

Semi-automated, Membrane-Based Protocol for DNA Isolation from Plants

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Abstract Many plant species are considered difficult for DNA isolation due to their high concentrations of secondary metabolites such as polysaccharides and polyphenols. Several protocols have been developed to overcome this problem, but they are typically time-consuming, not scalable for high throughput and not compatible with automation. Although a variety of commercial kits are available for plant DNA isolation, their cost is high and these kits usually have limited taxonomic applicability. In a previous study we developed an inexpensive automation-friendly protocol for DNA extraction from animal tissues. Here we demonstrate that a similar protocol allows DNA isolation from plants.

Keywords DNA isolation · Glass fiber plates · Plants · Automation · DNA barcoding

Abbreviations

CTAB cetyltrimethylammonium bromide
PVP polyvinylpyrrolidone
ILB insect lysis buffer
GuSCN guanidine thiocyanate
SDS sodium dodecyl sulfate

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Introduction

Isolation of high quality DNA remains a limiting step in large-scale plant molecular research. The presence of polysaccharide cell walls in plants is a significant obstacle to obtaining sufficient yields of high quality DNA. In addition to the extra steps required to grind or pulverize plant tissue to break the cell wall, the incomplete removal of polysaccharides or secondary metabolites, such as polyphenols, during DNA extraction often inhibits enzymatic reactions (i.e. PCR) and causes DNA degradation after long-term storage (Porebski et al. 1997; Schlink and Reski 2002; Sharma et al. 2002).

Common DNA isolation methods that rely on ethanol to precipitate DNA from extraction buffers are prone to these problems as polysaccharides often co-precipitate with DNA. Although methods are available that yield high quality plant DNA via binding to silica columns or beads in the presence of chaotropic salts, commercial kits that employ these methods are costly [\$2.00–\$4.00/per sample]. Consequently, researchers continue to modify existing inexpensive phenol-chloroform based methods, tailoring them to deal with problems such as excessive polysaccharides in specific groups of plants (Cheng et al. 2003; Sharma et al. 2002). Such methods are typically time consuming and the quality of DNA obtained can be inconsistent, due to incomplete removal of PCR inhibitors.

There is a need for inexpensive high-throughput DNA isolation methods that work well on a variety of plant species. This is particularly important for areas of research, such as DNA barcoding, where large number of plants from different taxonomic groups are analyzed, making it difficult to tailor methods to specific taxa. Few large-scale methods have been developed as an alternative to commercial kits. One approach employs silica fines on top of a filter plate (Elphinstone et al. 2003), and with minor modifications was successfully applied to DNA extraction from brown macroalgae (Hoarau et al. 2007). However, this approach is not fully compatible with automation. Methods for high throughput DNA isolation of animals (Ivanova et al. 2006) and fungi (Lamour and Finley 2006) have recently been developed that use glass fiber plates. These methods yield high quality DNA, are inexpensive (\$0.50/per sample), and are compatible with automated systems.

Here, we report a protocol for high throughput DNA isolation from plants developed by evaluating the performance of several buffer systems on both fresh and dried material under four lysis conditions, two binding conditions and two wash buffers. The procedure was optimized for a variety of plants including a number of recalcitrant species with the objective of producing a high throughput, inexpensive (i.e. \$0.55/sample), automation friendly method that consistently yields high quality DNA.

Materials and Methods

Plant Species

Twenty four mature plant leaf samples were collected fresh from the University of Guelph Arboretum or greenhouses (Table 1) on June 18, 2007. One portion of each

