**Something old, something new, a DNA preparation
procedure for long read genomic sequencing**

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Something old, something new, a DNA preparation procedure for long read genomic sequencing

Hongjun Wang and Sen Xu*

Department of Biology, University of Texas at Arlington, 76019, Texas, USA

*Correspondence: 501 S. Nedderman Dr, Arlington, TX, 76019. Email: sen.xu@uta.edu

Key words: CTAB, long read sequencing, third-generation sequencing, *Daphnia*

Running title: High molecular weight DNA for long read sequencing

16   **Abstract**

17   Extracting high molecular weight DNA is critical for successful long read DNA sequencing.
18   Here we present a DNA preparation procedure combining CTAB DNA extraction protocol and a
19   short read eliminator kit, which consistently produces high yields of high molecular weight DNA
20   in the freshwater microcrustacean *Daphnia*. This method only requires common chemicals and
21   equipment, providing researchers a viable alternative to DNA extraction kits that often require
22   specialized tools.

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23   **Text**

24   Generating sufficient amount of high molecular weight (HMW) DNA is a key for successfully
25   generating third generation, long read sequencing data (e.g., Oxford Nanopore and PacBio
26   sequencing). As DNA molecules can be easily fragmented during common in-house DNA
27   extraction protocols, commercial DNA extraction kits are often used for extracting HMW DNA.
28   To achieve the preservation of HMW DNA, these commercial kits often utilize gentle enzymatic
29   digestions (e.g., proteinase K) to release DNA from cells/tissues and use precipitation or high
30   affinity binding for purifying DNA molecules. However, these kits often require users to provide
31   extra specialized tools such as a magnetic rack or thermomixer for accomplishing certain steps in
32   the procedure, creating an undesirable barrier for researchers who do not have access to these
33   tools.

34   Here we describe a DNA preparation protocol that our research group routinely uses to
35   obtain high yields of HMW DNA from the freshwater microcrustacean *Daphnia pulex*, a widely
36   distributed species that serves as an important model system in ecology, evolution, toxicology,
37   and genomics. This procedure combines the CTAB extraction protocol of Doyle and Doyle
38   (1987) and a new short read eliminator kit. Requiring only common chemicals and laboratory
39   equipment, the CTAB method provides a low-cost alternative to commercial DNA extraction
40   kits, whereas the short read eliminator can be used to enhance sequencing results. We note some
41   simple modifications to the procedure of Doyle and Doyle (1987). First, for all steps that involve
42   aspirating or dispensing solutions containing tissues/DNA (excluding steps for adding chemical
43   reagents), we use wide bore pipettor tips that are cut from regular tips. These wide bore pipettor
44   tips help to reduce fragmentation of DNA molecules. Second, we used a much higher volume of
45   2-mercaptoethanol in our CTAB buffer than the original protocol of Doyle and Doyle (1987).

Adding extra 2-mercaptoethanol leads to the preservation of HMW DNA molecules, because as a reducing reagent 2-mercaptoethanol protects DNA from free oxidative radicals released from tissue and cells (Abbasi Kejani et al. 2009; Azmat et al. 2012). Third, because DNA is less soluble in isopropanol than in ethanol (Green & Sambrook 2017), we used isopropanol instead of 95% ethanol to precipitate DNA and increase final DNA yield (step 8 below), which was also used in the CTAB protocol from Athanasio et al (2016).

DNA extraction procedure

1. At 65 °C, CTAB buffer is warmed up (stored at 4 °C) containing 100mM Tris-HCl pH 8.0, 1.4 M NaCl, 20mM EDTA, 2% cetyltrimethylammonium bromide, and 0.2% 2-mercaptoethanol. After CTAB buffer becomes clear, an aliquot of buffer is prepared which is enough for processing all samples (500µl per sample) and additional 10µl 2-mercaptoethanol per ml is added to the CTAB buffer.
2. 100 *Daphnia* adults (from a lab culture), about 72 mg (wet weight), are transferred into one 1.5ml microcentrifuge tube with a pipette. 200ul homogenization buffer is added into the tube. The sample is ground gently with a plastic pestle. Then the pestle is washed with additional 300µl CTAB buffer into the tube.
3. The sample is incubated at 65 °C for 120 minutes in water bath.
4. The sample is centrifuged for 5 minutes at 17000 RCF in an Eppendorf centrifuge. Then 400-500µl supernatant is transferred into one new labeled 1.5 ml tube with wide pore pipettes. 500µl of chloroform : isoamyl alcohol (24:1, volume ratio) is added and the sample is gently mixed.
5. The sample is centrifuged at 17000 RCF at 4 °C. Then three layers appear with the mixture in the tube.

6. 5µl (10µg/µl) RNase is added into the upper layer that contains DNA. Avoid disturbing the middle layer containing remnants of tissue. Then the sample is incubated for 20 minutes at 37 °C.
7. The sample is gently mixed by inverting the tube 8-10 times and then centrifuged for 15 minutes at 17000 RCF at 4 °C.
8. The supernatant is transferred into a new 1.5ml tube with wide pore tips and 80% volume of cold 100% isopropanol (stored at -20 °C) is added into the supernatant. The sample is gently mixed by inverting the tube for 20 times. A cloudy solution will appear.
9. The sample is incubated for 2 hours (or overnight as a safe stopping point) at -20 °C and then centrifuged for 30 minutes at 17000 RCF at 4 °C.
10. The supernatant (the clear layer) is carefully discarded without disturbing the cloudy looking precipitated DNA. Then 1ml cold 70% ethanol (molecular grade, stored at -20 °C) is added into the tube. The sample is centrifuged for 15 minutes at 17000 RCF. The supernatant is carefully discarded after centrifuging.
11. The sample is left to air dry for 15-20 min at room temperature (20-25 °C). Then 50µl ultra-pure distilled RNase-free and DNase-free water or TE buffer are added into the tube to re-dissolve DNA.

