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A high-throughput plate method for nucleic acid extraction from beet leafhopper (Hemiptera: Cicadellidae) and potato psyllid (Hemiptera: Triozidae) for pathogen detection

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Plant pathogens that are transmitted by insect vectors cause considerable damage to crops when pests or pathogens are not detected early in the season and populations are not controlled. Knowledge of pathogen prevalence in insect pest populations can aid growers in their insect pest management decisions but requires the timely dissemination of results. This process requires that specimen capture, identification, nucleic acid extraction, and molecular detection of a pathogen(s) occur alongside a platform for sharing results. The potato psyllid (*Bactericera cockerelli*, Sulc; Hemiptera: Triozidae) and beet leafhopper (*Circulifer tenellus*, Baker; Hemiptera: Cicadellidae) transmit pathogens to potato and other vegetable or seed crops each season in the northwestern United States. While the potato psyllid has been tested for pathogen occurrence for the past decade, testing of the beet leafhopper is a new endeavor and substantially increases the specimen number that must be tested by our laboratories each season. To aid in the rapid processing of individual insect specimens, we optimized and validated a new high-throughput 96-well plate nucleic acid extraction method for use in place of a standard 1.5-ml single-tube extraction method. Processing efficiency, in terms of total specimens processed over a 2-day period, improved 2.5-fold, and the cost associated with processing a single sample was nearly cut in half with this newly developed plate nucleic acid extraction method. Overall, this method has proven to be an excellent tool for the rapid testing of large numbers of small, individual insect vectors to enable timely dissemination of data on pathogen prevalence to growers.

Key words: Liberibacter, phytoplasma, psyllid haplotyping

Introduction

Molecular techniques used to diagnose the presence of plant pathogens in insect vectors can provide accurate and timely reporting of potential pathogen presence in agroecosystems. However, a limitation of assessing pathogen incidence across a geographic region is that many samples must be processed every week. The feasibility of this is dependent upon the availability of supplies and reagents, laboratory equipment, and personnel to perform the field collections and molecular diagnostics. Given that platforms for sharing results to decision makers often exist for cropping systems (as does the case in our region and crop, at https://potatoes.decisionaid.systems/), routine reporting of pathogen prevalence throughout a growing season on these platforms can provide information that may assist growers in their pest management strategies by anticipating when pests or pathogens may arrive.

For over a decade, potato psyllids across the United States have been collected near potato fields using sticky traps and tested by molecular methods for "*Candidatus* Liberibacter solanacearum", the causal agent of the economically important zebra chip disease of potato (Goolsby et al. 2012, Munyaneza 2015). A modified small-scale nucleic acid extraction method that relies on Cetyltrimethyl ammonium bromide (CTAB, Zhang et al. 1998, Crosslin et al. 2006) has been routinely used for processing psyllid specimens because it yields a high quantity and quality of DNA that enables successful

pathogen detection and insect and pathogen population genotyping at relatively low cost (Goolsby et al. 2012, Swisher et al. 2014). In seasons with high insect pressure, psyllids are often tested in bulk of up to 30 specimens to ensure the timely processing and reporting of pathogen prevalence to growers (Crosslin et al. 2011).

In 2020, our laboratory sought to expand upon potato psyllid testing to include testing of the beet leafhopper, *Circulifer tenellus*, for the *Candidatus* Phytoplasma trifolii pathogen, Beet leafhopper transmitted virescence agent (BLTVA). The BLTVA pathogen causes potato purple top disease and subsequent tuber yield losses in the northwestern United States (Munyaneza et al. 2006), and populations of leafhoppers and BLTVA have been problematic over the past 5 yr (Wohleb 2023). From April to September of 2020, our lab received nearly 25,000 beet leafhoppers in addition to 3,500 potato psyllids from an insect monitoring network set up across the Columbia Basin of Washington State, with as many as 1,500 insect samples delivered weekly. Due to the high volume of samples, we were unable to provide timely results on pathogen testing of BLTVA from the beet leafhoppers, even with selecting a subset of samples to process each week. To achieve greater feasibility of disseminating pathogen prevalence in a timely manner required high-throughput methods.

The successful shift from a single-tube CTAB extraction method to a high-throughput plate extraction method required optimization and validation to ensure that DNA quantity and quality were not sacrificed for speed. The single-tube CTAB extraction method averaged 22.5 ± 8.8 and 54.4 ± 27.5 ng/µl of DNA from a single insect in a 50 µl final volume for sticky card captured potato psyllid and beet leafhopper, respectively (Swisher Grimm laboratory, unpublished). It routinely produces A_{260}/A_{280} values of 2.1 ± 0.07 and 2.0 ± 0.06 for psyllid and beet leafhopper, respectively. This paper details the optimization and validation process for the development of a high-throughput 96-well plate nucleic acid extraction protocol for pathogen detection and population genotyping from potato psyllid and beet leafhopper. Two different insect specimen grinding methods were validated to ensure that this high-throughput method could also be utilized in laboratories that do not have access to specialized equipment.

Materials and Methods

CTAB Extraction Method

The CTAB extraction protocol for insects by Zhang et al. (1998) with modifications by Crosslin et al. (2006) was used as a single-tube extraction method. Here, individual *Bactericera lobata* or *B. cockerelli* received from insect colonies maintained at the USDA-ARS research station in Wapato, WA, or *C. tenellus* collected from the Columbia Basin of Washington, were placed in 1.5 ml tubes. To each tube, 200 µl of CTAB buffer (Table 1) was added and incubated for 15 min in a 65 °C water bath. Insect samples were homogenized with sterile micropestles (USA Scientific, Ocala, FL), and 400 µl of CTAB buffer with β-mercaptoethanol was added to tubes and inverted 6 times to mix before incubating for 15 min in the 65 °C water bath. Tubes were then removed and left at room temperature for 2–3 min before adding 300 µl of cold chloroform (stored at 4 °C). The resulting solution was vortexed to form an emulsion and centrifuged at maximum speed (18,213 rcf) for 3 min. Approximately 500 µl of the upper aqueous phase was transferred, without disturbing the bilayer, to a new 1.5 ml tube containing 3 µl of 1:5 GlycoBlue (Thermo Fisher Scientific, Waltham, MA, USA), and 500 µl of ice-cold isopropanol (–20 °C) was added for nucleic acid precipitation. Tubes were inverted 10 times to mix and placed on ice for 10 min. Tubes were then centrifuged for 10 min at maximum speed, the supernatant was decanted, and nucleic acid pellets were washed with 500 µl of ice-cold 70% ethanol to remove residual salts. Pellets were dried at 37 °C to remove residual alcohol and resuspended in 50 µl water.

