Extraction and selection of high-molecular-weight DNA for long-read sequencing from Chlamydomonas reinhardtii

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Keywords: Algae DNA extraction, high-molecular weight DNA, long-read sequencing, size selection

Abstract

Recent advances in long-read sequencing technologies have enabled the complete assembly of eukaryotic genomes from telomere to telomere by allowing repeated regions to be fully sequenced and assembled, thus filling the gaps left by previous short-read sequencing methods. Furthermore, long-read sequencing can also help characterizing structural variants, with applications in the fields of genome evolution or cancer genomics. For many organisms, the main bottleneck to sequence long reads remains the lack of robust methods to obtain high-molecular-weight (HMW) DNA. For this purpose, we developed an optimized protocol to extract DNA suitable for long-read sequencing from the unicellular green alga *Chlamydomonas reinhardtii*, based on CTAB/phenol extraction followed by a size selection step for long DNA molecules. We provide validation results for the extraction protocol, as well as statistics obtained with Oxford Nanopore Technologies sequencing.

Introduction

In recent years, long-read sequencing technologies, such as the ones developed by Pacific Biosciences (PacBio) and Oxford Nanopore Technologies (Nanopore), have emerged as a solution to the pitfalls of short-read technologies in the detection of structural variants and in assembling repeated sequences and other complex regions (1). Additionally, because native DNA is used, long-read technologies can directly detect a variety of modified bases, including the most commonly studied methylated cytosines (2, 3). For their applications in genome assembly and structural variant detection, these technologies typically sequence DNA molecules ranging in size from kilobases to hundreds of kilobases as a continuous read. Reads traversing repeated sequences are necessary to correctly assemble neighboring regions, with longer reads enabling more contiguous genome assemblies. Today, the major bottleneck to sequence long reads comes from the ability to extract high-quality DNA devoid of polyphenol and polysaccharide contaminants with sizes compatible with this purpose. This is especially true for most plant tissues and algae cells, because polyphenols and polysaccharides are often co-extracted with DNA and can inhibit downstream applications such as sequencing (4, 5).

Chlamydomonas reinhardtii is a unicellular green alga that is widely used as a model organism to study photosynthesis and cellular motility (6), and is an organism of choice for biotechnological application, with many synthetic biology tools being currently developed (7, 8). In *C. reinhardtii*, as for other plants and algae, contending with phenolic and polysaccharide contaminants while preserving HMW DNA is a major challenge and requires an optimized protocol. PacBio and Nanopore sequencing have been performed on this organism, contributing to important advances in our understanding of its genome structure and content, base modifications and evolution (9-13). However, previous protocols did not include a size selection step, which can substantially enrich for longer molecules.

Here, we present a detailed protocol dedicated to efficiently extract and select HMW DNA from *C. reinhardtii* cells. The protocol minimizes DNA-shearing manipulations and comprises an additional step to enrich for HMW DNA. We validated the method by pulse-field gel electrophoresis (PFGE) and measurement of read length from Nanopore sequencing.

Materials and Methods

The protocol described here is also published on protocols.io, dx.doi.org/10.17504/protocols.io.8epv59j9jg1b/v2 and is included as supporting information file 1 with this article.

Nanopore sequencing

Sequencing libraries were prepared as per manufacturer's recommendations, using NEBNext companion module (E7180S, NEB) and Ligation Sequencing Kit SQK LSK-109 (Nanoporetech), except for the ligation time, which we increased to 30 min. For each run, 500 ng were loaded on MinION flow cells (R9.4.1, Nanoporetech) and sequenced for 6h to 16h, depending on flow-cell kinetics. Libraries were loaded at least twice, with 1h wash using the manufacturer's washing buffer (EXP-WSH004) between runs. Basecalling was performed using Guppy (version 4.3.4) with parameters set to "high accuracy".

