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Evaluation of Extraction Methodologies for Corn Kernel (*Zea mays*) DNA for Detection of Trace Amounts of Biotechnology-Derived DNA

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Sensitive and accurate testing for trace amounts of biotechnology-derived DNA from plant material requires pure, high-quality genomic DNA as template for subsequent amplification using the polymerase chain reaction (PCR). Six methodologies were evaluated for extracting DNA from ground corn kernels spiked with 0.1% (m/m) CBH351 (StarLink) corn. DNA preparations were evaluated for purity and fragment size. Extraction efficiency was determined. The alcohol dehydrogenase gene (*adh1*) and the CBH351 (*cry9C*, *35S* promoter) genes in the genomic DNA were detected using PCR. DNA isolated by two of the methods proved unsuitable for performing PCR amplification. All other methods produced some DNA preparations that gave false negative PCR results. We observed that cornstarch, a primary component of corn kernels, was not an inhibitor of PCR, while acidic polysaccharides were. Our data suggest that amplification of an endogenous positive control gene, as an indicator for the absence of PCR inhibitors, is not always valid. This study points out aspects of DNA isolation that need to be considered when choosing a method for a particular plant/tissue type.

KEYWORDS: ***Zea mays*; corn; genomic; DNA; extraction; polymerase chain reaction; PCR; PCR inhibition**

INTRODUCTION

The production of biotechnology-derived crops, especially maize and soybean, has increased in the United States over the past several years. One hundred and thirty million acres of land was used in 2001 worldwide for the commercial production of biotechnology-derived crops, mostly in the United States (*1*). Countries that import US grain have not approved some of the biotechnology-derived varieties that are approved in the United States. Establishing the presence or absence of biotechnology-derived crop material in shipments of plant material and/or processed foods requires sensitive, accurate, and reliable detection methods. Current methods involve either detection of a novel protein via antigen−antibody-based reactions or direct amplification of deoxyribonucleic acid (DNA) using the polymerase chain reaction (PCR). Recently, the European Union revised its food regulations to require labeling as genetically modified if more than 1%, on a mass basis, of a food product or raw ingredient is genetically modified (biotechnology-derived) (*2*). At the present time, corn (*Zea mays*) and soybeans (*Glycine max*) are the most important biotechnology-derived commodity crops in terms of quantity and variety of use in food products. As regulation regarding labeling of biotechnology-derived foods or ingredients is becoming more widespread internationally, testing will play a much larger role in ensuring compliance with governmental regulations and satisfying contractual agreements.

Amplification of DNA using PCR is often the method of choice by the European Union to detect sequences that are unique to the specific engineered gene. Accurate detection is dependent upon the specificity and sensitivity of the amplification protocol as well as the quality of the genomic DNA used in the PCR reaction. Conventional PCR can be technically challenging and is prone to erroneous conclusions of both false positive (contamination or nonspecific amplification products) and false negative results (attributable to DNA polymerase inhibitors that copurify with the target DNA).

DNA isolation from plant materials is a challenge. Cell walls hold plant tissues together and must be penetrated and dissolved via mechanical shearing and chemical means to release cell organelles. The tissue must be milled sufficiently to release adequate quantities of DNA but without resulting in excessive DNA degradation. In addition, plant tissues may contain secondary products that could interfere with the isolation of the

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genomic DNA or remain as contaminants that alter subsequent manipulations of the DNA, such as amplification efficiency in the PCR reaction. Polysaccharides and polyphenols, including tannins, can interfere with enzymatic reactions or even degrade the DNA (*3*−*5*). The prominent polysaccharide of corn is the starch contained in the endosperm of whole corn. The mean composition of seven Midwest corn hybrids was found to be 87.6% starch, of the total dry wt. (*6*).

In this study, genomic DNA was isolated from ground corn using a variety of commercial kits. The efficiency of the various methods to extract genomic DNA from ground corn was determined. DNA quality was assessed using wavelength scans, PCR amplification results, and determinations of DNA fragment size. Conventional corn was spiked gravimetrically to 0.1% (by mass) with a biotechnology-derived variety of corn, StarLink (CBH351, *cry9C*), determined to be 100% CBH351. We investigated commercial DNA extraction kits and a published protocol (CTAB/NaCl). The extractions were performed as recommended by the manufacturer. Genomic DNA, from spiked corn samples, was used as the template to amplify and detect the *35S* promoter and *cry9C* regions of the engineered gene plus an endogenous control gene, alcohol dehydrogenase I (*adh1*) using PCR. The effect of the particle size of the ground corn on extraction efficiency and PCR amplification results was investigated. We investigated the commonly accepted concept that successful amplification of an endogenous gene was indicative of a lack of PCR inhibitors in a DNA preparation. Mono- and polysaccharides were evaluated for their effect on PCR amplification of *adh1*. The goal of this study was not to identify the most efficacious method for DNA isolation from ground corn kernels but to identify issues that should be considered when the goal is the specific and consistent detection of trace amounts of a target DNA.

