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PROTOCOL NOTE

Modified CTAB protocols for high-molecular-weight DNA extractions from ferns

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Abstract

Premise: Efficient protocols for extracting high-molecular-weight (HMW) DNA from ferns facilitate the long-read sequencing of their large and complex genomes. Here, we perform two cetyltrimethylammonium bromide (CTAB)-based protocols to extract HMW DNA and evaluate their applicability in diverse fern taxa for the first time.

Methods and Results: We describe two modified CTAB protocols, with key adjustments to minimize mechanical disruption during lysis to prevent DNA shearing. One of these protocols uses a small amount of fresh tissue but yields a considerable quantity of HMW DNA with high efficiency. The other accommodates a large amount of input tissue, adopts an initial step of nuclei isolation, and thus ensures a high yield in a short period of time. Both methods were proven to be robust and effective in obtaining HMW DNA from diverse fern lineages, including 33 species in 19 families. The DNA extractions mostly had high DNA integrity, with mean sizes larger than 50 kbp, as well as high purity (A_{260}/A_{230} and $A_{260}/A_{280} > 1.8$).

Conclusions: This study provides HMW DNA extraction protocols for ferns in the hope of facilitating further attempts to sequence their genomes, which will bridge our genomic understanding of land plant diversity.

KEYWORDS

CTAB method, fern, HMW DNA extraction, nuclei isolation

Ferns were one of the last major plant lineages for which a whole genome was sequenced. The de novo assembly of typical fern genomes is particularly challenging due to their large size (Clark et al., 2016; Kuo and Li, 2019; Fujiwara et al., 2023) and surprising complexity (Zhong et al., 2022). The genomes of ferns in the order Salviniales were the first to be sequenced because of their unusually small size (Li et al., 2018), and long-read sequencing has made the assembly of additional fern genomes feasible (Fang et al., 2022; Huang et al., 2022; Marchant et al., 2022; Rahmatpour et al., 2023). The assembly of complex fern genomes requires a considerable amount of intact and pure DNA, namely high-molecular-weight (HMW) DNA. A PacBio library, for example, usually requires over 10 μg of HMW DNA averaging 30–50 kbp in size (Li and Harkess, 2018); therefore, establishing protocols to extract sufficient quantities of high-quality HMW DNA is essential for fern genome research.

For decades, researchers have observed that DNA extraction from ferns is particularly difficult. Dempster et al. (1999) noticed that DNA extraction methods used to tackle challenging flowering plants performed poorly on the maidenhair fern (*Adiantum capillus-veneris* L.). Previous DNA barcoding studies of ferns (e.g., De Groot et al., 2011) have relied on commercial DNA isolation kits to ensure the quality of DNA extracts from diverse fern lineages; however, while the incorporation of spin columns in most kits improves DNA purity, it also causes DNA fragmentation. Instead of using commercial kits, modified cetyltrimethylammonium bromide (CTAB)- or sodium dodecyl sulfate (SDS)-based methods have been adopted for HMW DNA extraction in various plant groups (Aboul-Maaty and Oraby, 2019; Li et al., 2020; Jones et al., 2021); however, none of these established protocols have been tested on ferns, whose tissues are usually rich in secondary metabolites (Vetter, 2018). Moreover, due to the

small body size of many fern species, there is often a limited amount of tissue per individual. For genome sequencing, acquiring a considerable amount of DNA from a single individual or clone is necessary to avoid mixing DNA from multiple individuals with non-identical genotypes, which usually complicates the downstream genome assembly.

In this study, we demonstrate two modified CTAB-based protocols for HMW DNA extraction in ferns, referred to as "standard" and "nuclei isolation," and apply them to samples from 33 diverse fern species across 19 families, as well as two lycophyte species. In contrast with a typical CTAB procedure (Doyle and Doyle, 1987; reviewed by Schenk et al., 2023), our protocols include modifications to minimize mechanical disruption (e.g., pulverization, pipetting, or vortexing) during the suspension or lysis steps (e.g., mixing with a CTAB buffer). In particular, we keep the ground tissue frozen and gently transfer it into CTAB or nuclei isolation buffer instead of grinding the tissue in the presence of these lysis buffers. Tissue debris is then removed through a filter. These modifications significantly reduce DNA shearing. The DNA yield of the standard protocol exceeded 8 μg of DNA and used less than 0.5 g of fresh leaf tissue. The nuclei isolation protocol adopts an initial step to isolate nuclei, which accommodates a greater input amount and ensures a high yield (>10 μg) of DNA. With both protocols, we successfully obtained pure HMW DNA products (as measured by the ratio of absorbance at 260 and 230 nm [A_{260}/A_{230}] and at 260 and 280 nm [A_{260}/A_{280}]; A_{260}/A_{230} and $A_{260}/A_{280} > 1.8$) with mean sizes longer than 50 kbp.