DIGSOL Extraction Method

A DIGSOL extraction method in a 96-well plate by Lagisz et al. (2010) was optimized in this study. For initial tests, the Lagisz 96-well plate extraction for insects was adapted for 1.5 ml tubes as follows. Individual *B. lobata* were homogenized with a sterile micropestle in 30 µl DIGSOL buffer (Table 1) freshly mixed with Proteinase K (Thermo Scientific, Waltham, MA, USA), vortexed, and incubated at either 37 °C overnight or at 55 °C for 3 h. After this initial incubation, 50 µl of 4M ammonium acetate was added to the tubes, vortexed, and incubated for another 30 min at room temperature. Following the second incubation, samples were centrifuged at maximum speed (18,213 rcf) for 15 min to pellet debris. As much supernatant was transferred as possible (50–70 µl) to a new 1.5 ml tube. To precipitate nucleic acids, 70 µl of 96% ethanol was added to the aqueous solution, inverted 6 times to mix, and centrifuged at maximum speed (18,213 rcf) for 15 min. The alcohol was then removed, and pellets were washed with 100 µl of 70% ethanol to remove residual salts. Tubes were centrifuged for 5 min at maximum speed (18,213 rcf), the alcohol was removed, and the open tubes were placed in a 95 °C heating block for 5 min to dry the pellets, following which the pellets were resuspended in 100 µl water.

The DIGSOL protocol was modified in several ways to improve nucleic acid yield. Modified factors included salts (50 µl of 4M ammonium acetate vs. 50 µl of 3M potassium acetate), alcohol

Table 1. Buffers used in this study

Buffer	Final Concentration	Amount for 25 ml
DIGSOL	20 mM EDTA	1 ml of 500mM EDTA pH 8.0
	50 mM Tris-HCl0	1.25 ml of 1M Tris pH 8.0
	0.4 mM NaCl	2 ml of 5M NaCl
	0.5% SDS	1.25 ml of 10% SDS
	20:1 mix ratio of 10mg/mL proteinase K	1.25 ml of [10mg/ml proteinase K]
		18.25 ml sterile H_2O
CTAB	2% CTAB (cetyltrimethyl ammonium bromide)	0.5 g of CTAB
	1.4 M NaCl	7 ml of 5 M NaCl
	20 mM EDTA	1 ml of 500mM EDTA pH 8.0
	100 mM Tris-HCl	2.5 ml of 1M Tris-HCl pH 8.0
	0.2% β-mercaptoethanol	50 µl of β-mercaptoethanol (add just before use)
		14.5 ml sterile H_2O

precipitants (70 µl 96% ethanol vs. 70 µl isopropanol), and adding 3 µl 1:5 GlycoBlue to the DNA precipitation step (see Table 2). Protocol optimizations were done in a series of tests. The initial 1.5 ml tube test compared the CTAB and DIGSOL protocols and included treatments with and without GlycoBlue (n = 5). Subsequent tests to assess salts and nucleic acid precipitant were done similarly (n = 5). A test using 8-tube PCR strips (USA Scientific, Ocala, FL) was conducted to assess cross-contamination in these smaller tubes and was used to compare the initial incubation time (3 h at 55 °C vs. overnight at 37 °C).

Optimization of 96-Well Plate Extractions

The DIGSOL protocol with modifications described earlier (3M potassium acetate, isopropanol, GlycoBlue, and resuspension in 50 µl water) was further optimized and validated in 96-well plates (TempPlate 96-well semi-skirt 0.2 ml PCR plates, USA Scientific, Ocala, FL) using 2 different grinding methods. For both methods, *B. lobata*, *B. cockerelli*, and/or *C. tenellus* specimens were placed in alternating wells along with no-template controls (where no specimen was added) in a grid-like fashion (Fig. 1).

For homogenization with pipette tip micropestles, all insects were ground in 10 µl DIGSOL buffer (Table 1) with freshly added proteinase K using an eight-channel pipette with grinding pipette tips. Sterile pipette tip micropestles were made by melting the tip of a 200 µl pipette tip such that a small bead for grinding would form (Lagisz et al. 2010). After insects were thoroughly ground, an additional 20 µl of DIGSOL buffer with Proteinase K was added to each well. The plates were sealed, vortexed, and briefly spun down using a microplate microcentrifuge (Benchmark Scientific, Sayreville, NJ, USA) to collect debris and buffer at the bottom of wells, then incubated either overnight at 37 °C or for 3 h at 55 °C (Lagisz et al. 2010).

Two tests were done in 96-well plates to determine the optimal method for high DNA extraction quality/quantity and lack of cross-contamination between wells using insects homogenized with micropestles. The first test using *B. lobata* specimens compared 2 sealing methods, by PCR film or by PCR 8-cap strips (n = 48). The second test was done to validate pathogen detection sensitivity of nucleic acids recovered by the DIGSOL 96-well plate extraction (n = 94) and CTAB 1.5-ml tube extraction (n = 46) on "*Ca.* L. solanacearum" in potato psyllid and BLTVA in beet leafhopper. Insects in the plate extractions alternated with no-template controls every other well to determine the occurrence of cross-contamination (Fig. 1). Assessment of cross-contamination was done using PCR targeting the CO1 gene of *B. lobata* and beet leafhopper, and using PCR targeting the "*Ca.* L. solanacearum" 16S gene in tests containing potato psyllids (due to potato psyllids originating from a near 100% infected colony).