Table 1 Plant species used for DNA extraction and sample locations for Fig. 3

N	Species	Wells on Fig. 3
1	<i>Opuntia rufida</i>	H1, H7
2	<i>Cattleya</i> sp.	G1, G7
3	<i>Portulacaria afra</i>	F1, F7
4	<i>Aloe zebrina</i>	E1, E7
5	<i>Dieffenbachia</i> sp.	D1, D7
6	<i>Rhododendron brachycarpum</i>	C1, C7
7	<i>Rhododendron fortunei</i>	B1, B7
8	<i>Asclepias syriaca</i>	A1, A7
9	<i>Equisetum arvense</i>	H3, H9
10	<i>Euphorbia esula</i>	G3, G9
11	<i>Impatiens capensis</i>	F3, F9
12	<i>Asimina triloba</i>	E3, E9
13	<i>Carya ovata</i>	D3, D9
14	<i>Acer platanoides</i>	C3, C9
15	<i>Vitis riparia</i>	B3, B9
16	<i>Quercus bicolor</i>	A3, A9
17	<i>Taxus canadensis</i>	H5, H11
18	<i>Ginkgo biloba</i>	G5, G11
19	<i>Pinus banksiana</i>	F5, F11
20	<i>Pinus armandii</i>	E5, E11
21	<i>Juniperus virginiana</i>	D5, D11
22	<i>Picea pungens</i>	C5, C11
23	<i>Larix decidua</i> 'pendula'	B5, B11
24	<i>Thuja occidentalis</i>	A5, A11

sample was used for DNA isolation within a few hours and a second portion was placed into a plastic bag filled with silica gel for drying (remaining fresh samples were frozen at -20°C). Tissue was homogenized with a TissueLyser (Qiagen GmbH, Hilden, Germany) using 3 mm Tungsten Carbide beads (Qiagen) at 30 Hz for 1–1.5 min. Lysis buffers were added before homogenization of fresh plant material and after homogenization of dried material.

Optimization Outline

The composition of each of the lysis, binding and wash buffers is described in Table 2. A summary of the DNA isolation experiments is provided in Table 3.

In a first series of experiments we tested 4 different lysis buffers (Table 2): cetyltrimethylammonium bromide (CTAB; Doyle and Doyle 1987), CTAB with addition of polyvinylpyrrolidone (PVP; CTAB+PVP), insect lysis buffer (Ivanova et al. 2006) with addition of PVP (ILB+PVP) and sodium dodecyl sulfate (SDS) digestion buffer (TES; Elphinstone et al. 2003) on three types of material (fresh, dried tissue and dried tissue without homogenization). A volume of 50 μl of each lysate was used to test two binding conditions with buffers PBB1 and PBM1 (with and without ethanol). At this optimization stage we used buffers PWB1 and WB1 for wash steps.

Based on the results of the first experiment, we evaluated the utility of a second wash step with WB1 or WB2 (75% ethanol) on dried material and orchid tissue preserved in RNAlater (Qiagen).

Table 2 Reagents for plant DNA extraction (optimal buffers are highlighted)

Buffer	Composition
Lysis buffers	
CTAB	2% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl
CTAB+PVP	2% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 2% PVP
ILB+PVP	700 mM GuSCN, 30 mM EDTA pH 8.0, 30 mM Tris-HCl pH 8.0, 0.5% Triton® X-100, 5% Tween-20, 1% PVP
TES	100 mM NaCl, 50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 0.5% SDS
Guanidine thiocyanate stocks	
GSS1	6 M GuSCN, 20 mM EDTA pH 8.0, 10 mM Tris-HCl pH 6.4, 4% Triton® X-100
GSS2	6 M GuSCN, 20 mM EDTA pH 8.0, 10 mM Tris-HCl pH 6.4
Binding buffers	
PBB1	40 ml of GSS1, 8 ml ddH₂O
PBB2	40 ml of GSS2, 8 ml ddH ₂ O
PBM1	50 ml of GSS1, 50 ml 100% ethanol
First wash buffers	
PWB1	50 ml of GSS1, 50 ml 100% ethanol
PWB2	50 ml of GSS2, 50 ml 100% ethanol
Second wash buffers	
PW1	60% ethanol, 50 mM NaCl, 10 mM Tris-HCl pH 7.4, 0.5 mM EDTA pH 8.0
PW2	75% ethanol

Finally, we tried a slight modification of the protocol with alternative binding and first wash buffers PBB2 and PWB2 (without Triton® X-100) in combination with CTAB and ILB+PVP lysis buffers on four dried homogenized plant samples.

Spin-Column Evaluation

To evaluate the suitability of the system for lower throughput operations we applied the optimized reagents to EconoSpin™ All-in-1 Mini Spin Columns for DNA/RNA extraction (Epoch Biolabs, Inc., Sugar Land, TX, USA).