METHODS AND MATERIALS

Sample Preparation. Two batches of experimental corn (Zea mays) material were prepared. For the experiments comparing the extraction methodologies, 1 kg of conventional kernels (Colorado Corn Co., Greeley, CO) was ground with a Pulverizette mill (Fritsh, Idar-Oberstein, Germany). The CBH351 (StarLink) corn was added to conventional corn at 0.1 wt %.

For experiments comparing samples of variable particle size, 250 g of conventional corn was spiked gravimetrically with CBH351 corn to 0.5 wt %. The mixture was pulverized in an Osterizer blender for 30 s at the highest speed setting. The sample was placed onto a brass mesh multilevel sieve series (W. S. Tyler, Cleveland, OH) with aperture sizes ranging from 2.8 to 0.3 mm^2. Particles were size-fractionated by shaking the sample for 2 min on a Fisher-Wheeler (Fisher Scientific, Chicago, IL) sieve shaker. Subsamples from each particle size group were divided in two. No further grinding was performed on the first subsample set prior to extracting genomic DNA ("non-mortar and pestle"). The second subsample set was ground for 3 min prior to extracting genomic DNA, using a mortar and pestle.

Particle Size Analysis. The particle size of the ground corn was evaluated using a Microtrac II laser particle size analyzer (Model 7997-20, Leeds-Northrup, Inc., North Wales, PA) with a working range of 0.7−700 μm. It measures particle size distribution in terms of the volume of particles in each of 20 size channels.

DNA Isolation Procedures. DNA was isolated from the ground corn using five commercial kits (methods A−E) plus a standard protocol (method F) that.utilizes cellular lysis and selective precipitation with cetyltrimethylammonium bromide (CTAB) (*7*). The amount of ground corn used in an individual isolation ranged from 50 to 200 mg. The same batch of corn (0.1% CBH351) was used for all isolations. The protocols were followed as indicated by the manufacturer without modification, except for the addition of RNase A (Qiagen, Inc., Valencia CA) to protocols that did not already include RNase treatment. The commercial kits will only be identified by the principle on which the DNA isolation is based. Kits were chosen on the basis of advertised suitability for the isolation of high molecular weight DNA from plant material.

Methods A and B consisted of cell lysis, precipitation of protein and polysaccharides, binding of the DNA to silica gel, washing, and elution.

Method C consisted of cell lysis and binding of DNA to magnetic beads, washing, and elution.

Method D consisted of cell lysis, protein precipitation, and DNA precipitation.

Method E consisted of cell lysis and selective DNA precipitation.

Method F (*7*) consisted of cell lysis and selective DNA precipitation with CTAB.

Four different individuals, working completely independently from one another, in two different laboratories prepared DNA using the selected methodologies and also conducted PCR assays on the material that they isolated. DNA was quantified by measuring absorbance at 260 nm, with either Gene Quant DNA calculator (Amersham-Pharmacia Biotech, Piscataway, NJ) or a Beckman DU650 spectrophotometer (Beckman-Coulter, Fullerton, CA), assuming 1 absorbance unit is equal to 50 μg/mL. Scans were conducted over a wavelength range of 220−320 nm. The spectrophotometer was blanked with the relevant final resuspension buffer.

For experiments on the effect of particle size on DNA extraction efficiency, 1 g of each subsample was used to extract genomic DNA with Qiagen Maxi Kits (Qiagen, Inc, Valencia, CA). Once the DNA was eluted from the column, an ethanol precipitation step was performed by adding 1/5 vol of ammonium acetate and twice volume of absolute ethanol. The genomic DNA was vortexed briefly and placed into a −20 °C freezer for approximately 20 min. The DNA was centrifuged for 10 min (12 500*g*), washed with 70% ethanol, centrifuged again, and resoubilized into 25−100 μL of sterile Nanopure water. DNA recovery was quantified using a GeneQuant DNA calculator (Amersham-Pharmacia). Approximately 100 ng was used in PCR reactions as described below.