For homogenization with beads, either a beet leafhopper or potato psyllid was placed in Qiagen 96-well microcollection tubes along with a 3-mm stainless steel grinding bead (OPS Diagnostics, Readington, NJ, USA). Following this, 30 µl of DIGSOL buffer was added to each well and plates sealed with Qiagen 96-well microcollection tube cap strips (Qiagen, Hilden, Germany) securely pressed down with a strip cap tool. Plates were then disrupted using the TissueLyser II (Qiagen, Hilden, Germany) at 30 Hz for 30 s, twice. Plates were flipped between disruptions to ensure uniform disruption between wells. Plates were then spun down in an Eppendorf 5810R centrifuge adapted with the A-4-DWP deep plate rotor (Eppendorf, Hamburg, Germany) with a short spin up to 657 rcf to collect buffer and insect debris into the bottom of the wells. Plates were then incubated overnight at 37 °C, only. After incubation, microcollection tube plates were briefly spun down in the Eppendorf 5810R (~532–657 rcf), to reduce possibility of cross-contamination between wells, and the contents transferred to a 96-well PCR plate (~30 µl).

After incubation, contents were transferred to a PCR plate in the case of the bead disruption method, and plates disrupted with the pipette tip micropestles were briefly spun down (to collect condensation). Regardless of homogenization method, 50 µl of 3M potassium acetate was added to each well, the plate was resealed, vortexed, and incubated at room temperature for an additional 30 min. After this incubation, plates were centrifuged at maximum speed (2473 rcf) for 15 min to pellet debris using a 96-well plate rotor (Eppendorf Centrifuge 5430 with A-2-MTP rotor, Framingham, MA, USA). Supernatant was transferred (~70 µl) to new PCR plates that had 3 µl of 1:5 GlycoBlue, and 70 µl of isopropanol was added. Plates were sealed with new PCR cap strips, inverted approximately 6 times to mix, and centrifuged at maximum speed (2,473 rcf) for 15 min. After DNA was pelleted, the supernatant was removed from plates by inverting over a waste container, and then pressing 3 times onto a dry section of a stack of clean paper towels to remove excess liquid. To wash, 100 µl of 70% ethanol was added to each well to remove residual salts, plates were resealed, and centrifuged at maximum speed (2,473 rcf) for 5 min. The alcohol wash was removed in the same method as with the supernatant by inverting and pressing onto a clean paper towel stack. Plates were dried at 95 °C for 5–8

Table 2. Single 1.5 ml tube tests were conducted using *B. lobata* insect specimens to compare the conventional CTAB extraction method with the Lagisz et al. (2010) method (DIGSOL) that was designed for a 96-well plate. Modifications were made to the salt, precipitant, and use of GlycoBlue. Values within tests that have different letters are significantly different according to Tukey's honestly significant difference

Test	Insect	Protocol	Protocol modifications			DNA ng/µl	Final Vol. µl	Pr(>F)
					GlycoBlue			
1.5ml tube test 1	*B. lobata*	DIGSOL			Yes	17.95 ± 7.76 a	100	0.006
		DIGSOL			No	13.59 ± 5.66 a	100	
		CTAB			Yes	40.4 ± 18.03 b	50	
			Salt	Precipitant	GlycoBlue			
1.5ml tube test 2	*B. lobata*	DIGSOL	Ammonium acetate	96% EtOH	Yes	11.32 ± 6.62	100	0.310
			Ammonium acetate	Isopropanol	Yes	9.8 ± 3.41	100	
			Potassium acetate	96% EtOH	Yes	15.3 ± 8.18	100	
			Potassium acetate	Isopropanol	Yes	18.88 ± 9.18	100	
1.5ml tube test 3	*B. lobata*	DIGSOL	Potassium acetate	Isopropanol	Yes	39.82 ± 21.04	50	0.951
		CTAB	Potassium acetate	Isopropanol	Yes	40.4 ± 18.03	50	