Table 3 Optimization outline

Experiment	Material	Lysis	Binding	1st wash	2nd wash	3rd wash
Lysis and binding optimization 1	Fresh homogenized	1,2,3,4	PBB1, PBM1	PW1	WB1	–
Lysis and binding optimization 2	Dried homogenized	1,2,3,4	PBB1, PBM1	PW1	WB1	–
Lysis and binding optimization 3	Dried, long incubation	1,2,3,4	PBB1, PBM1	PW1	WB1	–
Wash optimization and cross-contamination test	RNAlater, dried homogenized	1,3	PBB1	PW1	WB2	WB2
Triton® removal	Dried homogenized	1,3	PBB2	PW2	WB1 (WB2)	optional

Commercial Kit Protocol

For comparison with commercially available methods, the same dried homogenized samples were used for extraction with a Plant II DNA extraction kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany), hereafter called MN plant kit, with CTAB lysis buffer (PL1). A volume of 50 μ l of clarified lysate was used for binding. The amount of binding buffer was adjusted following manufacturer's instructions, and subsequent wash steps were the same as in the original protocol. DNA was eluted in 50 μ l of ddH₂O.

Manual Protocol

1. Add a 3 mm Tungsten Carbide Bead (Qiagen) and \sim 5 mm² of fresh or dried plant leaf tissue into each well of 1.1 ml PROgene Mini Tube System (Ultident Scientific, St. Laurent, QC, Canada), hereafter called tube strips. Work with one row at a time, keeping the rest of the tube strips covered with cap strips. If working with fresh material, add 200 μ l of lysis buffer before homogenization, if working with dry or frozen material, add 200 μ l of lysis buffer after homogenization.¹ Cover the tube strips with cap strips and homogenize tissue using TissueLyser (Qiagen) at 30 Hz twice for 30 s. Shake well, centrifuge at 1,000 \times g for 1 min and incubate at 65°C for 1 h on an orbital shaker.
2. Centrifuge at 1,500 \times g for 1 min and transfer 50 μ l of lysate into the wells of a 96-well non-skirted microplate able to hold 200 μ l (use a skirted microplate for automated method).
3. Add 100 μ l of binding buffer to each well using a multi-channel pipette.
4. Carefully and slowly mix three to four times by aspirating and dispensing 100 μ l and transfer 150 μ l of each lysate into a well in a 1 ml AcroprepTM 96-well plate with 1 μ m glass fiber media (Pall Life Sciences, Ann Arbor, MI, USA), hereafter called GF plate, sitting on a 2 ml square-well block. Seal the GF plate with clear PCR film and centrifuge at 5,000 \times g for 5 min to bind DNA to the GF membrane.
5. For the first wash step, add 200 μ l of First Wash Buffer to each well of the GF plate before sealing it and centrifuging at 5,000 \times g for 2 min.
6. For the second wash step, add 750 μ l of second wash buffer to each well of the GF plate before sealing it and centrifuging at 5,000 \times g for 5 min.
7. Remove the seal, place the GF plate on the lid of a tip box, and incubate at 56°C for 30 min to evaporate residual ethanol. Position a collar (PALL Cat. No 5225) on the collection microplate and place the GF plate on top. To release the DNA, add 50 μ l of ddH₂O (at 56°C) to each well of the GF plate before sealing it and incubate at room temperature for 1 min.
8. Place the assembled plates on a square well block to prevent cracking of the collection plate and centrifuge at 5,000 \times g for 5 min to collect the DNA eluate. Remove the GF plate and discard it.

¹ To reduce the probability of cross-contamination due to airborne plant material, place one strip of tubes in a separate rack during sampling and after homogenization. After homogenization open tubes carefully using the individual side tabs of each tube. Discard the lids, replace with new ones after addition of lysis buffer and return the strip to original rack.

9. Cover the DNA plate with aluminum PCR film and store at 4°C for short-term storage or at –20°C for the long term storage.

