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RESEARCH ARTICLE

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A fast, flexible and inexpensive protocol for DNA and RNA extraction for forest trees

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Abstract

Aim of study: DNA and RNA extraction are still one of the most important and challenging steps of many molecular genetics applications such as Next-Generation Sequencing technologies. In this study, traditional laboratory preparation protocols and commercially available nucleic acids extraction kits' features were combined into a procedure suitable for extraction of either DNA or RNA in 96-well plate format at high throughput.

Area of study: The study covers forest tree species from the United States of America.

Material and methods: The DNA and RNA protocol were tested on 27 species, including especially recalcitrant forest tree species, from five angiosperm and three gymnosperm families. DNA was also extracted from stored (from 2 to 6 years) silica-dried samples of 11 species of *Pinaceae*.

Main results: The spectrophotometric analysis of DNA and RNA showed that gymnosperms yielded lower quantity, but higher quality nucleic acids than angiosperms which have variable results among species. The quantity and quality of DNA from stored samples were generally lower than fresh silica-dried samples. The RNA results showed high-enough yield (6.6 to 8.8 RIN) for downstream analyses.

Research highlights: It was demonstrated that high quality and high molecular weight nucleic acids for Next-Generation Sequencing applications can be isolated from hundreds of samples from a wide range of taxonomic groups. The new protocol has features similar to both traditional laboratory and commercial extraction kits; is easy to set up in any molecular research laboratory, can be applied to a large number of samples (hundreds) in a working day, uses inexpensive reagents and supplies, and is compatible with automation.

Keywords: Angiosperms; gymnosperms; isolation protocol; nucleic acids.

Authors' contributions: experimental design and obtaining material: YK and LM. Performed the experiments: YK, LM and WK. Data analysis: YK, LM, and RW. Wrote the paper: YK, LM, RW and JF.

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Introduction

Next-Generation Sequencing (NGS) technologies (*e.g.* Illumina/Solexa, SOLiD technology, Ion Torrent technology, Pacific BioSciences, Helicos BioSciences and Oxford Nanopore Technology) are increasingly becoming popular and commonly applied technologies in projects that have large sample numbers such as linkage mapping (Khan & Korban, 2012), de novo assembly (Canales *et al.*, 2014), genetic structure and association

genetics (Chhatre *et al.*, 2013; Plomion *et al.*, 2014), and population genetics analysis (Neale & Kremer, 2011). NGS technologies have developed impressively fast in terms of run time, read length, per-base cost reduction, high-throughput genotyping, and data analysis and management during the last decade. NGS technologies differ from each other due to specific protocol combinations and different types of data production, but they basically include three main parts: library (template) preparation, sequencing and imaging, and data analysis and management. The success of all protocols for NGS technologies depends on high quality and high molecular weight nucleic acids (DNA and RNA) for library preparation. Therefore, nucleic acids extraction is one of the most important cornerstones of NGS technologies as in many other molecular genetics' applications (Wang & Szmidt, 2001; Xin & Chen 2012; Buermans & den Dunnen, 2014; van Dijk *et al.*, 2014).

There have been hundreds of protocols for nucleic acids extraction from various biological materials published in the literature over the last few decades. These protocols can be carried out using traditional laboratory preparation (homemade extraction protocol) or one of the many commercially available nucleic acids extraction kits (Semagn, 2014). Both approaches have advantages and disadvantages. Many traditional extraction protocols originated from hexadecyltrimethylammonium bromide (CTAB) (Doyle & Doyle, 1987; 1990) or sodium dodecyl sulfate (SDS)/ CTAB (Dellaporta *et al.*, 1983) methods which are based on chloroform extraction and isopropanol precipitation, respectively. High quality and high molecular weight nucleic acids can be obtained from traditional protocols, but these protocols can be time-consuming for large numbers of samples, labor-intensive and generally not suitable to automation (Csaikl *et al.*, 1998). Various commercially available nucleic acids extraction kits are preferred due to their ease of use, low- labor, and ability to produce relatively high-quality and quantity nucleic acids, but these kits can be expensive and have limited application to some taxonomic groups. Therefore, a combination of both approaches is needed to get high quality and high molecular weight nucleic acids from many different biological specimens in quick, low-labor and low-cost manner (Ivanova *et al.*, 2006; 2008; Healey *et al.*, 2014).

Nucleic acids extraction from plant tissues, especially from recalcitrant forest trees, is more difficult than from animal samples because of high levels of structural polymers and secondary metabolites such as polysaccharides (*e.g.*, cellulose, pectin and starch) and polyphenols (*e.g.*, tannins, flavonoids and lignin). These components hinder attempts to obtain high quality and high molecular weight nucleic acids for molecular studies such as restriction digestion, PCR, library preparation and sequencing (Shepherd *et al.*, 2002; Healey *et al.*, 2014). However, it is possible to get adequate yields of high-quality nucleic acids from forest trees. Factors affecting success include the plant part sampled (Kim *et al.*, 1997; Reynolds & William, 2004; Barzegari *et al.*, 2010), preparation and conservation methods (Tibbits *et al.*, 2006; Semagn, 2014), and the extraction protocol (Csaikl *et al.*, 1998; Le Provost *et al.*, 2007; Bashalkhanov & Rajora, 2008).