Polymerase Chain Reaction and Gel Electrophoresis. All qualitative PCR reactions (20 or 25 μL) were run on a Gene Amp 9700 PCR system (Applied Biosystems, Foster City, CA) or a MJ Research thermocycler (South San Francisco, CA) using Qiagen HotStarTaq Master Mix (Qiagen, Inc.), which contains Taq DNA polymerase, buffer, 1.5 mmol/L $MgCl_2$, and dNTPs. The primer concentration was 0.4 μmol/L, and 100 ng of genomic DNA was used in each reaction. The *35S* PCR primer pairs were 5′-GCTCCTACAAATGCCATCA-3′ and 5′-GATAGTGGGATTGTGCGTCA-3′ (*8*). Reactions for the detection of *35S* were run for a total of 50 cycles after an activation of 14 min at 95 °C. The cycling protocol included five cycles of 95 °C for 1 min, 57 °C for 1 min, 72 °C for 2 min, and 45 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min. The PCR reaction product was 196 bp, and its purity and size were determined by electrophoresis in a 1% or 2% agarose gel stained with ethidium bromide. The PCR protocol for *adh1* used the PCR primer pair 5′-ACACCCTCTCCAACACTCTC-3′ and 5′-GCAGTTCTAGGAGAAGTGGAGC-3′. Cycling parameters were identical to the conditions listed above, except the second set of cycling parameters was repeated 40 rather than 45 times. The PCR-amplified product was 645 bp. The primer sequences used for PCR amplification of the *cry9C* gene are proprietary (Bayer Crop Science). The PCR primers used in the assays were specific for the intended product. Additional nonspecific bands were not observed. Positive, negative, and no DNA controls were run with each batch of assays and performed correctly. The correctness of the PCR product for each assay type was ascertained by DNA sequencing using dye terminator chemistry on an ABI 373 DNA sequencer (Applied Biosystems).

A real time PCR protocol for *adh1* was used to evaluate the effect of various putative inhibitors on the reaction. Primer sequences were 5′-ACCACCAACCATACCCATAA-3′ and 5′-TAGGAGAAGTG-GAGCGAGA, which produces a smaller product than the assay described above. The reactions were run on a Light Cycler (Roche Diagnostics Corp., Indianapolis IN) and the product was 308 bp. The reactions were conducted using two different reagents, QuantiTect SYBR Green (Qiagen, Inc.) and Light Cycler-Fast Start DNA Master

Table 1. Detection of CBH351 (*cry9C*) by PCR in 1.0 g of Size-Sorted Ground Corn Kernel

	non-mortar and pestle			mortar and pestle		
sieve opening (μm)	no. of particles/ g	DNA recovered (μg)	positive PCR reaction for *cry9C*[a]	no. of particles/ g	DNA recovered (μg)	positive PCR reaction for *cry9C*[a]
>2800	37[b]	1.2 ± 1.1[c]	4/6[d]	175 049[e]	55.0 ± 29.4[f]	4/6[d]
2800–2380	67[b]	2.3 ± 1.6	4/6[d]	174 122[e]	51.4 ± 26.0	4/6[d]
2380–850	1 800[b]	8.6 ± 4.0	6/6	148 735[e]	74.5 ± 29.4	6/6
850–600	6 521[e]	10.0 ± 6.5	6/6	259 138[e]	83.0 ± 45.9	6/6
600–300	59 945[e]	41.5 ± 14.9	6/6	322 917[e]	77.4 ± 28.3	6/6
<300	527 106[e]	76.7 ± 10.8	6/6	3 762 569[e]	80.2 ± 6.1	6/6

[a] CBH351 constitutes 0.5% by mass of corn sample, a PCR reaction for the endogenous gene, *adh1*, was run on all DNA samples and was found to be positive in all cases. [b] Based on manual counts of a 1 g sample, repeated three times. [c] $n = 6$, error estimates represent 1 standard deviation for all samples, average recoveries for subsamples declared statistically significant at the 0.001 significance level. [d] 99% probability of four or fewer positives out of six tests performed. [e] Approximation based on average particle size calculated from laser particle size analysis. [f] $n = 6$, error estimates = 1 standard deviation, average recoveries for subsamples not declared statistically different at the 0.05 significance level.