METHODS AND RESULTS

Standard protocol for HMW DNA extraction

Young and fresh plant tissues, usually newly expanded foliar parts or greenish gametophytes, were used for the HMW DNA extractions. These tissues and organs were preferred because of their nuclei-rich meristems and relatively fewer secondary metabolites (Moreira and Oliveira, 2011). Additionally, the gametophyte tissues sampled here were clones all derived from a single haploid spore, meaning they were genetically identical and completely homozygous for the ease of whole genome sequencing (WGS) and assembly. We were therefore particularly interested in testing HMW DNA extraction methods on these gametophyte samples. Before the DNA extraction, the leaves or gametophyte tissues were harvested and subjected to darkness for 48 h to reduce the contents of carbohydrates, polysaccharides, and polyphenolic compounds (Li et al., 2020). The equipment, including spatulas, mortars, and pestles, was autoclaved and then baked at 180°C for 8 h before use. Fresh tissues of each sample, typically 0.2 to 0.3 g, were flash-frozen in liquid nitrogen in a mortar before being ground into powder using a pestle. The frozen tissue powder was transferred into a 5-mL tube containing 2 mL of CTAB using a spatula (Figure 1), rather than mixing CTAB with the tissue powder in the mortar. To remove the tissue debris, the CTAB–tissue mixture was filtered through a 30-μm filter (Sysmex Partec, Goerlitz, Germany) followed by incubation at 55°C for 10 min (Figure 1). These procedures are critical to reduce

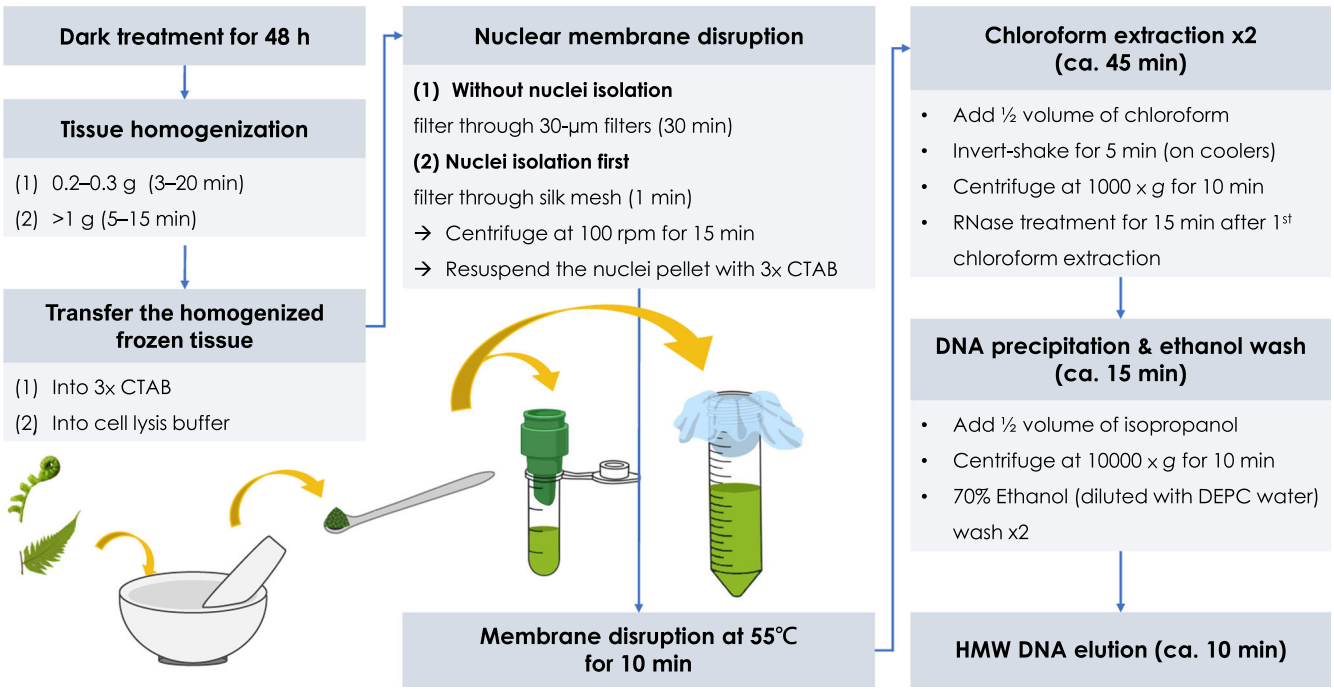

FIGURE 1 Flow diagrams of the two CTAB protocols for high-molecular-weight (HMW) DNA extractions. Steps identified as (1) represent the standard protocol with low tissue input and without nuclei isolation. Steps identified as (2) represent the nuclei isolation protocol for large tissue input including nuclei isolation.

