Comparing DNA extraction methods for successful PacBio HiFi sequencing: a case study of the freshwater mussel *Anodonta anatina* (Bivalvia: Unionidae)

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Abstract

High-quality reference genomes are increasingly recognized as essential resources in biodiversity genomics and conservation. However, successful DNA extraction and long-read sequencing remain highly organism-dependent. Molluscs, a diverse phylum of invertebrates, pose particular challenges due to the presence of inhibitory compounds and the difficulty of obtaining high-molecular-weight DNA, often necessitating careful optimization of extraction protocols. Here, we present a case study on the freshwater mussel *Anodonta anatina* (Bivalvia: Unionidae), evaluating two preservation methods, six DNA extraction protocols, and two post-extraction clean-up steps for their effects on DNA quality and PacBio HiFi sequencing yield from foot tissue of a single individual. The PacBio Nanobind and CTAB protocols produced high-quality DNA from fresh tissue but performed poorly on flash-frozen tissue in this system. Post-extraction clean-up generally degraded DNA and did not improve sequencing yield. Unexpectedly, the column-based Omega Mollusc Kit, although not designed for high-molecular-weight DNA, performed better than the PacBio-recommended Nanobind kit and the manual CTAB method on flash-frozen tissue. It generated high DNA quantity and purity, sufficient integrity for HiFi sequencing, and appeared to remove contaminants effectively. While the resulting DNA may be too fragmented for ultra-long read sequencing, the Omega Mollusc Kit offers a practical, cost-effective first approach for testing DNA extraction and PacBio sequencing in flash-frozen *A. anatina* foot tissue. When fresh tissue is available, Nanobind or CTAB were the best-performing options in our comparison. Overall, our results provide a practical starting point for protocol selection, while acknowledging that validation across other mollusc species, tissue types, and preservation methods remains important. This strategy could reduce the need for extensive protocol optimization and facilitate future mollusc genomics efforts.

Keywords

Mollusc genomics; inhibitors; reference genome

Introduction

The generation of reference genomes plays a crucial role in the conservation and management of biodiversity, providing a foundation for understanding species' evolutionary histories, adaptive potentials, and responses to environmental pressures (Formenti et al., 2022; Paez et al., 2022; Theissinger et al., 2023). Large-scale initiatives such as the Earth BioGenome Project (Lewin et al., 2018, 2022) with dedicated local centers, for instance the European Reference Genome Atlas (Mc Cartney et al., 2024), have emerged to coordinate the global effort to sequence and catalog genomes from all eukaryotic species. These projects are especially important for species-rich groups such as molluscs, one of the most diverse animal phyla with around 87,000 described extant species, with gastropods and bivalves being the two most species-rich classes (MolluscaBase eds. 2025).

Despite the high number of mollusc species, genomic resources for this phylum lag behind other species-rich taxa (Gomes-dos-Santos et al., 2020; Johansen and Davison, 2025). The reasons for this gap are multifaceted. While some challenges are attributed to genome complexity (e.g. heterozygosity and repeat content) (Halstead-Nussloch et al., 2024; Chen et al., 2025), a more pervasive issue involves the co-extraction of compounds that inhibit DNA polymerase activity during sequencing. Molluscs are known to produce polyphenolic proteins, mucopolysaccharides, and other biochemical contaminants that complicate the extraction of high-quality DNA, hampering efforts to obtain accurate and complete genomic data (Adema, 2021).

Accordingly, numerous DNA extraction protocols for molluscs have been developed not only to increase DNA yield, but also to address different classes of co-extracted inhibitors. In particular, CTAB-based methods have often been adopted because CTAB, especially under high-salt conditions, helps separate DNA from polysaccharides and associated contaminants, whereas other modified precipitation or purification steps have been introduced for tissues especially rich in mucus and mucopolysaccharides (Sokolov, 2000; Pereira et al., 2011; Arseneau et al., 2017; Chakraborty et al., 2020). Nevertheless, ensuring sufficient DNA quantity, integrity, and purity for successful sequencing remains challenging, because contaminants are not always completely removed during extraction and can compromise downstream enzymatic reactions even when DNA yield is high (Arseneau et al., 2017; Adema, 2021). Commercial kits specifically designed for molluscs, such as the E.Z.N.A. Mollusc & Insect DNA Kit (Omega Bio-tek), have been developed to address these challenges. These kits typically produce DNA with good yield and purity, making them a popular choice for routine DNA extractions. However, their efficacy is supposedly limited when long-read sequencing technologies are required (e.g. to assemble reference genomes) (Jaudou et al., 2022). The frequent use of centrifugation and column-based purification steps in these kits typically results in DNA fragmentation, which is not optimal for techniques such as Oxford Nanopore or PacBio where long DNA fragments (typically >50 kb) are usually required for *de novo* genome sequencing and assembly (Arseneau et al., 2017; Angthong et al., 2020). In addition, these approaches generally produce relatively low yields of high-molecular-weight (HMW) DNA (Kalendar et al., 2023). Nevertheless, this kit was

recently applied in the *Anodonta cygnea* genome project, where it enabled successful PacBio HiFi sequencing (Faust et al., 2025), suggesting that its utility for mollusc genome projects may be greater than previously assumed.

