy flour using CTAB and Qiagen extraction procedures as described under Materials and Methods. Samples were quantified by PG, and the equivalent of 1 μL was loaded into the corresponding wells. Additionally, DNA samples were diluted to 20 ng/μL, and equal amounts of 100 ng each were loaded into corresponding wells. Visualization of band intensities showed that Qiagen extracts generated greater discordance to lambda standards compared with CTAB extracts and that Qiagen-extracted soy samples were more degraded. Upon loading, DNA samples were size separated using an ethidium bromide containing TAE buffer and agarose gel electrophoresis. The band intensities were visualized as described under Materials and Methods.

in absolute quantities compared with PG_{TD700} and $PG_{Lum20/20}$ methods when using $corn_{4-6Qiagen}$ (three of three values were not statistically different) and $soy_{4-6CTAB}$ (two of three values were not statistically different) DNA extracted test samples as compared to $corn_{4-6CTAB}$ (three of three values were statistically different) and $soy_{4-6Qiagen}$ (three of three values were statistically different) test samples. Test samples were then analyzed using an Agilent 8453 spectrophotometer by measuring absorbance at A_{260} as described under Materials and Methods. $Corn_{4-6CTAB}$, $corn_{4-6Qiagen}$, $soy_{4-6CTAB}$, and $soy_{4-6Qiagen}$ test samples all showed statistically significant ($p < 0.001$) higher measurements when compared with any of the fluorescent dye methods of DNA quantification. Generally, A_{260} measurements on $corn_{4-6CTAB}$ samples were closer in magnitude to the fluorescent dye methods as compared with A_{260} measurements on $corn_{4-6Qiagen}$, $soy_{4-6CTAB}$, and $soy_{4-6Qiagen}$ DNA samples and fluorescent dye methods, with which there was as much as a 20.3-fold greater value in one instance (i.e., compare the ratio of A_{260} measurements to $PG_{ABI7500}$ on $soy_{4-6Qiagen}$ samples). These observations have been observed by others and suggest a hyperchromic effect due to DNA degradation in samples that provided highly discordant results.[14,32] The ratios of each quantification method, derived from average measurements generated by each method, are summarized in Table 4. The ratios were generated by taking the average values of each method (horizontally) and dividing each column (vertically). Values closest to 1.0 provided the best concordant results when any two quantitative methods were compared. The data revealed the best overall agreement between methods, with ratios closest to 1.0, when using $corn_{4-6CTAB}$ extracted DNA, and the least overall agreement between methods occurred with $soy_{4-6Qiagen}$ extracted DNA. The data also suggested that highly discordant results are possible in some instances when DNA quantification is

Table 4. Mean Ratio of DNA Quantification Values Using Extraction Methods (Ratio of Columns to Rows)