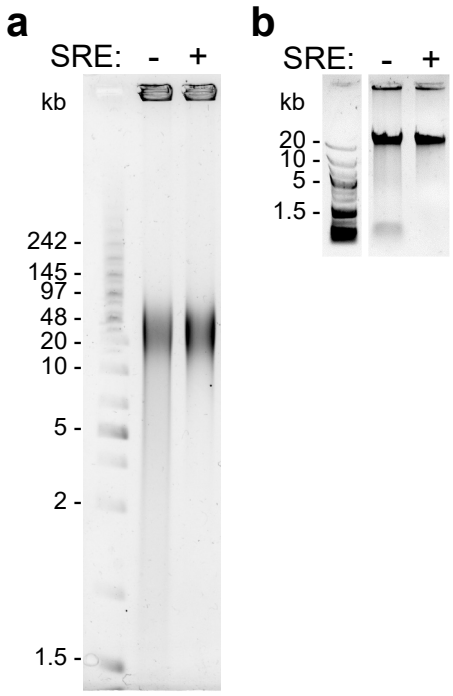
Results

We extracted genomic DNA following the presented protocol (S1 Supplementary Protocol) and applied size selection using the Short Read Eliminator (SRE) kit (Circulomics), an easy-to-use method that does not require dedicated devices which is based on a length-dependent precipitation of nucleic acids driven by polyvinylpyrrolidone crowding. Large amounts of small DNA fragments can be detrimental for long-read Nanopore sequencing (14), not only because the subsequent reads are short, but also because these molecules can outcompete the longer ones, both for adapter ligation and pore usage, thus yielding suboptimal results.

The size distribution of the extracted DNA was assessed by PFGE and Nanopore sequencing, with and without size-selection for HMW DNA. Samples were migrated in a pulse field,

stained by ethidium bromide and imaged with UV light (Fig. 1a). The DNA molecules extracted without size selection migrated as a large smear spread between approximately 1.5 and 150 kb. After size selection with the SRE kit, the upper part of the distribution remained unchanged while the low-molecular-weight fragments (< 10 kb) were visibly reduced. We made a similar observation after electrophoresis and staining of the samples in a 0.3% agarose gel (Fig. 1b).

Size-selection of DNA fragments before preparation of libraries for Nanopore sequencing reproducibly led to a substantially decreased number of shorter molecules and an enrichment of longer ones (Fig. 2a-c), without negatively affecting read quality (Fig. 2d) and with no effect on genome-wide sequencing depth (S3 Supplementary Figure). Size-selection doubled the mean read length, increased the N50 from 12 kb to 17 kb (20 kb in two other experiments), with reads in the top decile being longer than 21 kb (26 kb and 27 kb in two other experiments) (S2 Table). The longest molecules we sequenced were over 100 kb, which are instrumental for genome assemblies.



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105 **Figure 1.** Visualization of extracted genomic DNA size distributions. (a) PFGE using 0.5 µg
106 of DNA prepared with (+) or without (-) SRE size-selection, embedded in 30 µl of 0.5% low-
107 melting agarose plugs, migrated in a 1% SeaKem GTG agarose (Lonza) gel. The ladder is a
108 mix of PFG mid-range (N0342S, NEB) and GeneRuler 1 kb Plus (SM1331**,** ThermoFischer).
109 Electrophoresis conditions: 0.5X TBE (Tris Borate EDTA) buffer, 6 $V.cm^{-1}$, 120° angle, for
110 11h, switching time ramp from 1 to 60 seconds. Gel stained in ethidium bromide and imaged
111 with UV. (b) Standard gel electrophoresis (0.3% agarose) of the indicated samples. GeneRuler
112 1 kb Plus (SM1331**,** ThermoFischer) is used as the ladder.