Our method is a modified glass fiber plate protocol from The Canadian Centre for DNA Barcoding (CCDB, www.ccdb.ca) that combines the use of ground silica-dried or frozen samples with a guanidine thiocyanate (GuSCN) buffer, following by clean-up steps on a silica membrane that allows recovery of either DNA or RNA. The CCDB protocol is derived from a combination of Ivanova *et al.* (2006; 2008) and Whitlock *et al.* (2008) and has manual and robotic version of nucleic acids extraction from diverse biological groups (plants, fungi, echinoderms and mollusks) with 96-well plates. Although many other protocols use fresh plant samples with liquid nitrogen homogenization to extract DNA (Dellaporta *et al.*, 1983; Doyle & Doyle 1987; 1990; Ostrowska *et al.*, 1998; Telfer *et al.*, 2013; Healey *et al.*, 2014), our protocol uses specimens dried with silica gel which is one of the most common preservation (desiccation) methods in the case of forest trees (Chase & Hills, 1991; Semagn, 2014). Our protocol is based on binding of nucleic acids to a glass-fiber membrane (AcroprepTM 96-well filter plate) in the presence of a high concentration of GuSCN. GuSCN is a stronger chaotropic agent that contains potent cationic and anionic groups that form strong hydrogen bonds and can be used in the presence of a reducing agent (*e.g.* 2-mercaptoethanol, sodium bisulfite) to break protein disulfide bonds and in the presence of a detergent (*e.g.* sarkosyl) to disrupt hydrophobic interactions of lipid membranes. It is well known that guanidine-based solutions can be used successfully to extract DNA and undegraded RNA from different plant and animal tissues including ribonuclease-rich tissues. The initial version of a guanidine thiocyanate total RNA extraction method was based on Caesium Chloride (CsCl) gradient ultracentrifugation of the cell lysate (Chirgwin *et al.*, 1979). The discovery that total RNA remained soluble in an acidic aqueous phase and could be recovered by centrifugation allowed the elimination of the long ultracentrifugation step and reduced the required time significantly. This last protocol was described as a single step method of RNA isolation by acid guanidine thiocyanate-phenol-chloroform (Chomczynski & Sacchi, 1987). Our method has an optional step of chloroform-isoamyl alcohol extraction of the crude lysate that allows the recovery of higher-quality DNA from the upper aqueous phase, but this step is not required for recovery of high-quality total RNA. The purpose of this study is to optimize a procedure suitable for extraction of high quality and quantity DNA and RNA from recalcitrant forest trees, at low cost in labor, equipment and consumables, in high throughput 96-well plate format.

Material and Methods

Plant samples

Fresh mature leaf samples of 27 tree species from 15 genera were obtained from the JC Raulston Arboretum and CAMCORE program (North Carolina State University, NCSU), University of Washington Botanic Gardens, The United States National Arboretum (Washington D. C.) and, Hoyt Arboretum (Portland, Oregon). Stored silica-dried samples were provided by David Neale's research group at the University of California (Davis, California). Herbarium specimens were obtained from Dr. Richard Braham (NCSU, Forestry and Environmental Resources) and Dr. Alexander Krings (NCSU, Department of Plant and Microbial Biology). Detailed information of specimens is presented at Table 1. The fresh samples for RNA extraction were stored at -80 °C. DNA extraction was carried out after desiccation of fresh samples in silica.

Silica gel drying

The collected leaves or needles were cut into smaller pieces and put into 6 × 9 cm paper envelopes labelled with the sample name and number, and collection date. The paper envelopes were closed and placed into 10 cm × 16 cm plastic Ziploc bags. Nontoxic orange silica gel beads were added into the Ziploc bags. The amount of silica gel covered at least half of the paper envelope in the Ziploc bag or a 1:10 ratio of leaf tissue. The paper envelopes prevented samples from contacting the silica gel excluding dust from the silica gel beads. Ziploc bags were checked every day. The orange silica gel beads turned dark green when saturated with moisture were then replaced. Beads were replaced until the orange color remained stable. Drying leaves with silica gel takes time ranging from hours to days, depending on many factors such as amounts of leaf sample and silica gel, sample water content, and room and/or laboratory temperature and humidity. Silica gel can be reactivated by placing into an oven for 2-3 hours at 150-200 °C and reused many times (Chase & Hills, 1991). In this research, 8-10 young leaves and/or 3-4 mature leaves were totally dry after two days.

DNA and RNA extraction

As most laboratories currently use the 96-well plate format both for nucleic acids extraction and PCR, the present protocol describes processing of 384 samples (4 × 96) at once or more in a working day using silica-based membrane and 1.1 mL strip tubes with strip caps. The updated steps and improvements of protocol can be followed from a free protocol repository (protocols.io) website (https://www.protocols.io/view/easy-and-inexpensive-nucleic-acid-extraction-proto-5pwg5pe).

Chemicals, working solutions and consumables

- Lysis Buffer (LB) **for DNA:** 700 mM guanidine isothiocyanate (GuSCN), 30 mM EDTA pH 8.0, 30 mM Tris-HCl pH 8.0, 0.5% Triton X-100, 5% Tween-20, and 52 mM Na_2SO_3. Add Na_2SO_3 just prior to use.

- Lysis Buffer (LB) **for RNA:** 4 M GuSCN, 0.2 M sodium acetate pH 5.3, 25 mM EDTA, 2.5% PVP-10, 1% beta-mercaptoethanol. Add beta-mercaptoethanol just prior to use. Keep the buffer at 4 °C.

- Binding Buffer Stock (BBS): 6M GuSCN, 20 mM EDTA pH 8.0, 10 mM Tris-HCl pH 6.4, and 4% Triton X-100. Mix on magnetic stirrer with heater. If any re-crystallization occurs, pre-warm at 56 °C to dissolve before use.

- Protein Wash Buffer (PWB): 50 mL binding buffer was mixed with 50 mL ethanol (96%). PWB is stable at room temperature for a few months.

- Wash Buffer (WB): 60% ethanol, 50 mM NaCl, 10 mM Tris-HCl pH 7.4, 0.5 mM EDTA pH 8.0. Components should be mixed well and stored at -20 ºC.

- Chloroform:isoamylalcohol (24:1), cold ethanol (70% and 96% stored at -20 ºC), sodium lauroyl sarcosinate (sarkosyl), elution buffer (AE), and dry ice.

- 1.1 mL strip tubes with strip caps, 4 mm stainless steel balls, 96 Filter plate (1 mL, PALL Glass fiber plate, VWR-28148-622 catalog no, Pall Life Sciences, Ann Arbor, MI, USA), 96 Deep-well plate (1.64 mL, VWR-10011-944 catalog no), 96-well sealing mat, 96-well PCR plate, 100 ml reagent reservoir, and extended disposable tips (with/without filter).