	PG_{TD700}	$PG_{Lum20/20}$	$Hoescht_{TD700}$	$Hoescht_{Lum20/20}$	$PG_{ABI7500}$	A_{260}
A. $Corn_{4-6CTAB}$						
PG_{TD700}	–	1.02 ± 0.01	1.06 ± 0.04	1.06 ± 0.06	0.89 ± 0.03	1.42 ± 0.16
$PG_{Lum20/20}$	0.98 ± 0.01	–	1.04 ± 0.04	1.04 ± 0.04	0.88 ± 0.04	1.39 ± 0.17
$Hoescht_{TD700}$	0.94 ± 0.03	0.96 ± 0.04	–	1.00 ± 0.03	0.85 ± 0.03	1.41 ± 0.23
$Hoescht_{Lum20/20}$	0.94 ± 0.05	0.96 ± 0.05	1.00 ± 0.03	–	0.85 ± 0.02	1.33 ± 0.11
$PG_{ABI7500}$	1.12 ± 0.04	1.14 ± 0.04	1.18 ± 0.04	1.19 ± 0.03	–	1.58 ± 0.16
A_{260}	0.71 ± 0.09	0.73 ± 0.09	0.75 ± 0.07	0.76 ± 0.06	0.64 ± 0.07	–
B. $Corn_{4-6Qiagen}$						
PG_{TD700}	–	1.05 ± 0.01	1.11 ± 0.02	1.14 ± 0.03	0.92 ± 0.09	5.36 ± 0.85
$PG_{Lum20/20}$	0.95 ± 0.01	–	1.05 ± 0.01	1.09 ± 0.02	0.87 ± 0.08	5.07 ± 0.80
$Hoescht_{TD700}$	0.90 ± 0.01	0.95 ± 0.01	–	1.04 ± 0.01	0.83 ± 0.09	4.83 ± 0.74
$Hoescht_{Lum20/20}$	0.87 ± 0.02	0.92 ± 0.02	0.96 ± 0.01	–	0.80 ± 0.09	4.66 ± 0.70
$PG_{ABI7500}$	1.09 ± 0.10	1.15 ± 0.10	1.21 ± 0.12	1.26 ± 0.14	–	5.92 ± 1.43
A_{260}	0.19 ± 0.03	0.20 ± 0.04	0.21 ± 0.03	0.22 ± 0.03	0.18 ± 0.05	–
C. $Soy_{4-6CTAB}$						
PG_{TD700}	–	1.02 ± 0.005	1.43 ± 0.01	1.44 ± 0.02	0.88 ± 0.03	2.70 ± 0.50
$PG_{Lum20/20}$	0.98 ± 0.01	–	1.40 ± 0.02	1.41 ± 0.02	0.86 ± 0.04	2.64 ± 0.48
$Hoescht_{TD700}$	0.70 ± 0.01	0.72 ± 0.01	–	1.01 ± 0.02	0.62 ± 0.03	1.90 ± 0.36
$Hoescht_{Lum20/20}$	0.69 ± 0.01	0.71 ± 0.01	0.99 ± 0.01	–	0.61 ± 0.03	1.88 ± 0.36
$PG_{ABI7500}$	1.14 ± 0.04	1.16 ± 0.05	1.62 ± 0.56	1.63 ± 0.08	–	3.08 ± 0.59
A_{260}	0.38 ± 0.07	0.39 ± 0.07	0.54 ± 0.09	0.54 ± 0.10	0.33 ± 0.06	–
D. $Soy_{4-6Qiagen}$						
PG_{TD700}	–	1.02 ± 0.03	1.22 ± 0.01	1.28 ± 0.04	0.75 ± 0.05	15.2 ± 0.86
$PG_{Lum20/20}$	0.98 ± 0.03	–	1.22 ± 0.01	1.26 ± 0.01	0.74 ± 0.03	14.9 ± 0.40
$Hoescht_{TD700}$	0.82 ± 0.005	0.83 ± 0.03	–	1.07 ± 0.02	0.61 ± 0.04	12.4 ± 0.74
$Hoescht_{Lum20/20}$	0.78 ± 0.03	0.79 ± 0.01	0.95 ± 0.04	–	0.58 ± 0.02	11.8 ± 0.30
$PG_{ABI7500}$	1.34 ± 0.09	1.36 ± 0.05	1.64 ± 0.12	1.71 ± 0.06	–	20.3 ± 0.25
A_{260}	0.06 ± 0.005	0.07 ± 0.001	0.08 ± 0.005	0.08 ± 0.002	0.05 ± 0.001	–

Table 5. Efficiency Values

A. Corn SSIIb-3 qPCR Efficiency Values

	SSIIb-3					SSIIb-3			
Qiagen DNA extract	^aC_t $n = 3$	slope	PCR efficiency (%)	R^2	CTAB DNA extract	^aC_t $n = 3$	slope	PCR efficiency (%)	R^2
corn-4_{Qiagen}	25.28 ± 0.09	−3.34	99.0	0.999	corn-4_{CTAB}	24.81 ± 0.09	−3.71	86.0	0.997
corn-5_{Qiagen}	25.11 ± 0.04	−3.36	98.4	0.998	corn-5_{CTAB}	24.87 ± 0.06	−3.65	87.9	1.00
corn-6_{Qiagen}	25.25 ± 0.08	−3.37	98.0	0.998	corn-6_{CTAB}	24.87 ± 0.06	−3.70	86.2	0.999
mean ± SD	25.21 ± 0.09	−3.36 ± 0.02	98.5 ± 0.50	0.998	mean ± SD	24.85 ± 0.03	−3.69 ± 0.03	86.7 ± 1.0	0.999