the physical shearing of DNA caused by repetitive pipetting. The DNA extractions were then washed twice with a 1/2 volume of chloroform (details in Appendix 1). In between the chloroform washes, the aqueous extracts were treated with RNase A (100 mg/mL; Qiagen, Hilden, Germany) and incubated at room temperature for 10 min. After mixing a 1/2 volume of cool isopropanol (pre-chilled at −20°C) with the extractions, the mixtures were centrifuged under 13,000 × *g* at 4°C for 10 min to pellet the DNA. The DNA pellets were then washed twice with 70% ethanol and air-dried at room temperature for 3 min, before being dissolved in nuclease-free water. If the DNA extracts will be used immediately for sequencing, they should be stored at 4°C to avoid any further freeze-thawing; otherwise, −80°C is a better condition for their long-term storage.

Nuclei isolation protocol for HMW DNA extraction

We modified this protocol by increasing the amount of input tissue and adding an initial nuclei isolation step. This is likely to improve DNA purity by first separating the contaminants, such as polysaccharides, polyphenols, and other secondary metabolites, from the tissue (Li et al., 2020). About 6–8 g of fresh tissue per sample was ground as described for the standard protocol above, and the powdered tissue was mixed with 30 mL of nuclei isolation buffer in a 50-mL tube. We selected the ideal nuclei isolation buffer based on the taxon: either LB01 (Doležel et al., 1989, 2007), Beckman (Ebihara et al., 2005), or general-purpose buffer (GPB; Loureiro et al., 2007). The buffer selection depended on their flow cytometric performance in the same species or a close relative (e.g., Clark et al., 2016; Fujiwara et al., 2023). The tissue–buffer mixture was filtered through a 250-mesh (~58 μm) polyester silk screen printing mesh (Figure 1), which could accommodate a large flowthrough volume. After removing the tissue debris, the filtered mixture was centrifuged at 100 × *g* for 15 min at 4°C to pellet the nuclei. After carefully discarding the supernatant, the retained nuclei pellet was dissolved in 4 mL of CTAB solution (details in Appendix 2). The remaining procedures, including the lysis, chloroform wash, RNase treatment, DNA precipitation, and storage, were the same as described in the standard protocol.

Quality and quantity of HMW DNA

The DNA integrity was first evaluated by electrophoresis using a Tris acetate EDTA (TAE) 1% agarose gel. The purity of the DNA extractions was inferred using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) to measure their A_{260}/A_{230} and A_{260}/A_{280} values. The precise DNA concentration was determined using Quanti-Fluor (Promega, Madison, Wisconsin, USA). Eight recently extracted samples that had not been subjected to repeated freezing and thawing (Table 1) were selected for the examination of their DNA fragment sizes using a Fragment Analyzer 5200 (Agilent Technologies, Santa Clara, California, USA). The DNA recovery rate, defined as the total DNA yield (in micrograms) recovered per gram of fresh input tissue, was also calculated. Using our standard protocol, we extracted HMW DNA from 31 fern species and two lycophyte species (Appendix S1). Notably, due to the limited amounts of fresh tissue per individual, we were unable to test both the standard and nuclei isolation protocols on all samples, except for the two *Diplopterygium* (Diels) Nakai species.

Comparing the performance of the two protocols on the two *Diplopterygium* species, the nuclei isolation protocol likely generated DNA extracts with a greater purity and integrity than the standard method (Table 1); however, in the case of *D. blotianum* (C. Chr.) Nakai, we only checked the DNA fragment length after the bead cleanup, which might also shear DNA. No consistent pattern was found in the recovery rate of DNA between these two cases (Table 1). The qualitative comparisons with additional samples showed no obvious differences in the resultant DNA purity and recovery rates of DNA between the two protocols (Appendix S1). Applying both protocols to more fern species and samples in the future would facilitate rigorous statistical tests of their performances. Using the standard protocol, the DNA extraction of a batch comprising fewer than seven samples could take about 2 h. By contrast, the nuclei isolation protocol can yield 40–60-fold more DNA from a single run (Table 1) that usually takes only about 4 h. When sufficient fresh tissue can be sampled from one individual, we found the nuclei isolation protocol to be more efficient for obtaining sufficient HMW DNA for a WGS project. In addition, because the nuclei isolation protocol concentrates the nuclei and greatly reduces the volume of the CTAB mixture, DNA should not be pooled from multiple tubes or batch runs, as this potentially risks HMW DNA being sheared due to repeated pipetting and freeze-thawing cycles.