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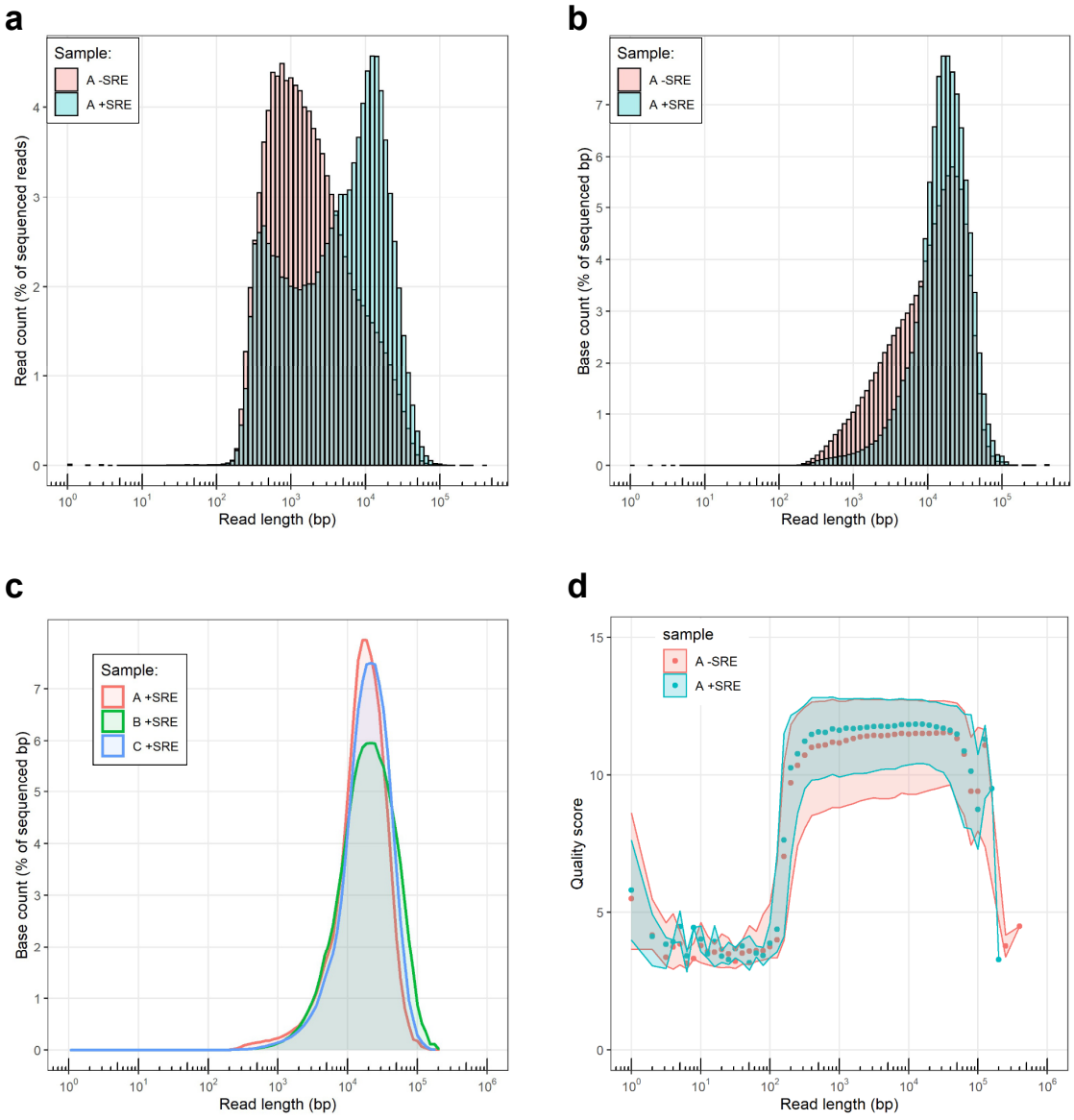

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115 **Figure 2.** Distributions of read length in Nanopore-sequenced datasets. (a-b) Count percentage
116 of (a) reads and of (b) bases as a function of read length obtained from genomic DNA of *C.*
117 *reinhardtii* (experiment "A", see Table S2) with or without size selection (+SRE and -SRE).
118 (c) Count of bases after size-selection (+SRE) as a function of read length obtained from three
119 different samples (see S2 Table and S4 Supplementary Figure). (d) Quality score for individual
120 reads, grouped into bins of 0.1 log unit for samples "A-SRE" and "A+SRE". The shaded areas
121 represent the values between the 1st and 3rd quartiles.