B. Soy Lectin qPCR Efficiency Values

	lectin					lectin			
Qiagen DNA extract	^aC_t $n = 3$	slope	PCR efficiency (%)	R^2	CTAB DNA extract	^aC_t $n = 3$	slope	PCR efficiency (%)	R^2
soy-4_{Qiagen}	23.90 ± 0.05	−3.43	95.7	0.999	soy-4_{CTAB}	23.19 ± 0.17	−3.64	88.2	0.998
soy-5_{Qiagen}	23.94 ± 0.03	−3.43	95.7	1.00	soy-5_{CTAB}	23.36 ± 0.07	−3.51	92.7	0.999
soy-6_{Qiagen}	23.74 ± 0.04	−3.36	98.4	1.00	soy-6_{CTAB}	23.25 ± 0.03	−3.58	90.2	0.998
mean ± SD	23.86 ± 0.11	−3.41 ± 0.04	96.6 ± 1.56	1.00	mean ± SD	23.27 ± 0.09	−3.58 ± 0.07	90.4 ± 2.25	0.998

^aC_t values generated from 100 ng DNA samples only.

performed with different DNA extraction methods on distinctive grain cultivars.

Agarose Gel Electrophoresis. DNA extracted $corn_{CTAB}$, $corn_{Qiagen}$, soy_{CTAB}, and soy_{Qiagen}, quantified by PG/Hoescht/A_{260}, were characterized by agarose gel electrophoresis. Amounts of DNA loaded were based upon quantification values derived from the PG_{TD700} method only. The equivalent of 1 μL of DNA per stock sample was loaded neat into individual wells of an 0.8% agarose gel. Then, each DNA sample was diluted to a constant concentration of 20 ng/μL, and equivalent amounts of 100 ng of DNA (5 μL per sample) were loaded into individual wells. Resulting band intensities were compared to band intensities from serially diluted λ reference standards. The results of gel electrophoresis data are shown in Figure 2. Upon visual inspection, a PG_{TD700} quantitative value of 100 ng of $corn_{4-6Qiagen}$ DNA per well provided a less intense band signal using ethidium bromide staining compared with $corn_{4-6CTAB}$, although equal amounts of DNA were loaded into individual wells of the gel (based upon the PG_{TD700} method). A markedly greater difference in band intensities was observed when equal amounts of 100 ng of DNA $soy_{4-6Qiagen}$ per well were compared with 100 ng of

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$soy_{4-6CTAB}$ DNA per well. Gel data revealed significantly more DNA degradation when using a Qiagen extraction method compared with CTAB for both corn and soy grain samples. Because CTAB-extracted DNA samples provided a greater amount of intact DNA compared with Qiagen, better concordance was observed with these samples when compared with the 100 ng λ DNA standard (where DNA was observed to also be intact). These data suggested that incongruous interpretations can exist when DNA quantified by PG or other quantification methods is compared with DNA characterized by gel electrophoresis, especially when significant amounts of DNA degradation are present.

PCR Amplification of SSIIb-3/Lectin Endogenous Control Genes. To evaluate reaction efficiencies of $corn_{CTAB}$, $corn_{Qiagen}$, soy_{CTAB}, and soy_{Qiagen} DNA by qPCR, a series of 2-fold dilutions were prepared and amplified by PCR as described under Materials and Methods. Slopes were generated based upon C_t values derived from the serially diluted reactions. The SSIIb-3 endogenous control gene was amplified for corn, whereas the lectin gene was amplified for soy.[6] A mean $PG_{TD700}/PG_{Lum20/20}$ dye empirical value of 100 ng of genomic DNA corresponding to a reference value of "100" was assigned on the instrument software. For purposes of obtaining slopes by this experimental approach, values of 100, 50, 25, 12.5, 6.25, and 0 from the dilution series were assigned accordingly. Individual samples were amplified in triplicate, and the slopes of the SSIIb-3/lectin C_t values versus concentration were calculated along with correlation coefficients (R^2). The slope, reaction efficiency, and R^2 values are summarized in Table 5. The data revealed mean qPCR efficiencies of 86.7 ± 1.0% for $corn_{CTAB}$, 98.5 ± 0.50% for $corn_{Qiagen}$, 90.4 ± 2.25% for soy_{CTAB}, and 96.6 ± 1.56% for soy_{Qiagen} by application of the following equation: efficiency = $[10^{(-1/\text{slope})} - 1]$ *100. Qiagen-extracted sample reaction efficiency exceeded CTAB-extracted sample reaction efficiency but simultaneously provided slightly greater variability in measured mean C_t values when Qiagen-extracted samples were compared with CTAB-extracted samples.