The two protocols were applied to a total of 33 fern taxa from 19 families, as well as two lycophyte species (Table 1, Appendix S1). Collectively, among a total of 68 samples, 54 (79.4%) were shown to have both A_{260}/A_{230} and A_{260}/A_{280} values above 1.8 when using the standard protocol; all but one sample achieved these values when adopting the nuclei isolation protocol. Both protocols can generate considerable amounts of HMW DNA; the recovery rates of DNA were comparable in both protocols. For most samples, more than 5 μg of HMW DNA can be recovered from 1 g of fresh tissue, although variation is observed among the fern species (Appendix S1). The eight samples selected for examination of DNA fragment size appeared to have high DNA integrity because their DNA bands had no smear, as revealed by capillary electrophoresis (Figure 2, Appendix S2). These HMW DNA fragments were an average of 53.1 kbp and a maximum of 77.25 kbp in length (Table 1, Appendix S2).

TABLE 1 Quality and quantity of the eight selected high-molecular-weight (HMW) DNA samples.

Taxon (Family)	Collection locality, voucher (herbarium)	Tissue	Protocol	Tissue weight (g)	A_{260}/A_{280}	A_{260}/A_{230}	DNA concentration (ng/μL)[a]	Amount of DNA yield (μg)	Recovery rate of DNA (μg/g)[b]	Main size (kbp)
Diplopterygium chinensis (Rosenst.) De Vol (Gleicheniaceae)	Taiwan, *Kuo4660* (TAIF)	Fresh leaf	Standard	0.33	1.93	2.02	129	8.385	25.61	47.54
			Nuclei isolation	12	1.96	2.10	1270	393.700	32.81	47.54
Diplopterygium blotianum (C. Chr.) Nakai (Gleicheniaceae)	Taiwan, *Kuo4659* (TAIF)	Fresh leaf	Standard[c]	0.301	1.84 (1.91[f])	1.87 (1.74[f])	48.6	2.673	8.72	45.15
			Standard[d]	0.26	1.88 (1.69[f])	2.26 (1.26[f])	128	8.320	32.29	NA
			Nuclei isolation	12	1.83	1.82	1120	179.200	14.93	59.38
Tectaria devexa (Kunze) Copel. (Tectariaceae)	Taiwan, *BH00033* (TAIF)	Fresh leaf	Standard	0.20	1.85	1.78	248	15.872	78.61	45.18
Deparia lancea (Thunb.) Fraser-Jenk. (Athyriaceae)	Taiwan, *Kuo4294* (TAIF)	Fresh leaf	Standard	0.20	1.91	1.99	1060	67.840	336.84	77.25
Sceptridium formosanum (Tagawa) Holub (Ophioglossaceae)	Taiwan, *Kuo4635* (TAIF)	Fresh leaf	Standard	0.23	2.06	2.16	565	22.600	98.73	52.39
Nephrolepis biserrata (Sw.) Schott (Nephrolepidaceae)	Taiwan, *Kuo4549* (TAIF)	Gametophyte	Standard[e]	1.23	1.69	1.48	87	8.700	7.09	48.50

Note: NA = not applicable; TAIF = Herbarium of Taiwan Forestry Research Institute.

[a]Measured using QuantiFluor.

[b]Amount of DNA divided by the starting weight of the sample.

[c]Final DNA product was cleaned using KAPA Hyper Pure Beads.

[d]Final DNA product was cleaned using Select-a-Size MagBeads.

[e]Precipitation with isopropanol mixed with high concentrations of salt.

[f]Values before bead cleanup.