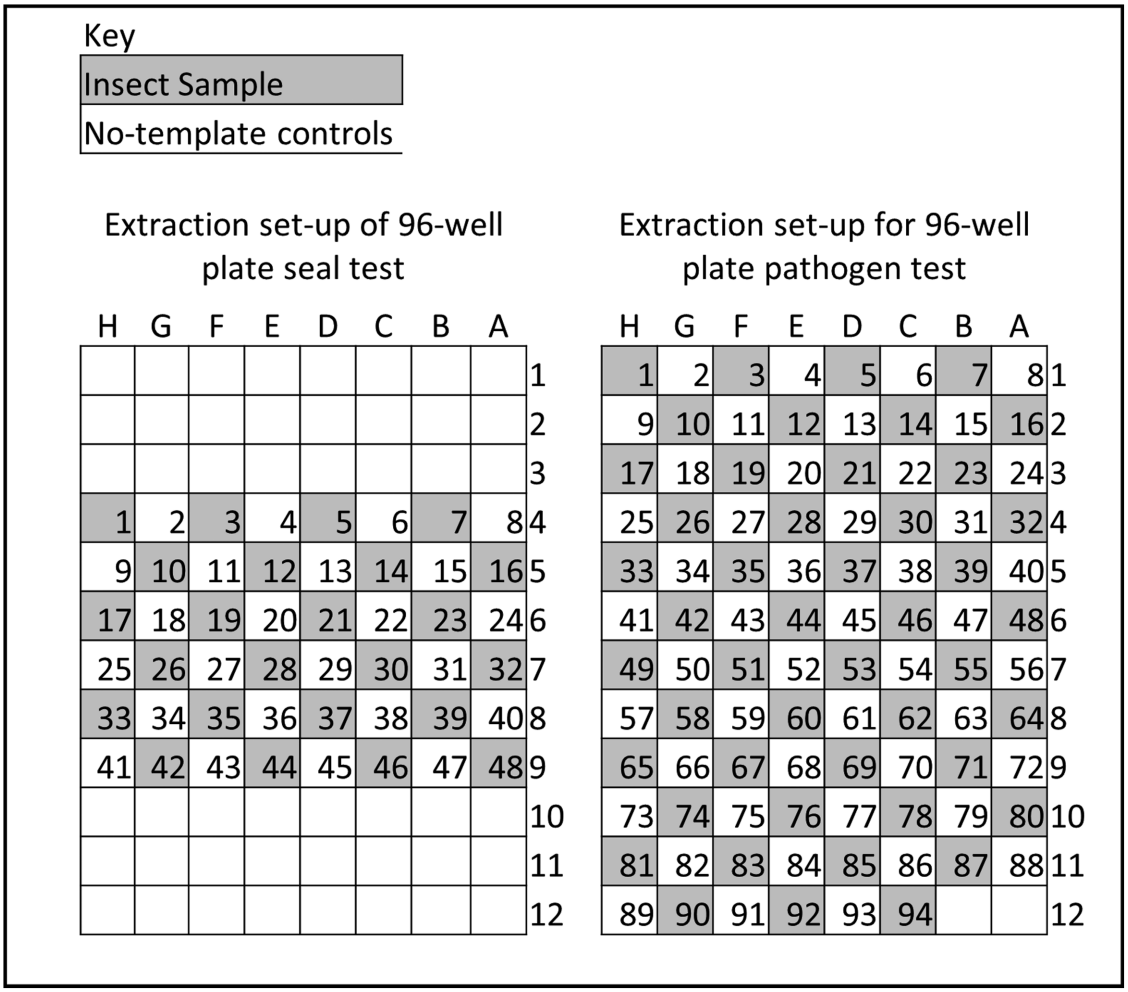

Fig. 1. Insect specimen placement in PCR strip and plate tests. No-template controls are empty insect-free wells interspersed throughout the strips and plates.

min on a heat block. Once nucleic acid pellets were dried, the pellets were resuspended in 50 µl H_2O.

Many tests were done with the TissueLyser to determine the best combination of bead size/material and duration of disruption for DNA yield and quality. All tests were performed on potato psyllids and beet leafhoppers collected either from sticky cards received from Washington State University cooperator, Carrie Wohleb or from greenhouse-maintained colonies at the USDA-ARS laboratory in Wapato, WA. Beads tested were a combination of sizes and materials which included 3/32″ stainless steel (Grainger, Lake Forest, IL, USA), 3-mm tungsten carbide (Qiagen, Hilden, Germany), 3-mm stainless steel (OPS Diagnostics, Lebanon, NJ, USA), and 5-mm stainless steel (Qiagen, Hilden, Germany). Disruption duration with the TissueLyser varied from a total of 30 s up to 60 s, at 30 Hz. TissueLyser tests were not done in full factorial design, the most rigorous bead/duration test was performed with 3-mm stainless steel beads at a total of 30, 40, and 60 s (with plates turned around halfway through this duration) on potato psyllids and beat leafhoppers obtained from both sticky cards and greenhouse cultures (n = 12).

Determination of Extraction Yield, Quality, and Pathogen Detection

All DNA samples were measured for quantity and quality using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA); pure DNA is considered to have an A_{260}/A_{280} value of 1.8. In addition to measuring sample DNA concentration and quality, PCR was performed and visualized by gel electrophoresis to confirm successful DNA extraction of insect samples. PCR and gels were also performed on no-template controls to check for contamination between wells in the PCR strip and plate tests. PCR of the mitochondrial CO1 gene with universal primer pairs LCO 1490 (GGTCAACAAATCATAAAGATATTGG) and HCO 2198 (TAAACTTCAGGGTGACCAAAAAATCA) was used to confirm successful DNA extraction of *B. lobata* (Folmer et al. 1994). The thermal cycling conditions for CO1 were as follows: 95 °C for 1 min, 40 cycles of 95 °C for 15 s, 48 °C for 1 min, and 72 °C for 30 s, followed by 72 °C for 7 min. To confirm presence of "*Ca.* L. solanacearum" in DNA extracted from potato psyllid, traditional PCR was performed using Lso primers OA2 (GCGCTTATTTTTAATAGGAGCGGCA) and OI2C (GCCTCGCGACTTCGCAACCCAT) (Jagoueix et al. 1996, Liefting et al. 2009, Crosslin et al. 2011). The thermal cycling conditions for "*Ca.* L. solanacearum" were as follows: 94 °C for 2 min, 40 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min, followed by 72 °C for 5 min. For confirming the presence of BLTVA phytoplasma, a nested PCR protocol was used with primers PHY-P1 (AAGAGTTTGATCCTGGCTCAGGATT) and PHY-P7 (CGTCCTTCATCGGCTCTT), followed by PHY-Fu5 (CGGCAATGGAGGAAACT) and BLTVA-int (GATGATTTTAGTATATATAGTCC) (Deng and Hiruki 1991,

Lorenz et al. 1995, Schneider et al. 1995, Smart et al. 1996, Crosslin et al. 2006). Thermal cycler conditions for both PHY-P1/PHY-P7 and PHY-Fu5/BLTVA-int primer pairs were as follows: 94 °C for 2 min, 30 cycles of 94 °C for 15 s, 55 °C for 90 s, and 72 °C for 90 s, followed by 72 °C for 5 min. Standard conventional PCR using Go Taq polymerase (Promega, Madison, WI, USA) was used as described in Crosslin et al. (2011), and reactions were visualized on a 1.5% agarose gel with ethidium bromide staining.

A comparison of pathogen detection was conducted on beet leafhopper for BLTVA and on potato psyllid for "*Ca*. L. solanacearum" between the single-tube CTAB (n = 46) and 96-well plate DIGSOL extraction method (n = 47). For DIGSOL, both homogenization methods were used for potato psyllids, but only the pipette tip micropestle was used for the beet leafhoppers. For these single-tube CTAB and plate DIGSOL extractions, potato psyllids were obtained from "*Ca*. L. solanacearum"-infected colonies (near 100% infection), while beet leafhoppers were from sticky traps placed in a single field in the Columbia Basin (received from Washington State University cooperator, Carrie Wohleb). These specific beet leafhoppers were chosen from a single field site that, from previous testing, had a high incidence of BLTVA. It was expected for the beet leafhoppers that comparable rates of BLTVA infection would be seen from both the CTAB single-tube and DIGSOL plate extraction methods. All plate extractions were set up with no-template controls interspersed with insect specimens; no-template controls were assessed for cross-contamination using PCR to detect "*Ca*. L. solanacearum" (potato psyllid) or cytochrome oxidase I (beet leafhopper).