After DNA extraction, we ran pulse field gel electrophoresis using a Sage Science Pippin Pulse power supply and Owl™ D2 Wide-Gel Electrophoresis System to check the fragment length distribution of extracted DNA samples (for running parameters see **Table 1**). We also removed short DNA fragments in the samples using the Short Read Eliminator Kit (Circulomics, Baltimore, MD) and then ran the samples on a 1% agarose gel.

Using this procedure, we consistently get high yields of high molecular weight DNA (**Table 2**). Most DNA molecules are > 48.5 kb (kilo basepair) and longest molecules reaching ~1 million basepairs (**Figure 1**), despite the presence of shorter DNA molecules. Compared to the observation of Doyle and Doyle (1987) that the original method generates DNA fragments larger than 30 kbp, our modification further improves the DNA fragment length distribution. After short fragment removal, the majority of DNA fragments are over 48 kb (**Figure 2**), and the DNA yield and quality are ready for library preparation for long read sequencing (**Table 3**). A Nanopore sequencing run was performed using the prepared samples. The mean read length is 6479 bp, with the longest reads reaching ~50kb (**Figure 3**; SRA accession no. PRJNA742705). The read length is comparable to published datasets (e.g., Aganezov et al. 2020). Further size selection can be performed on the input DNA and sequencing library to enhance sequence read lengths.

Although we have only experimented this method on the microcrustacean *Daphnia*, the original method has proven to be effective for a variety of plant tissues (Doyle & Doyle, 1987). Furthermore, an efficient CTAB DNA extraction method was also developed by Chakroborty et al. (2020) for non-marine molluscs (2020). Thus, our modified method is likely useful for extracting HMW DNA from plant and other types of tissues too. Although for other types of tissues and organisms the efficacy of this method remains to be tested, we suggest that researchers consider testing this CTAB method (<\$0.1 per sample) before settling on a commercial DNA extraction kit that cost a few dollars per sample.

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114   **Competing interests**

115   The authors declare no competing interests.

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141 **Figure captions**

142 **Figure 1.** Pulse field gel image of DNA samples extracted from *Daphnia pulex* using CTAB
143 protocol. Samples, S1 (1.2 ug), S2 (0.8 ug), and S3 (0.5 ug) remain in a high molecular weight.
144 Two molecular ladders, M1, 0.5 ug (Bio-Rad, catalog no.1703635) and M2, 0.5 ug (NEB,
145 catalog no. N3239S), were loaded on the right most lanes.

146 **Figure 2.** 1% agarose gel image of DNA samples, S1 (0.6 ug), S2 (0.8 ug), and S3 (1.1 ug) after
147 removal of short fragments, with molecular ladder M, 0.5 ug (NEB, Cat# N3239S) loaded on the
148 left most lane.

149 **Figure 3.** Read length distribution of DNA sequences from a MinIon nanopore sequencer using
150 the DNA prepared with the current method. The mean read length is 6479 bp.

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153   **Table 1.** Parameters for running pulse field gel electrophoresis using Pippen Pulse.

Parameters	Setting
A. Forward Time at start of run	300 msec
B. Reverse Time at start of run	100 msec
C. Increment added to A at each step	30 msec
D. Increment added to B at each step	10 msec
E. Increment added to C at each step	30 msec
F. Increment added to D at each step	10 msec
G. Number of steps per cycle	45 msec
Running time	12 hrs
Voltage	75V

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157 **Table 2.** DNA quality metrics for samples S1, S2, and S3 after CTAB extraction. Approximately
158 100 *Daphnia pulex* adults were used for DNA extraction of each sample.
159 NanoDrop™ Microvolume UV-Vis Spectrophotometer and Qubit 4 Fluorometer were used to
160 test the DNA quality.

	Nanodrop (ng/μl)	Qubit (ng/μl)	260/280	260/230
S1	404.5	321.2	1.85	2.03
S2	273.3	217.8	1.94	2.16
S3	314.7	239.4	1.92	2.21

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Table 3. DNA concentration and quality metrics for samples S1, S2, and S3 after treatment with the short read eliminator kit. 9 ug was input with short fragments elimination treatment for each sample. NanoDrop™ One/One^C Microvolume UV-Vis Spectrophotometer and Qubit 4 Fluorometer were used to test the quality metrics.

	Nanodrop (ng/μl)	Qubit (ng/μl)	260/280	260/230
S1	95.1	54.7	1.81	2.17
S2	82.4	45.2	1.97	2.23
S3	91.8	44.3	1.84	2.05

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Figure 1. Pulse field gel image of DNA samples extracted from *Daphnia pulex* using CTAB protocol. Samples, S1 (1.2 ug), S2 (0.8 ug), and S3 (0.5 ug) remain in a high molecular weight. Two molecular ladders, M1, 0.5 ug (Bio-Rad, catalog no.1703635) and M2, 0.5 ug (NEB, catalog no. N3239S), were loaded on the right most lanes.

137x350mm (118 x 118 DPI)

Figure 2. 1% agarose gel image of DNA samples, S1 (0.6 ug), S2 (0.8 ug), and S3 (1.1 ug) after removal of short fragments, with molecular ladder M, 0.5 ug (NEB, Cat# N3239S) loaded on the left most lane.

17x32mm (600 x 600 DPI)

Figure 3. Read length distribution of DNA sequences from a MinIon nanopore sequencer using the DNA prepared with the current method. The mean read length is 6479 bp.

296x209mm (300 x 300 DPI)