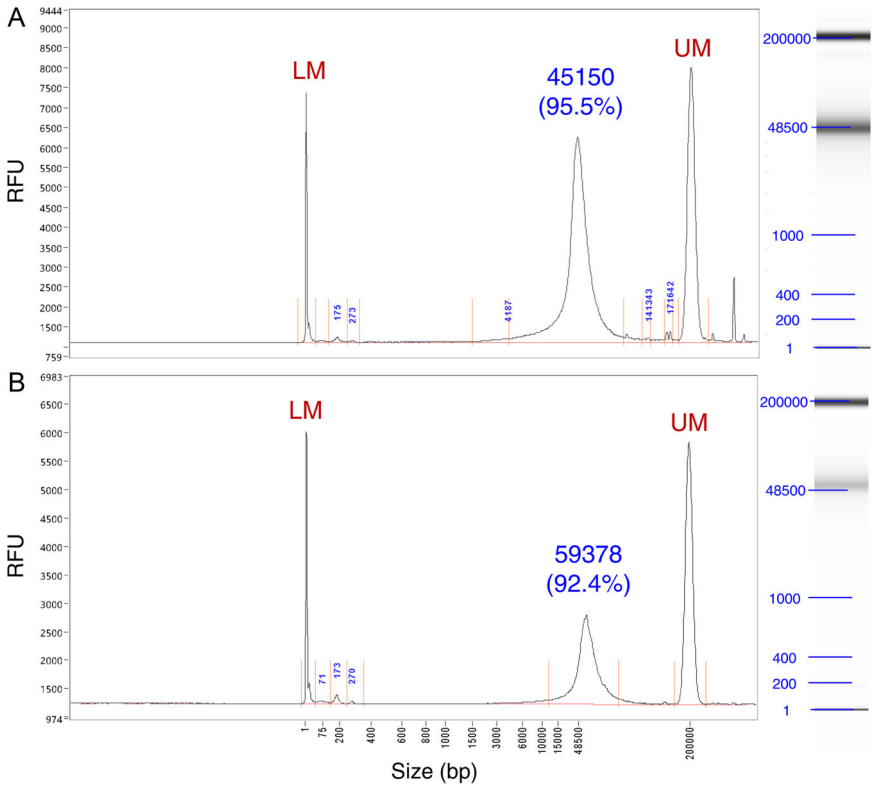

FIGURE 2 Integrity and size of the high-molecular-weight (HMW) DNA extractions from *Diplopterygium blotianum*. (A) Using the standard protocol, the fragment length profile of the HMW DNA product had a main peak at 45.150 kbp. (B) Applying the nuclei isolation protocol, the main peak of HMW DNA fragments was observed at 59.378 kbp. The main peak accounts for 95.5% and 92.4%, respectively, of whole DNA fragment distribution. The HMW DNA fragments from both protocols were largely intact, with almost no smearing on an agarose gel following electrophoresis. LM, lower marker; RFU, relative fluorescence units; UM, upper marker.

Bead-based cleanup and high-salt precipitation

Magnetic beads were sometimes used to clean the extracted DNA products. We used KAPA HyperPure Beads (Roche, Basel, Switzerland) and Select-a-Size DNA MagBead (Zymo Research, Irvine, California, USA) to clean two HMW DNA samples extracted from *D. blotianum* (Table 1). The volume ratio of beads to sample and the cleanup procedures followed the manufacturer's protocols. The post-cleanup A_{260}/A_{230} and A_{260}/A_{280} values indicated that the beads improved the purity of the DNA extractions (e.g., A_{260}/A_{230} increased from 1.26 to 2.26; Table 1). Some established protocols also use magnetic beads to purify and concentrate HMW DNAs (Mayjonade et al., 2016; Russo et al., 2022). The cleaned extractions retained DNA fragments of acceptable length for long-read sequencing (*D. blotianum* in Table 1), although we did not compare the DNA sizes before and after the cleanup. In the case of the *Nephrolepis biserrata* (Sw.) Schott gametophyte, a high-concentration salt solution (1/10 volume of 3 M NaOAc) was added to the cool isopropanol to reduce the precipitation of polysaccharides (Nishii et al., 2022). With this high-salt treatment, the A_{260}/A_{230} of the resultant DNA extraction was improved to 1.49, while the A_{260}/A_{230} ranged from 0.29 to 1.11 in samples without any treatment.

Long-read sequencing

Applying our nuclei isolation protocol, the HMW DNA extracts from the lycophyte *Isoetes taiwanensis* De Vol were sequenced and performed well on the nanopore platform (Oxford Nanopore Technologies, Oxford, United Kingdom) (Wickell et al., 2021). One run generated a total of 66 Gbp of data from 2629 out of 3000 active channels, with a N50 read length of 14.64 kbp. Our standard protocol was also applied in a recent study to sequence the genome of the fern *Marsilea vestita* Hook. & Grev. using MinION nanopore sequencing (Rahmatpour et al., 2023). In this case, more than 500 out of 512 active channels produced 34 Gbp of long reads, reaching a N50 read length of 25.8 kbp, from which chromosome-level assemblies were successfully generated. These results indicate that HMW DNA extractions using our CTAB-based protocols are of high purity and integrity, and also meet the needs for library construction and sequencing using a nanopore system.