Equipment

- Tissue lyser or grinder (mixer mill), tissue lyser adapter set, plate centrifuge, balances, spectrophotometer or fluorescent plate reader for nucleic acid measurement, oven (range between 60 ºC to 200 ºC), autoclave, refrigerator and freezers (+ 4 ºC, - 20 ºC and - 80 ºC), heaters and stirrers, single and multi-channel pipettes of different volumes, water bath, fume hood, scissors, and forceps.

Table 1. Comparison of DNA yield from Angiosperm and Gymnosperm families extracted from dry leaf tissue and quantified by Nanodrop and Picogreen methods. ± indicates standard deviations.

Angiosperm													
Family	Species	Nanodrop								Picogreen		Age (Year)	Replicate number
		Weight (mg)		DNA yield (ng/uL)		Absorbance				DNA yield (ng/uL)			
						OD 260/280		OD 260/230					
Altingiaceae	*Liquidambar styraciflua*	50.80	±0.19	420.18	±151.01	1.62	±0.08	0.67	±0.12	40.06	±12.28	<1	8
Fagaceae	*Quercus myrsinifolia*	50.79	±0.27	165.86	±36.42	1.56	±0.08	1.08	±0.16	23.13	±5.07	<1	8
Myrtaceae	*Corymbia calophylla*	50.55	±0.25	77.08	±13.32	1.67	±0.04	0.63	±0.07	13.82	±9.55	<1	8
	Corymbia torreliana	50.61	±0.32	140.33	±17.71	1.60	±0.03	0.79	±0.08	34.07	±10.14	<1	8
	Eucalyptus globulus	50.76	±0.30	115.86	±48.43	1.47	±0.04	0.13	±0.03	0.96	±0.14	<1	5
	Eucalyptus urophylia	50.95	±0.08	110.40	±30.98	1.73	±0.05	0.68	±0.19	29.40	±15.40	<1	8
Salicaceae	*Populus monticola*	50.89	±0.34	513.61	±80.28	1.87	±0.02	1.00	±0.09	58.82	±10.07	<1	8
Sapindaceae	*Acer palmatum*	50.36	±0.31	849.00	±105.84	1.24	±0.02	0.77	±0.04	81.66	±11.57	<1	8
Gymnosperm													
Araucariaceae	*Araucaria angustifolia*	50.89	±0.29	263.18	±47.36	1.78	±0.06	0.79	±0.17	60.17	±14.48	<1	8
Cupressaceae	*Cupressus arizonica*	50.74	±0.21	434.58	±101.38	1.46	±0.13	0.90	±1.54	45.47	±11.73	<1	12
	Juniperus rigida	50.74	±0.26	233.81	±39.20	1.57	±0.04	0.48	±0.07	72.60	±10.71	<1	12
	Sequoia sempervirens	50.74	±0.31	182.48	±43.53	1.82	±0.02	0.53	±0.15	76.54	±18.05	<1	8
Pinaceae	*Abies alba*	50.78	±0.22	238.52	±40.41	1.89	±0.01	0.47	±0.06	60.41	±7.54	<1	12
	Abies alba	50.78	±0.38	116.68	±6.35	1.66	±0.01	0.25	±0.03	54.63	±6.67	6	4
	Abies fraseri	51.03	±0.50	317.14	±35.94	1.89	±0.02	0.60	±0.07	81.06	±13.18	<1	8
	Cedrus atlantica	50.91	±0.28	216.28	±24.75	1.86	±0.02	0.62	±0.07	60.49	±8.92	<1	8
	Larix decidua	50.92	±0.42	1604.24	±176.52	1.96	±0.02	0.70	±0.02	112.17	±10.93	<1	5
	Larix decidua	28.37	±3.01	501.43	±148.88	1.72	±0.04	0.48	±0.04	69.55	±14.60	6	3
	Picea abies (Maxwellii)	50.73	±0.16	447.13	±67.25	1.83	±0.03	0.73	±0.11	115.51	±14.66	<1	12
	Picea abies (Maxwellii)	50.38	±0.30	105.93	±24.88	1.62	±0.03	0.25	±0.11	43.06	±16.72	6	4
	Picea glauca	51.04	±0.33	288.21	±98.96	1.61	±0.06	0.50	±0.14	66.35	±22.11	<1	8
	Picea orientalis	50.75	±0.18	438.55	±36.10	1.91	±0.01	0.48	±0.03	68.59	±7.83	<1	6
	Pinus albicaulis	51.18	±0.37	257.46	±59.27	1.68	±0.05	0.41	±0.06	79.24	±17.18	<1	12
	Pinus albicaulis	50.00	±0.44	101.65	±13.29	1.74	±0.01	0.31	±0.13	50.64	±10.44	3	4
	Pinus cembra	50.57	±0.16	131.35	±21.65	1.79	±0.02	0.38	±0.04	48.58	±11.98	<1	12
	Pinus cembra	46.90	±3.01	143.50	±31.44	1.74	±0.02	0.41	±0.10	49.02	±12.92	6	4
	Pinus elliottii	50.74	±0.27	131.83	±27.17	1.79	±0.03	0.38	±0.07	62.12	±12.22	<1	12
	Pinus elliottii	50.28	±0.14	245.70	±40.91	1.93	±0.01	0.94	±0.14	18.74	±5.21	5	4
	Pinus lambertiana	50.58	±0.13	175.68	±32.96	1.81	±0.01	0.41	±0.06	67.16	±12.56	<1	12
	Pinus lambertiana	50.33	±0.28	156.98	±56.72	1.84	±0.02	0.37	±0.19	26.32	±10.14	6	4
	Pinus mugo	50.68	±0.25	281.77	±50.63	1.83	±0.03	0.50	±0.07	59.23	±11.21	<1	12
	Pinus mugo	50.63	±0.56	146.28	±40.63	1.91	±0.03	0.32	±0.10	42.15	±13.29	6	4
	Pinus monticola	51.13	±0.22	263.11	±47.15	1.85	±0.01	0.53	±0.08	101.35	±14.12	<1	12
	Pinus monticola	34.77	±1.13	270.60	±30.16	1.80	±0.04	0.61	±0.22	89.72	±5.67	6	3
	Pinus taeda	50.59	±0.15	239.38	±42.14	1.86	±0.02	0.55	±0.08	105.61	±15.43	<1	12
	Pinus taeda	50.00	±0.15	160.18	±23.37	1.74	±0.02	0.38	±0.12	32.45	±9.65	4	4
	Pinus taeda	50.28	±0.23	167.83	±32.39	1.64	±0.01	0.26	±0.03	40.29	±7.21	6	4
	Pseudotsuga menziesii	50.81	±0.18	267.76	±36.39	1.82	±0.01	0.49	±0.06	102.26	±13.54	<1	12
	Pseudotsuga menziesii	49.93	±0.51	143.95	±20.33	1.66	±0.02	0.39	±0.13	31.08	±8.15	2	4