High-resolution melting (HRM) analysis was conducted on a subset of potato psyllid specimens, extracted in the 96-well format, as described in Swisher et al. (2013) using primer pair COI F3 (TACGCCATACTAGCAATCGG) and COI meltR (TGAAATAGGCACGAGAATCAA), and primer pair COI meltF (GGATTCATTGTTTGAGCACATC) and COI meltR. The LightCycler 480 II (Roche Applied Science, Indianapolis, IN, USA) and the LightCycler Gene Scanning software were used for analyses. Control psyllids of central, western, and northwestern haplotypes were used from previous single-tube CTAB extractions of potato psyllids maintained at the USDA-ARS laboratory in Wapato, WA.

Statistical Analyses

Differences in DNA concentration and quality between treatments were statistically analyzed by ANOVA using the "lm" function in R. Mean separations for significant effects were conducted when appropriate using Tukey's HSD post hoc test (part of the "Agricolae" package in R). All data analysis was performed using R statistical software in R Studio.

Results

Optimization and Validation of the 96-Well Extraction Protocol

Initial analyses of the Lagisz et al. (2010) high-throughput nucleic acid extraction protocol were done using *B. lobata* specimens in single-tube extractions to compare this method to the conventional single-tube CTAB method used routinely in our laboratory. The CTAB protocol had a higher concentration of nucleic acids across samples (40.4 ± 18.03 ng/µl) compared to the Lagisz method with a 3-h incubation at 55 °C following insect grinding with micropestles (Table 2). This was true both without (13.59 ± 5.66 ng/µl) and with (17.95 ± 7.76 ng/µl) GlycoBlue coprecipitant added to ensure adequate visualization of the nucleic acid pellets to prevent loss during the wash step. The CTAB method resuspends nucleic acid pellets in 50 µl H_2O, while the Lagisz et al. (2010) method resuspends in 100 µl buffer, indicating that our modified Lagisz et al. (2010) method may generate nearly comparable yields to the CTAB method if resuspended in the lower volume.

Modifications were made to the Lagisz et al. (2010) high-throughput extraction protocol in an attempt to increase the extraction yield, again in single tubes. Here, 4M ammonium acetate was substituted with 3M potassium acetate, and 96% ethanol was substituted with isopropanol for precipitation of nucleic acids. GlycoBlue coprecipitant was again used to allow visualization of the nucleic acid pellet during the wash steps, and the initial incubation was done overnight at 37 °C instead of 3 h at 55 °C (both incubation options are described in Lagisz et al. 2010). Although not statistically significant, these modifications numerically improved the average nucleic acid yield from 11.32 ± 6.62 ng/µl (ammonium acetate and ethanol) to 18.88 ± 9.18 ng/µl (potassium acetate and isopropanol), but the yield was still lower than the CTAB method with an average yield of 40.4 ± 18.03 ng/µl (Table 2).

The average nucleic acid concentration was increased to 39.82 ± 21.04 ng/µl using 3M potassium acetate, isopropanol, GlycoBlue, an initial 3-h incubation at 55 °C, and re-suspending in just 50 µl of H_2O (Table 2). This new protocol was subsequently tested in PCR strips to mimic the 96-well plate well size (Fig. 1). With these modifications, a final comparison was made between the 2 incubation treatments in PCR 8-tube strips: overnight at 37 °C and 3 h at 55 °C. There was no significant difference in nucleic acid yield between incubation treatments (P = 0.836) (Table 3).

Following our single-tube and incubation assays, we next validated our modified Lagisz protocol in a 96-well plate using *B. lobata* in a grid-like format of insect specimens interspersed with no-template controls (Fig. 1 and Lagisz et al. 2010), homogenized with pipette tip micropestles. For this, 2 different sealing methods were used: sealing film (used by Lagisz et al. 2010) and PCR 8-strip caps. There was a significant difference between treatments for nucleic acid yield. Average nucleic acid yield was 11.59 ± 10.49 ng/µl with the sealing film and 37.46 ± 27.16 ng/µl with the PCR strip cap (P < 0.001) (Table 3). Extraction quality was determined by assessing A_{260}/A_{280} ratio. The average A_{260}/A_{280} ratio was not significantly different between sealing methods (P = 0.139), with 1.91 ± 0.26 and 1.82 ± 0.10 for the extractions done using the sealing film and the PCR strip caps, respectively. PCR amplification of the insect CO1 gene was used to determine if cross-contamination occurred in the no-template controls. There were 3 instances of cross-contamination with sealing film, but no evidence of contamination using the PCR strip caps.

The efficiency of our modified high-throughput extraction method was compared to the conventional CTAB single-tube nucleic acid extraction protocol with beet leafhopper and potato psyllid that are routinely analyzed for pathogen detection in our lab (Table 3). As with our initial 96-well extraction and PCR test using *B. lobata*, our analysis was done by interspersing no-template controls among insect specimens to assess cross-contamination (Fig. 1). No contamination was seen in the no-template controls in the beet leafhopper or potato psyllid plates (Table 3). For the beet leafhopper, mean DNA concentration of samples extracted using our modified method (with pipette tip micropestles) was 60.87 ± 31.49 ng/µl compared to the CTAB extraction method of 51.9 ± 22.59 ng/µl (P = 0.186). For potato psyllid, mean DNA concentration generated with our modified method was 32.5 ± 15.37 ng/µl compared to 29.15 ± 7.46 ng/µl with the CTAB single-tube extraction method (P = 0.119). Quality of nucleic acids extracted by our high-throughput method

Table 3. PCR strip and plate tests were conducted using *B. lobata*, *B. cockerelli* (potato psyllid), and *C. tenellus* (beet leafhopper) to compare the conventional CTAB extraction method with the modified Lagisz et al. (2010) DIGSOL method designed in this study for use in a 96-well plate. Comparisons were made between initial incubation (3 h at 55 °C vs. overnight at 37 °C) and between sealing method on the plate (PCR film vs. individual PCR cap strips)