$Corn_{CTAB}$, $corn_{Qiagen}$, soy_{CTAB}, and soy_{Qiagen} DNA extracts, containing empirically determined concentrations of 100 ng of DNA each (mean from $PG_{TD700}/PG_{Lum20/20}$ dye methods), were used in qPCR to evaluate probabilities from F tests in the analysis of variance. The interaction between the extraction method and sample was significant for corn ($p < 0.05$) but was not significant for soybean. The interaction data suggested that differences observed for $corn_{Qiagen}$ compared with $corn_{CTAB}$ were not the same from sample to sample. $Corn_{Qiagen}$ extractions provided statistically significant higher C_t values compared with $corn_{CTAB}$, and similar observations were declared for soy_{Qiagen} compared with soy_{CTAB}. For corn and soy, differences in C_t values were calculated from their respective means to be 0.36 ($corn_{Qiagen} - corn_{CTAB}$) and 0.59 ($soy_{Qiagen} - soy_{CTAB}$). Because qPCR is calculated on the basis of a log scale, $2^{\Delta Ct}$ provided an estimate of the fold differences in copy numbers between Qiagen- and CTAB-extracted samples. The data revealed 1.3- and 1.5-fold differences for corn and soy, respectively, for samples that contained equal amounts of DNA in the qPCR that was based upon a PG method of DNA quantification.

DISCUSSION

This study evaluated various methods of DNA quantification and extraction used to obtain and quantify DNA prior to initiating PCR using corn and soy grains as models. Food manufacturers and retailers will use GE testing to label products as non-GE and to demonstrate compliance with food and feed labeling laws.[4,39] Protein- and DNA-based methods are the most commonly used technologies to quantify and/or detect the presence or absence of GE traits.[5,33] Decisions as they relate to grain trade rely upon analytical measurements generated by qPCR, particularly for demonstrating compliance with legislative mandates and meeting contractual obligations.[4,39] qPCR is generally the technology of choice for official laboratories testing the GE content of grains, especially in Europe.[34,39] DNA quantification is an essential component, used in conjunction with qPCR, when testing for GE traits.[10,14] Although technical capabilities continue to improve, challenges still exist with this evolving technology.[24] The analytical process must take into account a multifaceted, yet practical, approach that (1) obtains representative samples, (2) provides uniformity in sample preparations, (3) facilitates efficient DNA extraction and accurate quantification, (4) yields highly purified DNA of utmost quality and known quantity, (5) minimizes matrix effects, (6) offers uniformity in sample setup procedures, and (7) incorporates appropriate reference control samples.[15,16,35] Competency with qPCR relies upon quality assurance policies that include validated methods, appropriate estimates of measurement uncertainty, availability of calibrated equipment, demonstrable proficiency in testing capabilities, knowledge of method limitations, and having well-characterized sample preparations.[5,36−38] Generating repeatable and accurate analytical measurements whether from rapid, simple-to-use techniques or from sophisticated laboratory analyses is key in the identification of method processes that can be used in conjunction with qPCR. This study compared several different common DNA quantification methods that are used in conjunction with qPCR when testing for GE traits in corn and soy. The data revealed that corn and soy DNA preparations were stable during the duration of the time course of these studies. $Corn_{CTAB}$ and $corn_{Qiagen}$ DNA extracts produced statistically similar quantification measurements by PG_{TD700}, $PG_{Lum20/20}$, $Hoescht_{TD700}$, and $Hoescht_{Lum20/20}$ methods, suggesting that these extraction and quantification methods can be used interchangeably to quantify corn DNA samples prior to initiating qPCR. Conversely, quantification measurements were less reliable with soy_{Qiagen} DNA extracts when PG was compared to Hoescht, although similar values were generated with a TD 700 instrument and a Luminometer 20/20 instrument when using the same fluorescent dye (i.e., PG_{TD700} vs $PG_{Lum20/20}$ and $Hoescht_{TD700}$ vs $Hoescht_{Lum20/20}$). Soy_{CTAB} DNA extracts that were quantified using different instruments but the same fluorescent dye (PG_{TD700} vs $PG_{Lum20/20}$ or $Hoescht_{TD700}$ vs $Hoescht_{Lum20/20}$) provided statistically similar quantitative measurements, but discordant and statistically significant differences were consistently observed in replicate sample sets when measurements with different dyes were compared (PG_{TD700} with $Hoescht_{TD700}$ or $PG_{Lum20/20}$ with $Hoescht_{Lum20/20}$). These data suggested that PG and Hoescht fluorescent dyes are not equivalent methods and cannot be used interchangeably when soy_{CTAB} DNA extracts are quantified. Generally, for both corn and soy with both extraction methods, the extracted DNA quantified by the TD 700 or Luminometer 20/20 gave significantly different results compared with $PG_{ABI7500}$ or A_{260} spectrophotometric methods. Aliquots of $corn_{CTAB}$, $corn_{Qiagen}$, soy_{CTAB}, and soy_{Qiagen} extracted DNA were characterized and quantified using agarose

