Evaluation of the Synergy™ Rapid Plant DNA Isolation Chemistry

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Abstract

A new chemistry (Synergy™ Plant DNA Extraction Kit) for the isolation of DNA from plant tissues was evaluated and compared to traditional CTAB and DNeasy® methods. These existing methods produce quality DNA, but at the expense of yield (DNeasy®) or with the use of hazardous chloroform (CTAB). Synergy™ combines bead beating with an extraction matrix that disrupts the sample while simultaneously acting as a solid phase adsorbent to capture contaminants, including polyphenols and protein. DNA isolation from *Anthurium*, corn, cotton, grape, rice, rye, sorghum, soybean, and wheat has demonstrated that the Synergy™ chemistry provides higher yields than both CTAB and DNeasy® methods. Purities, as measured by 260/280 and 260/230 ratios, are comparable between the extraction methods. DNA generated by the three methods, as well as the Synergy™ lysate, were all successfully amplified by PCR. Using Synergy™ lysate as a DNA source greatly simplifies sample preparation for plant PCR. The time required to process samples using the Synergy™ protocol is between 5 and 30 minutes, which is, at most, 1/3 the time required for both the CTAB and DNeasy® protocols. An analysis shows that Synergy is about half the cost per sample than DNeasy® while avoiding the issues associated with using chloroform with CTAB.

Introduction

The isolation of DNA from plant tissues is complicated due to the presence of polysaccharides, polyphenols, and polyphenol oxidases in plant homogenates, which can be detrimental to downstream manipulations of DNA (Varma et al., 2007). Non-commercial methods for isolating DNA from plant tissues have long relied on extracting homogenized tissues with CTAB (cetyl trimethylammonium bromide) based buffers to circumvent these contaminants. CTAB-based DNA isolation procedures aim to separate polysaccharides and DNA by selectively precipitating these species at different salt concentrations (Murray and Thompson, 1980). In the presence of CTAB, polysaccharides precipitate at higher concentrations of NaCl, while DNA precipitates at lower concentrations. A second concern associated with isolating plant DNA is the high concentrations of polyphenols and associated polyphenol oxidases, which can cause browning of DNA preparations and hinder further manipulation of the DNA. The addition of polyvinylpyrrolidone to extraction buffers was found to bind the polyphenols and prevent oxidation (Rogers and Bendich, 1985).

CTAB procedures have evolved so that good yields of quality DNA can be obtained from even the most problematic plant samples, such as pine and grape. However, practical issues (i.e., chloroform and long incubations times) inherent to CTAB procedures have lead researchers to seek alternative methods. Chloroform is required for extractions when using CTAB. Unlike when these methods were developed (in the 1980s), the knowledge of the hazardous nature of chloroform now means its use is restricted in many industrial and academic laboratories where safety protocols are tightly monitored. In addition, incubation times are typically longer with CTAB methods. It can take several hours to complete a protocol. The laborious nature of CTAB protocols has thus spawned the commercialization of alternative DNA isolation kits.

Most commercial kits used for isolating DNA from plants rely on spin columns that capture the nucleic acids from cleared homogenates. A typical spin column procedure involves homogenizing the sample dry (either cryogenically or at room temperature), adding an extraction buffer, removing debris by filtration or centrifugation, and then binding the DNA to a chromatography medium (usually silica) in the spin column. The DNA is cleaned using a series of wash buffers and is subsequently collected from the column in an elution buffer, a process accomplished with the use of a centrifuge. Spin column procedures can be relatively fast compared to CTAB methods, but it can

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still take up to an hour or longer to complete. There can be up to six buffers used in plant DNA isolation kits, accompanied by as many as seven centrifugation steps.