CONCLUSIONS

This is the first study to demonstrate how CTAB-based methods perform for HMW DNA extractions from diverse fern species, as well as two lycophytes. Our CTAB protocols

incorporate some minor but critical modifications to minimize DNA shearing during the extraction. The two protocols, "standard" and "nuclei isolation," were designed for small and large amounts of input tissue, respectively, the availability of which can depend on the size of the plant individuals and the availability of living tissue sources. Although direct comparisons between the two protocols were limited here, overall both protocols performed similarly in terms of the resultant DNA purity, integrity, and recovery rates. They both yielded HMW DNA extracts with A_{260}/A_{230} and A_{260}/A_{280} values above 1.8, indicating high purity; an average fragment size >45 kbp; and a yield of >10 µg when using 2 g of fresh tissue. In our comprehensive test with diverse fern species across 19 families, nearly 80% of extractions met this criterion of DNA purity (A_{260}/A_{230} and A_{260}/A_{280} values > 1.8) using the standard protocol. From the comparisons of the two *Diplopterygium* species, the nuclei isolation protocol appeared to perform better than the standard one in terms of the resultant DNA purity and integrity; however, to conclusively determine the optimal protocol, we encourage future researchers to further test both methods on more fern samples and taxa. Most importantly, our protocols provide a solution for preparing adequate quantities of HMW DNA with sufficient quality for the long-read sequencing of these plants, which is essential for a chromosome-level genome assembly. Finally, in combination with investigations into genome sizes in different fern lineages (Kuo and Li, 2019), we hope that the HMW DNA extraction protocols presented here can facilitate further attempts at WGS of these plants and help to bridge our genomic understanding of land plant diversity (e.g., 10KP project; Cheng et al., 2018).

AUTHOR CONTRIBUTIONS

All authors carried out the experiments. P.-J.X. prepared the first draft. L.-Y.K. and P.-J.X. revised the manuscript. All authors approved the final version of the manuscript.

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DATA AVAILABILITY STATEMENT

All supporting data are provided in the article and the Supporting Information.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Appendix S1. Sample and voucher information for the taxa used for high-molecular-weight DNA extraction.

Appendix S2. Integrity and size distribution of the high-molecular-weight DNA extractions from the selected samples.

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Appendix 1: Standard protocol for high-molecular-weight (HMW) DNA extraction for ferns and lycophytes.

Reagents

3× Cetyltrimethylammonium bromide (CTAB)
β-Mercaptoethanol (β-ME; MilliporeSigma, Burlington, Massachusetts, USA)
Polyvinylpolypyrrolidone (PVPP; MilliporeSigma)
Liquid nitrogen in a Dewar flask
100% chloroform
RNase A (Qiagen, Hilden, Germany)
75% ethanol (diluted with diethylpyrocarbonate [DEPC]-treated water or nuclease-free water)
100% cool isopropanol (pre-chilled at −20°C)
Isopropanol mixed with 3 M NaOAc (optional)
Nuclease-free water

Equipment and supplies

15-mL Falcon tubes (DNase and RNase free)
Mortar and pestle (autoclaved before use)
Lab spatulas (autoclaved before use)
5-mL, 2-mL, and 1.5-mL Eppendorf tubes (DNase and RNase free)
1000-µL and 200-µL wide-bore pipette tips
30-µm CellTrics filters (Sysmex Partec, Goerlitz, Germany)
Thermal shaker (or incubator)
Refrigerated centrifuge
Cooling racks

Buffer setup

3× CTAB buffer (pH 8.0)
30 mg/mL CTAB (w/v)
0.1 M Tris HCl
0.02 M EDTA
1.4 M NaCl

Procedure

0. Preparation before extraction

Step 0.1 Sample newly expanding fronds or gametophytes and subject them to darkness for 48 h. We usually sealed the samples in plastic bags, wrapped them in paper, and refrigerated at 4°C to keep the samples fresh during the dark treatment.

Step 0.2 Bake the autoclaved spatulas and mortars and pestles at 180°C for 8 h to eliminate contamination by nucleases.

Step 0.3 Prepare 2 mL of 3× CTAB buffer for each sample and add 5 µL of β-ME and 4 mg of PVPP per mL of CTAB buffer. Make the buffer in a 15-mL Falcon tube and dispense 2 mL into a 5-mL tube.

Step 0.4 Precool the centrifuge to 4°C.

Step 0.5 Preheat the incubator (with adapter for 2-mL tubes) to 55°C.

1. Freeze and pulverize the tissue

Step 1.1 Weigh 0.2–0.3 g of fresh tissue and put in a mortar.

Step 1.2 Add two scoops of liquid nitrogen into the mortar and immediately pulverize the tissue with the pestle. The homogenized tissue should be kept frozen.

Step 1.3 Use a spatula to transfer the frozen tissue powder to a 5-mL tube containing the 2 mL of CTAB buffer. Do not add the CTAB buffer into the mortar. Invert-shake the 5-mL tubes to mix the tissue powder with the CTAB buffer and place the tubes on a cooling rack.

2. Remove the tissue debris and lyse the cells

Step 2.1 Filter the matrix through a 30-µm CellTrics filter into 2-mL tubes. Collect no more than 1 mL of filtrate in each tube and separate into two tubes if necessary.

Step 2.2 Incubate the samples at 55°C for 10 min. Invert-mix the samples every 3 min.