122

Acknowledgements

We thank Samuel O'Donnell for his help in the initial development of this protocol.

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Supporting information

S1 Protocol. Step-by-step protocol, also available on protocols.io:

dx.doi.org/10.17504/protocols.io.8epv59j9jg1b/v2

S2 Table. Summary statistics for 6 DNA preparations and sequencing experiments.

Major limiting outputs are shown in red.

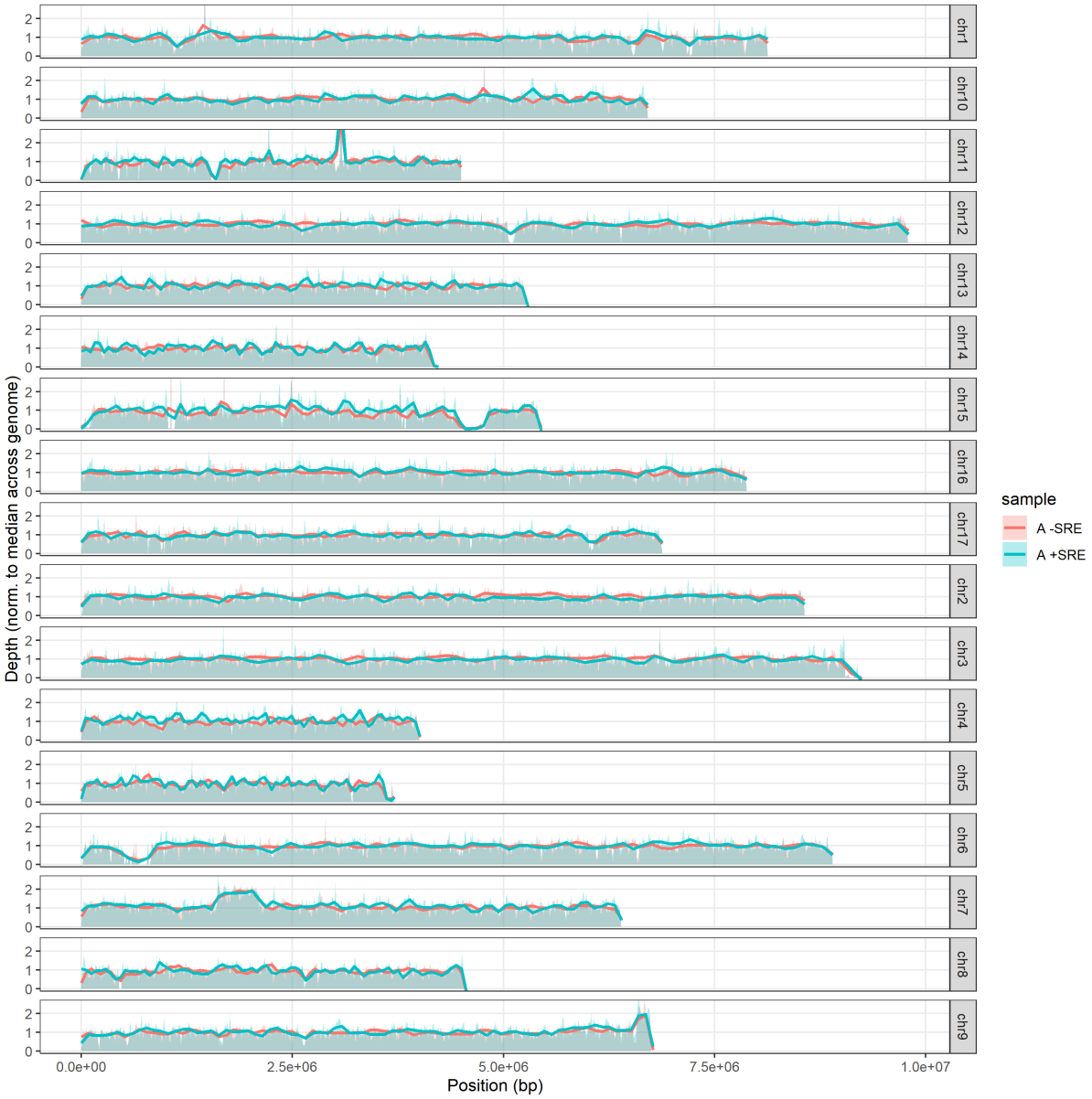
Sample	CLiP library [a]	DNA extraction	Library preparation	Read count	Base count (Gb)	Read length quantiles (kb)			Median read length weighted by base count ("N50") (kb)	Top 3 longest reads [b] (kb)		
						Median	Q90	Q99				
A +SRE	LMJ.RY0402.077111	This protocol	LSK109 +barcodes	232 076	2,0	5,0	21	44	17	142	121	120
A -SRE		This protocol (except SRE)	LSK109	850 066	3,6	1,5	12	34	12	127	117	112
B +SRE	CC-4533 (cMJ030)	This protocol	LSK109	280 614	3,1	6,7	26	64	20	213	201	191
B -SRE		Monarch HMW DNA kit [c]	LSK109	177 794	1,1	2,5	15	53	15	199	177	175
C +SRE	LMJ.RY0402.209904	This protocol	LSK110	344 223	3,9	8,0	27	54	20	175	166	161
C -SRE		DNeasy Plant/Tip100 [d]	LSK109	81 078	0,6	3,0	22	54	21	121	119	108