Extraction protocol

A randomized layout of the samples was designed to reduce position effects during homogenization. One stainless steel grinding ball (4 mm diameter) was put into empty strip tube plates (eight tubes per strip). Each strip was labelled according to the 96-box order (from 1 to 12) for both DNA and RNA extraction. For DNA extraction, 1-3 mature leaves or 8-10 young (seedlings) leaves (dependent on species) were weighed (about 50 mg) and added to strip tubes in order. If dried plant material was small (about 0.5-1.5 cm), it was directly placed into strip tubes. Otherwise, it was cut down into smaller pieces and added to tubes. Cutting fresh plant material into smaller pieces during drying period is preferable to cutting dried samples into smaller pieces during preparation of sample strip tubes. Cutting fresh leaves into small pieces increases the surface area of the leaf that is exposed to the silica gel and speeds up the drying process. Dried plant samples have an electrostatic charge that may cause problems during weighting, cutting and adding samples to tubes. 96-well plates were prepared for DNA extraction from freshly dried samples of 27 species with a minimum of three replicates per species. A 96-well plate was also prepared with stored (2-6 years) and freshly dried samples of 11 species.

For RNA extraction, frozen leaf samples (about 50 mg) from - 80 ºC were used. During the plate preparation, one sample was weighted and placed into a plate on dry ice. The amounts for the other samples of the same species were estimated to reduce the time required to prepare the plate of samples for RNA extraction. 96-well plates were prepared for RNA extraction from 27 species. After putting the plant material into strip tubes, another grinding ball was added on top of the plant material in the 96 tubes for both the RNA and DNA plates. Before starting the extraction procedure, the following steps were carried out: the water bath (incubator for RNA) was warmed to 65 ºC, all buffers were checked, Na_2SO_3 (and beta-mercaptoethanol for RNA) was added to LB and mixed by inversion, AE was placed at 50 ºC in oven or incubator for at least 1 hour prior to use. For RNA extraction, 96 tubes with 10 µL sarkosyl (20%) were prepared and placed at 4 ºC before extraction. When two 96-well plates were handled, each plate was handled by a different researcher. All steps in the protocol could be carried out at room temperature (with RNA on ice) unless different conditions are specified (*e.g.*, store at -20 ºC about one hour). The first four steps of the protocol are relatively different for DNA and RNA extraction. The last six steps of protocol are slightly modified for RNA extraction: these modifications are indicated in bold.

1. **DNA and RNA:** The samples were ground 5 (2 for RNA) minutes (min) at 25-30 hertz (hz) in a mixer mill (MM300 Retsch, Hann, Germany). The position of the plates was rotated and ground a second time until the samples became a fine homogenized powder.
   RNA: 500 µL cold LB was added to each tube on ice. The samples were mixed 1 min at 30 hz and centrifuged at 5000 rpm for 1 min. 500 µL supernatant was transferred to tubes which contain 10 µL 20% sarkosyl and maintained at 4 °C.
2. **DNA:** 600 µL LB was added to each tube. The samples were incubated at 65 °C about 1.5 hour, and ground for 5 min at 25 hz every 30 min. The samples were centrifuged at 5000 rpm for 10 min.
   RNA: 500 µL cold LB was again added to each tube (rest of homogenate) on ice. The samples were mixed 1 min at 30 hz and centrifuged at 5000 rpm for 1 min. 500 µL supernatant was transferred to previously described tubes with 20% sarkosyl to obtain about 1 mL final volume. The samples were incubated at 65 ºC and mixed manually by inversion 2-3 times about 10 minutes.
3. **DNA:** 400-500 µL of lysate was transferred to a new 96 tube series. 600 µL or 1 volume of chloroform:isoamylalcohol (24:1) was added to each tube. The samples were mixed well by gently inverting the plate and centrifuged at 5000 rpm for 10 min.
   RNA: The samples were immediately placed on ice after 65 °C incubation and kept there about 3 min. 500 µL lysate was transferred to a 96 deep-well plates and filled to 1 mL with 96% ethanol. The solution was mixed by pipetting and maintained at 4 °C.
4. **DNA:** The supernatant (250-300 µL) was carefully transferred into 1 µm glass fiber Pall plate (Pall Life Sciences, Ann Arbor, MI, USA), taking care to avoid the aqueous/organic layer interface. 500 µL cold ethanol (96% from -20 ºC) was added and plates were sealed. Pall plates were kept in -20 ºC for 45-60 min.
   RNA: All the solution (1 mL lysate-ethanol) was transferred to a Pall plate and kept at -20 ºC for 40 min. The plate was centrifuged at 5000 rpm for 1 min at 4 ºC. The rest of the lysate was transferred to a 96 deep-well plate and 500 µL ethanol was again added. The solution was mixed by pipetting and transferred to the Pall plate. The plate was kept at -20 ºC for 30 min.
5. **DNA and RNA:** The Pall plates were centrifuged over an S-Block at 5000 rpm for 5 min or until all liquid had gone down **(for RNA 1 min or no more than 4 min at 4 ºC)**. Flow-through was discarded in GuSCN waste.
6. **DNA and RNA:** 500 µL PWB was added and centrifuged at 5000 rpm for 5 min **(RNA 1 min at 4 ºC)**. Flow-through was discarded in GuSCN waste. **This step was repeated.**