Automated Protocol:

1. Prepare lysates using the manual protocol and transfer 50 µl of lysate into wells of a skirted microplate able to hold 150 µl.
2. Load the deck of a Biomek FXP liquid-handling unit (Beckman Coulter Inc., Fullerton, CA, USA) with labware and reagents.
3. Add 100 µl of binding buffer robotically to each of the 96 wells in the plate and incubate at room temperature for 4 min.
4. Mix each lysate by repeatedly (12×) withdrawing and re-injecting 100 µl of it. Transfer 125 µl of each lysate into a GF plate sitting on a 36 mm collar positioned on the vacuum manifold. Apply a vacuum of 23 In Hg for 2 min and discard the filtrate.
5. Add 180 µl of first wash buffer to each well and place the plate under vacuum for 3 min.
6. For the second wash step, add 220 µl of second wash buffer to each well and apply vacuum for 2 min.
7. For the third wash step, add 660 µl of second wash buffer to each well and apply vacuum for 10 min to dry the plate.
8. Place the GF plate on a 2-ml square-well block and centrifuge at 5,000×g for 2 min to remove residual wash buffer.
9. Incubate all plates at 56°C for 20–30 min to evaporate residual ethanol.
10. Elute in the centrifuge as in manual protocol.

DNA Quantification

DNA quantity and quality was evaluated using a NanoDrop® spectrophotometer ND-1000 (NanoDrop® Technologies, Wilmington, DE, USA). In addition, 10 µl of each DNA extract was loaded and visualized on a 2% agarose gel using an E-Gel96® Pre-cast Agarose Electrophoresis System (Invitrogen).

PCR Amplification

To evaluate the suitability of the DNA isolates for PCR we used primers targeting the COI barcode region (Hebert et al. 2003), Cox1-42F 5' GGATCTTCTCCAC TAACCACAAA 3' (Cho et al. 1998) and a newly designed Cox1-ajf699R 5' CCG AAAGAGATGCTGGTATA 3' and primers targeting a portion of the chloroplast region *rbcL* (Lledo et al. 1998) 1f 5'ATGTCACCACAAACAGAAAC 3' and 724r 5' TCGCATGTACCTGCAGTAGC 3'. Primers for the universal plastid region (Presting 2006) p23SrV_F1 5' GGACAGAAAGACCCTATGAAGCTT 3' and p23Srv_R1c 5' TCAGCCTGTTATCCCTAGAGTAAC 3' were used as an additional marker to evaluate DNA extractions from *Equisetum*.

All PCR reactions had a total volume of 12.5 µl and included: 6.25 µL of 10% trehalose, 2.00 µl of ultra pure water, 1.25 µl 10× PCR Platinum Taq buffer

[500 mM KCl, 200 mM Tris–HCl (pH 8.4)], 0.625 μ l MgCl₂ (50 mM) (Invitrogen, Carlsbad, CA, USA), 0.125 μ l of each primer (0.01 mM), 0.0625 μ l of each dNTP (10 mM), 0.3 U of Platinum DNA Polymerase (5 U/ μ l) (Invitrogen), and 2.0 μ l of DNA template. The thermocycle profile COI-1 consisted of 94°C for 2 min, 35 cycles of 94°C for 30 s, 51°C for 40 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. The *rbcl* thermocycle profile differed only by annealing at 52°C.

PCR products were visualized on a 2% agarose gel using an E-Gel96® Pre-cast Agarose Electrophoresis System (Invitrogen). PCR success was evaluated separately for each treatment (array of 24 wells) as the percentage of wells containing a PCR product. Bidirectional sequencing was done using the BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3730xl Genetic Analyzer (Applied Biosystems; Hajibabaei et al. 2005). Bidirectional sequences were assembled in SeqScape v. 2.1.1 (Applied Biosystems) and manually edited.

As an additional quality control 16 μ l of DNA extracted from dried material using CTAB buffer for lysis was digested overnight at 37°C with *EcoRI* (New England BioLabs, Ipswich, MA, USA).

Results and Discussion

The best PCR results for both COI and *rbcl* from fresh plant material were observed with either the regular CTAB buffer or the ILB buffer with addition of PVP, and the use of binding buffers without ethanol. Agarose gel electrophoresis of DNA extracts and Nanodrop measurements revealed that use of the binding buffer PBM1 containing ethanol resulted in sheared DNA and lower yields indicating co-precipitation of polysaccharides even in the presence of high salt concentrations (Fig. 1). The average PCR success was also significantly lower—67% vs 97% for COI and 67% vs 96% for *rbcl* (Fig. 2). The major difference in PCR success was observed with CTAB and TES lysis buffers. Extraction with CTAB buffer yielded only 25% vs 100% for COI and 50% vs 100% for *rbcl*, while extraction with TES buffer—54% vs 96% for COI and 37.5% vs 97% for *rbcl* (corresponding to binding with or without ethanol).