SYBR Green I (Roche Diagnostics Corp.). Additional $MgCl_2$ (1 mM) was added to the Roche reagent mixture along with 50 ng of genomic DNA. After a 15 min activation step at 95 °C, 40 cycles were run (95 °C for 15 s, 59 °C for 20 s, 72 °C for 40 s). Readings for SYBR Green fluorescence were taken at the end of each cycle at 82 °C for the Roche reagent reactions and 80 °C for the Qiagen reagent reactions. Carbohydrates were tested as possible PCR inhibitors. These included glucuronic acid (CAS 14984-34-0), dextran (CAS 9004-54-0), dextran sulfate (CAS 9011-18-1), starch (CAS 9005-25-8), and alginic acid (CAS 9005-38-3) and were added as concentrates to the reaction. Acidic polysaccharides and glucuronic acid were added as Na^+ salts and were tested to verify that there was no effect on the pH of the reaction.

Corn Genomic DNA Size Analysis. The fragment size range of the isolated DNA was investigated using field inversion gel electrophoresis (FIGE). Samples (1−3 μg of DNA) were run in a 1% agarose gel with 0.5 × TBE buffer [22.5 mmol/L tris(hydroxymethyl)-aminomethane, 22.5 mmol/L boric acid, 0.5 mmol/L ethylenediaminetetraacetic acid, tetrasodium salt] for 30 min at 200 V. This was followed by a field reversal sequence of three units of time in one direction and one unit in the reverse (Hoefer SwitchBack Pulse Controller, PC500, Hoefer Scientific Instruments, San Francisco, CA) at 100 V with a ramp of 1−13 s over 13 h. A sizing ladder of 0.1−200 kbp was used to calibrate the sizes of the DNA.

RESULTS

The corn kernel is a complex array of tissues and cell types. The highest levels of DNA are found in embryo tissue, while endosperm and pericarp contain significantly lower levels. Prokish et al. developed a model that predicts DNA recovery on the basis of particle size and concentration distribution for certified reference materials (*9*). This model predicts that DNA recovery is highest with smaller particle size fraction starting material. We determined the relationship between particle size and PCR amplifiability in corn kernels, gravimetrically spiked to 0.5% CBH351. Corn samples were blended and particle size separated using a multilevel sieve series as described in Methods and Materials. Genomic DNA was extracted from 1 g of starting material for each particle size subsample. **Table 1** shows the relationship between particle size and DNA extraction efficiency. Genomic DNA recovery increased as a function of decreasing particle size for each subsample. Corn tissues including pericarp, endosperm, and embryo contain different levels of DNA. To test whether DNA extraction efficiency results from an unequal distribution of these tissues in the starting material, subsamples were ground with a mortar and pestle into a fine powder and DNA extractions were repeated. The number of particles representing a 1 g sample for each subsample are shown in **Table 1**. These data showed a relatively consistent recovery of corn genomic DNA with mortar and pestle treated subsamples (columns 4 and 5) and relatively large standard deviations. The observed differences in DNA recovery might be caused by surface and interfacial tensions between detergent and different compositions of the particle size fractions of the subsample.

To determine whether *adh1* and *cry9C* genes could be amplified using PCR from these subsamples, 100 ng of corn genomic DNA was added to PCR reactions. The results showed that successful amplification of the *cry9C* gene was a function of particle size (**Table 1**). Identical results were obtained, regardless of whether the sample was mortar and pestle treated or not. On the basis of these findings, particle sizes of 850 μm or less, when spiked at a 0.5% level, were representative of sample starting material, while samples consisting of large particles were not consistently representative. DNA extraction efficiency was lower for larger particle size samples, and all samples amplified consistently and reliably for the *adh1*, endogenous control gene. The likely explanation for the negative results with some samples containing the largest particles is sampling variability. Statistical probabilities suggest that some samples did not contain particles from a CBH351 kernel.

The batch of ground corn used for experiments comparing DNA extraction methodologies was analyzed for particle size using a Microtrac II laser particle size analyzer as described in Methods and Materials, and a mean diameter of 212 μm was determined. From this value, the mean number of particles per 100 mg sample was calculated to be 1.54×10^4 with a particle mass of 6.5 μg. When a lot contains 0.1% concentration of biotechnology-derived particles, then a sample of 4603 particles has a 99% probability of containing at least one biotechnology-derived particle. A sample of 6905 particles has a 99.9% probability of containing at least one biotechnology-derived particle. Thus, a sample of 100 mg ground corn with a mean of 1.54×10^4 particles would be representative of a sample derived from the 0.1% (m/m) biotechnology-derived corn in conventional corn and would permit detection of a trace amount of engineered DNA.