7. **DNA and RNA:** 750 µL WB was added and centrifuged at 5000 rpm for 5 min **(RNA 1 min at 4 ºC)**. Flow-through was discarded in GuSCN waste. **This step was repeated.**
8. **DNA and RNA:** 750 µL cold 70% ethanol (from -20 ºC) was added and centrifuged at 5000 rpm for 5 min **(RNA 1 min at 4 ºC)**. Flow-through was discarded in GuSCN waste. **This step was repeated.**
9. **DNA and RNA:** The PALL plates were centrifuged at 5000 rpm for 10 min without addition of any solution for drying **(RNA 1 min at 4 ºC)**. The PALL plate of RNA was kept at 4 ºC for 10 min without sealing the top.
10. **DNA and RNA:** The DNA and RNA PALL plates were placed on a 96-PCR plate. 50 µL warm elution buffer (AE 50%) was added to the DNA PALL plate and kept 5 min at room temperature. The RNA was eluted with 50 µL RNase free water and kept on ice for 5 min. The PALL plate and 96-PCR plate block were centrifuged at 5000 rpm for 5 min **(RNA 1 min at 4 ºC)**. The 96-PCR plate was capped and stored at -20 ºC and **-80 ºC** for DNA and **RNA**, respectively.

Technical Hints and Tricks

The following step numbers are the numbers of the above protocol.

Step 1. If fresh plant material will be used for DNA extraction, precooling of boxes in liquid nitrogen before grinding helps to get a nice powder. It was not used in our experiment. It is also useful for dry samples. For RNA extraction, samples should be kept on dry ice and grinding adapters should be used after freezing in liquid nitrogen if there is not a cooling system for the homogenizer.

Step 2. If fresh plant material will be used directly for extraction, it is recommended to add LB before homogenization. When opening the strip tube caps after dry material homogenization, it is possible to get cross contamination. To minimize and/or prevent cross contamination with dried material, strip tubes can be transfer to another box one by one and cap strips can be replaced with new ones. During the incubation at 65 °C, put a heat resistant pad under the 96-well plates.

Step 3. Chloroform:isoamyl alcohol should be kept in a glass or solvent-resistant reservoir and added in fume hood. If initial trials result in poor quality of DNA, repeating the chloroform:isoamyl alcohol extraction step may improve DNA quality.

Step 4. It is easy to use deep-well plates for centrifugation of PALL plates. Used deep-well plates can be cleaned, stored and used many times.

Step 9. If the odor of ethanol is detected from the PALL plates, plates can be kept 10-15 min in a fume hood to air-dry (for DNA and RNA) or incubated 5 min at 37 °C (for DNA).

DNA and RNA quantification

Quantity, purity and quality of nucleic acids were measured using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The A260/A280 and A260/A230 absorbance ratios were used to determine quantity and quality of nucleic acids. Double strand DNA concentration was also measured using a Quant-iT Picogreen plate reader. RNA samples were cleaned and concentrated by the RNA Clean and Concentrator-5 TM kit from Zymo Research. DNA and RNA integrity were measured with Agilent 2100 Bioanalyzer with Pico chips (Agilent Technologies, Waldbronn, Germany).

Results

Variation in quality and quantity of extracted DNA and RNA was found among families and species within families (Table 1). The average concentration of DNA extracted from angiosperm plant samples was 308.05 ± 42.33 ng/µL, and the average ratio of absorbance at 260 nm to 280 nm was 1.60 ± 0.03 (Table 2). DNA extractions from gymnosperm samples had lower average concentrations (297.53 ± 20.61), but higher quality as assessed by A260/A280 ratio (1.78 ± 0.01) (Table 2). The DNA yield from about 50 mg of dried foliage ranged between 3.9 µg (*Corymbia calophylla*) and 42.5 µg (*Acer palmatum*) with 14.9 µg average for all species. The most diverse quality and quantity variation was seen between angiosperm species (Table 1).

The amount and quality of DNA from stored *Abies*, *Picea* and *Pseudotsuga* samples were lower than freshly dried samples. Variation was seen between stored and freshly dried *Pinus* DNA samples in terms of quality and quantity, but they generally were in the range or very close to the ideal A260/A280 ratio of 1.8-2.2 (Tables 1 and 2).

The spectrophotometric analysis of RNA yield and quality showed similar patterns to the DNA results (Table 3); on average, the concentrations of extracted RNAs were higher for angiosperms (174.45 ± 42.97 ng/µL) compared to gymnosperms (171.33 ± 14.70 ng/µL), but A260/A280 ratio suggestion higher quality RNA for gymnosperms (1.79 ± 0.03) relative to angiosperms (1.53 ± 0.05) (Table

Table 2. Summary of DNA yield from Angiosperm and Gymnosperm families. Nanodrop and Picogreen measurements, OD 260/280 and 260/230 were obtained from dry-leaf tissue. ± indicates standard deviations.

Angiosperm											
			Nanodrop						Picogreen		Sample number
Family	Weight (mg)		DNA yield (ng/uL		Absorbance				DNA yield (ng/uL)		
					OD 260/280		OD 260/230				
**Altingiaceae* (1)	50.80	±0.19	420.18	±151.01	1.62	±0.08	0.67	±0.12	40.06	±12.28	8
**Fagaceae* (1)	50.79	±0.27	165.86	±36.42	1.56	±0.08	1.08	±0.16	23.13	±5.07	8
**Myrtaceae* (4)	50.55	±0.25	77.08	±13.32	1.67	±0.04	0.63	±0.07	13.82	±9.55	8
**Salicaceae* (1)	50.61	±0.32	140.33	±17.71	1.60	±0.03	0.79	±0.08	34.07	±10.14	8
**Sapindaceae* (1)	50.76	±0.30	115.86	±48.43	1.47	±0.04	0.13	±0.03	0.96	±0.14	5
General Angiosperm	50.95	±0.08	110.40	±30.98	1.73	±0.05	0.68	±0.19	29.40	±15.40	8
Gymnosperm											
**Araucariaceae* (1)	50.89	±0.29	263.18	±47.36	1.78	±0.06	0.79	±0.17	60.17	±14.48	8
**Cupressaceae* (3)	50.74	±0.14	296.26	±45.41	1.59	±0.06	-0.03	±0.58	63.41	±7.63	32
**Pinaceae* (27)	50.81	±0.06	299.57	±24.12	1.82	±0.01	0.51	±0.02	79.05	±3.83	155
***Pinaceae* (11)	47.55	±1.00	179.80	±18.35	1.75	±0.02	0.41	±0.02	44.16	±3.74	46
General Gymnosperm	50.81	±0.06	297.53	±20.61	1.78	±0.01	0.43	±0.10	75.71	±3.37	195