Despite adherence to established DNA quality control measures, such as achieving appropriate Nanodrop ratios, good DNA integrity, and quantity, as well as successful library preparation for long-read sequencing platforms like PacBio, the expected sequencing outputs are not always met. For example, cases have been reported where sequencing results yielded shorter reads and lower output than anticipated, despite following correct protocols (Mollusc Genomics Conference 2024, pers. comm.). In such cases, there may still be unknown contaminants co-purified with the DNA, which could potentially inhibit the performance of the high-fidelity polymerase, ultimately compromising the sequencing output. Unfortunately, such issues remain largely undetectable prior to sequencing, as both the DNA and libraries may appear high-quality based on standard QC metrics. As a result, incorporating additional post-extraction DNA clean-up steps may help eliminate residual contaminants and improve sequencing performance. Finally, it has been shown that tissue preservation method (e.g. flash freezing in liquid nitrogen and preservation at -80°C; preservation in ethanol 100%; or using fresh tissue directly) can also influence the outcome of DNA extractions (Alberola-Mora et al., 2025), with flash freezing in liquid nitrogen being the current gold standard for *de novo* reference genome projects (Reichel et al., 2026).

To date, systematic studies that compare long-read sequencing output from different preservation methods and DNA extraction protocols remain uncommon, with some recent exceptions (Angthong et al., 2020; Alberola-Mora et al., 2025). This is a significant gap in the context of large-scale genome initiatives where optimizing genomic resources for non-model organisms such molluscs is critical. In this study, we address this gap by conducting a comparison of various factors that can influence DNA extraction and sequencing success in the freshwater mussel *Anodonta anatina* (Bivalvia: Unionidae), a species notorioulsy difficult to extract high-molecular weigth DNA from (Faust et al., 2025). Specifically, using foot tissue from the same individual, we: 1) Compare two different preservation methods (flash freezing in liquid nitrogen; fresh tissue); 2) Compare six DNA extraction protocols; 3) Evaluate the impact of two additional DNA cleanup steps applied before library preparation; and 4) Assess the sequencing output from five libraries sequenced on one full PacBio Revio cell. Our goal was to provide a comprehensive evaluation of the factors influencing genome sequencing in a freshwater mussel, thereby offering practical guidance for improving genomic resource generation in molluscs and other non-model organisms.

Methods

Sampling and molecular species identification

A collection permit (Nr. 24091) was obtained from Canton Zürich (Switzerland) and five adult individuals of the duck mussel *Anodonta anatina* were collected on the 5th of August 2024 by snorkeling in Türlersee, Switzerland (GPS coordinates: 47.265602, 8.510178). They were brought back to the laboratory and kept alive in the Eawag experimental facility

124 Aquatikum until processing. They were maintained in an open-water circuit at 19°C and fed
125 twice a week using a mixture of lab-grown algae. Given the morphological plasticity of
126 *Anodonta* mussels (Riccardi et al., 2020), species identification was initially based on shell
127 morphology and subsequently confirmed by sequencing a COI barcode. Briefly, a small
128 tissue biopsy was taken from the foot of each of the five individual mussels and DNA was
129 extracted using the E.Z.N.A Tissue DNA kit (Omega Bio-tek) according to the manufacturer's
130 instructions. DNA integrity, quantity, and purity were assessed using NanoDrop Eight. A
131 fragment of the COI mitochondrial gene was amplified with forward primer LCO22me2: 5'-
132 GGTCAACAAAYCATAARGATATTGG-3' and reverse primer HCO700dy2: 5'-
133 TCAGGGTGACCAAAAAAYCA-3' (Walker et al., 2006). PCR cycling conditions were: an initial
134 step of 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 48°C for 40 s, 72°C for 60 s
135 and then a final extension of 72°C for 10 min. Amplified PCR products were purified and
136 Sanger sequenced by Microsynth AG, Balgach, Switzerland. The COI sequences of the five
137 *Anodonta* individuals were compared to the NCBI GenBank database using the Basic Local
138 Alignment Search Tool (BLAST) to confirm morphological species identification.
139
140 *Comparison of DNA extraction methods and quality control*
141 All DNA extractions were performed using ~30 mg of foot tissue from a single *Anodonta*
142 *anatina* individual to eliminate inter-individual variability and isolate the effects of
143 methodological factors. This design allowed us to focus on methodological rather than
144 biological sources of variation, but did not allow inter-individual variation in extraction
145 performance to be quantified. We tested the impact of two variables on DNA extraction
146 success (Fig. 1): 1) Preservation method: fresh tissue vs. flash-frozen in liquid nitrogen and
147 subsequently preserved at -80°C; 2) DNA extraction method: six protocols, five of which
148 being specifically designed to extract high-molecular weight (HMW) DNA (Table 1). For both
149 preservation methods, tissues were processed immediately upon sampling: fresh foot tissue
150 was transferred directly into lysis buffer after biopsy, and tissues intended for the frozen
151 treatment were dissected and flash-frozen in liquid nitrogen immediately after sacrifice.
152 Each extraction condition was performed in triplicate, except for the GenomicTip and
153 MegaLong protocols with frozen tissue, which were conducted in duplicate. All DNA
154 extractions were conducted following the manufacturer's instructions or using a dedicated
155 protocol (Table 1). For the Nanobind and Omega kits, tissue homogeneisation was
156 performed with a TissueRuptor II (QIAGEN). For the Omega kit, a single final elution step
157 was conducted. DNA extractions using the six protocols were first performed on fresh foot
158 tissue, as this allowed non-lethal sampling (small biopsy) and kept the individual alive. Then,
159 the specimen was killed and remaining tissues (foot, gills, and whole body) were flash-
160 frozen in liquid nitrogen and stored at –80°C. Subsequent extractions from flash-frozen foot
161 tissue were conducted using the six DNA extraction methods after approximately three
162 months at –80°C. Finally, to evaluate the effect of clean-up on DNA quality, we applied two
163 commercial clean-up kits (Zymo and PowerClean) only to fresh-tissue extracts from the two
164 top-performing column-free methods (CTAB and Nanobind), while limiting additional