The efficiency of extraction of DNA from corn using the five commercially available kits and the CTAB/NaCl method is reported in **Table 2**. A statistical analysis of variance test on the data found a statistically significant interaction between methods and investigators. This means that the difference between methods was not always consistent from one investigator to the next. Methods A and B (ion exchange on silica gel) gave the lowest yields (mass DNA/ mass of corn), while the method F (CTAB) gave the highest yield. DNA recovery ranged

Table 2. Comparison of DNA Isolation Methods for Efficiency of Extraction of Corn Kernel DNA

	yield (ng DNA/mg corn)[a]				A_{260nm}/A_{280nm}[a]			
method	1[b]	2[c]	3[b]	4[b]	1	2	3	4
A	13 ± 1[d]	23 ± 2	20 ± 2	7 ± 1	1.7	1.4	1.8	1.8
B	15 ± 5	14 ± 1	36 ± 5	26 ± 34	2.0	1.5	1.3	1.9
C	46 ± 25	42 ± 12	35 ± 5	33 ± 7	1.4	1.3	1.7	1.7
D	43 ± 4	46 ± 27	21 ± 3	77 ± 7	1.6	1.4	1.7	1.6
E		84 ± 11	27 ± 7	184 ±121		1.3	1.7	1.4
F	143 ± 65	54 ±15	104 ± 26	161 ± 65	2.0	1.6	1.8	2.0

[a] Four investigators (1–4) conducted multiple isolations of DNA from ground corn kernels. [b] Results were from six replicates of each method. [c] Results were from three replicates of each method. [d] One standard deviation.

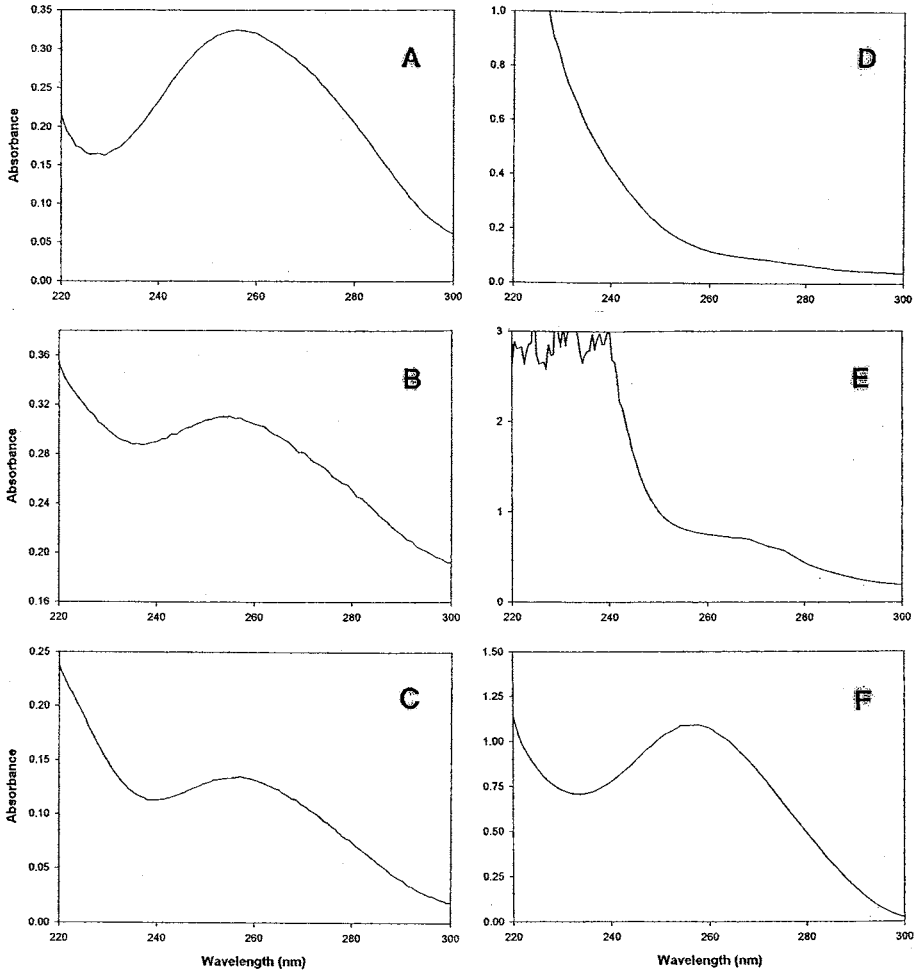

Figure 1. Wavelength scans (220–300 nm) of genomic DNA isolated from ground corn using Methods A–F. The same batch of finely ground corn kernels was used in all isolations. The final solution used for solubilizing the DNA served as a blank for the scan.

from 1.6 to 11 μg, based on the average yield for each method and the starting mass of ground corn used, and is sufficient for PCR amplification protocols. While the differences among investigators varied by method, method C (magnetic particles) showed the most consistent yield across investigators.