gel electrohporesis and ethidium bromide staining. Equal amounts (100 ng/well) of DNA, based upon PG_{TD700} method of DNA quantification, were loaded onto an agarose gel. The agarose gel data suggested that $corn_{Qiagen}$ and soy_{Qiagen} DNA had lesser amounts of DNA compared with 100 ng reference standards and 100 ng amounts of $corn_{CTAB}$ and soy_{CTAB} samples, even though equal amounts of DNA (based on PG_{TD700} quantification values) were loaded onto the gel. Interestingly, when equal amounts of 100 ng of corn DNA (based upon PG_{TD700} quantification method) were dispensed into qPCR, C_t values provided a 1.3-fold greater amount of the SSIIb-3 target when $corn_{CTAB}$ was compared to $corn_{Qiagen}$. Similarly, when equal amounts of 100 ng of soy DNA (based upon PG_{TD700} quantification method) were dispensed into qPCR, C_t values provided a 1.5-fold greater amount of the lectin target when soy_{CTAB} was compared to soy_{Qiagen}. Collectively, these data suggested that agarose gel electrophoresis should be used in conjunction with other methods of DNA quantification, but not exclusively. When using agarose gel electrophoresis as a method of DNA quantification, it becomes challenging to interpret a true copy number of amplifiable targets that are actually present in the qPCR reaction, if a significant amount of DNA degradation is observed on the gel. Intact DNA can generally be obtained from minimally processed food samples, whereas structural damage to DNA occurs as products become more processed.[14−16]

Findings in this study demonstrate some of the limitations regarding the prerequisite of DNA quantification prior to initiating qPCR in corn and soy extracted DNA. On the basis of the results of this study, it would be interesting to determine how DNA extractions and quantification methods compare for other grain matrices including rice, flaxseed, wheat, and canola. Initiatives that advance progress and implement confidence in analytical measurements used for detecting GE traits in grains and oilseeds will continue to facilitate trade by providing standardization in the marketplace.

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Notes

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ABBREVIATIONS USED

qPCR, real-time quantitative PCR; C_t, crossing threshold; USDA-GIPSA-TSD, U.S. Department of Agriculture−Grain Inspection, Packers and Stockyards Administration−Technical and Sciences Division; FAM/TAMRA, 6-carboxyfluorescein/carboxymethylrhodamine; CTAB, hexadecyltrimethylammonium bromide; PG, Picogreen reagent; SSIIb-3, starch synthase; ISO, International Standards Organization; GE, genetically engineered.

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