[a] https://www.chlamylibrary.org and reference (15).

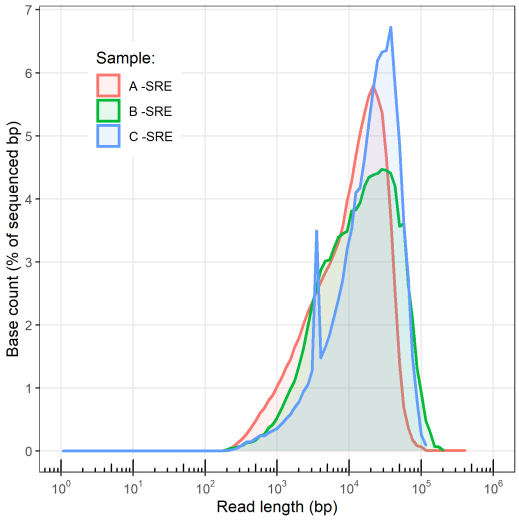
[b] with quality > 7.

[c] as per manufacturer's protocol (Monarch® HMW DNA Extraction Kit for Tissue Cat. no. T3060L, New England Biolabs).

[d] cell lysis using DNeasy Maxi Plant (Cat. no. 68163, Qiagen) as in (16) and purification using Genomic-tip 100/G (Cat. no. 10243, Qiagen), then AMPure beads (Cat. no. A63880, Beckman Coulter).



S3 Supplementary Figure. Sequencing depth normalized to the median across the whole genome of the sequencing reads for all chromosomes, using DNA obtained with (+) or without (-) SRE size selection.



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185 **S4 Supplementary Figure.** Count percentage of bases as a function of read length with

186 alternative sample preparations without size selection (-SRE). See Table S2 for details.

187 Sample C was sequenced in the presence of control DNA ("DNA CS" from Oxford Nanopore

188 sequencing), which peaked at 3 kb.

189