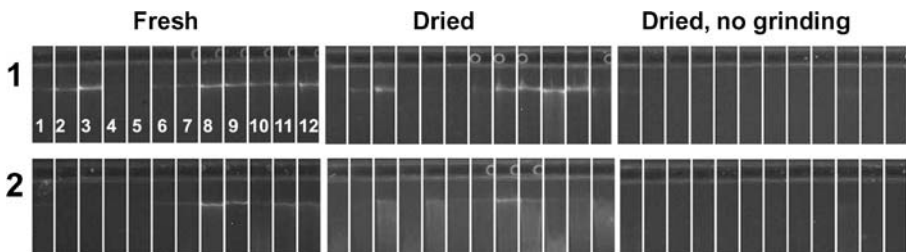


Fig. 1 Agarose E-gel96® images of DNA extracted with different treatments. 1 Binding without ethanol, 2 binding in the presence of ethanol. Columns 1–3 on each image correspond to CTAB, 4–6 to CTAB+PVP, 7–9 to ILB+PVP and 10–12 to TES lysis buffer. The following plants are presented on the image (from left to the right for each lysis buffer system): *Opuntia rufida*, *Equisetum arvense* and *Taxus canadensis*

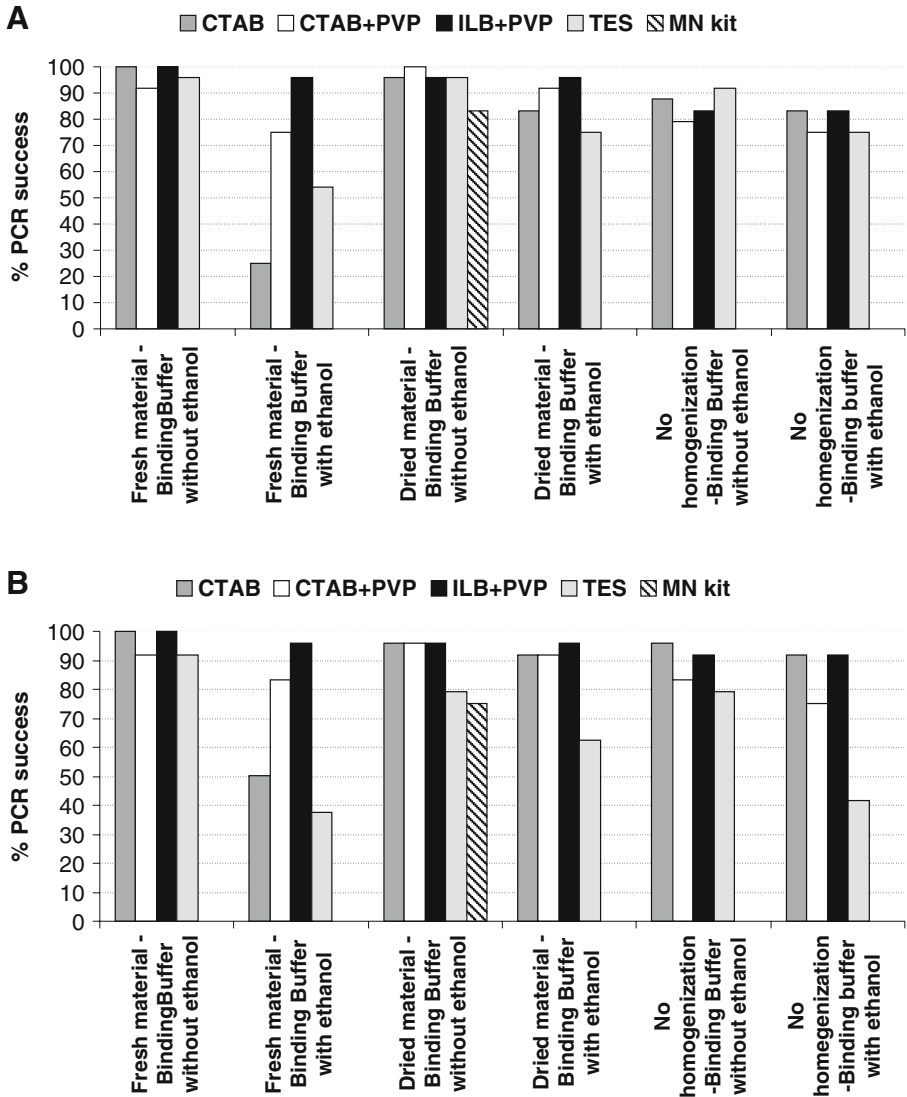


Fig. 2 Difference in PCR success for DNA extracted from homogenized material (fresh and dried) and from dried material without homogenization using four lysis buffers and two binding conditions (see Tables 2 and 3 for details). MN kit was used as a control for dry homogenized material. **a** PCR success with COI, **b** PCR success with *rbcL*

For dried homogenized plant material, the binding buffer without ethanol yielded high molecular weight DNA (Fig. 1) and also had high PCR success (Fig. 2). Binding in the presence of ethanol resulted in sheared DNA contaminated with polysaccharides (Fig. 1), and significantly lower PCR success except for samples lysed in ILB+PVP (Fig. 2). Samples that were simply incubated overnight in lysis buffer without prior homogenization resulted in completely sheared DNA—high molecular weight DNA was not visible on an agarose gel (Fig. 1) and lower PCR