Test	Insect	Protocol	Initial incubation	DNA ng/µl	Pr(>F)	A260/A280	Pr(>F)	Cross-contamination
PCR strip test 1	*B. lobata*	DIGSOL	3 hours at 55°C	22.66 ± 9.25	0.836	1.87 ± 0.06	0.035	0
			Overnight at 37°C	21.30 ± 14.31		1.74 ± 0.14		0
			Plate seal					
96-well plate test 1	*B. lobata*	DIGSOL	PCR film	11.59 ± 10.49	<0.001	1.91 ± 0.26	0.139	3
			PCR cap strips	37.46 ± 27.16		1.82 ± 0.10		0
96-well plate test 2	Potato Psyllid	DIGSOL	PCR cap strips	32.5 ± 15.37	0.119	1.98 ± 0.04	0.039	0
	(*Bactericera cockerelli*)	CTAB	N/A (1.5ml tube)	29.15 ± 7.46		1.96 ± 0.05		N/A
	Beet Leafhopper	DIGSOL	PCR cap strips	60.87 ± 31.49	0.186	1.90 ± 0.05	<0.001	0
	(*Circulifer tenellus*)	CTAB	N/A (1.5ml tube)	51.90 ± 22.59		1.96 ± 0.04		N/A

was determined by assessing A_{260}/A_{280} ratio. For the beet leafhopper extracted with our method (including PCR strip caps for sealing), the average ratio was 1.90 ± 0.04, while the average ratio using the CTAB extraction method was 1.96 ± 0.04 ($P < 0.001$). For the potato psyllid extracted with our method, the average ratio was 1.98 ± 0.04, while the average ratio using the CTAB extraction method was 1.96 ± 0.05 ($P = 0.039$).

To reduce the time and resources required for pipette tip micropestle homogenization of insect specimens, bead homogenization with a TissueLyser was used as an alternative. Here, insect source and bead disruption optimization assays were conducted. In general, whether an insect was collected on sticky cards from the field or live captured from greenhouse-maintained colonies, insect source did not have an effect of DNA yield or quality when homogenized by TissueLyser with a 3-mm stainless steel bead (Table 4). The exception was the DNA quality from potato psyllids captured on sticky cards which had an A_{260}/A_{280} value of 1.62 ± 0.19 compared to colony potato psyllids which had an A_{260}/A_{280} of 1.69 ± 0.10 ($P = 0.048$). TissueLyser disruption optimization results with a 3-mm stainless steel bead are in Table 4. For both potato psyllids and beat leafhoppers, disruption duration had a strong effect on DNA yield ($P = 0.005$ and $P < 0.001$, respectively). For quality of DNA, disruption duration had a significant effect on beet leafhopper ($P < 0.001$) and a marginal effect for potato psyllids ($P = 0.096$). A significant interaction was not found between disruption duration and insect source for either DNA yield or quality. A total disruption duration of 60 s at 30 Hz had the best combination of DNA yield and quantity for both beet leafhopper (67.11 ± 24.22 ng/µl and 1.78 ± $0.08_{260/280}$) and potato psyllids (28.75 ± 16.6 ng/µl and 1.70 ± $0.12_{260/280}$) (Table 4).

A final analysis was done that compared the CTAB single-tube protocol to the results of the optimized DIGSOL with micropestle homogenization and DIGSOL with TissueLyser homogenization (3-mm stainless steel beads at 30 Hz for a total of 60 s) for both beet leafhopper and potato psyllid DNA yield and quality, with yield and quality averaged across insect source. There were significant differences between optimized protocols in terms of DNA yield and quality for beet leafhopper, and in DNA quality for potato psyllids (Fig. 2). There was no significant difference in DNA yield between optimized protocols for potato psyllids.

Pathogen Detection

Sticky card beet leafhoppers were tested for BLTVA, the causal agent of potato purple top, and the no-template controls were tested for detection of insect CO1 gene. Colony reared potato psyllids and interspersed no-template controls were tested for the zebra chip pathogen, "*Ca.* L. solanacearum". There was no evidence of cross-contamination in no-template controls for either the beet leafhoppers or potato psyllids, regardless of the homogenization method used, confirming that our modified high-throughput methods were effective. Comparison of pathogen incidence was done from these plate samples and single-tube CTAB extractions. For BLTVA testing of the beet leafhoppers, 29 of 47 specimens (61.7%) extracted with our modified Lagisz et al. (2010) 96-well plate method (with pipette tip micropestle homogenization) were BLTVA-positive, while 23 of 46 specimens (50%) extracted with the CTAB method tested positive. This difference is likely due to randomness in sample selection, not extraction method, as beet leafhoppers were arbitrarily selected from a single field site for both extraction methods. All potato psyllid samples from our modified Lagisz et al. (2010) extraction method (with either pipette tip micropestle or bead homogenization) and the conventional CTAB method were confirmed positive for "*Ca.* L. solanacearum" with PCR.

Psyllid Haplotyping using HRM Analysis

HRM analysis of the psyllid COI mitochondrial gene was conducted to compare psyllid haplotypes between specimens extracted using the CTAB method and those extracted with our modified Lagisz et al. (2010) plate method using either pipette tip micropestle disruption or bead disruption of the insect tissue. Using the COI meltF and COI meltR primer pair that identifies the western psyllid haplotype, melting peaks were similar between the extraction methods for all 3 psyllid populations assessed (central, northwestern, and western). A slight shift in melting peak to a lower temperature (~0.2 °C) was seen with samples extracted using our modified Lagisz et al. (2010) method when the COI F3 and COI meltR primers were used to identify the central psyllid haplotype. This shift was more prevalent in samples subjected to the pipette tip micropestle disruption as compared to those subjected to the bead disruption.

Discussion

Our goal was to develop a rapid and inexpensive 96-well plate insect extraction protocol with high nucleic acid yield and quality for conducting subsequent pathogen diagnostic assays. Using a 96-well plate extraction method of Lagisz et al. (2010) as a starting point, we modified the protocol to ensure that extraction yield and quality were similar to those produced by the standard CTAB 1.5-ml tube extraction protocol used for beet leafhopper and potato psyllid.