Though commercial kits for plant DNA isolation are widely used, it appears that ease of use and speed are the primary sought-after attributes as compared to CTAB methods. An examination of eight different procedures for DNA isolation from pine needles found that three commercial kits were equally as or less effective than several CTAB protocols (Telfer et al., 2013). These commercial spin column kits either gave higher DNA yield coupled to lower purity or vise versa. Li et al. (2008) compared a CTAB protocol to the DNeasy® kit for the isolation of total DNA for plant virus detection and found that CTAB provided much greater DNA yields, while the DNeasy® kit provided greater purity. The extraction of DNA from potatoes and potato-derived products using seven commercial kits and a CTAB method also demonstrated that CTAB produced the highest yields (Smith et al., 2005). However, the Kingfisher and Magnesil KF methods, both based on capturing DNA with magnetic particles, were able to select genomic DNA preferably where the other five kits (Wizard®, DNeasy®, Roche I, Hi-Pure, Mo-Bio) and CTAB method extracted total DNA. With the heterogeneous nature of plant tissues, no one method was superior for isolating DNA when multiple plant species were tested.

CTAB methods repeatedly produce greater yields of plant DNA, but lack the safety, ease, and speed of commercial spin column based kits. Thus, if a rapid and alternative extraction method was substituted for chloroform extraction, CTAB methods could be greatly improved. It was hypothesized that solid phase extraction methods, similar to those used in chromatographic sample preparation, could replace the chloroform extraction step from CTAB protocols. This idea was extrapolated to include the homogenization step by proposing that the solid phase extraction matrix can also serve as the homogenization media. Consequently, OPS Diagnostics developed extraction chemistry for plant DNA isolation that combines a proprietary homogenization matrix and buffer system that eliminates the need for chloroform. By combining steps, significant time is saved and labor is reduced.

The resulting chemistry developed from this idea, labeled Synergy™, employs a surface modified grinding matrix to capture impurities along with buffer chemistry that precipitates polysaccharides. Following homogenization, DNA remains in solution while cellular debris and unwanted contaminants are removed by centrifugation. The data presented below summaries this chemistry developed by OPS Diagnostics scientists and collaborators.
In order to assess the effectiveness of this new chemistry, the Synergy™ Plant DNA Extraction Kit was evaluated for DNA isolation on *Anthurium*, corn, cotton, grape, rice, rye, sorghum, soybean, and wheat. DNA yields, limits, and purity were compared to a standard CTAB method as well as a DNeasy® kit.

Materials and Methods

Samples: A total of nine plant species were used to assess three different DNA isolation procedures (Table 1). The specimens were obtained from the OPS Diagnostics Sample Library and consisted of *Anthurium*, corn, cotton, grape, rice, rye, sorghum, soybean, and wheat. Samples were taken from fresh leaf tissue by using a paper punch. Tissues were processed immediately upon sampling.

Table 1. Species of samples used for comparison of DNA extraction methods.

Common Name	Species	Source	Sample
Anthurium	*Anthurium andraeanum*	Westland Greenhouses	Leaf
Corn	*Zea mays*	Local farm	Leaf
Cotton	*Gossypium hirsutum*	Lehle Seeds	Leaf
Grape	*Vitis baco noir*	Local vineyard	Leaf
Rice	*Oryza sativa*	Cornell University	Leaf
Rye	*Secale cereale*	Lehle Seeds	Leaf
Sorghum	*Sorghum bicolor*	Lehle Seeds	Leaf
Soybean	*Glycine max*	Lehle Seeds	Leaf
Wheat	*Triticum aestivum*	Lehle Seeds	Leaf

Homogenization Methods: All samples were homogenized via bead beating using one of several homogenizers, namely the HT Mini™, HT 24™, or Mini G™, with the following parameters:

HT Mini™ – 4000 rpm for 60 seconds
HT 24™ – 4200 rpm for 70 seconds
Mini G™ – 1500 rpm for 190 seconds

Samples were processed at room temperature in 2 ml disruption tubes, either with or without extraction buffer.