DNA was scanned over the range of 220−320 nm, and representative samples are shown in **Figure 1**. Scans were very consistent within methods. DNA should give a symmetrical peak with a maximum at 260 nm. Large absorbance below 260 nm is seen in the wavelength scans of DNA isolated by methods D and E, suggesting the presence of some contaminants (*10*). If absorbance at 240 nm is higher than that at 260 nm, it is very likely that the contaminant(s) may overlap the absorbance of DNA, leading to an overestimation of the DNA content, when measured by absorbance at 260 nm.

The ability to amplify an endogenous gene has been used as a way to verify that a DNA preparation is of sufficient purity to support detection of biotechnology-derived DNA. We tested whether this was a reasonable hypothesis. PCR reactions were run on each sample of isolated DNA to test for the detection of the endogenous *adh1* gene, plus the *35S* promoter and the *cry9C* gene present in CBH351 corn (**Table 3**). There are some DNA samples that would have been judged unsuitable for PCR amplification of biotechnology-derived genes, based on the experimental observation that the endogenous *adh1* assay did not produce a PCR product. Samples generating this type of result would ordinarily be reanalyzed.

Table 3. PCR Assays Conducted on Corn DNA Containing 0.1 % CBH351 for Investigators 1–4

	1			2			3			4		
method	adh1	35S	cry9C	adh1	35S	cry9C	adh1	35S	cry9C	adh1	35S	cry9C
A	6/6	6/6	0/6	3/3	3/3	3/3	6/6	3/6	6/6	5/5	**2**/5	**2**/5
B	6/6	4/4	5/6	3/3	0/3	3/3	6/6	6/6	6/6	6/6	6/6	6/6
C	5/6	3/3	5/6	3/3	3/3	3/3	6/6	6/6	6/6	6/6	6/6	**2**/6
D	1/6	5/6	4/6	3/3	3/3	3/3	5/6	5/6	**0**/6	2/5	4/5	**2**/5
E				0/3	0/3	0/3	3/6	3/6	**1**/6	0/6	2/6	0/6
F	6/6	3/3	2/4	3/3	3/3	3/3				6/6	6/6	**4**/6

[a] Three separate types of assays were conducted for amplification of an endogenous gene, *adh1* and CBH351 DNA sequences (*35s* promoter and cry9c gene). All assays should give positive results. The notation indicates the number of positive results out of the total number of individual samples tested. Numbers that are underlined highlight results where one or more event assays were negative when the *adh1* assay was positive.

But more concerning were the "false negative" results as indicated by the bold numbers in **Table 3**. These indicate DNA samples that tested positive for *adh1* but were negative for either *35S* or *cry9C*. Examples of "false negatives" were observed with every DNA isolation method employed. The "false negatives" were more abundant with the *cry9C* specific detection method when compared to the *35S* assay. The percentage of *total* negative assays was the same for *adh1* and *35S* (20%), while *cry9C* had twice as many (41%). The reason for the decreased sensitivity of the *cry9C* method as compared to *35S* may be

due to the fact that CBH351 carries two copies of the *35S* promoter as compared to just one target copy of the *cry9C* gene or the *cry9C* primer assay is not as sensitive. Both the conventional and CBH351 corn was tested for the presence of all other approved and commercialized corn events in the United States and found to be negative (unpublished results). This was done to verify that no additional biotechnology-derived DNA that also used the *35S* promoter as part of the construct was present.

Commercial DNA isolation methods that rely on selective binding of the DNA to a solid matrix performed better with ground corn (methods: A, 25%; B, 6%; C, 10% negative results) than the two methods that rely on selective precipitation (methods: D, 50% and E, 81% negative results). The CTAB/NaCl method, which also relies on selective precipitation, performed better than the commercial reagents (method: F, 10% negative results).