*Number in brackets indicate the number of species in each family
** Samples older than 1 year old

4). There was variation among plant families and species within families in terms of quantity and quality of RNA (Tables 3 and 4). The bioanalyzer results of selected species from angiosperm and gymnosperm show that RNA concentration and integrity were high-enough (6.6 to 8.07 RIN) for downstream analyses (Table 5).

Discussion

Nucleic acids quantity and quality

The amounts of extracted DNA were similar to (Kim *et al.*, 1997; Shepherd *et al.*, 2002; Tibbits *et al.*, 2006; Telfer *et al.*, 2013) or higher (Csaikl *et al.*, 1998; Bashalkhanov & Rajora, 2008) than previously reported values for forest tree species. Many of the angiosperm species used for this study are recalcitrant trees that accumulate high amounts of phenolic and polysaccharides within their leaves. The quality of DNA in this study was found lower than previously reported studies for *Acer*, *Quercus* (Lefort & Douglas, 1999), and *Corymbia* species (Healey *et al.*, 2014). During the optimization of the present protocol, *Abies fraseri* and *Pinus taeda* were selected due to their usage in different ongoing projects in our laboratory. Both species yielded excellent results in terms of quality and quantity, as did many other *Pinaceae* species (Tables 1 and 2).

The RNA quality and quantity were similar to (Chang *et al.*, 1993; Kiefer *et al.*, 2000; Le Provost *et al.*, 2007) or lower (Claros & Canovas, 1998) than previously reported studies. The differences between angiosperm and gymnosperm nucleic acids quality and quantity results can be attributed to differences among species in mature leaf tissue complexity (Semagn, 2014; Kiefer *et al.*, 2000), differential homogenization of samples (Drabkova *et al.*, 2002) and species specificity of protocol (Claros & Canovas, 1998; Csaikl *et al.*, 1998; Ostrowska *et al.*, 1998; Telfer *et al.*, 2013).

The results of the A260/A230 ratio was under the ideal ratio for all studied samples of DNA and RNA extraction. These low A260/A230 values may be due to high levels of carbohydrates and phenolic in forest tree leaves (Ostrowska *et al.*, 1998; Healey *et al.*, 2014) and/or the use of high concentrations of chaotropic guanidine thiocyanate salt in the protocol (Ivanova *et al.*, 2008; Telfer *et al.*, 2013). Polysaccharides and polyphenols co-precipitate with nucleic acids in the presence of ethanol, and guanidine isothiocyanate salts absorb at 230 nm by spectrophotometer (Ivanova *et al.*, 2008; Telfer *et al.*, 2013). Guanidine salts and ethanol are both used in the washing steps of this protocol (see Material and Methods).

Comparison of isolation protocols

One of the biggest differences between the present protocol and some other protocols is the use of silica-dried plant samples for DNA extraction. Some other protocols use fresh plant samples or other preservation methods like blotter paper (another type of desiccant), freezing methods (liquid nitrogen and dry ice), and preservative

Table 3. RNA yield from Angiosperm and Gymnosperm families extracted from frozen leaf tissue (~50 mg) and quantified by Nanodrop method. ± indicates standard deviations.

Angiosperm								
				Nanodrop				
Family	Species	RNA yield (ng/uL)		OD 260/280		OD 260/230		Replicate number
Fagaceae	*Quercus myrsinifolia*	131.70	±35.47	1.41	±0.18	0.44	±0.15	8
Myrtaceae	*Corymbia torreliana*	271.99	±73.16	1.47	±0.11	0.55	±0.12	8
	Eucalyptus globulus	136.75	±64.75	1.39	±0.12	0.29	±0.13	8
	Eucalyptus urophylia	201.06	±35.79	1.78	±0.09	0.77	±0.14	8
	Corymbia calophylla	78.73	±22.61	1.52	±0.10	0.28	±0.08	16
Salicaceae	*Populus monticola*	19.94	±4.66	1.37	±0.14	0.18	±0.07	8
Sapindaceae	Acer palmatum	476.69	±312.52	1.77	±0.13	0.91	±0.29	8
Gymnosperm								
Araucariaceae	*Araucaria angustifolia*	70.85	±28.75	1.51	±0.17	0.36	±0.18	8
Cupressaceae	*Cupressus arizonica*	23.24	±6.20	1.32	±0.10	0.20	±0.04	8
	Juniperus rigida	70.84	±22.94	1.68	±0.12	0.39	±0.12	8
	Sequoia sempervirens	68.13	±19.13	1.66	±0.10	0.31	±0.13	8
Pinaceae	*Abies alba*	256.64	±44.38	2.03	±0.01	0.92	±0.14	8
	Abies firma	227.81	±71.50	1.85	±0.09	0.58	±0.17	8
	Abies fraseri	90.14	±19.72	1.74	±0.13	0.52	±0.10	8
	Cedrus atlantica	153.93	±38.81	1.94	±0.03	0.66	±0.19	8
	Larix decidua	67.38	±22.48	1.15	±0.11	0.41	±0.17	8
	Picea abies (Maxwellii)	175.88	±53.29	1.89	±0.09	0.81	±0.11	8
	Picea glauca	192.34	±43.54	1.89	±0.06	0.65	±0.15	8
	Picea orientalis	331.50	±194.49	1.84	±0.10	0.65	±0.20	8
	Pinus albicaulis	187.66	±30.66	1.95	±0.09	0.96	±0.14	8
	Pinus cembra	229.66	±34.43	2.02	±0.01	±1.11	±0.15	8
	Pinus elliottii	106.54	±37.34	1.77	±0.08	±0.56	±0.23	8
	Pinus lambertiana	185.24	±31.49	1.89	±0.10	0.94	±0.19	8
	Pinus monticola	335.50	±96.34	1.89	±0.12	1.03	±0.21	8
	Pinus mugo	329.84	±64.39	2.00	±0.02	1.29	±0.24	8
	Pinus taeda	166.61	±26.31	1.88	±0.13	0.66	±0.14	8
	Pseudotsuga menziesii	156.81	±42.57	1.88	±0.11	0.69	±0.22	8