3. Extract nucleic acids from proteins and other metabolites using chloroform

Step 3.1 Add 0.5 mL of 100% chloroform to each 2-mL tube. Place the samples on a cooling rack and invert-shake for 5 min.

Step 3.2 Centrifuge the samples at 13,000 × *g* at 4°C for 10 min. Transfer the supernatant to new 2-mL tubes using wide-bore pipette tips.

Step 3.3 Add 1 µL of RNase to each tube, invert-shake gently, and incubate at room temperature for 15 min. Do not vortex the samples.

Step 3.4 Repeat Step 3.2, but transfer the supernatant into 1.7-mL tubes using wide-bore pipette tips (less than 0.8 mL sample per tube).

4. Precipitate the HMW DNA

Step 4.1 Add a 1/2 volume of cool 100% isopropanol to each tube. Invert-mix the solution. Cool high-salt isopropanol can be used for some challenging samples.

Step 4.2 Centrifuge the samples under 10,000 × *g* at 4°C for 10 min. If pellets are not seen, centrifuge for another 10 min. Discard the supernatant.

5. Ethanol wash

Step 5.1 Add 200 µL of 70% ethanol (diluted with nuclease-free water) and gently suspend the pellet.

Step 5.2 Centrifuge the samples under 10,000 × *g* at 4°C for 10 min. Discard the supernatant.

Step 5.3 Repeat Steps 5.1 and 5.2. Spin down the tubes and remove the ethanol completely using a pipette.

6. Elute the HMW DNA

Step 6.1 Dry the pellets under a chemical hood at room temperature for 3–5 min. Do not overdry the pellets.

Step 6.2 Dissolve the pellets in each tube in 15–25 µL of nuclease-free water at room temperature for about 10 min and flip the tubes gently to ensure the pellets are well dissolved. Do not vortex the samples.

Step 6.3 If DNA sequencing will be conducted immediately after the extraction, the HMW DNA should be stored at 4°C to avoid unnecessary freeze-thaw cycles. Otherwise, keep one tube or 10-µL aliquot per sample for the quality/quantity examination and preserve the rest of the sample at −80°C for long-term storage.

Appendix 2: Nuclei isolation protocol for high-molecular-weight (HMW) DNA extraction for ferns and lycophytes.

Reagents

Nuclei isolation buffer (LB01, general-purpose buffer [GPB], or Beckman buffer)
Polyvinylpyrrolidone (PVP-40; MilliporeSigma, Burlington, Massachusetts, USA)
3× Cetyltrimethylammonium bromide (CTAB)
β-Mercaptoethanol (β-ME; MilliporeSigma)
Polyvinylpolypyrrolidone (PVPP; MilliporeSigma)
Liquid nitrogen in a Dewar flask
100% chloroform
RNase A (Qiagen, Hilden, Germany)
75% ethanol (diluted with diethylpyrocarbonate [DEPC]-treated water or nuclease-free water)
100% cool isopropanol (pre-chilled at −20°C)
Isopropanol mixed with 3 M NaOAc (optional)
Nuclease-free water

Equipment and supplies

100-mL laboratory glass bottle (autoclaved before use)
50-mL Falcon tubes (DNase and RNase free)
Mortar and pestle (autoclaved before use)
Lab spatulas (autoclaved before use)
5-mL, 2-mL, and 1.5-mL Eppendorf tubes (DNase and RNase free)
1000-µL and 200-µL wide-bore pipette tips (DNase and RNase free)
250-mesh (~58 µm) polyester silk screen printing mesh
Thermal shaker (or incubator)
Refrigerated centrifuge
Cooling racks
Ice box with ice

Buffer setup

3× CTAB buffer (pH 8.0)
30 mg/mL CTAB (w/v)
0.1 M Tris HCl
0.02 M EDTA
1.4 M NaCl

One of three types of nuclei isolation buffer:

Beckman stock buffer (pH 7.5) (Ebihara et al., 2005)
1% (v/v) Triton X-100
50 mM Na_2SO_3
50 mM Tris-HCl
ddH_2O (the solvent)

LB01 buffer (pH 7.5) (Doležel et al., 2007)
15 mM Tris-HCl
2 mM Na_2EDTA
0.5 mM spermine tetrahydrochloride
80 mM KCl
20 mM NaCl
0.1% (v/v) Triton X-100
ddH_2O (the solvent)

GPB (pH 7.0) (Loureiro et al., 2007)
0.5 mM spermine · 4HCl
30 mM sodium citrate
20 mM 4-Morpholinepropane sulfonate (MOPS)
80 mM KCl
20 mM NaCl

0.5% (v/v) Triton X-100
ddH_2O (the solvent)

Procedure

0. Preparation before extraction

Step 0.1 Sample newly expanding fronds or gametophytes and subject them to darkness for 48 h. We usually sealed the samples in plastic bags, wrapped them in paper, and refrigerated at 4°C to keep the samples fresh during the dark treatment.