The failure to amplify could be due to (a) DNA degradation, (b) insufficient DNA in the PCR assay, or (c) copurifying inhibitors. We tested for DNA degradation by determining the range of fragment size of the DNA isolated using the six methods. DNA was size-sorted using field inversion electrophoresis in agarose gels, stained with ethidium bromide, and compared to markers that ranged from 2 to 200 kbp. DNA fragments from 23 to 48 kbp were prominent with DNA prepared by each method, suggesting that the DNA was not excessively sheered and was suitable for amplification. DNA quantity could have been overestimated using absorbance at 260 nm, especially in the case of methods D and E, where the wavelength scan indicated significant absorbance below 260 nm (**Figure 1**). The PCR reactions were supposed to contain 100 ng of corn genomic DNA. This represents 4×10^4 copies of the genome (*11*) or approximately 40 copies of the *cry9C* gene (if present in one copy/genome) and 80 copies of the *35S* promoter in the case of 0.1% CBH351 in conventional corn. Overestimation of the DNA quantity by 10-fold would reduce the theoretical number of copies to only 4. So a gross overestimation of the amount of DNA added to a PCR reaction could account for the lack of amplification of CBH351 genes, but not the endogenous *adh1* gene, suggesting that inhibitors are present in the PCR reaction.

Polysaccharides, especially starch, are described as inhibitors of the PCR reaction (*12*). Corn kernels contain high levels of starch in the endosperm (*6*). Plant cell walls may be another source of contaminating polysaccharides. Plant cell walls contain neutral and acidic polysaccharides. The cellulose scaffold in plant cell walls is cross-linked by xyloglucans and glucuronoarabinoxylans (GAXs) (*13*). GAXs are the major cross-linking polymers in the grass family, of which corn (*Zea mays*) is a member. GAXs are 1→4 linked polyxylans with 1→2 linked branches of arabinose and glucuronic acid. In addition, plant cell walls contain pectins, a diverse group of hydrophilic polysaccharides that are rich in galacturonic acid.

Two neutral polysaccharides, starch and dextran, and three acidic mono- and polysaccharides, glucuronic acid, alginic acid and dextran sulfate, were tested for their ability to inhibit PCR amplification. These compounds were chosen because they mimic cell wall components that might copurify with genomic DNA during an isolation procedure. Alginic acid, found naturally in brown algae, is an uronic acid polysaccharide commonly used as an emulsifier in processed foods. In these experiments, a real time assay for *adh1* was used with SYBR Green as the fluorescent dye detector. **Table 4** shows that the neutral polysaccharides, starch (2% final concentration), and dextran (4% final concentration) were not inhibitory to the PCR reaction. At a 2% final concentration, starch is observable because it creates a haze in the reaction and changes the reaction consistency to that of a loose gel at room temperature. In contrast, the acidic polysaccharides (dextran sulfate, alginic acid) and glucuronic acid were inhibitors of *adh1* amplification at low concentrations. Assays were run with two different master reagents (see Methods and Materials) and the level of inhibition was generally the same, except for glucuronic acid, where assays run with the Qiagen reagent mixture were inhibited at a lower concentration.

Table 4. Effect of Mono- and Polysaccharide Additives on *adh1* PCR Amplification

	no effect on PCR[a]		complete inhibition of PCR[b]	
additive	Roche	Qiagen	Roche	Qiagen
glucuronic acid			20–40 mM	10 mM
alginic acid			0.02%	0.02%
dextran	4%	4%		
dextran sulfate			0.001%	0.001%
corn starch	2%	2%		

[a] For those additives that did not interfere with amplification, the highest concentration tested is indicated. [b] Where inhibition of the PCR reaction was seen, the minimum amount required for complete inhibition of the PCR reaction is indicated.

DISCUSSION

DNA extraction methods should be cost- and time-effective, especially in situations where the handling of large numbers of samples is required. The quality of DNA is critical to consistent, reliable, and accurate results. The most important factors are integrity and purity. It is necessary to carefully evaluate the suitability of a method. In the case of trace amounts of biotechnology-derived genomic DNA, the goal can be to detect material that may be present, as in the study here, in only one copy per thousand copies of a haploid genome.

In this study we evaluated the particle size of the material we were extracting and calculated the number of particles in 100 mg of ground corn, the mass recommended for the commercial DNA isolation methods used. The fineness of the grind was sufficient to be representative of the corn sample used in these experiments, as long as the sample was homogeneous and the extraction of DNA from all particles was of similar efficiency. Particle size should also be considered when picking a sample size to extract and a method for grinding corn.