sample consumption. For each extraction method, the three replicate extracts were first pooled, and each pooled sample was then divided into three aliquots: one aliquot was cleaned with the Zymo kit, one with the PowerClean kit, and one was left untreated as a no-clean-up control.

DNA quality control included three metrics: 1) Yield (Qubit dsDNA High Sensitivity kit); 2) Purity (Nanodrop 260/280 and 260/230 ratios); 3) Integrity (Agilent TapeStation gDNA ScreenTape). A DNA extraction was considered successful if it met all of the following thresholds: 1) Yield: >1 µg total DNA; 2) Purity: 260/280 ratio between 1.8 and 2.0; 3) Integrity: largest TapeStation peak >15 kb. 260/230 ratio was not used as a formal threshold because it was not consistently predictive of downstream sequencing success in our workflow.

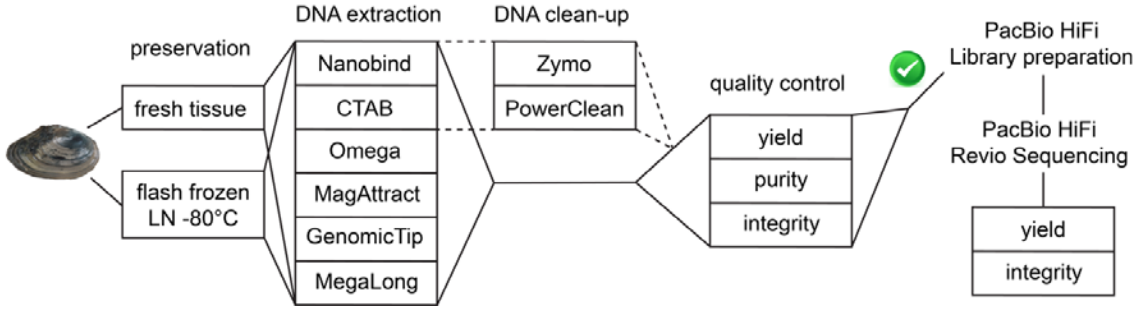


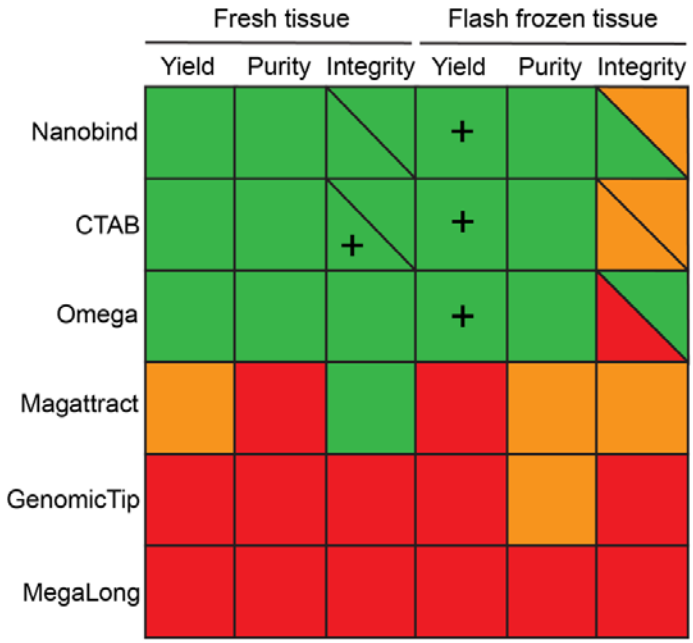
Figure 1: Experimental design for comparing DNA extraction methods and sequencing output in the freshwater mussel *Anodonta anatina*. All DNA extractions were performed from foot tissue of a single individual. DNA quality control at the DNA extraction laboratory was based on Qubit yield, NanoDrop purity, and TapeStation integrity; DNA integrity of selected samples was re-assessed at the sequencing center using a Fragment Analyzer prior to library preparation. LN: liquid nitrogen. See Table 1 for details regarding DNA extraction methods.