Step 0.2 Bake the autoclaved spatulas and mortars and pestles at 180°C for 8 h to eliminate contamination by nucleases.

Step 0.3 Prepare 30 mL of nuclei isolation buffer for each sample and add 5 μL of β-ME, 40 mg of PVP-40, and 1 μL of RNase per mL of nuclei isolation buffer. Make the buffer in a 100-mL glass bottle and dispense 30 mL into a 50-mL Falcon tube. Place the tubes on ice.

Step 0.4 Prepare 4 mL of 3× CTAB buffer for each sample and add 5 μL of β-ME and 4 mg of PVPP per mL of CTAB buffer. Make the buffer in a 50-mL Falcon tube.

Step 0.5 Precool the centrifuge to 4°C.

Step 0.6 Preheat the incubator (with adapter for 2-mL tubes) to 55°C.

1. Freeze and pulverize the tissue

Step 1.1 Weigh 6–8 g of fresh tissue and put in a mortar.

Step 1.2 Add two scoops of liquid nitrogen into the mortar and immediately pulverize the tissue with the pestle. The homogenized tissue should be kept frozen.

Step 1.3 Use a spatula to transfer the frozen tissue powder to a 50-mL Falcon tube containing 30 mL of nuclei isolation buffer. Invert-shake the 50-mL tubes to mix the tissue powder with the nuclei isolation buffer and place the tubes on a cooling rack.

Step 1.4 Repeat Steps 1.2 and 1.3 until all the materials are pulverized into powder.

2. Remove the tissue debris and isolate nuclei

Step 2.1 Filter the matrix through a 250-mesh polyester mesh into 50-mL tubes.

Step 2.2 Centrifuge the filtered extractions at 100 × *g* at 4°C for 15 min.

Step 2.3 Carefully transfer the supernatant to another 50-mL tube and avoid disturbing the pellet. Keep the tube containing the pellet on ice.

Step 2.4 Centrifuge the supernatant at 100 × *g* at 4°C for 15 min again to gather more nuclei. (optional)

Step 2.5 Add 4 mL of CTAB buffer to dissolve the pellet in the 50-mL tube. Transfer the solution into 2.0-mL tubes (dispense up to 1.5 mL sample per tube).

Step 2.6 Incubate the samples at 55°C for 10 min. Invert-mix the samples every 3 min.

3. Extract nucleic acids from proteins and other metabolites using chloroform

Step 3.1 Add 0.5 mL of 100% chloroform to each 2.0-mL tube. Place the samples on a cooling rack and invert-shake for 5 min.

Step 3.2 Centrifuge the samples at 10,000 × *g* at 4°C for 10 min. Transfer the supernatant to new 2-mL tubes using wide-bore pipette tips.

Step 3.3 Add 1 μL of RNase A (100 mg/mL) to each tube, invert-shake gently, and incubate at room temperature for 15 min. Do not vortex the samples.

Step 3.4 Repeat Step 3.2, but transfer the supernatant into 1.5-mL tubes using wide-bore pipette tips (less than 0.8 mL sample per tube).

4. Precipitate the HMW DNA

Step 4.1 Add a 1/2 volume of cool 100% isopropanol to each tube. Invert-mix the solution. Cool high-salt isopropanol can be used for some challenging samples.

Step 4.2 Centrifuge the samples under 13,000 × *g* at 4°C for 10 min. If pellets are not seen, centrifuge for another 10 min. Discard the supernatant.

5. Ethanol wash

Step 5.1 Add 200 μL of 70% ethanol (diluted with nuclease-free water) and gently suspend the pellet.

Step 5.2 Centrifuge the samples under 10,000 × *g* at 4°C for 10 min. Discard the supernatant.

Step 5.3 Repeat Steps 5.1 and 5.2. Spin down the tubes and remove the ethanol completely using a pipette.

6. Elute the HMW DNA

Step 6.1 Dry the pellets under a chemical hood at room temperature for 3–5 min. Do not overdry the pellets.

Step 6.2 Dissolve the pellets in each tube in 15–25 μL of nuclease-free water (roughly 80–120 μL after pooling together per sample) at room temperature for about 10 min and flip the tubes gently to ensure the pellets are well dissolved. Do not vortex the samples.

Step 6.3 If DNA sequencing will be conducted immediately after the extraction, the HMW DNA should be stored at 4°C to avoid unnecessary freeze-thaw cycles. Otherwise, keep one tube or 10-μL aliquot per sample for the quality/quantity examination and preserve the rest of the sample at −80°C for long-term storage.