success (Fig. 2). However, less difference was observed between the two binding conditions suggesting that lower amounts of contaminating polysaccharides are released from the intact tissue. Our previous experiments with the extraction of dried plants without disruption (data not shown) indicate that longer incubation time (up to 48 h) could dramatically improve PCR success. Overall, two lysis buffers CTAB and ILB+PVP showed the highest yields of DNA and best PCR success, but the optimal binding conditions (without ethanol) were more important for a successful extraction than the lysis buffer choice.

In our experiments all plant species were successfully amplified with COI and *rbcL* primers. However, *Equisetum* produced double-banded faint amplicons with the COI primer pair (Fig. 3, wells H3 and H9). With alternate primers targeting a universal plastid region (Presting 2006) and *rbcL* (Lledo et al. 1998) we were able to amplify *Equisetum* from most isolations (data not shown). Most of *Cattleya* extracts derived from dried material failed to produce amplicons for both COI and *rbcL*, probably due to strong oxidizing enzymes causing DNA damage or contamination with polyphenols. By contrast, fresh tissue (data not shown), as well as tissue preserved in RNAlater, yielded high quality DNA suitable for PCR (Fig. 3, wells G1, G7).

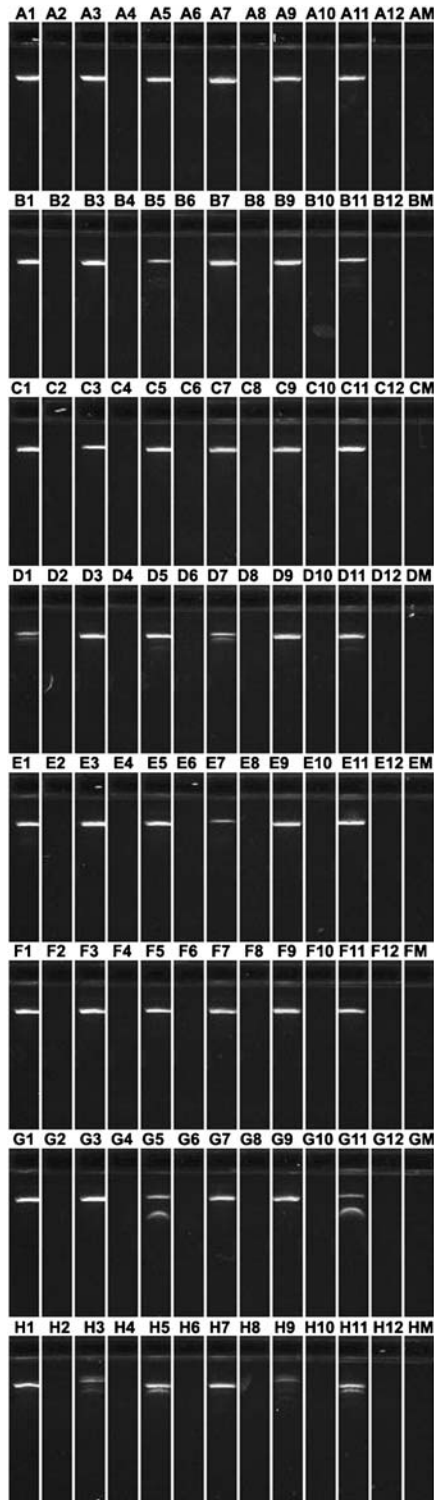
Our protocol outperformed a single-tube commercial plant DNA isolation kit (MN), but we did not test the alternate lysis buffer PL2 recommended by the manufacturer for some recalcitrant species.