CTAB Extraction Protocol: Plant tissue (approximately 50 mg) was placed in a 2 ml disruption tube with 500 µl of CTAB buffer (2% hexadecyltrimethyl ammonium bromide (Sigma H6269), 100 mM Tris-HCl (Sigma T3253), 1.4 M NaCl (Sigma S9888), 20 mM EDTA (Sigma ED2SS)) and 2.8 mm stainless steel grinding balls. The sample was processed under conditions described above. The homogenate was transferred to a 55°C bath for 15 min. and then centrifuged for 5 min. at 10,000 g. The supernatant was transferred to a new 2 ml tube and an equal volume of chloroform/isoamyl alcohol (24:1) was added and vortexed for 5 seconds. The sample was centrifuged for 1 min. at 10,000 g to separate the phases. The upper aqueous phase was transferred to a new tube where $1/10^{th}$ volume 7.5 M ammonium acetate was added and mixed by vortexing for five seconds. The DNA is precipitated by adding 0.7 volume cold isopropanol followed by incubation at -20°C for 15 min. The tube was centrifuged at 10,000 g for 10 min. The supernatant was decanted without disturbing the pellet, which was washed with 500 µl ice cold 70% ethanol. The ethanol was decanted with the residual alcohol being removed by drying in a SpeedVac. The pellet was processed long enough to remove alcohol, without overly drying the DNA. The DNA was dissolved in 20 µl TE buffer (10 mM Tris, pH 8, 1 mM EDTA). The sample at times needed warming to dissolve the pellet completely.

Synergy™ Plant DNA Extraction Kit Protocol: The Synergy™ kit can be used with samples homogenized at both ambient temperature and cryogenically. For this study, samples were homogenized at room temperature, using 50 mg of leaf tissue and 500 µl of homogenization buffer per 2 ml disruption tube containing Synergy™ grinding resin and a grinding satellite. The tube was capped and placed in the HT Mini™ or HT 24™ bead beater and processed as described above. The sample was subsequently centrifuged at 10,000 g for 5 minutes. The supernatant (about 250 µl) was transferred to a new tube and 5 µl RNase was added to the lysate, followed by a 5 minute incubation period at room temperature. At this point the DNA could be used directly for PCR or be further purified by alcohol precipitation with the addition of 0.7 volume of cold (-20°C) isopropanol, followed by a 15 minute incubation period at -20°C. The DNA was pelleted by centrifugation at 10,000 g for 10 min. The pelleted DNA was washed twice with 500 µl of 70% ethanol and then briefly dried in a SpeedVac. The DNA was resuspended in 20 µl of TE buffer prior to analysis or use.

DNeasy® Extraction Protocol: Using the product protocol as a guideline, 50 mg of leaf tissue was placed in a 2 ml disruption tube along with one 6 mm zirconium oxide grinding satellite (OPS Diagnostics, product number GSZO 006-1000-05), which was then homogenized in the HT Mini™ for 1 minute at 4000 rpm. Buffer AP1 (400 µl) was then added to the ground tissue followed by 4 µl of RNase A. The sample was mixed by briefly vortexing and then incubated at 65°C for 10 minutes, periodically inverting the tube during incubation. Buffer P3 (130 µl) was added to the lysate, followed by mixing and incubation on ice for 5 minutes. The lysate was cleared by centrifuged for 5 minutes at 10,000 g and then the supernatant was transferred to a QIAshredder, which was centrifuged with the filtrate being collected in a 2 ml tube. Buffer AW1 (1.5 volumes) was added to the filtrate and then mixed by pipetting. Up to 650 µl of the solution was transferred into a DNeasy® Mini spin column, and centrifuged at 10,000 g for 1 minute (the flow-through fraction was discarded). Remaining sample was applied to the spin column in the same manner. The adsorbed DNA was washed by adding 500 µl of buffer AW2 followed by centrifuging at 10,000 g for 2 min. To elute DNA from the spin column, 100 µl of Buffer AE was added followed by incubation at room temperature for 5 minutes. The DNA was collected by centrifuging the spin column inserted in a collection tube for 1 minute at 10,000. The elution step was repeated and the eluted DNA fraction pooled.