184 **Table 1:** List of the DNA extraction methods and DNA clean-up kits used in this study. HMW: high-molecular weight.
185

Kit or protocol name	Purpose	Short name	Kit price (CHF)	Number of preps	Price per prep (CHF)	Link
PacBio Nanobind panDNA kit	HMW DNA extraction for animals	Nanobind	315	24	13.1	https://www.protocols.io/view/sanger-tree-of-life-hmw-dna-extraction-manual-moll-14egn36nyl5d/v1
CTAB for molluscs	HMW DNA extraction for molluscs	CTAB	237	~3000	0.08	https://www.protocols.io/view/ctab-dna-extraction-protocol-for-mollusks-e6nvwjpr2lmk/v1
Omega Bio-tek E.Z.N.A. Mollusc & Insect DNA Kit	DNA extraction for mollusc	Omega	480	200	2.4	https://omegabiotek.com/product/e-z-n-a-mollusc-dna-kit/
Qiagen MagAttract	HMW DNA extraction	MagAttract	350	48	7.3	https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/magattract-hmw-dna-kit-48
Qiagen Genomic-tip 100G	HMW DNA extraction	GenomicTip	422	25	16.9	https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/qiagen-genomic-tips?catno=10223
G-Biosciences MegaLong	HMW DNA extraction	MegaLong	768	25	30.7	https://www.gbiosciences.com/MegaLong/MegaLong-359
Qiagen DNeasy PowerClean Pro Cleanup Kit	DNA clean-up	Powerclean	362	50	7.2	https://www.qiagen.com/us/products/discovery-translational-research/dna-rna-purification/dna-purification/dna-clean-up/dneasy-powerclean-pro-cleanup-kit
ZYMO Genomic DNA Clean & Concentrator-10	DNA clean-up	Zymo	133	25	5.3	https://zymoresearch.eu/collections/genomic-dna-clean-concentrator-gdcc/products/genomic-dna-clean-concentrator-25

186 *PacBio library preparation and sequencing*
187 A total of 15 DNA extractions from eight treatments were brought to the Lausanne Genomic
188 Technologies Facility (GTF) sequencing platform in Lausanne (Switzerland) for quality
189 control, PacBio library preparation and sequencing. Integrity of DNA was first assessed using
190 a 5200 Fragment Analyzer (Agilent Technologies). Based on quality control, a total of six
191 DNA extractions representing six treatments were selected for PacBio library
192 preparation. DNA fragments below <10 kb were partially removed with a short-read
193 eliminator (SRE) kit (PacBio). DNA of Omega_frozen1 sample was not sheared because it
194 was already fragmented enough. For the five remaining DNA extractions, DNA was sheared
195 in a Megaruptor 3 DNA shearing system (Diagenode) with a target mean fragment length of
196 15-20 kb. Fragments profile was assessed by capillary electrophoresis on a Fragment
197 Analyzer using an HS large fragment 50 kb kit (Agilent Technologies). Repair and A-tailing of
198 DNA fragments, SMRTbell adapters ligation, nuclease treatment and removal of fragments
199 <3kb were performed according to the SMRTbell prep kit 3.0 protocol (PacBio). Primer
200 annealing and polymerase binding were performed using the PacBio Revio SPRQ polymerase
201 kit including a DNA internal control according to the manufacturer's protocol. Sequencing
202 run was set up using SMRT Link v25.01. The six libraries were sequenced in HiFi mode in a
203 PacBio Revio platform using Revio SPRQ Sequencing plate and one 25M ZMW SMRT cell
204 with a 30-hour movie time and 2 hours of pre-extension time. Unfortunately, the initial run
205 failed due to a technical issue with the PacBio instrument and had to be repeated. Due to
206 the exhaustion of the CTAB_fresh_Zymo1 library, only five libraries were ultimately
207 sequenced on a single 25M ZMW SMRT cell.
208
209 **Results**
210 *Molecular species identification, comparison of DNA extraction performance and DNA clean-*
211 *up*
212 Using COI barcodes, all five analyzed individuals were assigned to *Anodonta anatina*,
213 confirming the morphological species identification. A single representative individual
214 (AE01) was subsequently used for all DNA extraction tests. Full QC metrics for all extractions
215 are provided in Table S1. Three high-molecular weight (HMW) DNA extraction protocols—
216 MagAttract, GenomicTip, and MegaLong—failed to produce DNA of sufficient quality,
217 regardless of whether fresh or flash-frozen tissue was used. These protocols did not meet
218 the minimum quality criteria for yield, purity, or integrity (Fig. 2; Table S1). In particular, the
219 GenomicTip and MegaLong kits yielded almost no detectable DNA, and therefore none of
220 these extracts were selected for sequencing.
221 In contrast, the remaining three protocols—Nanobind, CTAB, and Omega—produced
222 satisfactory DNA yield, purity, and integrity from fresh tissue, with CTAB yielding the highest
223 integrity (Fig. 2; Table S1). As expected, the high-molecular-weight extraction methods
224 (Nanobind and CTAB) generated DNA with greater integrity than the column-based Omega
225 kit. Nonetheless, the Omega kit still produced relatively long fragments (approximately 18–
226 31 kb; Table S1), sufficient for several long-read sequencing applications. Interestingly, all