When working in 96-well format, special care should be taken while handling plant samples. The first set of experiments to determine the optimal binding conditions was carried out following standard protocols for animal tissue (Ivanova et al. 2006). However, dried plant material can easily become airborne during the sampling procedure, or after tissue disruption and prior to the addition of lysis buffer. When opening the lids, there is potential for material to be dislodged and enter another tube. In our case, sequencing of PCR products for COI revealed four cross-contamination events with dried material and one cross-contamination event with fresh plant samples. Cross contamination during the processing of fresh samples was likely due to insufficient instrument sterilization between samples. Remaining cases of cross-contamination with dried samples required protocol modifications to exclude the potential for airborne dispersal of dried samples.

To reduce the probability of cross-contamination in subsequent experiments we placed one strip of tubes in a separate rack during sampling and after homogenization. After homogenization we opened them carefully using the individual side tabs of each tube. The lids were discarded and replaced with new ones after addition of lysis buffer and the strip returned to original rack. To ascertain whether cross-contamination was still occurring, wells containing plant samples were separated by blank wells filled solely with corresponding lysis buffer. These simple procedures resulted in no further cross-contamination based on PCR and sequencing results (Fig. 3).

Polysaccharides and polyphenols tend to co-precipitate with DNA in the presence of ethanol and can potentially inhibit subsequent enzymatic reactions (Pandey et al. 1996). Increased salt concentration helps to keep polysaccharides in solution during the precipitation step (Crowley et al. 2003), while PVP removes polyphenols from solution (Salzman et al. 1999). In a previous study on DNA isolation from animal

Fig. 3 PCR cross-contamination test on DNA extracted from the dried homogenized material (except orchid preserved in RNAlater) with optimized binding and sampling procedure and two washes with 75% ethanol. Columns 1–6 correspond to CTAB lysis buffer, columns 7–12 to ILB+PVP lysis buffer (odd rows are samples, even rows are blank wells)



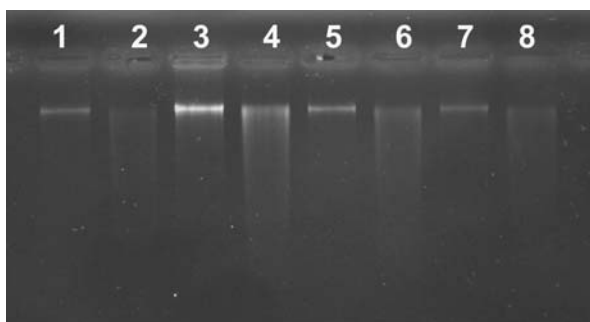
tissues (Ivanova et al. 2006) we employed binding of DNA to glass fiber in the presence of guanidine thiocyanate (GuSCN) and ethanol. Here we evaluated the use of binding buffers without ethanol to avoid co-precipitation of polysaccharides onto the glass fiber membrane. Binding buffers containing GuSCN are widely used in silica-based protocols (Boom et al. 1990; Hoss and Paabo 1993; Oliveri et al. 2006) and have been proven to be more effective than other chaotropic salts (Rohland and Hofreiter 2007).