Table 4. Comparison of insect collection source (sticky card vs. colony) and bead disruption duration for both potato psyllid and beet leafhopper specimens using 3-mm stainless steel beads. For comparison of insect specimen source (sticky card vs. colony), values averaged across disruption duration. For comparison of disruption duration (30, 40, or 60 s), values are averaged between collection sources. Values within tests that have different letters are significantly different according to Tukey's honestly significant difference

Insect	Collection Source	DNA ng/µl		Pr(>F)	A260/A280		Pr(>F)
Potato Psyllid *(Bactericera cockerelli)*	Sticky Card	21.43 ± 10.43		0.138	1.62 ± 0.19		0.048
	Colony	25.69 ± 14.91			1.69 ± 0.10		
Beet Leafhopper *(Circulifer tenellus)*	Sticky Card	61.06 ± 38.38		0.447	1.68 ± 0.11		0.634
	Colony	55.68 ± 26.65			1.68 ± 0.12		
	Disruption Duration (s)						
Potato Psyllid *(Bactericera cockerelli)*	30	17.13 ± 7.9	a	0.005	1.65 ± 0.12		0.096
	40	24.80 ± 10.35	ab		1.61 ± 0.20		
	60	28.75 ± 16.6	b		1.70 ± 0.12		
Beet Leafhopper *(Circulifer tenellus)*	30	38.44 ± 14.44	a	<0.001	1.57 ± 0.09	a	<0.001
	40	69.55 ± 43.88	b		1.68 ± 0.08	b	
	60	67.11 ± 24.22	b		1.78 ± 0.08	c	

Homogenization of the insect specimens was successful using either pipette tip micropestles or beads, enabling the protocol to be utilized in laboratories that do not have access to a Qiagen TissueLyser machine, or similar equipment, that is required for homogenization with beads.

With this optimized and validated protocol for the modified Lagisz et al. (2010) method (designated as the DIGSOL plate extraction method), processing speed of insect samples from our monitoring network has been greatly increased. Using the traditional CTAB single-tube extraction method, 360 specimens can be processed over 2 days. In comparison, our modified DIGSOL 96-well plate extraction method allows 736 specimens to be processed over 2 long days using pipette tip micropestles for homogenization, or 920 specimens to be processed over 2 normal days using beads for homogenization. In addition to this increase in processing efficiency, having nucleic acid extractions in a 96-well plate format has also increased our molecular diagnostic pathogen screening, as it no longer requires opening individual 1.5-ml tubes to retrieve the DNA samples for PCR analysis, and transfer of samples to 96-well PCR plates for testing is seamless.

In addition to this increase in processing efficiency the plate extraction method is also cost-effective compared to the single-tube extraction protocol. The plate extraction with pipette tip micropestles costs less both in terms of plastics and reagents used and is overall cheaper by our estimates of cost per sample ($0.43 vs. $1.53, respectively). Though the plate extraction cost per sample does increase substantially with the use of TissueLyser bead disruption, to an estimate of $0.87 per sample, it is still less than the estimate for the single-tube extraction. This decrease in the cost of plastics and reagents is further supplemented by the reduction in labor per sample.

Although the numerical increase in DNA ng/µl with our DIGSOL 96-well plate extraction method was significant only in beet leafhoppers extracted with TissueLyser disruption (with 3-mm stainless steel beads at 30 Hz for a total of 60 s), results indicate that the modified plate extraction method is comparable to the 1.5-ml tube CTAB extraction method. Our optimized DIGSOL protocol also compared well to the DNA yield of insects reported by Lagisz et al. (2010). Lagisz et al. validated their protocol on red flour beetle (*Tribolium castaneum*) and pea aphid (*Acyrthosiphum pisum*) which are comparable to potato psyllid and beet leafhopper in size, respectively. They reported their DNA yield as total DNA in sample (µg/insect) with an average of 0.8 ± 0.30 in red flour beetle and 3.67 ± 2.38 in pea aphid, compared to our validation on potato psyllid (1.63 ± 0.77 µg/insect) and beet leafhopper (3.04 ± 1.57 µg/insect), respectively. Like Lagisz et al. (2010) we saw large variation in DNA yield between individual insects. It is possible this variation could be due to between well differences in how thorough grinding of samples was. In the case of beet leafhoppers trapped on sticky cards, variation in insect quality could be a reason for large variation in DNA ng/µl between individual insects as some individuals are mangled, smashed, missing body parts, etc. However, PCR and gel electrophoresis was able to detect target DNA in as low as –0.1 DNA ng/µl (according to spectrophotometer reading, which is inaccurate below 2.0 ng/µl).

Initially, multiple sizes and types of beads were tried with the TissueLyser. These ranged from 2.3- to 5-mm beads and included both stainless steel and tungsten carbide. We found that for the size of insects we used a 5-mm grinding bead was too large, with specimens remaining intact during a 60-s disruption. Though we did find initial success with a 2.3-mm bead in disrupting insect tissue, such a size of bead was not compatible with the 96-well bead dispenser we acquired (which was designed for 3-mm beads). We also had issues with beads that were made of tungsten carbide, which appeared to react with the DIGSOL buffer during incubation, staining the solution a murky gray color and resulting in abnormal spectrophotometry curves and A_{260}/A_{280} values downstream (both in insect samples and no-template controls). We also tried 3-h incubations with the TissueLyser microcollection tube 96-well plate, but the tube caps used for that part of the procedure would pop off at 55 °C, resulting in evaporation and cross-contamination. For these reasons, TissueLyser optimization with respect to bead size and incubation method, was limited to 3-mm stainless steel beads and an overnight incubation at 37 °C. Similar to how TissueLyser cap strips would pop off at 55 °C, with micropestle disruption the film seal would fail during the initial incubation (either the 55 °C for 3 h or the 37 °C overnight) compared to cap strips. This resulted in evaporation and cross-contamination, and is the likely explanation for why the cap strip sealing method was 3-fold higher in final DNA concentration as compared to the film sealing method.