227 three methods yielded substantially more DNA when using flash-frozen tissue, but this
228 increase came at the expense of integrity, which was markedly reduced compared to
229 extractions from fresh tissue (Fig. S1). Actually, the Omega kit did not meet our integrity
230 threshold on flash-frozen material (largest TapeStation peak >15 kb). However, because the
231 DNA profiles showed a clean and promising fragment distribution, we proceeded to submit
232 these extracts to the sequencing center for further quality assessment. Some extracts,
233 particularly CTAB from fresh tissue, showed relatively low A260/A230 ratios that may reflect
234 residual carryover of contaminants (Table S1). However, because this metric was not
235 consistently predictive of library preparation or sequencing success in our dataset, it was
236 not used as a formal exclusion criterion.
237 To further assess potential improvements in DNA quality, we subjected extracts obtained
238 with the two most promising column-free protocols (Nanobind and CTAB) from fresh tissue
239 to additional clean-up steps using two commercial kits (Zymo and PowerClean; Fig. 3).
240 Unexpectedly, the Zymo clean-up reduced the purity of Nanobind DNA, whereas CTAB-
241 derived DNA remained stable after the same treatment. In both cases, the PowerClean
242 protocol markedly degraded the DNA, rendering it unsuitable for sequencing. However,
243 DNA integrity after the Zymo clean-up remained sufficient for downstream long-read
244 sequencing.
245



246
247 **Figure 2. DNA extraction performance across methods.**
248 Thresholds for successful extraction were defined as: yield >1 µg total DNA ("+" indicates >10 µg across three
249 replicates), purity with an A260/A280 ratio of 1.8–2.0, and integrity with the largest TapeStation peak >15 kb
250 ("+" indicates largest TapeStation peak >60 kb across three replicates). Color coding: green = success in all
251 replicates; orange = success in one to two replicates; red = no success. Extractions from five treatments
252 (Nanobind fresh and frozen, CTAB fresh and frozen, Omega frozen) were submitted to the sequencing facility,
253 where DNA integrity was confirmed on a Fragment Analyzer. Integrity values are shown as diagonals: lower

left = TapeStation results from the extraction laboratory; upper right = Fragment Analyzer results from the sequencing center.

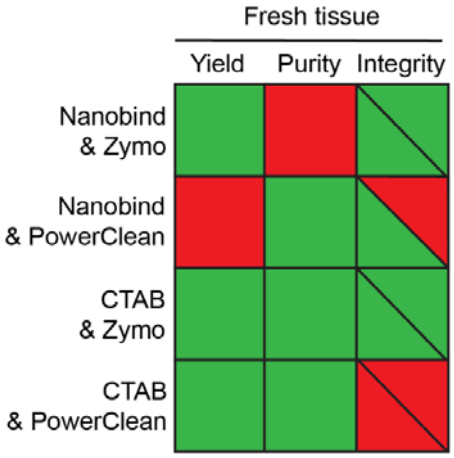


Figure 3: DNA quality metrics after additional clean-up step. Color coding: green = success. Red = no success. All four extractions were submitted to the sequencing facility, where DNA integrity was confirmed on a Fragment Analyzer. Integrity values are shown as diagonals: lower left = TapeStation results from the extraction laboratory; upper right = Fragment Analyzer results from the sequencing center.

DNA extraction quality control, library preparation and PacBio sequencing at the sequencing center

Based on the quality control conducted at the DNA extraction laboratory in Dübendorf, 15 DNA extractions representing eight independent treatment conditions were selected for further quality control, library preparation, and sequencing at the GTF platform in Lausanne (Table 2). During the second round of quality control, substantial discrepancies in DNA integrity values were observed between instruments: the TapeStation (Eawag Dübendorf) and the Fragment Analyzer (GTF Lausanne) produced several inconsistent results (Fig. S2). These differences likely reflect both known platform-specific biases—since the TapeStation is known to slightly overestimate DNA fragment lengths—and most likely post-extraction DNA degradation (Fig. 2; Fig. 3).

For instance, Nanobind extractions from flash-frozen tissue displayed relatively high integrity when analyzed at the extraction laboratory but were found to be completely degraded upon re-evaluation at the sequencing facility (Fig. 2; Table 2; Fig. S2). Interestingly, this degradation was not observed for Nanobind extractions from fresh tissue, which retained their integrity across both analyses. A comparable pattern was found for CTAB extractions, where DNA from frozen tissue appeared substantially degraded at the sequencing center, while DNA from fresh tissue remained stable (Fig. S2). In contrast, no evidence of post-extraction degradation was detected for DNA obtained with the Omega kit; in fact, integrity values were relatively higher when assessed with the Fragment Analyzer (~22-24 kb) compared to the TapeStation (~13-14 kb), which was unexpected.

286 **Table 2:** Quality control summary of the 15 DNA extractions brought to the sequencing facility GTF. Extraction protocol, tissue preservation and potential clean-up steps
287 are included in the sample name. All DNA extractions were performed using foot tissue from the same *A. anatina* individual. Technical replicates are numbered 1-3. The
288 size detection limit of the genomic DNA Tapestation is 60,000 bp. Tapestation QC was performed in the extraction laboratory in Dübendorf. Fragment Analyzer QC was
289 performed at the sequencing facility GTF in Lausanne. *Samples used for library preparation. [1] yield calculated from Qubit concentration.