Average DNA concentrations from our protocol were comparable to those obtained with the MN kit, ranging from ~15 to 35 ng/ μ l. Average 260/280 absorbance ratio was 1.6 for MN kit, but ranged from 1.1 to 1.5 with our extraction protocol, depending on the lysis buffer used. However, a strong absorption spike at 220–230 nm was observed with our protocol, which interfered with the accuracy of NanoDrop[®] readings. A similar absorption profile was obtained for blank samples, suggesting that the source of this absorbance spike was not due to the co-purification of polysaccharides, but rather to a component of one of the reagents. Both thiocyanate salts and Triton[®] X-100 absorb at 230 nm. The incorporation of an additional wash step with WB2 or WB1 or removal of Triton[®] X-100 from the binding and first wash buffers did not significantly improve the spectral characteristics of isolations, but in some cases reduced the average yield (data not shown). Moreover, an insoluble white precipitate was formed after the addition of binding buffer without Triton[®] X-100 to lysates containing the CTAB buffer and this subsequently clogged wells on the filter plate. The leftovers of this precipitate were observed in the wells even after subsequent wash steps. Finally, no high molecular weight DNA was observed on an agarose gel (data not shown). In contrast, similar precipitates that form after addition of Binding Buffer containing Triton[®] X-100 to CTAB are easily soluble after a few mixing cycles and are fully removed during subsequent wash steps. Therefore, initial binding and wash conditions were considered optimal.

In spite of their lower 260/280 absorption ratios, DNA extracts obtained with our protocol were consistently more successful for PCR than these obtained from MN kit (Fig. 2). As well, DNA extracts were successfully digested with *Eco*R I (Fig. 4).

DNA isolations using the optimal lysis and binding buffers (CTAB and ILB+PVP) with single spin-columns also yielded high molecular weight DNA, successful PCR and restriction digests. Hence with a marginal increase in cost (\$0.20) our

Fig. 4 *Eco*R I digestion of DNA extracted from dried plants with CTAB lysis buffer and binding buffer containing Triton[®] X-100. 1, 2 *Asclepius syriaca*, 3, 4 *Opuntia rufida*, 5, 6 *Pinus armandii*, 7, 8 *Thuja occidentalis*. Odd numbers correspond to untreated DNA, even numbers to DNA digested with *Eco*R I



protocol is easily adapted to spin columns which are convenient for low throughput applications.

One of the central objectives of our work was to develop a protocol compatible with the automated DNA extraction methods available for animal tissues (Ivanova et al. 2006) currently employed at the CCDB. Compared to other protocols, our method does not require a precipitation stage (Lamour and Finley 2006) or chloroform extraction (Hoarau et al. 2007) before binding. Our Biomek FX (Beckman Coulter) is equipped with three recirculating reservoirs containing binding and two wash buffers. In the plant protocol we use animal binding mix as a first wash buffer, while the second wash buffer is the same. Therefore, the plant binding buffer without ethanol (PBB1) is the only additional reagent and it could be easily accommodated by placing a regular reservoir on the deck and making only the minor changes in the Biomek script. Interestingly, the plant protocol with CTAB lysis buffer with addition of Proteinase K allowed successful DNA extraction from gastropods which had failed with regular animal protocols (Ivanova et al. 2006; Dirk Steinke, personal communication). In addition, our preliminary results indicate that the plant protocol with CTAB buffer also works well for fungi and lichens (Isabelle Meusier, Natalia Ivanova, pers. comm.).

The protocol developed here has a minimal number of steps, is suitable for DNA recovery from recalcitrant species and can be integrated into DNA barcoding facilities employing automated liquid handling robotic devices for high throughput DNA isolation. The only additional step beyond that required for animal DNA isolation protocols is a tissue homogenization step that can be easily performed in a 96-well format using the TissueLyser (Qiagen) or any similar instrument. To date, the robotic version of protocol has been successfully used for more than 3,000 samples including 394 species of Canadian flora in a parallel trial with Qiagen DNeasy 96 kit (Kevin Burgess, Prasad Kesanakurti pers. comm.) and 300 species of diverse tropical plants from Costa Rica (Isabelle Meusier, pers. comm.) with highly positive results.

Because the automated part of the protocol takes just 15–20 min on Biomek FX, a high-throughput facility equipped with this equipment can easily extract twenty 96-well plates per 8-h day, given that tissue sampling is done on a previous day. By contrast, the manual protocol requires an hour for every two plates after the lysis stage. Aside from speed, our work has identified a protocol for DNA extraction that matches high-performance commercial kits, but delivers these results for just \$0.55 per sample.

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