One thing that was a concern in the DIGSOL plate extraction is the amount of salt that carries over to the final solution, as there is no step in the protocol that removes excess EtOH wash with a fine tipped pipette like there is in the CTAB protocol. This was a concern for us as we use HRM analysis on potato psyllid DNA to determine and compare haplotypes of potato psyllids. Melt peaks are highly influenced by salt concentrations in DNA samples (Swisher Grimm personal observation) and we found that there were slight degree

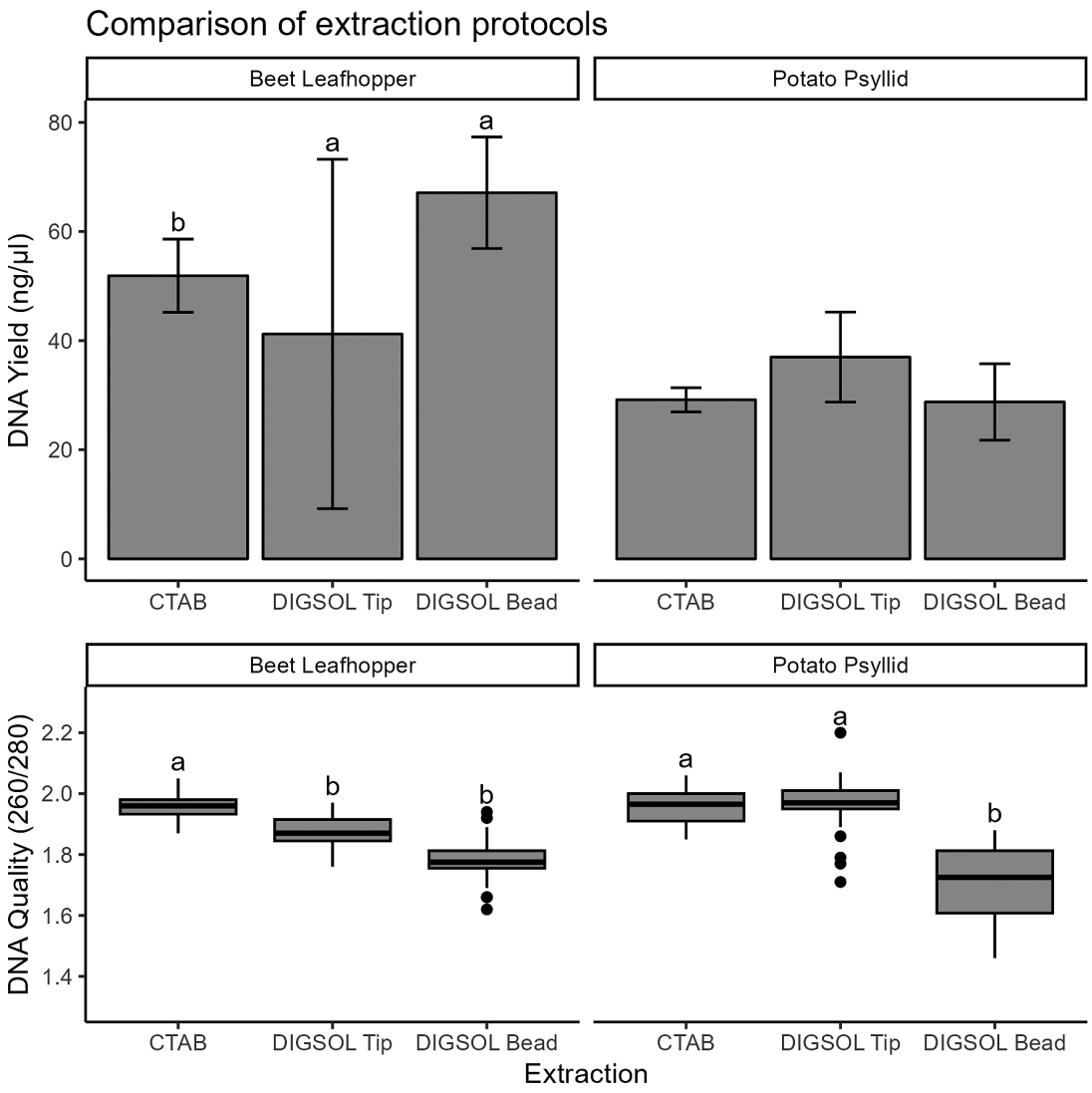

Fig. 2. Comparison of extraction protocols on DNA quantity/quality of beet leafhopper and potato psyllids. Values of bars and box plots are averaged across insect source for each insect. Bars or box plots with different letters within treatments are significantly different according to Tukey's honestly significant difference.

shifts in haplotype controls extracted by our DIGSOL plate method when compared to controls extracted by the standard CTAB 1.5 ml tube method. This degree shift could be falsely construed as a new haplotype if the tested samples were extracted by a different method than the control and so it will be important that tested samples and controls are extracted by the same method.

Insects used in this study came from colonies maintained at the USDA-ARS laboratory in Wapato, WA, or from insect sticky traps from the field in the Columbia Basin production area of central Washington State, USA. Whereas the *B. lobata* was only live captured and frozen prior to extractions, both the potato psyllid and beet leafhopper samples were either live captured from colonies or collected via yellow sticky traps, stored at –20 °C and removed before extraction. Insect "source" was consequently analyzed for an effect on DNA yield and quality in the TissueLyser optimization, which was only significant in terms of lower DNA quality for sticky card captured potato psyllids, with no effect on yield. Overall, we were able to demonstrate that our new DIGSOL 96-well plate extraction method can successfully extract colony insect specimens and field-caught insects exposed to harsher environmental conditions or sticky trap residues.

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Author Contributions

Christopher Gorman (Formal analysis [Lead], Investigation [Lead], Methodology [Lead], Validation [Lead], Writing – original draft [Lead], Writing – review & editing [Equal]), David Crowder (Funding acquisition [Equal], Writing – review & editing [Supporting]), and Kylie Swisher Grimm (Conceptualization [Lead], Funding acquisition [Equal], Methodology [Supporting], Project administration [Lead], Resources [Lead], Supervision [Lead], Writing – original draft [Supporting], Writing – review & editing [Equal])

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