Sample name	Treatment	Yield[1] [ug]	Qubit [ng/µl]	Nanodrop [ng/µl]	A260/A280	A260/A230	Largest peak Tapestation [bp]	Largest peak Fragment Analyzer [bp]
*CTAB_fresh1	1	4.8	48.4	122.5	1.94	1.62	>60,000	48,313
*CTAB_fresh_Zymo1	2	1.9	41.6	53.7	1.88	3.07	51,900	47,003
CTAB_fresh_Powerclean 1	3	1.5	33	57.9	1.84	1.92	12,973	147
*Nanobind_fresh1	4	7.5	74.6	159.8	1.86	2.24	>60,000	47,751
*Nanobind_fresh_Zymo 1	5	1.6	36.2	40.4	1.76	3.16	>60,000	40,640
Nanobind_fresh_Powerc lean1	6	0.6	13.4	16.3	1.92	1.04	17,352	8,557
*Omega_frozen1	6	18.8	125	209.7	1.84	2.17	13,080	24,733
Omega_frozen2	6	93.8	625	902.8	1.87	2.34	13,519	22,861
Omega_frozen3	6	75.8	505	1,111.6	1.86	2.37	14,075	22,300
Nanobind_frozen1	7	90	600	920.8	1.93	2.34	27,603	3,810
Nanobind_frozen2	7	38.1	254	493.5	1.96	2.33	>60,000	3,953
Nanobind_frozen3	7	124.5	830	974.9	1.91	2.39	22,985	>60,000
*CTAB_frozen1	8	42.6	284	461.3	1.92	2.71	18,259	21,177
CTAB_frozen2	8	41.6	277	457.5	1.93	2.72	10,900	84
CTAB_frozen3	8	39.9	266	426.7	1.92	2.76	14,120	12,813

290

291 Following quality control at GTF, six samples (marked with asterisks in Table 2) were
292 selected for library preparation. Because the initial run failed and used up the
293 CTAB_fresh_Zymo1 library (see methods), only five libraries were ultimately sequenced. The
294 second sequencing run was successful, yielding a total of 91 Gbp, which is in the range of
295 expected output of a PacBio Revio cell (Table 3). One library (CTAB_frozen1) produced
296 shorter reads, likely due to more extensive shearing compared to other samples, despite
297 using the same shearing parameters. This resulted in reduced HiFi read length and overall
298 yield. In contrast, the remaining libraries produced yields between 18 and 22.9 Gbp, with
299 Omega_frozen1 achieving the highest output. A trade-off between HiFi read length and
300 quality was observed, with shorter reads generally having higher quality. Nevertheless, even
301 the longest reads (mean ~13 kbp) maintained excellent quality (Q38). Overall, all five
302 libraries were successfully sequenced, and read length distributions closely mirrored the
303 respective library fragment sizes, indicating the absence of sequencing inhibitors.
304
305 **Table 3:** PacBio HiFi sequencing output on one Revio cell for each of the five libraries.

Sample Name	HiFi Reads	HiFi Read Length (mean, bp)	HiFi Read Quality (median, QV)	HiFi Yield (Gbp)
CTAB_fresh1	1,792,289	10,087	Q41	18.08
Nanobind_fresh1	1,493,698	12,371	Q38	18.48
Nanobind_fresh_Zymo1	1,469,733	13,051	Q38	19.18
Omega_frozen1	1,957,907	11,698	Q39	22.91
CTAB_frozen1	2,002,334	6,347	Q47	12.71
Not barcoded	51,897	11,226	Q30	0.58
Total	**8,767,858**	**10,796**	**Q40**	**91.94**

306
307 **Discussion**
308 *Tissue preservation and extraction protocol shape extraction outcomes*
309 Molluscs present well-known challenges for DNA extraction due to high levels of mucus and
310 endogenous inhibitors (Adema, 2021). In this study, we evaluated different strategies for
311 extracting high-molecular-weight (HMW) DNA from the freshwater mussel *Anodonta*
312 *anatina*, a species for which we previously encountered difficulties in generating sufficient
313 quality data for PacBio sequencing (Faust et al., 2025). While our results are based on a
314 single species, we believe they provide valuable insights likely applicable to other unionids
315 and possibly to other mollusc species.
316 We found that tissue preservation had a critical influence on the performance of the top
317 three HMW DNA extraction protocols. This effect was evident both in terms of yield and
318 integrity. First, yield differed markedly between preservation methods, with approximately
319 ten times more DNA recovered from flash-frozen tissue compared with fresh tissue. One
320 possible explanation is that rapid freezing partially disrupted cellular membranes through
321 intracellular ice-crystal formation (Muldrew and McGann, 1990). Therefore, flash freezing
322 prior to extraction may have weakened tissue structure and improved lysis efficiency during
323 DNA extraction, resulting in higher DNA recovery. While we did not explicitly test this