solutions (CTAB, ethanol, and isopropanol) to conserve plant material (Semagn, 2014). Forest tree species generally require more complex extraction methods than annual plants in terms of preservation and special grinding procedures (Shepherd *et al.*, 2002). Mechanical disruption (homogenization) of plant material is the first essential part of the extraction process. Many extraction protocols commonly use liquid nitrogen for grinding of fresh samples (Csaikl *et al.*, 1998; Lutz *et al.*, 2011). Although grinding with liquid nitrogen in a mortar provides uniformly ground plant material for forest trees (such as pines), it is one of the most time- and labor- consuming parts of extraction protocols, and has limitations when handling multiple samples in parallel (Drabkova *et al.*, 2002). Also, laboratories need many freezers (-20 °C and/or -80 °C) to store large numbers of fresh samples.

Silica-dried samples have many advantages for plant species. Transporting plant samples domestically or internationally is very easy with the silica gel procedure. Sample transportation with silica gel is more practical, reliable, safer and inexpensive than freezing and preservative methods. Leaf sample drying of forest trees in silica gel is generally considered more effective than other methods due to rapid desiccation, inexpensive and, reusable characteristics of silica gel (Chase & Hills, 1991; Semagn, 2014). Homogenization of silica gel dried samples are easier, safer and faster than liquid nitrogen grinding. Also, it is possible to handle hundreds or thousands of dried samples in a mixer mill in a working day (Table 6, Drabkova *et al.*, 2002). Another advantage of the silica gel procedure is that dried samples in paper envelopes can be stored at room temperature in a moisture free office and/or laboratory many years. In this paper, it was demonstrated that high quality and high molecular weight DNA may be obtained from silica gel dried leaves/needles samples such

Table 4. Summary of RNA yield from Angiosperm and Gymnosperm families. Nanodrop measurement, OD 260/280 and 260/230 were obtained from frozen leaf tissue. ± indicates standard deviations.

Angiosperm								
			Nanodrop					
Family	RNA yield (ng/uL)		OD 260/238		OD 260/230		Sample number	
**Fagaceae* (1)	131.70	±35.47	1.41	±0.18	0.44	±0.15	8	
*Myrtaceae (4)	153.45	±24.69	1.54	±0.06	0.43	±0.06	40	
*Salicaceae (1)	19.94	±4.66	1.37	±0.14	0.18	±0.07	8	
*Sapindaceae (1)	476.69	±312.52	1.77	±0.13	0.91	±0.29	8	
General Angiosperm	174.45	±42.97	1.53	±0.05	0.46	±0.06	64	
Gymnosperm								
**Araucariaceae* (1)	70.85	±28.75	1.51	±0.17	0.36	±0.18	8	
**Cupressaceae* (3)	54.07	±10.73	1.55	±0.07	0.30	±0.06	24	
**Pinaceae* (16)	199.59	±17.32	1.85	±0.03	0.78	±0.05	128	
General Gymnosperm	171.33	±14.70	1.79	±0.03	0.68	±0.04	160	

*Number in brackets indicate the number of species in each family

Table 5. Bioanalyzer results showing RNA concentration and integrity. ± indicates standard deviations.

	Family	Species	RNA yield (ng/uL)	RIN	
Angiosperm	*Myrtaceae*	*Corymbia calophylla*	21.00	6.63	±0.26
		Eucalyptus globulus	45.33	8.03	±0.18
Gymnosperm	*Pinaceae*	*Abies fraseri*	96.33	8.07	±0.03
	Cupressaceae	*Sequoia sempervirens*	10.00	6.80	±0.31

as *Pinus* sp. after many years (Tables 1 and 2). It has reported that silica-dried samples have higher quality DNA than the preservative solution methods (Semagn, 2014). Sarkinen *et al.* (2012) reported that PCR success of silica gel dried leaves was 100% for all studied regions. However, they found that success of PCR using four other drying methods varied depending on amplified regions and drying method.

Nucleic acids extraction methods have an impressive effect on quality, quantity and purity of extracts. Also, speed of method, labor, extraction cost and broad taxonomic applicability are affected by the extraction protocol. The present protocol has advantages than other protocols, especially for comprehensive projects that have large numbers of samples. This protocol allows DNA extractions from many samples (hundreds) in a working day. RNA can also be extracted from four 96-well plates simultaneously with minor modifications of the DNA protocol (see Material and Methods). One extraction (4 × 96) process takes about 5 hours. If a laboratory only has one water bath and one plate centrifuge for four plates, another four plates could be extracted after each incubation period in the same day for DNA extraction. The present protocol has simpler and cheaper steps (see Material and Methods) than most other protocols (Table 6). High quality and high molecular weight DNA and RNA can be obtained from recalcitrant forest tree species which need more complex nucleic acids extraction methods than annual plants (Shepherd *et al.*, 2002; Le Provost *et al.*, 2007; Healey *et al.*, 2014; Valledor *et al.*, 2014).