Each DNA isolation method was performed many times on the same material. With every method there were some DNA preparations that failed to amplify one or more of the target genes. What was observed in this study applies only to the ground corn used in these investigations. Each tissue type and plant species differ in chemical composition, resulting in unique extraction and purification problems. Many variations on extractions of plant tissues have been developed in order to obtain suitable DNA where severe and specific problems have been encountered. These include situations where the polysaccharide content was very high and difficult to remove or where polyphenols bound and degraded DNA but could be eliminated with additions such as polyvinylpyrollidone or poly(ethylene glycol) (*14*, *15*).

The methods that employ binding of DNA to a solid matrix (silica gel or magnetic particles) performed better in PCR reactions than the selective precipitation methods, with the exception of method F (CTAB/NaCl). Zimmerman et al. (*16*)

observed that similar binding methodologies produced smaller quantities but better quality DNA from soybean. The wavelength scans of DNA isolated using these methods (**Figure 1A−C**) showed symmetrical peaks and reduced absorbance at wavelengths <260 nm, indicating cleaner preparations than some other methods. Nonetheless, there were some false negative results with all three of these methods, suggesting that preparations may be contaminated with potential PCR inhibitors and so require additional purification step(s). Ethanol or 2-propanol precipitation, chloroform/isoamyl alcohol extraction, addition of proteases, or some other measure may be required in order to obtain consistent and accurate target DNA amplification (*7*). Method F (CTAB) worked as well as the DNA binding methodologies, while the other two selective precipitation methods were generally not appropriate for corn kernels.

Failure of the PCR reaction to amplify the target DNA sequence could be the result of degraded DNA, insufficient target DNA, or DNA contaminated with inhibitors. Field inversion gel electrophoresis (FIGE) showed that the DNA isolated from corn by all the methods had a broad range of fragment sizes but with a significant proportion of large nucleic acid fragments ranging from 23 to 48 kbp. Thus, the DNA had sufficient integrity to support amplification. Insufficient target DNA is not likely to be the culprit, with the possible exception of three DNA preparations. If the PCR amplification was performed on DNA that was not representative of the sample (conventional corn DNA plus CBH351 DNA), then neither of the engineered DNA target PCR assays should have worked. This only occurred with three DNA samples (**Table 2**, method A, investigator 4). Investigator 4 obtained negative results for three of five method A DNA preparations for *35S* and *cry9C* while all five were positive for *adh1* (**Table 3**). The same samples had the lowest efficiency of extraction for method A in general and all methods in total (**Table 3**). There is the possibility that there was little or no CBH351 DNA was present in the samples.

The bulk of the false negative results are most likely due to the presence of inhibitors that inactivate Taq DNA polymerase or otherwise interfere with the PCR reaction. While starch is a major component of corn (*6*), the data in **Table 4** indicate that starch and other neutral polysaccharides are unlikely to interfere with the amplification process. Low concentrations of acidic polysaccharides can be quite inhibitory (**Table 4**). The test substances were not identical to what is found in corn, but were suitable surrogates. It would be of interest to investigate corn-derived acidic polysaccharides for their presence, if any, in DNA preparations and their effect on the PCR process.

In this study we utilized a traditional and still commonly used DNA quantification method, absorbance at 260 nm. The accuracy of this method is limited by sensitivity as well as possible interference by compounds coextracted with DNA. This is illustrated by wavelength scans of DNA (D, E) shown in **Figure 1**. There are additional methods, based on fluorescent dyes that intercalate into DNA, that are in use for quantification of DNA. While there are described chemicals that interfere with these methods also, they may offer more accurate quantification of DNA than the traditional method and should be investigated.

An important conclusion of this work is that successful amplification of an endogenous gene is not a guarantee that the DNA is of sufficient purity for amplification and detection of a gene present in many fewer copies as would be the case for 0.1% biotechnology-derived DNA. With every DNA extraction method employed in this study, there were some extracted DNA samples where either the *35S* promoter or *cry9C* (or both) were not detected by PCR, but the *adh1* gene was detected. To choose an appropriate method for a given tissue, experiments similar to what is reported here would be useful for determining consistency of performance for detection of trace quantities of biotechnology-derived genes. The final method should provide consistent results, regardless of the investigator. Additional purification steps may be required for plant genomic DNA prior to amplification using PCR (see ref 17 for a recent review). Wavelength scans provide information for evaluation of the quality of a DNA preparation. Quantification of the extracted DNA is important and methods other than absorbance at 260 nm should be investigated and employed. Finally, well-mixed samples need to be ground to a sufficient fineness to be representative of the entire sample.

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