DNA Concentration and Purity: DNA concentration was determined using fluorescent dye that binds to double stranded DNA. The QuantiFluor dsDNA System (Promega, Madison, WI) was used to assess the DNA concentration of extracted samples. Fluorescent measurements were made on a Glomax Multi Junior Detection System (Promega, Madison, WI). Lambda control DNA was used to establish a standard curve. DNA purity was determined using a Spectra Max 190 microplate reader (Molecular Devices, Sunnyvale, CA) using SoftMax Pro 5.4.1 software. Measurements were made in 96 well UV transparent microplates (Product Number 655801, Greiner Bio-One, Monroe, NC). DNA samples were diluted 1:100 in TE buffer and measured at 230, 260 and 280 nm, which were used to calculate the 260/280 and 260/230 ratios.

Amplification of DNA: PCR was used to assess whether the Synergy™, CTAB, and DNeasy® purification processes would provide sufficiently pure DNA for detection and amplification. Primer sets for four of the plant species were known to produce positive PCR products when using the CTAB purification method. PCR was composed of 85.5 µl of water, 10 µl of 10X buffer, 2 µl of dNTP, 1 µl of forward primer, 1 µl of reverse primer, 1 µl of the template, and 0.5 µl of Taq polymerase. PCR primers and conditions for each primer set are listed in Table 2. Agarose gel electrophoresis (2% agarose in 1X TAE buffer) was used to assess PCR product size as measured against a known 100-1000 bp marker (Denville Scientific, CB-4229-2).

Table 2. Plant species, targets, conditions and PCR primer pairs used for amplification.

Plant	Target	Primer Pair	Cycling Conditions
Corn	Chloroplast 23S rRNA (Z00028.1), product length: 363 bp	Forward: 5'-TGTACCCGAAACCGACACAG-3' Reverse: 5'-GATCGTTACGCCTTTCGTGC-3'	Denature: 95°C, 1 min. Anneal: 52°C, 1 min. Extend: 72°C, 1 min. Cycles 35
Grape	Chloroplast psbA-trnK intergenic spacer (HQ108323.1), product length: 325 bp	Forward: 5'-CACGACCTTGGCTATCAACTAC-3' Reverse: 5'-GTATTCGGCGGTTCCCTATTC-3'	Denature: 95°C, 1 min. Anneal: 50.3°C, 1 min. Extend: 72°C, 1 min. Cycles 35
Rice	Microsatellite Sequence RM171 (http://gramene.org), product length: 325 bp	Forward: 5'-AACGCGAGGACACGTACTTAC-3' Reverse: 5'-ACGAGATACGTACGCCTTTG-3'	Denature: 95°C, 1 min. Anneal: 49.6°C, 1 min. Extend: 72°C, 1 min. Cycles: 35
Sorghum	Gamma Kafirin, promoter MML 4 region (AY294252.1), product length: 291 bp	Forward: 5'-AGGCCGGAGAGAGGACAATA-3' Reverse: 5'-ATGTGCATAGGCGTACGAGG-3'	Denature: 95°C, 1 min. Anneal: 52°C, 1 min. Extend: 72°C, 1 min. Cycles: 35

Results and Discussion

Performance: A comparison of the CTAB, DNeasy®, and Synergy™ DNA isolation methods showed that all three methods can generate quality DNA, despite significant variations in protocol. Ease of use, yields, and purity were not consistent between methods. The most notable difference, regardless of sample type, was the varying effort required to process a sample. The Synergy™ process was rapid compared to both the DNeasy® and CTAB methods, taking only 5 minutes to yield a lysate suitable for PCR or less than 30 minutes for alcohol precipitated DNA. Upon centrifugation, the Synergy™ extraction/grinding matrix rapidly removed most contaminants as compared to standard grinding matrix after grinding (Fig. 1).

Figure 1. Comparison of supernatant from Synergy™ grinding media (left) to standard grinding media (right).

Yield and Purity: Nine different plant samples were tested, and, in all cases, the Synergy™ kit yielded the greatest amount of DNA (Table 3). Surprisingly, CTAB and DNeasy® were about split on which method produced the second highest yield of DNA. This is contrary to many of the previous reports, which indicated CTAB methods yield more DNA than the DNeasy® method. On-the-other-hand, the Synergy™ protocol recovered approximately half (250 µl of 500 µl) of the homogenization buffer used for processing, yet total DNA yields still exceed those of CTAB and DNeasy®. It is unknown whether half of the DNA is lost with the remaining buffer or whether the DNA is excluded from the pellet and into the supernatant.