Flexibility of present protocol

The present protocol requires a small amount of plant material for DNA extraction (from 28 to 51 mg, Tables 1 and 2). DNA can be extracted from fresh and dried specimens. Scalable features allow easy adaptation to different laboratories. DNA and RNA are isolated with small modifications of the same protocol which is a big advantage in terms of chemicals and solutions preparation and cost. It has 10 easy steps to get high quality and enough quantity of nucleic acids from forest trees for NGS library preparation (Table 6). The extraction (lysis) and washing buffers are changeable according

Table 6. Comparison of present protocol with common manual version of traditional and commercial DNA extraction protocols

Protocol	Tissue amount (mg)	Extraction buffer *	Sample number	Extraction time (hour)	Per sample cost ($)	RNase A/ Proteinase K	Taxonomic groups	References
Dellaporta *et al.* (1983)	>1000	SDS/CTAB	24-48	3-5	1.0-2.0	None (RNase A)	Plants	Itself; Csaikl *et al.*, 1998
Doyle & Doyle (1987)	>1000	CTAB	24	4-5 or overnight	1.0-3.0	RNase A	Plants	Itself; Sarkinen *et al.*, 2012
Doyle & Doyle (1990)	>1000	CTAB	24	2-3 or overnight	1.0-3.0	RNase A	Plants	Ostrowska *et al.*, 1998
Ivanova *et al.* (2006)	500	SDS	2 × 96	Overnight	0.50	Proteinase K	Mostly animals	Itself
Ivanova *et al.* (2008)	50-100	CTAB/PVP/ GuSCN	2 × 96	2-3	0.55	None	Plants	Itself; Kesanakurti *et al.*, 2011
Whitlock *et al.* (2008)	3	SDS	2 × 96	2-3 (half automated)	>1.0	Both	Plants and animals	Itself
The CCDB protocol	50-100	CTAB/ GuSCN	2 × 96	3-4	<0.50	Proteinase K	Plants, fungi, echinoderms and mollusks	www.ccdb.ca Ashfaq *et al.*, 2013
Qiagen DNeasy 96 Plant kit	25-100	Manufacturer buffer	2 × 96	1-2	2.0-4.0	RNase A	Plants	www.qiagen.com Shepherd *et al.*, 2002
Present study	28-51	GuSCN	4-12 × 96	5-8	<0.50	None	Plants	Itself

*SDS: Sodium dodecyl sulfate, CTAB: Cetyl trimethylammonium bromide, PVP:Polyvinylpyrrolidone, GuSCN: Guanidine thiocyanate.

to species and laboratory requirements. It requires only standard laboratory equipment, consumables and small number of chemicals. It is mostly processed at room temperature for DNA extraction and it can be rapidly established in any nucleic acids-based laboratory. The homogenization and washing steps of the present protocol can be adjusted according to species and type of plant tissue such as leaves, bark and fruit. For example, if soft tissue is used, the steps and time for homogenization, incubation and washing may be reduced. Proteinase K and RNase A are not used in the original present protocol steps. But they can be added to protocol according to desired nucleic acids quality, quantity and long-term storage of the samples.

The present protocol is based on 96-well silica-membrane plates, allowing the use of multi-channel pipet which reduces labor, pipetting time and error. The high throughput nucleic acids extraction of present protocol is a big advantage for per sample cost which can be reduced to less than other protocols (Table 6) for projects that have large sample numbers. Also, low cost of per sample can reduce the need for plant bulking or DNA pooling. The present protocol was tested in different 96-well plates with different researchers, and results of all plates were very similar in terms of quality and quantity of nucleic acids. The results from different researchers and species suggest that this protocol yields reproducible results. These features should allow the protocol to be adapted to many different taxonomic groups working laboratories, especially for forest trees.

Future actions and outputs

The protocol described here is designed to optimize nucleic acids extraction for many laboratories which use high number of species and samples from different taxonomic groups for DNA barcoding and NGS technologies. Beside the extraction protocol, features of the plant material such as type, age and quality of tissue may affect nucleic acids yield and purity due to, for instance, secondary metabolite accumulation which is a very common problem for forest trees and inhibits downstream applications (Ostrowska *et al.*, 1998). Mature tree leaves were generally used for DNA and RNA extraction in optimizing the present protocol. The use of seedlings or young parts of plants usually increases nucleic acids quality and quantity (Claros & Canovas, 1998). Some samples of herbaria-preserved plant materials were also used for DNA extraction with the present protocol; the DNAs extracted from these samples were highly fragmented and low quality according to agarose gel visualization (not shown here). The herbaria-preserved specimens have some problems such as low amount of suitable material and low yield of nucleic acids (Drabkova *et al.*, 2002). It would be worthwhile to optimize the present protocol if it is to be used for low amounts of herbaria-preserved plant material (from 28 to 51 mg, Tables 1 and 2), in order to get high quality and quantity DNA from herbarium specimens, which are a treasure of rare and/or extinct species (Staats *et al.*, 2013).

NGS technologies have generally been applied to highly domesticated forest tree species which belong to

four families (*Pinaceae*-gymnosperm and *Salicaceae*, *Myrtaceae* and *Fagaceae*-angiosperms) and seven genera within these families (*Pinus*, *Picea*, *Pseudotsuga*, *Populus*, *Eucalyptus*, *Quercus* and *Castanea*). Samples of all these except *Castanea* were included in this protocol development. These families and genera are a very small part of forest tree species which includes at least 100.000 species (Abril *et al.*, 2011; Neale & Kremer, 2011). The present protocol is also very valuable to isolate nucleic acids from the untouched diversity treasure of recalcitrant forest trees. The chemicals and some steps in the method reported here are based on the CCDB protocol, which is also effective at extracting DNA from animal specimens (Whitlock *et al.*, 2008). Therefore, it would be worthwhile to try this protocol method on animal species from different taxonomic groups in the future.

In conclusion, the present method for high-throughput extraction of DNA and/or RNA reported here is a combination of commercially available kits and traditional homemade protocols. This combination provides advantages from both protocols: the present protocol is quick and easy to apply to many samples (hundreds) in a working day, is inexpensive, requires only modest laboratory equipment, compatible to automation, yields high quality and sufficient quantity of DNA and RNA from recalcitrant forest trees.

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