324 mechanism, similar effects of increased DNA yield after flash-freezing have been previously
325 reported (Stark et al., 2020; Salis et al., 2025).
326 Second, we observed clear differences in DNA integrity between fresh and flash-frozen
327 tissue extracted using the Nanobind and CTAB protocols. While both protocols produced
328 high-quality and stable DNA from fresh tissue, DNA obtained from flash-frozen tissue
329 initially showed relatively high integrity at the DNA extraction laboratory in Dübendorf but
330 exhibited strong post-extraction degradation when re-assessed at the sequencing center in
331 Lausanne, in some cases declining from ~18–28 kb or even >60 kb on the TapeStation to
332 only ~4 kb or less on the Fragment Analyzer. This occurred despite identical handling of all
333 samples—storage at −20 °C after extraction and quality control, and transport on dry ice.
334 The cause of this degradation remains unclear. One possibility is that residual compounds,
335 such as DNases, remained active in extracts from frozen tissue, though this explanation
336 remains speculative. Nevertheless, these findings demonstrate a clear advantage of
337 extracting DNA from fresh tissue when feasible. We acknowledge, however, that this is
338 often impractical—particularly in field-based or conservation genomic contexts—where
339 optimized methods for preserved material remain indispensable (Reichel et al., 2026).
340 Among the flash-frozen samples, the Omega kit consistently yielded DNA of moderate
341 fragment length (~13–14 kb on the TapeStation; ~22-24 kb on the FragmentAnalyzer) and
342 showed high reproducibility across replicates. Importantly, no post-extraction degradation
343 was detected, suggesting that any residual compounds potentially responsible for
344 degradation in the Nanobind and CTAB extractions were effectively removed by the Omega
345 protocol. When applied to fresh tissue, the Omega kit also produced consistent results,
346 yielding DNA of moderate fragment length (~18–31 kb) and generally high purity. Together,
347 these findings highlight the robustness and versatility of the Omega kit, which performed
348 reliably across both fresh and flash-frozen samples. An important limitation of this study is
349 that all extractions were performed from foot tissue only. We chose foot tissue because it
350 enables standardized sampling and, for fresh material, non-lethal biopsy while keeping the
351 animal alive. However, tissue choice may itself influence extraction outcomes, because
352 molluscan tissues are often rich in mucus, polysaccharides, and other compounds that can
353 co-purify with DNA and inhibit downstream enzymatic reactions (Adema, 2021). Our results
354 should therefore be interpreted as a comparison of extraction performance for *A. anatina*
355 foot tissue, rather than as a general ranking of methods across all molluscan tissues.
356
357 *High-molecular weight DNA kits do not guarantee better results*
358 In our study, three commercial HMW DNA kits (MagAttract, GenomicTip, Megalong) failed
359 to yield usable DNA, from both fresh and flash-frozen tissues. However, because
360 performance may depend strongly on species, tissue type, and preservation method, these
361 negative results should not be interpreted as a general failure of these kits across molluscs.
362 In contrast, we successfully extracted high-quality DNA using a manual CTAB protocol, the
363 Nanobind kit, and the Omega kit—though success depended strongly on tissue
364 preservation. These results emphasize that dedicated HMW kits are not necessarily superior

365 to manual or general-purpose protocols in molluscs and may require organism-specific
366 optimization. Factors such as cost, processing time, and consistency should be considered
367 when choosing a protocol. For some samples, NanoDrop concentrations differed
368 substantially from Qubit measurements, likely due to residual contaminants affecting
369 absorbance-based quantification. We therefore considered NanoDrop values mainly as a
370 complementary purity metric, while sequencing suitability was assessed primarily from
371 Qubit yield, fragment integrity, and downstream library preparation success.
372 Because mollusc DNA extractions are notoriously prone to inhibition by co-extracted
373 contaminants, we tested additional post-extraction clean-up steps using PowerClean and
374 Zymo kits to further remove residual inhibitors after standard DNA extraction. The
375 PowerClean kit was unsuitable for genomic DNA, leading to severe degradation. In contrast,
376 the Zymo kit preserved fragment length more effectively. However, clean-up did not provide
377 clear added value, as libraries prepared from unpurified extractions (including from
378 relatively dirty samples like CTAB_fresh1) sequenced successfully. In our case, additional
379 clean-up of DNA extracts prior to PacBio library preparation did not provide additional
380 benefits, although we cannot exclude that other clean-up strategies may be useful in
381 different contexts.
382 We also note that the poor performance of some HMW extraction kits may partly reflect the
383 specific properties of the tissue used here. Although foot tissue is practical for repeated and
384 partly non-lethal sampling, kit performance observed here may not fully translate to other
385 tissue types such as mantle, gill, or adductor muscle, which could differ in inhibitor load and
386 tissue composition. Future work comparing multiple tissue types within the same individual
387 would help disentangle protocol-specific from tissue-specific effects.
388 Ultimately, we generated high-quality PacBio data from five different DNA extraction and
389 preservation combinations. Despite pooling all libraries on the same SMRT cell, we observed
390 no evidence that inhibitors from one sample compromised sequencing success in others.
391 Overall read yield was consistent across treatments, suggesting that inhibitor removal was
392 generally successful, and that the selected protocols are robust enough for long-read
393 sequencing applications in *A. anatina.*
394
395 *Recommendations for a new mollusc genome project*
396 We showed that several HMW DNA extraction kits failed to produce DNA of sufficient
397 quality for PacBio sequencing. Some protocols (PacBio Nanobind and CTAB) were successful,
398 but their performance depended strongly on tissue preservation, leading to high-quality
399 DNA only using fresh tissue. We would therefore recommend using one of these protocols if
400 fresh tissue is available. It is also noteworthy that the manual CTAB protocol requires
401 considerably longer hands-on time, while the Nanobind kit is comparatively rapid and user-
402 friendly. Because we focused on *A. anatina*—a species notoriously difficult for DNA
403 extraction (Faust et al., 2025)—rather than testing multiple species, we reduced
404 confounding factors while generating results that provide a useful case study for mollusc

genome projects, although their applicability across species, tissue types, and preservation methods should be interpreted cautiously.