Table 3. Comparative DNA yields from Synergy™, CTAB, and DNeasy® methods. Values are displayed in ng/50 mg leaf tissue.

Sample	CTAB Method	DNeasy® Method	Synergy™ Method
Anthurium	452	830	1170
Corn	1653	1300	1800
Cotton	1600	750	2800
Grape	76	62	84
Rice	2120	488	2330
Rye	822	1980	2800
Sorghum	1730	704	2300
Soybean	292	480	612
Wheat	2060	1800	2870

The traditional measurement for DNA purity is to determine absorbance at both 260 and 280 nm and then calculate the 260/280 ratio. DNA preparations lacking protein contamination will have a ratio of 1.8; preparations with protein contamination absorb strongly at 280 nm due to aromatic rings on amino acid side chains and will lower this ratio. However, with plants, a second concern when isolating DNA is the contamination by polysaccharides and polyphenols. Both polyphenols and polysaccharides absorb strongly in the 230 nm range, thus measuring DNA solutions at 230 nm can also provide useful information as to DNA purity. Pure DNA has a 260/230 ratio of 2.0 and above. A lower ratio is indicative of some degree polysaccharide and polyphenol contamination.

Data from the comparative isolation shows that the Synergy™ chemistry was very effective at removing plant contaminants as seven of the nine plants extracted yielded 260/230 ratios above 2.0 (Table 4). Both the CTAB and DNeasy® methods were also effective with each yielding five ratios above 2.0. In general, all three methods were very effective at generating clean DNA. A similar result can be seen with the 260/280 ratios, with the three methods generating ratios very close to 1.8. All samples tested had ratios between 1.72 and 1.94. Lower ratios signify protein contamination, though these measurements are all reasonable. Higher ratios may be the result of some residual phenolics from the sample, but again the ratios are all relatively close to 1.8.

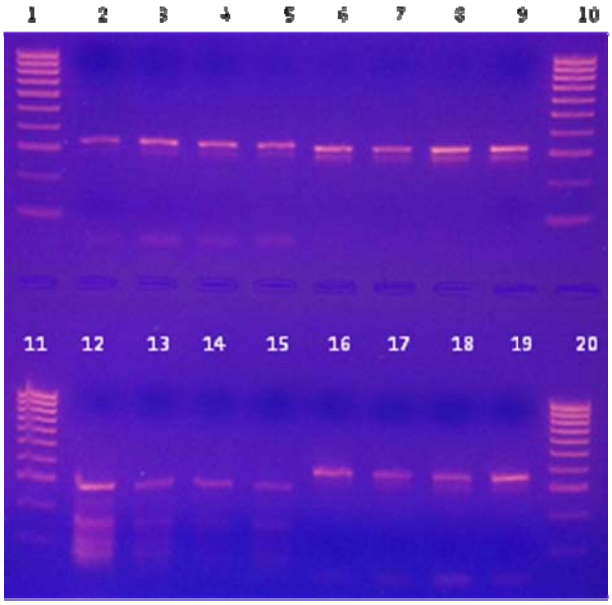
Table 4. Comparative purities of DNA isolated using Synergy™, CTAB, and DNeasy®.

	260/280			260/230		
Sample	**CTAB**	**DNeasy®**	**Synergy™**	**CTAB**	**DNeasy®**	**Synergy™**
Anthurium	1.82	1.81	1.85	1.96	1.97	2.03
Corn	1.90	1.87	1.74	1.94	1.96	2.06
Cotton	1.86	1.76	1.86	1.97	2.01	2.01
Grape	1.77	1.83	1.84	2.03	1.96	1.98
Rice	1.74	1.94	1.83	1.99	2.07	2.04
Rye	1.72	1.83	1.85	2.03	2.03	2.03
Sorghum	1.76	1.72	1.78	2.03	1.98	1.97
Soybean	1.86	1.90	1.76	2.05	2.04	2.04
Wheat	1.89	1.78	1.83	2.07	2.06	2.05

Amplification: The Synergy™ chemistry was originally designed so that plant DNA could be rapidly isolated for PCR applications. Consequently, the ability to amplify targeted DNA by PCR was a critical aspect of this study. To assess whether the isolated DNA could be effectively amplified, four previously developed PCR reactions for rice, grape, sorghum, and corn were performed (Table 2). Template DNA was isolated by each of the three methods being evaluated, as well as cleared lysate from the Synergy™ method.