In the present study, the Omega kit consistently produced DNA of sufficient quality for PacBio sequencing, especially for flash-frozen tissue. The same kit has been successfully applied in the *Anodonta cygnea* genome project using flash-frozen tissue (Faust et al., 2025). In another genome project conducted in our lab, we also obtained high-quality PacBio HiFi data from an RNAlater-preserved *A. exulcerata* sample stored at –80 °C using this kit – we however acknowledge that this is an anecdotal observation. Although ethanol-preserved tissues were not tested in this study, their suitability for PacBio HiFi sequencing using the Omega kit remains to be systematically evaluated.

We are aware that the Omega kit was not initally designed for long-read sequencing, and therefore fragment sizes are relatively modest. Hence, small adjustments to PacBio library preparation can - or should - be performed to increase the length of the resulting sequencing data. For example, in the present study, DNA was not sheared and was used directly in library preparation after the standard SRE step. In the above-mentioned *A. exulcerata* project, the sequencing platform further recommended a BluePippin purification step to remove small fragments (<10 kb) after PacBio library preparation. Nevertheless, such adjustments are straightforward, add little cost compared with months of DNA extraction optimization, and ensure effective sequencing output. In all cases, we recommend close contact and open communication with the experienced sequencing platform conducting library preparation and sequencing.

For new mollusc genome projects, it may often be most practical to begin with broadly reliable strategies rather than investing heavily at the outset in expensive HMW extraction kits and extensive protocol optimization. This is especially relevant because some of the dedicated HMW kits tested here (MagAttract, GenomicTip, and MegaLong) were among the most expensive options, yet did not yield DNA of sufficient quality, whereas the CTAB protocol and the Omega kit were substantially more cost-effective and performed better in the context of our study. Furthermore, the choice of DNA extraction method was strongly influenced by tissue preservation in *Anodonta anatina*. For fresh foot tissue, the PacBio Nanobind and manual CTAB protocols consistently produced the highest DNA integrity and are therefore the preferred options in this context. For flash-frozen tissue, which is commonly used in field collections, the column-based Omega kit performed unexpectedly well, providing sufficient DNA quantity, purity, and fragment length for PacBio HiFi sequencing despite not being designed for HMW extraction. To our knowledge, this kit has also yielded high-quality PacBio data in three *Anodonta* species (Faust et al., 2025). The Omega kit does have limitations—adequate tissue input is needed to allow size selection, short fragments may require removal (e.g., via BluePippin), and it is not suitable when ultra-long reads are required. In such cases, Nanobind or CTAB remain better choices. Overall, our findings provide a practical decision framework for *A. anatina* foot tissue: Nanobind or CTAB are good options when high-quality fresh tissue is available, while the Omega kit represents an efficient and cost-effective first option for flash-frozen material. More broadly, this

approach may help guide initial protocol selection in other mollusc genome projects, although species-, tissue-, and preservation-specific validation remains important.

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Data availability

PacBio HiFi whole-genome sequencing data are available from the European Nucleotide Archive (ENA) under BioProject accession PRJEB105293. The dataset comprises five barcoded libraries and one set of unassigned reads generated from a single sequencing flow cell, all derived from the same BioSample (SAMEA120807678), with sequencing runs deposited as ERR16075080 and ERR16075215–ERR16075219.

Author contributions

MG: Investigation, Supervision, Writing – review and editing.
UL: Data curation, Investigation.
PGDF: Funding acquisition, Writing – review and editing.
AATW: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Visualization, Writing – original draft, Writing – review and editing.

Supplementary material

Figure S1: Tapestation profiles of all DNA extractions, treatments and replicates from foot tissue of a single *Anodonta anatina* individual. Profiles are shown for both fresh and flash-frozen tissue across all extraction methods. Successful extractions were defined in the main text as those meeting thresholds for DNA yield (>1 µg total DNA), purity (A260/A280 ratio 1.8–2.0), and integrity (largest TapeStation peak >15 kb). Profiles with a dominant high-molecular-weight peak were considered more suitable for long-read sequencing, whereas smeared profiles or profiles dominated by low-molecular-weight fragments indicated reduced DNA integrity. Panels: **A.** Nanobind extractions. **B.** CTAB extractions. **C.** Omega extractions. **D.** MagAttract extractions. **E.** GenomicTip extractions. **F.** MegaLong extractions. **G.** Nanobind and CTAB extractions after DNA clean-up.

Figure S2: TapeStation and Fragment Analyzer profiles of the 15 DNA extractions from foot tissue of a single *Anodonta anatina* individual that were submitted to the sequencing center in Lausanne, Switzerland. For each sample, the left panel shows the TapeStation profile generated at the extraction laboratory and the right panel shows the Fragment Analyzer profile generated at the sequencing center. These profiles were used to compare DNA integrity across instruments and to assess sample suitability for PacBio library preparation.

566 Panels: **A.** CTAB extractions using fresh tissue. **B.** Nanobind extractions using fresh tissue. **C.**
567 Omega extractions using frozen tissue. **D.** Nanobind extractions using frozen tissue. **E.** CTAB
568 extractions using frozen tissue.
569
570 **Table S1:** Quality control results of all DNA extractions, treatments and replicates.
571
572 **Table S2:** Quality control results of DNA extractions following DNA clean-up steps.
573