All DNA extracted from the four plants could be amplified by PCR regardless of the method used to isolate the DNA (Fig. 2). The grape PCR reactions (lanes 6-9) yielded very clean DNA, while the sorghum PCR displayed several secondary products. This may certainly be the function of the reaction itself and not the DNA. It is important to note that the starting concentration of template (i.e., copy number) is different in each sample because a unit of volume (µl) and not mass was used for each reaction. The sorghum/Synergy™ PCR (lane12) was very robust, which could be linked to high concentration of template. Interestingly, the reactions using Synergy™ lysate (lanes 3, 7, 13, and 17) generated product comparable to the CTAB and DNeasy® methods, but generally less robust

than the complete Synergy™ method that included alcohol precipitation. Consequently, the original goal of developing Synergy™ as a rapid and easy method for quickly isolating plant DNA for PCR seems to have been achieved.



Gel contents:

1- Marker (100-1000 bp)
2- Rice Synergy™ IPA precipitated
3- Rice Synergy™ lysate
4- Rice CTAB
5- Rice DNeasy®
6- Grape Synergy™ IPA precipitated
7- Grape Synergy™ lysate
8- Grape CTAB
9- Grape DNeasy®
10- Marker (100-1000bp)
11- Marker (100-1000bp)
12- Sorghum Synergy™ IPA precipitated
13- Sorghum Synergy™ lysate
14- Sorghum CTAB
15- Sorghum DNeasy®
16- Corn Synergy™ IPA precipitated
17- Corn Synergy™ lysate
18- Corn CTAB
19- Corn DNeasy®
20- Marker (100-1000bp)

Figure 2. PCR of rice, grape, sorghum, and corn using Synergy™, CTAB, and DNeasy® isolated DNA.

Cost Analysis: Synergy™ and DNeasy® are both purchased as kits, so price-to-price comparison is relatively easy. Synergy™ currently costs about $2.25 per sample, while the DNeasy® is over $4.15 per sample. Cost of ancillary reagents is approximately the same; both protocols require alcohol precipitation, with the alcohol being supplied by the user. From a fixed cost perspective, the time required to isolate DNA using Synergy® chemistry is far less the DNeasy®, which translates into increased user efficiency and reduced wear on the researcher. CTAB protocols, in general, are homegrown methods which are less expensive than DNA isolation kits, but are hindered by the use of chloroform and numerous steps involved in the process. Additionally, chloroform is classified as a carcinogen and therefore has significant costs associated with hazardous materials, such as waste disposal and regulatory costs. CTAB protocols also have many time consuming steps, which lower user efficiency.

Conclusion

The Synergy™, CTAB, and DNeasy® chemistries are effective for isolating quality DNA from plant leaf tissue, though the Synergy™ method requires less manipulation by the researcher and less time from start to finish. With the nine plant leaf tissues tested, Synergy™ produced the greatest yield for all samples. The purity of the DNA as measured by 260/280 and 260/230 ratios were comparable between the three methods tested. The amplification of corn, rice, grape, and sorghum were all achieved using the DNA isolated by Synergy™, CTAB, and DNeasy®, however cleared lysate from the Synergy process was also used to generate PCR products. Regarding cost, Synergy™ was found to be about half the price of DNeasy®, while avoiding the risks and inconvenience of using chloroform associated with CTAB. Overall, the Synergy™ chemistry has allowed for the development of a rapid, cost effective method for isolating quality DNA as compared to widely used alternatives.

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